



# WJG

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EDITORIAL

# Risk for colorectal cancer in ulcerative colitis: Changes, causes and management strategies

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## INTRODUCTION AND EPIDEMIOLOGY

Since the first report of an inflammatory bowel disease (IBD) case associated with colorectal cancer (CRC) by Crohn and Rosenberg<sup>[1]</sup>, significant efforts have been made to elucidate this presumed association. Nowadays, the association between IBD and the increased risk for CRC is widely accepted. Although CRC, complicating ulcerative colitis and Crohn's disease, accounts only for 1%-2% of all cases of CRC in the general population, it is considered a serious complication of the disease and accounts for approximately 10%-15% of all deaths in IBD patients<sup>[2]</sup>. The age at diagnosis of CRC associated with IBD is 15-20 years earlier compared to sporadic cancers. In the meta-analysis by Eaden *et al*<sup>[3]</sup>, the average age at diagnosis was 43.2 years. Similarly, in a recent publication from Eastern Europe<sup>[4]</sup>, it was found to be 50.9 years, 10-15 years younger compared to sporadic CRC cases from the same area (62.2 years)<sup>[5]</sup>. According to US<sup>[6]</sup> and Canadian<sup>[7]</sup> publications, almost two-thirds of the affected patients were males, yet results are conflicting<sup>[4,8]</sup>. In addition, the frequency of multiple CRCs is higher than in patients with sporadic CRC<sup>[9]</sup>.

The increased risk of CRC in UC is almost a universal finding<sup>[3,10-13]</sup>, yet the extent of this risk varies considerably with differences in study design and geographic area. The initial reports were published by tertiary gastroenterology centers, thus the high risk detected might have been a consequence of referral bias and overinterpretation due to the high percentage of extensive and chronically active cases in these cohorts. Results of population-based studies are more reliable; however, several geographical and ethnic differences have been noted.

The report of the meta-analysis by Eaden *et al*<sup>[3]</sup> in 2001 was one of several milestones in this subject. The incidence of UC-associated CRC was estimated based on 116 articles involving 54 478 patients in whom 1698 CRC cases were detected. This was a mixture of referral center-based, hospital-based, and population-based studies of variable methodological qualities and levels of detail. The reported incidence was higher in the US

## Abstract

The risk of colorectal cancer for any patient with ulcerative colitis is known to be elevated, and is estimated to be 2% after 10 years, 8% after 20 years and 18% after 30 years of disease. Risk factors for cancer include extent and duration of ulcerative colitis, primary sclerosing cholangitis, a family history of sporadic colorectal cancer, severity of histologic bowel inflammation, and in some studies, young age at onset of colitis. In this review, the authors discuss recent epidemiological trends and causes for the observed changes. Population-based studies published within the past 5 years suggest that this risk has decreased over time, despite the low frequency of colectomies. The crude annual incidence rate of colorectal cancer in ulcerative colitis ranges from approximately 0.06% to 0.16% with a relative risk of 1.0-2.75. The exact mechanism for this change is unknown; it may partly be explained by the more widespread use of maintenance therapy and surveillance colonoscopy.

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**Key words:** Ulcerative colitis; Colorectal cancer; Risk factors; Surveillance; Chemoprevention

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**Table 1** Risk of colorectal cancer in recent population-based studies

Study	Location	Observed period	UC cohort size	Follow-up (person-years)	CRC	Annual crude incidence (%)	Cumulative incidence at 30 years (%)
North America							
Bernstein <i>et al</i> <sup>[7]</sup>	Manitoba, Canada	1984-1997	2672	19655	36 CRC	0.16	NR
Bernstein <i>et al</i> <sup>[7]</sup>	Manitoba, Canada	1984-1997	2672	19655	13 rectum	0.06	NR
Jess <i>et al</i> <sup>[8]</sup>	Olmsted County, USA	1940-2004	378	5567	6	0.10	2
Europe							
Winther <i>et al</i> <sup>[16]</sup>	Copenhagen County, Denmark	1962-1987	1160	22290	13	0.06	2.1
Palli <i>et al</i> <sup>[15]</sup>	Florence, Italy	1978-1992	689	7877	10	0.12	NR
Lakatos <i>et al</i> <sup>[4]</sup>	Veszprem, Hungary	1974-2004	723	8564	13	0.15	7.5

NR: Not reported.

and UK than in Scandinavia. Only limited data were available from other European countries, for example Eastern Europe<sup>[14]</sup>. Based on the 41 studies, the duration of UC was 3 (2-4) cases per 1000 person-years, equaling an annual risk of 0.3% or 1 in 333 patients. However, this calculation did not take into account varying degrees of annual risk based on the duration of UC. The well known risk figures at 10 years' (1.6%), 20 years' (8.3%), and 30 years' disease (18.4%) duration were derived from 19 studies that reported CRC incidence according to UC duration at a 10-year interval. In other words, one would expect to diagnose CRC in almost one in five individuals with UC after 30 years' disease duration.

The new, independent population-based studies suggest, however, a lower incidence rate (Table 1)<sup>[4,7,8,15-17]</sup>. During the follow-up of 689 UC patients in Florence between 1978 and 1992, ten new CRC cases were reported<sup>[15]</sup>, equaling a yearly incidence of 0.13%. Bernstein *et al*<sup>[7]</sup> reported 36 colon and 13 rectal cancers in 2672 patients, the annual risks of colon and rectal cancer being 0.16% and 0.06%, respectively, in a follow-up of 19 665 person-years. In an inception cohort from Denmark between 1962 and 1987, only 13 cases of CRC were reported among 1160 patients with UC, and followed up for 22 290 person-years, yielding an annual risk of 0.06%<sup>[16]</sup>. The 30-year cumulative CRC risk was 2.1%. The rate of surgery in Denmark is among the highest reported worldwide. A much smaller cohort of 378 patients with UC diagnosed between 1940 and 2001 in Olmsted County were followed up for 5567 person-years, leading to detection of six CRCs<sup>[8]</sup>. The crude annual incidence was 0.1%, while the cumulative risk after 30-years' disease duration was as low as 2.0%. The authors of the study concluded that, in general, the risk of CRC is not increased in UC, only in patients with extensive disease. Finally, despite one of highest incidence rates for sporadic CRC, and a much lower non-CRC related colectomy rate (3.4%), the incidence of UC-related CRC was only moderately increased in a Hungarian population-based study. The cohort consisted of 723 individuals diagnosed with UC over a 30-year period and followed up for 8564 person-years. The cumulative risk for CRC during the follow-up of these 723 UC patients was 0.6% after a disease duration of 10 years, 5.4% after 20 years, and 7.5% after 30 years, with an overall incidence

rate of 1.52/1000 person-years. Somewhat contradictory, Terdiman *et al*<sup>[18]</sup> still report a significantly increased risk for both UC and CD ( $OR_{CRC}$ , 6.72-6.60) based on insurance database reports in 364 IBD-CRC and 1172 IBD patients. Similarly intriguing data were presented at the 2008 European Crohn's and Colitis Organization Congress. Based on the Dutch National Registry, almost 50% of all IBD-associated CRC cases developed in patients with Crohn's disease<sup>[19]</sup>. How these reports might influence current surveillance strategies is unclear.

Therefore, these population-based studies would indicate a much lower UC-related CRC incidence rate, ranging from 1/500 to 1/1600 patients annually. The causes for this change remain unclear, but possibilities include more widespread institution of surveillance colonoscopy and a higher prevalence of patients on maintenance therapy. An additional option may be that the population-based acquisition of the data, in other words, the study design per se, is at least partially responsible for the apparent decline in the incidence rates. In these studies, the proportion of severe or extensive cases was much lower compared to that reported by tertiary centers<sup>[3,17]</sup>.

The risk of CRC can also be expressed in relative terms, as standardized morbidity ratio (SMR; observed cancers in a UC cohort divided by expected cancers, with expected rates derived from the general population) or an incidence rate ratio (IRR; observed incidence of cancers in a UC cohort divided by observed incidence of cancers in a control cohort of the general population). The SMR or IRR values in the new population-based studies were only moderately increased in Denmark, Canada, and Italy. Compared to the general population, the risk varied between 1.05 and 2.75. Although these studies suggest that the relative risk of CRC is considerably lower than previously described, some would argue that the low rates of CRC observed in these studies are the successful result of timely and appropriate access to good health care, including maintenance therapies, surveillance colonoscopy, and surgery<sup>[20]</sup>. This is further corroborated by the data arising from a large colonoscopic surveillance program at St. Mark's Hospital between 1970 and 2001<sup>[17]</sup>. Six hundred patients with extensive UC were followed up for 5932 person-years. The cumulative probability of CRC in UC patients undergoing surveillance was only 7.6% after

30 years. Linear regression suggested that CRC incidence declined over the course of the study, supporting a role for surveillance in decreasing the risk for CRC. In addition, although the prognosis of UC-related CRC cases has generally been considered to be worse compared to sporadic cases, the experience from the study at St. Mark's Hospital<sup>[17]</sup> is much better. In 600 patients during 5932 patient-years of follow-up, 30 patients (5%) developed CRC, with a 5-year survival rate of 73.3%. Similarly high 5-year survival rates were reported from the Mayo Clinic<sup>[21]</sup> (55%) and from Eastern Europe<sup>[4]</sup> (68.4% at 5 years and 10 years).

The incidence of colorectal dysplasia in UC is even more difficult to determine than the incidence of CRC. There is considerable interobserver variability as well as a lack of uniform definitions. In addition, underlying inflammation might influence the diagnosis of dysplasia. Two population-based studies have examined the incidence of colorectal dysplasia in UC. In Sweden<sup>[22]</sup>, 52/204 patients (24%), including 66% with pancolitis developed dysplasia at some points in their disease course, during follow-up with a median of 16.5 years. Lower incidence rates were reported from the US<sup>[23]</sup>, where adherence to surveillance was lower. A total of 22 dysplastic lesions were diagnosed. In concordance, relatively low incidence rates were reported from the St. Mark's Hospital<sup>[17]</sup>. During a follow-up of 5080 person-years, the cumulative probability of dysplasia at 20 years was 7.7% and 15.8% at 30 years. Low grade dysplasia developed in 7.8%, while high grade developed in 3.2% of the patients. In addition, polypoid dysplasia was revealed in 3.3% and sporadic adenoma in 5.3% of the patients.

## RISK FACTORS FOR COLORECTAL CANCER IN ULCERATIVE COLITIS

The most important risk factors for UC-associated CRC are disease duration and extent. The possible mechanisms include chronic inflammation and as the duration of chronic bowel inflammation increases, so does the risk for colorectal dysplasia and CRC. In some studies, this annual risk rises exponentially with a duration beyond 30 years. This has led some guidelines to recommend surveillance colonoscopy every 1 year to 3 years between years 8 and 20 and every 1 year to 2 years thereafter<sup>[24]</sup>. In contrast, some of the recently published population-based studies could not demonstrate a clear-cut relation in UC duration<sup>[8]</sup>, and cancer risk or the gradual increase of the risk was much lower<sup>[4,7]</sup>.

In the landmark trial by Ekbom *et al*<sup>[12]</sup>, more than 3000 UC patients were followed up and the risk for CRC increased gradually from 1.7-fold in proctitis and 2.8-fold in left-sided colitis to 14.8-fold in pancolitis, compared to the general population. Most studies including the meta-analysis by Eaden *et al*<sup>[11]</sup>, have come to similar conclusions. The overall prevalence of CRC among patients with UC in all 116 studies was 3.7%, but when restricted to the 35 studies that stratified their analyses by extent of UC, the prevalence of CRC among patients with ex-

tensive involvement rose to 5.4%.

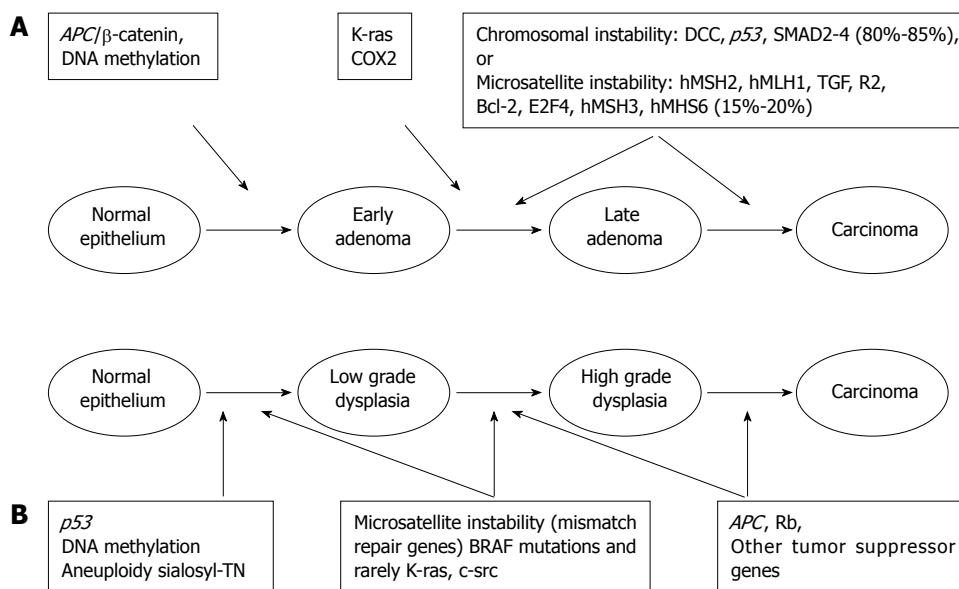
Important risk factors include primary sclerosing cholangitis (PSC)<sup>[25-27]</sup>, family history of CRC<sup>[28]</sup>, whereas the role of other factors, such as age at onset of UC, frequency of flare-ups, severity of inflammation<sup>[29]</sup>, "backwash ileitis"<sup>[30]</sup>, smoking, medical therapy used (5-amino-salicylates, azathioprine)<sup>[31]</sup>, is more controversial.

There is some debate as to whether patients with an early onset of colitis have a higher risk than patients with a later onset. Ekbom *et al*<sup>[12]</sup> identified the age below 15 years at onset as an independent risk factor for CRC. The cumulative risk for CRC after a disease duration of 35 years was 40% in extensive colitis if the disease started before the age of 15 years, while it was 25% if the disease onset was between 15 years and 39 years of age. Furthermore, disease onset during childhood was also established as an independent risk factor in the meta-analysis<sup>[3]</sup>. In contrast, other studies could not confirm this finding<sup>[4,32]</sup>; for example the age at onset above 30-40 years was associated with a higher risk in an American study, compared with a group of patients with onset below 20 years of age<sup>[33]</sup>. Since disease duration may be longer, theoretically, the risk of CRC should be greater in patients whose disease begins during early childhood.

Several studies have recognized PSC as a risk factor for CRC in UC patients<sup>[4,27,34,35]</sup>; however, this was not proven in all studies<sup>[36]</sup>. In one of the first reports, Broome *et al*<sup>[37]</sup> noted in a case-control study that the prevalence of PSC was 28% in the 17 patients with UC investigated with colorectal dysplasia or DNA aneuploidy *vs* 0 in the 55 patients without precancerous abnormalities. In the study by Kornfeld *et al*<sup>[35]</sup>, the cumulative CRC risk was 33% at 20 years and 40% at 30 years after UC diagnosis. In addition, the percentage of right-sided CRCs was higher in the subgroup of colitis patients with PSC<sup>[34]</sup>. In the study by Shetty *et al*, right-sided CRCs were observed in 76% of patients. The mechanism by which PSC induces CRC remains unclear. It has been hypothesized that alterations in the bile salt pool and a high concentration of bile acids in the colon may, at least partially, be responsible for the increased risk, but evidence also supports a strong association between PSC and quiescent to mild pancolitis. Colonic disease activity was milder in the 29 patients with PSC-IBD compared to the 58 patients with extensive UC without PSC in the study by Lundqvist *et al*<sup>[38]</sup>. Furthermore, patients with PSC were twice as likely to have never required corticosteroid treatment (52% *vs* 24%). In another study, patients with PSC-IBD were significantly less likely to require a proctocolectomy, in comparison to patients with extensive UC without PSC<sup>[27]</sup>.

The role of smoking in the development of UC-associated CRC has been a focus of controversy. Nonetheless, it has been identified as a risk factor for sporadic CRC<sup>[39]</sup>. In UC, an additive protective role might be hypothesized, as it might attenuate the inflammation and prevent relapses.

Finally, until recently, no direct evidence was available to support a link between the severity of inflammation



**Figure 1** Summary of genetic alterations in sporadic colorectal cancer (A) and colitis-associated colorectal cancer (B). The timing of p53 and APC mutations is different; unlike in sporadic neoplasia, mutations and LOH in p53 are early events in UC-associated CRCs. The opposite was reported for APC mutations<sup>[42]</sup>.

and the risk for CRC. A number of case-control studies with negative result relied on indirect outcome measures such as hospitalization, frequency of diagnostic testing, and frequency of symptomatic exacerbations<sup>[40]</sup>. Rutter *et al*<sup>[29]</sup> retrospectively reviewed endoscopic and pathologic reports of 68 UC patients with CRC or dysplasia and 136 UC patients without CRC, and assigned a severity score to each segment of the colon for each colonoscopy. The endoscopic (OR, 2.5; 95% CI, 1.4-4.4) or histologic (OR, 5.1; 95% CI, 2.4-11.1) scores were associated with the risk of CRC in univariate analysis. In addition, the histologic score was identified as an independent risk factor for CRC even after adjustment for confounding variables. This association was recently confirmed in a report from the Texas University<sup>[41]</sup>. During follow-up, 15 UC patients progressed to advanced neoplasia (high-grade dysplasia or colorectal cancer), and 65 progressed to neoplasia (low-grade dysplasia, high-grade dysplasia, or colorectal cancer). Univariate and multivariate analysis demonstrated significant relationships between histologic inflammation over time and progression to advanced neoplasia (HR, 2.2-3.4).

## GENETICS OF ULCERATIVE COLITIS ASSOCIATED COLORECTAL CANCERS

Both genetic and environmental factors contribute to the pathogenesis of CRC in IBD. Most sporadic CRC cases arise from a preceding adenoma (adenoma-carcinoma cascade) associated with unique genetic mutations. IBD-related cancers, however, are associated with a partially different genetic background<sup>[42]</sup>. The increased risk is thought to be an acquired event in IBD, although common inherited factors (e.g. glycosylation of mucin) have been proposed as a link between both forms of IBD and CRC<sup>[43]</sup>.

Complete elucidation of the mechanism of UC-CRC carcinogenesis will require further investigations; however, chronic inflammation is thought to be the most

important driving mechanism. Although the same three molecular pathways that have been described for sporadic colon carcinogenesis [loss of heterozygosity (LOH), microsatellite instability (MSI) and CpG methylator phenotype (CIMP)] are also found in colitis-associated neoplasms, yet the timing and frequency of some of the key genetic changes are different (Figure 1), possibly due to the different main driving mechanisms.

Changes in DNA methylation and microsatellite instability are also frequently found at an early stage in UC-associated CRCs. The prevalence in dysplasia-cancer cases ranges from < 1% to 70%<sup>[44-46]</sup>, for example hypermethylation of p14ARF occurs in approximately 30% of cases with dysplasia<sup>[47]</sup>. Unlike sporadic MSI-H (MSI-high) CRCs, MSI-H IBD CRCs present with heterogeneous mismatch repair defects involving MLH1, MSH2, MSH6, or PMS2, and a low frequency of MLH1 promoter methylation. They exhibit frequent BRAF but no KRAS mutations and frameshift mutations in genes containing coding repeat sequences. IBD patients exhibiting MSI-H present at younger age at diagnosis, and there is neither female predominance nor right-sided predominance<sup>[48]</sup>. In UC patients with MSI-H CRCs, MSI could already be demonstrated 2-12 years prior to the diagnosis of CRC in about 25% of the cases<sup>[49]</sup>. In contrast, hypermethylation of different target genes is a relatively rare event (MINT1, 2, 31, hMLH1, p16, p14, MGMT, HPP1, SFRP1, ERa and LINE-1)<sup>[50]</sup>. Thus MSI seems to be an important mechanism in UC-related carcinogenesis, at least in a subset of UC-CRC cases.

Loss of heterozygosity is a frequent but late event, primarily affecting SMAD4 and DCC loci (PACAP at 18p or DCC, SMAD2, SMAD4, GALNR at 18q-n)<sup>[51]</sup>. Unlike in sporadic CRCs, IBD-associated cancer mutations and LOH in p53 are early events that can already be found in macroscopically normal looking mucosa<sup>[52-54]</sup>. In contrast, mutations in k-ras are relatively infrequent. Since mutations in k-ras are believed to occur in sporadic adenoma cases<sup>[55]</sup>, this may partially explain the flat growth pattern of CRCs in IBD. Mutations in k-ras are

associated with polyp formation, which might explain why neoplasias in IBD are usually flat. Mutations of APC are infrequent (0%-3%), late events in UC, often occurring only in HGD or cancers<sup>[56]</sup>. Genetic events in DALM (dysplasia-associated lesion or mass) are similar to those observed in other UC-CRC cases including changes in MSI and LOH<sup>[57]</sup>.

Recently, an association was reported by the Mayo Clinic<sup>[58]</sup> between the G308A TNF $\alpha$  polymorphism and the risk of UC-associated CRC in 114 UC-CRC cases and matched controls, further corroborating the importance of chronic inflammation in CRC pathogenesis.

## HOW CAN WE DECREASE THE RISK OF CRC IN IBD?

The positive association between UC and CRC raises several practical questions. The causes behind the changing trends in UC-related CRC epidemiology are complex. One key element may be the early diagnosis and treatment of precancerous lesions by colonoscopic surveillance or sometimes prophylactic colectomy, while the third option is primary chemoprevention. Nowadays, prophylactic colectomy is obsolete. Nonetheless, the high colectomy rate, especially in Scandinavian countries has been associated with lower CRC risks<sup>[16]</sup>. There are however, obvious changes in the patient management, also in Scandinavia. In the new population-based cohorts, a decrease in the colectomy rate can be observed<sup>[59]</sup>.

## ENDOSCOPIC SURVEILLANCE IN IBD

Endoscopic surveillance remains an important but often disputed cornerstone of IBD management<sup>[60]</sup>. Colonoscopic surveillance to detect dysplasia and/or cancer is routinely indicated in compliant UC patients in clinical practice. Surveillance colonoscopy may permit earlier detection of CRC, with a correspondingly improved prognosis; however, unequivocal evidence is lacking that surveillance colonoscopy prolongs survival in patients with UC. Based on previous epidemiological data, international guidelines of the Crohn's and Colitis Foundation of America (CCFA)<sup>[24]</sup> and very recently the European Crohn's and Colitis Organisation (ECCO)<sup>[61]</sup> suggest a relatively strict surveillance policy. Whether these recommendations require adjustments in light of new epidemiological data that suggest a much lower CRC incidence remains questionable. Many factors (e.g. cost effectiveness, geographical differences, access to endoscopy and pathology) need to be considered. Nonetheless, it is possible that the lower incidence rates reported in recent population-based studies are at least partly a consequence of the vigorous surveillance programs.

As of today, the guidelines regarding the surveillance of CRC in UC patients can be summarized as follows: (1) Surveillance endoscopy should be performed in remission; (2) Initial screening colonoscopy should be performed in each patient after a 8-10 year disease duration, partly to reassess disease extent; (3) Regular surveil-

lance should begin after 8-10 years for pancolitis and after 15-20 years for left-sided disease. There should be a decrease in the screening interval with increasing disease duration (from 2 to 1 year). No surveillance is indicated in proctitis; (4) Two to four random biopsy specimens, every 10 cm, should be taken from the entire colon, with additional samples of suspicious areas. Alternatively methylene blue or indigo carmine chromoendoscopy can be offered for appropriately trained endoscopists and is superior to random biopsies in the detection rate of neoplastic lesions; and (5) Patients with primary sclerosis cholangitis represent a subgroup at higher risk, thus surveillance should be performed annually from the time of PSC diagnosis.

The use of random biopsies is being increasingly criticized. Since the reports by Rutter *et al*<sup>[62]</sup> and Rubin *et al*<sup>[63]</sup>, we know that dysplasia (71.7%-77.3%) and cancer (89.3%-100%) were macroscopically visible during colonoscopies in UC patients without PSC. Thus, the cost of additional random biopsies is difficult to justify. On the other hand, random biopsies visualize only 1% of total colonic mucosa surface area, promoting a high sampling error. In a retrospective analysis, it was demonstrated that the probability of detecting dysplasia was 90% if 33 and 95% if 56 random biopsies were taken<sup>[64]</sup>, with current guidelines for dysplasia surveillance recommending a minimum of 33 biopsies. In addition, almost half of patients with dysplasia initially detected in flat mucosa were later diagnosed to have colorectal cancer in the colectomy specimen<sup>[65]</sup>. Furthermore, almost one-third of patients with low grade dysplasia progressed to high grade dysplasia or cancer during follow-up. In the most recent meta-analysis, low-grade dysplasia was found to be associated with a 9-fold increased risk of developing CRC and a 12-fold risk of developing advanced neoplasia<sup>[66]</sup>. However, because some follow-up studies of patients with low-grade dysplasia have shown a low rate of CRC development (2%-10% during a 10-year follow-up)<sup>[67]</sup>, it seems there is a reasonable compromise to continue surveillance with extensive biopsy sampling at shorter intervals (e.g. 3-6 mo) in those who will adhere strictly to the surveillance program. In summary, a patient with low-grade dysplasia in flat mucosa should be offered proctocolectomy or repeat surveillance biopsies within 3-6 mo, while high-grade dysplasia in flat mucosa and adenocarcinoma are indications for proctocolectomy.

Raised lesions on a background of UC have been traditionally referred to as dysplasia-associated lesion or mass (DALM). Until recently, this finding had been considered an absolute indication for colectomy. It is increasingly recognized, however, that some of these raised lesions may resemble sporadic adenomas and that they may be treated by endoscopic resection<sup>[68]</sup>, if polypectomy can be performed safely and completely, without any dysplasia present in the adjacent mucosa in patients who will adhere to strict surveillance program afterwards.

The detection of CRC by surveillance is still not very

**Table 2** Summary of studies investigating the chemopreventive effect of sulfasalazine and 5-ASA therapies in ulcerative colitis

Study	Study design	n	Drug studies	Principal outcome
Pinczowski <i>et al</i> <sup>[31]</sup>	Case-control	298	Sulfasalazine > 3 mo	OR <sub>CRC</sub> : 0.38 (95% CI, 0.2-0.69) patients administered sulfasalazine
Moody <i>et al</i> <sup>[101]</sup>	Case-control	175	Sulfasalazine < 6 mo	10-fold elevated risk in non-exposed patients (30% vs 3%)
Eaden <i>et al</i> <sup>[40]</sup>	Case-control	102	Sulfasalazine, mesalamine regular use	OR <sub>CRC</sub> : 0.25 (95% CI, 0.13-0.48) in regular users
Lindberg <i>et al</i> <sup>[102]</sup>	Cohort study	143	Sulfasalazine > 6 mo	Non-significant decrease of risk (34% vs 44%)
Bernstein <i>et al</i> <sup>[87]</sup>	Case-control	373	5-ASA	Non-significant elevation of risk in patients exposed
Rutter <i>et al</i> <sup>[29]</sup>	Case-control	204	5-ASA	Non-significant elevation of risk of dysplasia in patients exposed
Rubin <i>et al</i> <sup>[90]</sup>	Case-control	124	5-ASA > 1.2 g regular use	OR <sub>CRC</sub> : 0.28 (95% CI, 0.09-0.85) in regular users

encouraging. Almost half of 92 CRC cases identified in surveillance programs in 1916 UC patients were in advanced stages (Dukes' C or D)<sup>[69]</sup> and only 12% of CRC cases were identified during surveillance colonoscopies in earlier studies. In concordance, a meta-analysis by the Cochrane group in 2006<sup>[70]</sup> failed to demonstrate a benefit for surveillance programs in preventing CRC-related death in UC (OR, 0.81, 95% CI, 0.17-3.83), but authors included only two studies in their final analysis. Furthermore, in the largest and most meticulous screening programs<sup>[17]</sup> reported to date, involving 600 patients, 2627 colonoscopies, 5932 patient-year of follow-up and a caecal intubation rate of 98.7% without significant complications, 16 of 30 cancers were interval cancers.

The diagnosis of dysplasia is demanding. During a period of active disease, it is almost impossible to differentiate between inflammation and true dysplasia. Furthermore, a significant interobserver variation was reported for the detection of dysplasia, and an agreement concerning low-grade dysplasia may be as low as 43%. Therefore, because of important prognostic implications, any case of dysplasia should be confirmed by an experienced pathologist<sup>[71,72]</sup>.

Targeted biopsies represent an alternative to random biopsies. All studies have confirmed an improved yield of surveillance colonoscopy by dye spraying (e.g. methylene blue or indigo carmine). When applied, random biopsies of apparently normal mucosa had no additional value compared to targeted biopsies obtained after dye staining of the mucosa. In the study by Rutter *et al*<sup>[73]</sup>, the clinical accuracy of consecutive, random (*n* = 2904) and targeted (indigocarmine: 157) biopsies was compared. Nine dysplastic lesions were diagnosed at chromoendoscopy, while no dysplasia was detected in random biopsies and no additional lesions were detected. Separate prospective studies have arrived at similar conclusions<sup>[74,75]</sup>, including the study by Hurlstone *et al*, who compared magnified chromoendoscopic surveillance in 350 UC patients and 350 disease-extent matched controls on traditional surveillance using random biopsies. Sixty-nine dysplastic lesions were identified by chromoendoscopy, compared with only 24 dysplastic lesions in the traditional surveillance group (*P* < 0.001). The diagnostic yield for detecting a dysplastic lesion increased in these studies by 3-4.5 folds and comparable diagnostic yields from chromoendoscopy have been obtained with both methylene blue and indigo carmine. In fact, Marion *et al*<sup>[76]</sup> was the first to identify a significant increase in the total number of patients (not

just lesions) with dysplasia following chromoendoscopy (1.5-fold).

Currently there are only limited data available regarding the role of advanced endoscopic techniques [e.g. narrow band imaging (NBI), fluorescence endoscopy, optical coherence tomography or confocal laser endomicroscopy]<sup>[77,78]</sup>. In a study by Kiesslich *et al*<sup>[79]</sup> endomicroscopy or colonoscopy were performed in 153 UC patients. Despite a significantly lower number of biopsies taken in the targeted group, the number of identified dysplastic lesions increased by 4.7 folds. In a very recent report by the same group<sup>[80]</sup>, confocal chromoscopic endomicroscopy was superior to chromoscopy alone for the detection and characterization of intraepithelial neoplasia in chronic ulcerative colitis.

In contrast, the use of NBI<sup>[81]</sup> did not improve the diagnostic accuracy compared to conventional colonoscopy. Although more lesions were identified using NBI, an almost equal number of dysplastic foci were identified and missed by both methods. Despite the promising results, further studies are needed before the use of these advanced techniques can be suggested in clinical practice.

## CHEMOPREVENTION IN IBD-IS IT POSSIBLE?

Given the theory that chronic inflammation is the driving force behind malignant transformation, the possibility exists for the use of maintenance anti-inflammatory therapy as primary chemoprevention<sup>[82]</sup>. The ideal chemopreventive agent would be safe, effective at preventing neoplastic progression, inexpensive, and able to prevent flares and control disease activity and symptoms. 5-aminosalicylic acid (5-ASA), including mesalamine and sulfasalazine, are attractive candidates; they are safe, relatively inexpensive, and effective maintenance therapy. Mesalamine is a potent anti-inflammatory drug exhibiting strong scavenger capacity, affecting *in vitro* NF-κB activity and apoptosis at least partly by increasing peroxisome proliferator-activated receptor-γ (PPAR-γ) expression<sup>[83-85]</sup>.

However, data supporting this possible chemopreventive effect are somewhat conflicting (Table 2). Promising results were produced by a case-control study by Eaden *et al*<sup>[40]</sup>, who identified 102 cases of CRC from a population of UC patients treated at a combination of academic and community-based gastroenterology practices. Overall

the use of any 5-ASA compound was associated with a 75% decreased risk of CRC (95% CI, 0.13-0.48). Among all 5-ASA compounds, mesalamine use was associated with the greatest degree of protection, providing the most benefit at doses greater than 1.2 g/d (OR, 0.09; 95% CI, 0.03-0.28). Sulfasalazine use was associated with a smaller protective effect, which was statistically significant only at doses of 2 g/d or more (OR, 0.41; 95% CI, 0.18-0.92). Of note, however, > 2 outpatient visits/year, use of systemic steroids, and regular colonoscopic surveillance were also associated with the decreased risk. If sulfasalazine is used, folate supplementation is mandatory, since folate deficiency alone might increase the risk of CRC in IBD<sup>[86]</sup>. In contrast, other studies, including the one by Bernstein *et al*<sup>[87]</sup>, failed to demonstrate a protective effect even after stratifying for dose and duration of therapy. Since controlled trials would necessitate withholding a drug used for remission maintenance by randomizing some patients into the placebo arm, these studies will never be performed due to ethical reasons. In addition, based on available data and statistical power analysis, theoretically, the follow-up of a total of 5260 patients for at least 10 years would be necessary to demonstrate a positive effect for 5-ASA vs placebo, if the estimated risk of cancer is approximately 0.51<sup>[88]</sup>. A much larger sample size would be required if a comparison with placebo would not be an option and the primary objective would be to assess a dose response.

A more recent meta-analysis has provided a systematic overview of the studies available with respect to 5-aminosalicylates<sup>[89]</sup>. A total of nine studies, three cohort and six case-control studies, were included. Half of the studies found significantly diminished incidence of colon cancer in aminosalicylate users, with an adjusted summary OR covering all trials of 0.51 (95% CI, 0.37-0.69). Only two studies focused on dysplasia incidence and 5-ASA use was not associated with lower risk of dysplasia (OR, 1.18). Nonetheless, the combination of both endpoints, dysplasia and cancer, again resulted in an OR of 0.51 (95% CI, 0.38-0.69). Surprisingly, the results were similar in short-term users (2-6 mo) or long-term users (2-20 years) with an OR of 0.56 and 0.50, respectively. An even lower OR was obtained in patients on a dose of 1.2 g/d or more (OR, 0.19-0.28). These conclusions were confirmed by a very large British study in regular mesalamine users with an OR of 0.31. In contrast, in the subgroup of CD patients, although numbers were small, the OR was 1.66, indicating no preventive effect for 5-ASA. Therefore, evidence in UC may not be simply extrapolated to CD. In the editorial<sup>[65]</sup> of this meta-analysis, the authors concluded that 5-ASA is a probable chemopreventive agent based on safety and current maintenance use. Reports following the meta-analysis were also contradictory. Investigating 96 cases and matched controls, Rubin *et al*<sup>[90]</sup> found that aminosalicylate use of 1.2 g/d or more was associated with a 72% reduction in the odds of dysplasia/CRC (OR, 0.28; 95% CI, 0.09-0.85). A trend for dose response was also reported ( $P = 0.056$ ). Of note, the OR for doses between 1.2-2.4 g/d was 0.19, while for doses  $\geq 2.4$  g/d

it was 0.48. In contrast, no benefit for 5-ASA use was found in a study based on two large claim databases<sup>[18]</sup>. Among patients with IBD (364 CRC cases, 1172 controls), exposure to 5-ASA therapy of any dose or duration during the 12 mo preceding CRC diagnosis was not associated with a reduced risk of CRC (OR, 0.97; 95% CI, 0.77-1.23). However, there was a trend toward a decreased risk of CRC with increasing number of mesalamine prescriptions ( $\geq 5$  prescriptions) in the previous year, though statistical significance was not achieved (trend,  $P = 0.08$ ). No long-term prescription data were analyzed.

The chemopreventive role for ursodeoxycholic acid (UDCA) therapy in PSC is well-supported by both experimental data and clinical studies<sup>[91,92]</sup>. In a post hoc analysis of a randomized clinical trial, three patients (10%) initially assigned to UDCA developed colorectal dysplasia or CRC compared to eight patients initially assigned to placebo (35%)<sup>[92]</sup>. The relative risk of dysplasia or CRC was 0.26 in the UDCA group.

There is only limited evidence for the role of other anti-inflammatory agents. In the study by Eaden *et al*<sup>[40]</sup>, 5% of cases and 19% of controls used systemic steroids (durations unknown), which was associated with a statistically significant reduction in CRC risk among UC patients (OR, 0.26; 95% CI, 0.01-0.70). No dose response effect was demonstrated. Results are, however, conflicting as in the later study by Lashner *et al*<sup>[93]</sup>, a positive effect by at least 6 mo of azathioprine or 6-mercaptopurine therapy could not be demonstrated. Similarly, no preventive effect (HR, 1.06-1.3) of azathioprine/6-mercaptopurine use could be demonstrated during an 8-year follow-up in a more recent study<sup>[94]</sup>. Of note, azathioprine was reported to be associated with a 4-fold elevated risk of lymphomas in some previous studies<sup>[95]</sup> and a recent meta-analysis<sup>[96]</sup>. Although IBD itself was not associated with an increased risk for lymphoma<sup>[97]</sup>, disease severity cannot be excluded as a confounding variable. Finally, no data are available on anti-TNF agents in this context. Risk for malignancy (RR, 1.1; 95% CI, 0.71-1.63) and lymphoma (RR, 1.3; 95% CI, 0.36-5.03) was not increased in CD according to the TREAT (TREAT-Crohn's therapy, resource, evaluation, assessment and tool) registry<sup>[98]</sup> and some other studies<sup>[99]</sup>. Somewhat in contrast, a recent meta-analysis<sup>[100]</sup> on the safety of TNF inhibitors in rheumatoid arthritis patients has shown a significantly increased risk of malignancies (OR, 3.29; 95% CI, 1.09-9.08). Many of the patients however, received a combination of anti-TNF and methotrexate, and only a single patient with colorectal cancer (rectal) was reported.

## CONCLUSION

The risk of colorectal cancer for any patient with ulcerative colitis is known to be elevated, and was estimated to be 2% after 10 years, 8% after 20 years and 18% after 30 years of disease. Recent population-based studies published within the past 5 years suggest that this risk has decreased over time, despite the relatively low

frequency of colectomies. The crude annual incidence rate of colorectal cancer in ulcerative colitis ranges from approximately 0.06% to 0.16% with a relative risk of 1.0-2.75. Risk factors for cancer include extent and duration of ulcerative colitis, primary sclerosing cholangitis, a family history of sporadic colorectal cancer, severity of histologic bowel inflammation, and in some studies, young age at onset of colitis. Complete elucidation of the mechanism of UC-CRC carcinogenesis will require further investigations; however, chronic inflammation is thought to be the most important driving mechanism. Although the same three molecular pathways that have been described for sporadic colon carcinogenesis [loss of heterozygosity (LOH), microsatellite instability (MSI) and CpG methylator phenotype (CIMP)] are also found in colitis-associated neoplasms, yet the timing and frequency of some of the key genetic changes are different. The exact mechanism for this change in epidemiology trends of UC-associated CRC is unknown. One key element may be the early diagnosis and treatment of precancerous lesions by colonoscopic surveillance or sometimes prophylactic colectomy, while a third option is primary chemoprevention. Nowadays, prophylactic colectomy is obsolete. Increasing amount of evidence is now available supporting an improved yield of surveillance colonoscopy by targeted biopsies. The method of choice or data regarding the role of advanced endoscopic techniques is under investigation. Another important factor contributing to the changing trends in epidemiology may be the more widespread use of maintenance therapy by aminosalicylates and UDCA in patients with PSC, making 5-ASAs a probable chemopreventive agent based on safety profile and current maintenance use. As of today, based on available literature and international guidelines, the use of surveillance endoscopy and maintenance chemopreventive therapy should be advised and discussed in patients with ulcerative colitis.

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EDITORIAL

## IgG4-related sclerosing disease

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## Abstract

Based on histological and immunohistochemical examination of various organs of patients with autoimmune pancreatitis (AIP), a novel clinicopathological entity of IgG4-related sclerosing disease has been proposed. This is a systemic disease that is characterized by extensive IgG4-positive plasma cells and T-lymphocyte infiltration of various organs. Clinical manifestations are apparent in the pancreas, bile duct, gallbladder, salivary gland, retroperitoneum, kidney, lung, and prostate, in which tissue fibrosis with obliterative phlebitis is pathologically induced. AIP is not simply pancreatitis but, in fact, is a pancreatic disease indicative of IgG4-related sclerosing diseases. This disease includes AIP, sclerosing cholangitis, cholecystitis, sialadenitis, retroperitoneal fibrosis, tubulointerstitial nephritis, interstitial pneumonia, prostatitis, inflammatory pseudotumor and lymphadenopathy, all IgG4-related. Most IgG4-related sclerosing diseases have been found to be associated with AIP, but also those without pancreatic involvement have been reported. In some cases, only one or two organs are clinically involved, while in others, three or four organs are affected. The disease occurs predominantly in older men and responds well to steroid therapy. Serum IgG4 levels and immunostaining with anti-IgG4 antibody are useful in making the diagnosis. Since malignant tumors are frequently suspected on initial presentation, IgG4-related sclerosing disease should be considered in the differential diagnosis to avoid unnecessary surgery.

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**Key words:** Autoimmune pancreatitis; IgG4; IgG4-

## INTRODUCTION

Since Yoshida *et al*<sup>[1]</sup> proposed the concept of autoimmune pancreatitis (AIP) in 1995, many cases have been reported in Western countries, as well as in Japan, and AIP has become a distinct entity recognized worldwide. Although the precise pathogenesis or pathophysiology of AIP remains unclear, many clinical, radiological, serological and histopathological characteristics are obvious. In patients with AIP, serum IgG4 levels are frequently and significantly elevated, and various extrapancreatic lesions are present<sup>[2]</sup>. Based on histological and immunohistochemical examination of various organs of AIP patients, we have found dense infiltration of IgG4-positive plasma cells and CD4- or CD8-positive T lymphocytes, as well as fibrosis in the peripancreatic retroperitoneal tissue, bile duct wall, gallbladder wall, periportal area of the liver, salivary glands, and the pancreas. Furthermore, all of the extrapancreatic lesions associated with AIP, such as sclerosing cholangitis, sclerosing sialadenitis, and retroperitoneal fibrosis, show infiltration of abundant IgG4-positive plasma cells<sup>[2-5]</sup>. Both the pancreatic and extrapancreatic lesions of AIP respond well to steroid therapy<sup>[6-8]</sup>.

Therefore, we proposed the existence of a novel clinicopathological entity, IgG4-related sclerosing disease, and suggested that AIP is a pancreatic lesion of this systemic disease. Many recent reports of multorgan, inflammatory, mass-forming lesions with increased numbers of IgG4-positive plasma cells affirm that AIP may have a systemic component<sup>[2,3,8]</sup>. On <sup>18</sup>F-fluorodeoxyglucose positron emission tomography (FDG-PET) performed in AIP patients, abnormal FDG uptake

**Table 1** Clinicopathological findings of IgG4-related sclerosing disease

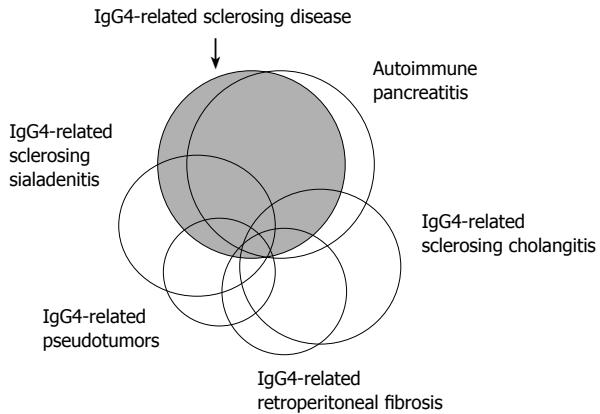
Clinicopathological findings	
Systemic disease characterized histopathologically by extensive IgG4-positive plasma cell infiltration of various organs together with T lymphocytes	
Major clinical manifestations are apparent in the organs in which tissues fibrosis with obstructive phlebitis is pathologically induced	
Pancreas	Autoimmune pancreatitis
Bile duct	IgG4-related sclerosing cholangitis
Gallbladder	IgG4-related sclerosing cholangitis
Salivary gland	IgG4-related sclerosing cholangitis
Retroperitoneum	IgG4-related retroperitoneal fibrosis
Kidney	IgG4-related tubulointerstitial nephritis
Lung	IgG4-related interstitial pneumonia
Prostate	IgG4-related prostatitis
Some inflammatory pseudotumors (liver, lung and hypophysis) may be involved in this disease	
Occasional association with lymphadenopathy	
Elderly male preponderance	
Frequent elevation of serum IgG4 levels	
Favorite response to steroid therapy	
Differentiation from malignant tumor is important	
Precise pathogenesis and pathophysiology remain unclear	

has been observed in various extrapancreatic lesions<sup>[9]</sup>. Furthermore, many IgG4-related sclerosing diseases of organs other than the pancreas have been recently reported. Although the nomenclature differs, IgG4-related sclerosing disease has been noted in hepatology, cholangiology, rheumatology, urology, nephrology, respirology, endocrinology, pathology, and radiology, as well as pancreatology. Based on our experience with 50 AIP patients, this review focuses on the clinical, laboratory, imaging, and histopathological features of IgG4-related sclerosing disease, including AIP.

## IgG4-RELATED SCLEROSING DISEASE

IgG4-related sclerosing disease is a systemic disease characterized by extensive IgG4-positive plasma cells and T-lymphocyte infiltration of various organs. Clinical manifestations are apparent in the pancreas, bile duct, gallbladder, salivary gland, retroperitoneum, kidney, lung and prostate, in which tissue fibrosis with obliterative phlebitis is pathologically induced (Table 1). AIP is not simply pancreatitis, but it is a pancreatic disease that is indicative of IgG4-related sclerosing disease. Most IgG4-related sclerosing diseases have been found to be associated with AIP, but IgG4-related sclerosing diseases without pancreatic involvement have been reported. Some inflammatory pseudotumors may be involved in this disease. In some cases, only one or two organs are clinically involved, while in others, three or four organs are affected (Figure 1). The disease occurs predominantly in older men, is frequently associated with lymphadenopathy, and responds well to steroid therapy. Serum IgG4 levels and immunostaining with anti-IgG4 antibody are useful in making the diagnosis. The precise pathogenesis and pathophysiology of IgG4-related sclerosing disease remain unclear. Since malignant tumors are frequently suspected on initial presentation, IgG4-related sclerosing disease should be considered in the differential diagnosis to avoid unnecessary surgery<sup>[2,3,8]</sup>.

Multifocal fibrosclerosis is an uncommon fibroproliferative systemic disorder with multiple manifestations,



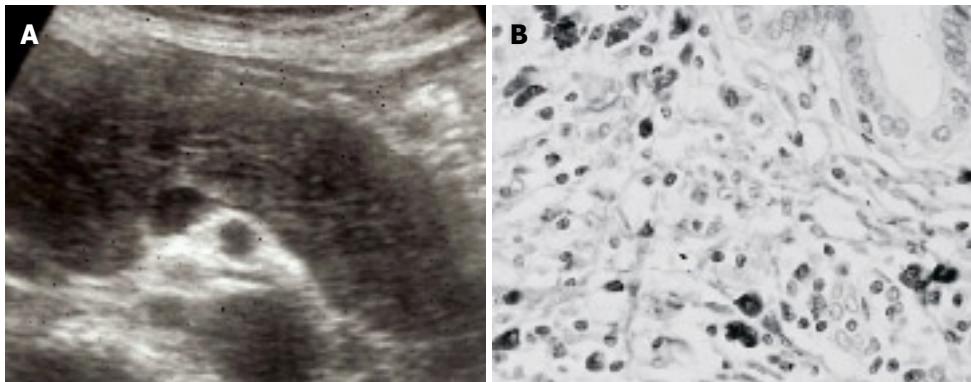
**Figure 1** Schematic illustration showing the relationship between IgG4-related sclerosing disease, AIP, IgG4-related sclerosing cholangitis, IgG4-related sclerosing sialadenitis, IgG4-related retroperitoneal fibrosis, and IgG4-related pseudotumors.

including sclerosing cholangitis, salivary gland fibrosis, retroperitoneal fibrosis, Riedel's thyroiditis, and fibrotic orbital pseudotumor<sup>[10]</sup>. As the histopathological findings of these disorders are similar, fibrotic changes with lymphoplasmacytic infiltration and occasional phlebitis, it is suggested that they are all interrelated and probably different manifestations of a common disorder of fibroblastic proliferation. Several cases of pancreatic pseudotumor or chronic pancreatitis associated with multifocal fibrosclerosis have been reported. The histopathology of the extrapancreatic lesions associated with AIP strongly suggests that multifocal fibrosclerosis is an IgG4-related sclerosing disease<sup>[5]</sup>.

## AIP

### Concept and clinical features

AIP is a unique form of pancreatitis in which autoimmune mechanisms are suspected to be involved in the pathogenesis. The histopathological findings of AIP include marked lymphoplasmacytic infiltration with fibrosis in the pancreas, known as lymphoplasmacytic



**Figure 2** AIP. (A) Diffuse hypoechoic enlargement of the pancreas on ultrasonography; (B) Dense infiltration of IgG4-positive plasma cells in the pancreas.

sclerosing pancreatitis. AIP occurs more commonly in older men. In our series, the mean age of the patients was 66.5 years (range, 25–83 years), and the male-to-female ratio was 4:1. The major clinical symptom is obstructive jaundice, due to associated sclerosing cholangitis (74% in our series). Failure of pancreatic exocrine or endocrine function is frequently seen. Up to 50% of AIP patients present with glucose intolerance. Diabetes mellitus and AIP are simultaneously diagnosed in many cases, but some cases show exacerbation of pre-existing diabetes mellitus with the onset of AIP<sup>[11]</sup>.

Recently, AIP with neutrophilic infiltration of the pancreatic duct epithelium has been reported by American<sup>[12]</sup> and European<sup>[13]</sup> pathologists. Their patients showed different clinicopathological features from AIP to those defined in Japan, as follows: no predilection for older men, a frequent association with inflammatory bowel disease, and a weaker association with other sclerosing diseases. In Japan, young AIP patients are more likely to have abdominal pain and serum amylase elevation than middle-aged or elderly patients<sup>[14]</sup>. Further, international surveys of a larger series of clinically relevant AIP subtypes are needed.

### Pathogenesis

Levels of serum IgG4 are frequently elevated, and they are particularly high in AIP. Dense infiltration of IgG4-positive plasma cells is seen in various organs of AIP patients. These findings suggest that IgG4 plays a major role in the pathogenesis of AIP, although the trigger for the IgG4 elevation or its pathogenetic role in AIP has not been clearly established.

Although the actual effector cells of AIP have not been clearly delineated, there are an increased numbers of activated CD4- and CD8-positive T cells bearing HLA-DR among the peripheral blood lymphocytes and in the pancreas of AIP patients. In 2001, Okazaki *et al*<sup>[15]</sup> reported that the number of CD4+ T cells producing interferon- $\gamma$  in peripheral blood and its secreted level were significantly higher in AIP patients than in controls, whereas the number of interleukin (IL)-4-producing CD4+ cells was not increased in AIP patients. They concluded that AIP may be mediated by a Th1-predominant immune reaction, similar to that in Sjogren's syndrome or primary sclerosing cholangitis (PSC).

There are many findings that support the involvement of immunological mechanisms in AIP, but target

antigens for AIP have not been detected. Given the preponderance of the disease amongst elderly males and the marked, dramatic response to oral steroid therapy, the pathogenesis of AIP may not involve an autoimmune mechanism, but other mechanisms, such as an allergic reaction. Zen *et al*<sup>[16]</sup> have reported that the expression of Th2 cytokines (IL-4, IL-5, and IL-13) and regulatory cytokines (IL-10 and transforming growth factor- $\beta$ ) was up-regulated in the affected tissues of patients with IgG4-related sclerosing pancreatitis and cholangitis. They have suggested that the predominant Th2 and regulatory immune reactions in this disease might reflect an allergic mechanism in its pathogenesis.

### Diagnosis

It is of utmost importance that AIP be differentiated from pancreatic cancer, as some patients with AIP in whom pancreatic cancer is suspected undergo unnecessary laparotomy or pancreatic resection. Since there is currently no diagnostic serological marker for AIP, diagnosis should be on the basis of the presence of a combination of abnormalities unique to AIP. In 2002, the Japan Pancreas Society established the Diagnostic Criteria for AIP<sup>[17,18]</sup>, which were revised in 2006<sup>[19]</sup>. In 2006, two new sets of diagnostic criteria for AIP were proposed, one in Korea and one in the USA.

Japanese criteria are based on the minimum consensus features of AIP, to minimize the risk of misdiagnosing pancreatic cancer. Revised criteria consist of three items: (1) radiological imaging showing diffuse or segmental narrowing of the main pancreatic duct with irregular walls and diffuse or localized enlargement of the pancreas; (2) laboratory data demonstrating abnormally elevated levels of serum gammaglobulin, IgG, or IgG4, or the presence of autoantibodies; and (3) histological examination of the pancreas showing lymphoplasmacytic infiltration and fibrosis. The diagnosis of AIP is made when either all three criteria are present, or criterion 1 together with either 2 or 3 is present. In addition to the Japanese criteria, the Korean criteria include the patient's response to steroid therapy and the presence of extra-pancreatic lesions.

Radiologically, pancreatic enlargement is usually hypoechoic, sometimes with scattered hyperechoic spots on ultrasonography (Figure 2A). On dynamic computed tomography (CT), there is delayed enhancement of the enlarged pancreatic parenchyma. Typical AIP cases

show diffuse enlargement of the pancreas, the so-called sausage-like appearance. Since inflammatory and fibrous changes involve the peripancreatic adipose tissue, a capsule-like rim surrounding the pancreas, which appears as a low density on CT, is detected in some cases. Pancreatic calcification or a pseudocyst is rarely seen. Cases of focal enlargement of the pancreas are sometimes difficult to differentiate from pancreatic cancer. Endoscopic retrograde cholangiopancreatography discloses an irregular, narrow (< 3 mm in diameter) main pancreatic duct. In patients with segmental narrowing, absence of upstream dilatation of the main pancreatic duct is characteristic<sup>[20]</sup>.

In our series of AIP patients, hypergammaglobulinemia (> 2.0 g/dL) and elevated serum IgG levels (> 1800 mg/dL) were detected in 34% and 56%, respectively, while autoantibodies, including antinuclear antibody and rheumatoid factor, were present in 44% and 16%.

Serum IgG4 levels are significantly and specifically high (> 135 mg/dL) in AIP patients. The sensitivity of elevated serum IgG4 levels is reported to be 67%-95%<sup>[21,22]</sup>. Although the measurement of serum IgG4 levels is useful for differentiating between AIP and pancreatic cancer, it should be noted that some patients with pancreatic cancer also have elevated serum IgG4 levels. Ghazale *et al*<sup>[23]</sup> have reported that serum IgG4 levels were elevated above 140 mg/dL in 13/135 (10%) patients with pancreatic cancer.

Histologically, dense lymphoplasmacytic infiltration with fibrosis is detected in the pancreas of AIP patients. Immunohistochemically, infiltrating inflammatory cells in the pancreas consist of CD4- or CD8-positive T lymphocytes and IgG4-positive plasma cells (Figure 2B). Lymphoid follicles are occasionally formed. The pancreatic duct is narrowed by periductal fibrosis and lymphoplasmacytic infiltration. Another characteristic histological finding is obliterative phlebitis that involves minor and major veins, including the portal vein. Such an inflammatory process extensively and markedly involves the contiguous soft tissue and peripancreatic retroperitoneal tissues.

#### Treatment and prognosis

The dramatic response to corticosteroids is well-known in AIP, but a standard steroid therapy regimen has not been established. Before steroid therapy is started, endoscopic or percutaneous transhepatic biliary drainage must be carried out in cases with obstructive jaundice, and glucose levels must be controlled in cases with diabetes mellitus. Oral prednisolone is usually started at 30-40 mg/d, and then it is tapered by 5 mg every 1-2 wk. Serological and imaging tests are followed periodically after commencement of steroid therapy. Usually, pancreatic size is normalized within a few weeks, and biliary drainage becomes unnecessary after 1-2 mo. Patients in whom complete radiological improvement is documented can stop their medication. To prevent relapse without complete discontinuation of steroids, continued maintenance therapy with prednisolone 5 mg/d is sometimes required. In half of steroid-treated patients,

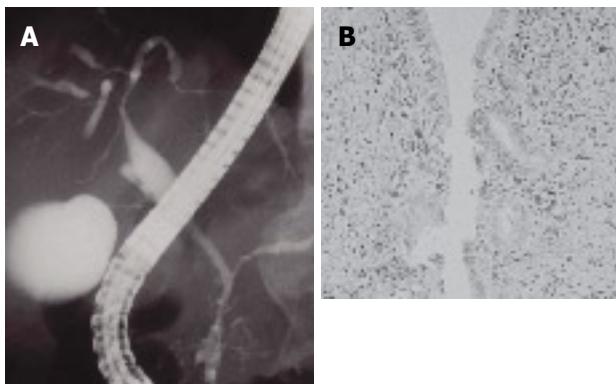
impaired exocrine or endocrine function improves. Some AIP patients relapse during maintenance therapy or after steroid medication is stopped; they should be retreated with high-dose steroid therapy. The indications for steroid therapy in AIP include obstructive jaundice due to stenosis of the bile duct, or the presence of other associated systemic diseases, such as retroperitoneal fibrosis<sup>[24-26]</sup>.

The long-term prognosis of AIP is not well known. Recurrent attacks of AIP resulting in pancreatic stone formation have been reported<sup>[27,28]</sup>.

## IgG4-RELATED SCLEROSING CHOLANGITIS

Sclerosing cholangitis is a heterogeneous disease that may be associated with choledocholithiasis, biliary tumor, or infection. Sclerosing cholangitis of unknown origin is called PSC. PSC is progressive despite conservative therapy, and it involves the intra- and extrahepatic bile ducts, and results in liver cirrhosis. The effect of steroid therapy is questionable, and liver transplantation currently provides the greatest hope for a possible cure. PSC occurs from 30 to 40 years of age and is frequently associated with inflammatory bowel disease<sup>[29,30]</sup>. Pancreatography is not abnormal in most PSC cases<sup>[31]</sup>.

IgG4-related sclerosing cholangitis is included within the sclerosing cholangitis group. This form is frequently associated with AIP and occasionally with other diseases such as sclerosing sialadenitis, all of which fall within the category of IgG4-related sclerosing disease. However, IgG4-related sclerosing cholangitis is not associated with inflammatory bowel disease. In many AIP cases, stenosis is located in the lower part of the common bile duct, but thickening of the common bile duct wall is sometimes detected even in the segment in which abnormalities are not clearly observed upon cholangiography. When stenosis is found in the intrahepatic or the hilar hepatic bile duct, the cholangiographic appearance is very similar to that of PSC (Figure 3A)<sup>[31,32]</sup>. Many cases of IgG4-related sclerosing cholangitis have been described with isolated biliary tract involvement in the absence of pancreatic disease<sup>[33,34]</sup>. Elevation of serum IgG4 is frequently observed in patients with IgG4-related sclerosing cholangitis, and it responds dramatically to steroid therapy, unlike PSC. Clinically, patients with IgG4-related sclerosing cholangitis are older at diagnosis than those with PSC. Patients with IgG4-related sclerosing cholangitis present more abruptly with obstructive jaundice, whereas obstructive jaundice is rarely present in PSC patients. The histological appearance is similar to that in the pancreas of AIP patients: transmural fibrosis, dense lymphoplasmacytic infiltration of the bile duct wall, along with lymphoplasmacytic infiltration and fibrosis in the periportal area of the liver, and obliterative phlebitis. Despite the dense periluminal inflammation, the biliary epithelium is usually intact, in contrast to PSC, in which mucosal erosion is often present. Furthermore, unlike in PSC, the inflammatory process is often more dense at the periphery of the duct. Neutrophils, commonly



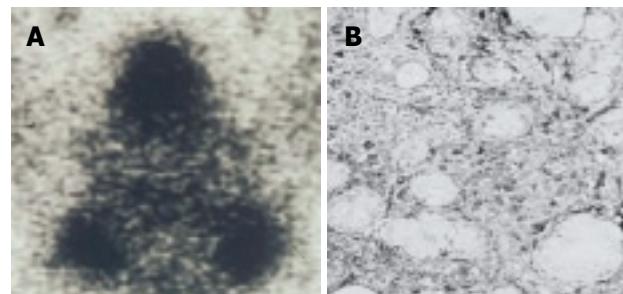
**Figure 3** IgG4-related sclerosing cholangitis. (A) Stenosis of the intrahepatic bile duct, similar to that in PSC; (B) Dense infiltration of IgG4-positive plasma cells in the bile duct wall.

seen in PSC, are not a feature of this disease. Immunohistochemically, infiltration of abundant IgG4-positive plasma cells is detected in the bile duct wall (Figure 3B), in contrast to PSC. Given the age at onset, associated diseases, pancreatic findings, response to steroid therapy, prognosis, and IgG4-related serological and immunohistochemical data, it can be said that IgG4-related sclerosing cholangitis is a different disease, and distinct from PSC<sup>[35,36]</sup>.

### IgG4-RELATED SCLEROSING SIALADENITIS

Sclerosing sialadenitis has been referred to as Kuttner's tumor, due to its presentation as a firm swelling of the salivary gland that is difficult to differentiate from a neoplasm<sup>[37]</sup>. Mikulicz's disease is a unique condition that refers to bilateral, painless and symmetrical swelling of the lacrimal, parotid and submandibular glands<sup>[38]</sup>. Although Mikulicz's disease has been considered a subtype of Sjögren's syndrome, there are several differences between the two diseases. Patients with Mikulicz's disease lack anti-SS-A and anti-SS-B antibodies, but frequently have elevated serum IgG4 levels. Infiltration of many IgG4-positive plasma cells into the lacrimal and salivary glands has been detected in Mikulicz's disease.

Swelling of the salivary glands was present in 24% of our AIP patients (Figure 4A), and it was associated with cervical or mediastinal lymphadenopathy. Swelling of the salivary glands and the lymph nodes improves after steroid therapy. In the salivary glands of these patients, dense infiltration of IgG4-positive plasma cells and fibrosis were detected (Figure 4B). Kitagawa *et al*<sup>[39]</sup> have reported that dense infiltration of IgG4-positive plasma cells was detected in the salivary glands of 12 patients with sclerosing sialadenitis (Kuttner's tumor). Five of these patients had associated sclerosing lesions in extrasalivary glandular tissue, such as in AIP, while the remaining seven patients had only salivary gland involvement. Thus, many cases of sclerosing sialadenitis, including Kuttner's tumor and Mikulicz's disease, may be salivary gland lesions of IgG4-related systemic disease<sup>[40,41]</sup>.



**Figure 4** IgG4-related sclerosing sialadenitis. (A) Bilateral swelling of submandibular glands (Gallium scintigraphy); (B) Dense infiltration of IgG4-positive plasma cells in the salivary gland.

### IgG4-RELATED RETROPERITONEAL FIBROSIS

Retroperitoneal fibrosis generally presents in a non-specific manner with malaise, fatigue, fever and weight loss. The pathognomonic feature of retroperitoneal fibrosis is a thick retroperitoneal fibrotic mass that covers the abdominal aorta and compresses the ureters<sup>[42]</sup>. The process of fibrosis can result in obstruction of the ureters and renal failure, or signs and symptoms may be related to the encasement or entrapment of other structures by the inflammatory mass, such as hydronephrosis. Retroperitoneal fibrosis has many causes, although in about 70% of cases, the cause is unknown<sup>[43]</sup>.

Retroperitoneal fibrosis was present simultaneously or metachronously in 8% of our AIP patients. Dense infiltration of IgG4-positive plasma cells and obliterative phlebitis were found in the pancreas and the retroperitoneal fibrous mass. Both the retroperitoneal fibrosis and AIP resolved after steroid therapy<sup>[44]</sup>. Neild *et al*<sup>[43]</sup> have reported the histological findings of 12 patients with idiopathic retroperitoneal fibrosis, which showed, to varying degrees, fibrosis and intense inflammatory cell infiltration with T lymphocytes and IgG4-positive plasma cells, as well as venulitis and obliterative arteritis. Biopsy of the mass after steroid therapy revealed decreased infiltration of IgG4-positive plasma cells. Some cases of retroperitoneal fibrosis are the retroperitoneal lesions of IgG4-related systemic disease. Recently, a 52-year-old man with retroperitoneal and mediastinal fibrosis without AIP was reported to have elevated serum IgG4 levels<sup>[45]</sup>.

### IgG4-RELATED TUBULOINTERSTITIAL NEPHRITIS

Tubulointerstitial nephritis may be related to various causative factors, including infection, and allergic, toxic and autoimmune conditions. Tubulointerstitial nephritis is sometimes associated with AIP, but there have been a few cases of tubulointerstitial nephritis showing high serum IgG4 levels without notable pancreatic lesions<sup>[46-50]</sup>. Some AIP cases show several nodular lesions in the kidney that mimic metastatic tumors<sup>[46,47]</sup>. Takahashi *et al*<sup>[48]</sup>

have reported that, in 40 AIP patients, 14 (35%) had renal involvement (12 with parenchymal involvement and five with extraparenchymal involvement). The renal lesions regressed after steroid therapy, but they progressed without steroid therapy. Immunohistochemically, dense infiltration of IgG4-positive plasma cells was detected in the renal interstitium. Furthermore, in some AIP cases, membranous nephropathy that showed IgG4-positive deposits in the glomeruli or tubular basement membrane have been reported<sup>[49]</sup>. These IgG4-positive lesions decreased with improvement of renal function after steroid therapy.

### IgG4-RELATED INTERSTITIAL PNEUMONIA

Interstitial pneumonia, with X-ray findings such as an interstitial pattern, ground-glass appearance, and honeycombing, is sometimes associated with AIP<sup>[51-53]</sup>. On high-resolution CT of the lung, dense alveolar consolidation and air bronchograms in bilateral perihilar regions have been reported<sup>[51]</sup>. Some cases have respiratory failure, and steroid therapy is effective in improving respiratory function and radiological findings. In biopsy specimens, dense infiltration of IgG4-positive plasma cells is detected in the thickened alveolar septum<sup>[51-53]</sup>. Hirano *et al*<sup>[53]</sup> have reported that 4/30 AIP patients had pulmonary involvement during follow-up.

### IgG4-RELATED SCLEROSING CHOLECYSTITIS

In our series, thickening of the gallbladder was detected on US and/or CT in 32% of AIP patients. Dense infiltration of IgG4-positive plasma cells and lymphocytes, as well as transmural fibrosis, was detected in the gallbladder wall of 6/8 patients examined<sup>[54]</sup>.

### IgG4-RELATED PROSTATITIS

IgG4-related prostatitis has recently been reported in patients with and without AIP. Patients show lower urinary tract symptoms, and prostate enlargement is evident on digital rectal examination. Significant FDG uptake by the prostate has been demonstrated. The symptoms and radiological findings improve after steroid therapy. Histologically, the prostate shows dense infiltration of IgG4-positive plasma cells and lymphocytes, obliterative phlebitis, and gland atrophy with dense fibrosis<sup>[55-57]</sup>.

### IgG4-RELATED INFLAMMATORY PSEUDOTUMOR OF THE LIVER, LUNG AND HYPOPHYSIS

Inflammatory pseudotumors occur in almost all major organs. An inflammatory pseudotumor is characterized histologically as an irregular proliferation of myofibroblasts intermixed with an infiltrate of inflammatory cells,

mainly lymphocytes and plasma cells. One inflammatory pseudotumor is categorized as a plasma cell-rich type, plasma cell granuloma<sup>[58]</sup>. Although its pathogenesis is not known, a close relationship between plasma cell granuloma and IgG4-positive plasma cells has recently become obvious<sup>[5]</sup>.

Some IgG4-related inflammatory pseudotumors of the liver<sup>[54,59,60]</sup> and lung<sup>[61]</sup>, which are characterized by dense infiltration of IgG4-positive plasma cells and lymphocytes intermixed with fibrosis and obliterative phlebitis, have been recently reported in patients with or without AIP. Four cases of hypophysitis in association with AIP have been reported; they all presented with hypopituitarism and swelling of the pituitary lesion<sup>[62-65]</sup>. In one case<sup>[62]</sup>, abundant infiltration of IgG4-positive plasma cell was demonstrated in the pituitary tumor. Steroid therapy is effective for these IgG4-related inflammatory pseudotumors.

### IgG4-RELATED LYMPHADENOPATHY

In a study using gallium-67 scintigraphy, pulmonary hilar gallium-67 uptake was found in 41/51 (80.4%) patients with AIP<sup>[66]</sup>. In our series, abdominal lymphadenopathy of up to 2 cm in diameter was observed in 5/8 patients at laparotomy, and cervical or mediastinal lymphadenopathy of up to 1.5 cm in diameter was observed on CT in 33% of our AIP patients. In all these cases, the lymphadenopathy disappeared after steroid therapy. Dense infiltration of IgG4-positive plasma cells was detected in all abdominal and cervical lymph nodes. When bilateral hilar lymphadenopathy is marked, sarcoidosis is suspected clinically<sup>[4]</sup>.

Other reported lesions associated with AIP are immune thrombocytopenic purpura<sup>[67,68]</sup>, autoimmune sensorineural hearing loss<sup>[68]</sup>, hypothyroidism<sup>[69]</sup>, anosmia<sup>[70]</sup>, and loss of taste<sup>[70]</sup>.

### CONCLUSION

IgG4-related sclerosing disease is a new clinicopathological systemic entity. It is characterized by extensive IgG4-positive plasma cells and T-lymphocyte infiltration of various organs, and major clinical manifestations are apparent in the organs, in which tissues fibrosis with obliterative phlebitis is pathologically induced. As steroid therapy is effective, accurate diagnosis is necessary.

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REVIEW

## Evolving management of colorectal cancer

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## INTRODUCTION

Colorectal cancer is a common cause of morbidity and mortality. Although the basic principles of screening, surgical resection when possible, and adjuvant therapy when indicated remain valid, considerable new information offers the possibility of substantially improving outcomes for such patients in the future. This review will briefly summarize current epidemiologic and prognostic information about this disease for context, and then will focus on new approaches to surgery, adjuvant therapy, the management of established metastasis, and the prevention of metastasis. Although screening for colorectal neoplasm is critical for prevention, early diagnosis, downstaging, and improved survival, this subject has been extensively reviewed elsewhere<sup>[1-4]</sup> and is beyond the scope of the current review.

## INCIDENCE AND PREVALENCE

Colorectal cancer is the third most common cancer and the third leading cause of cancer related mortality in the United States<sup>[5]</sup>. Colorectal cancer is also very common in Western Europe, Australia and New Zealand, whereas the age standardized incidence rate of colorectal carcinoma is very low in India and Africa<sup>[6,7]</sup>. There seems to be an association of higher incidence rates in colorectal cancer with increasing affluence<sup>[8]</sup>. Over the past decade, colorectal cancer rates have modestly decreased or remained level. Until age 50, men and women have similar incidence and mortality rates; after age 50, men are more vulnerable<sup>[5]</sup>. Colorectal cancer is generally a malignancy associated with the elderly, with a mean age at diagnosis of 73 years<sup>[9]</sup>. In the Netherlands, statistics showed that a peak incidence of colorectal cancer for both men and women occur between the age of 70-79 years<sup>[10]</sup>. Before the age of 75 years, men and

## Abstract

This article reviews recent advances in surgical techniques and adjuvant therapies for colorectal cancer, including total mesorectal excision, the resection of liver and lung metastasis and advances in chemoradiation and foreshadows some interventions that may lie just beyond the frontier. In particular, little is known about the intracellular and extracellular cascades that may influence colorectal cancer cell adhesion and metastasis. Although the phosphorylation of focal adhesion kinases and focal adhesion associated proteins in response to integrin-mediated cell matrix binding ("outside in integrin signaling") is well described, the stimulation of cell adhesion by intracellular signals activated by pressure prior to adhesion represents a different signal paradigm. However, several studies have suggested that increased pressure and shear stress activate cancer cell adhesion. Further studies of the pathways that regulate integrin-driven cancer cell adhesion may identify ways to disrupt these signals or block integrin-mediated adhesion so that adhesion and eventual metastasis can be prevented in the future.

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women in the Netherlands have a 4.67% and 3.34% cumulative incidence to develop colorectal cancer<sup>[11]</sup>. By the age of 70 years, at least 50% of the western population will develop some form of colorectal tumor, spanning the spectrum from an early benign polyp to an invasive adenocarcinoma.

## STAGE OF COLORECTAL DISEASE

The stage of disease is one of the most important prognostic factors for survival in patients with colorectal cancer. It is therefore clinically significant to know the relative incidence for each stage of the disease. The incidence of Stage I disease in the United States has increased over the past years due to better screening and is currently around 30%. This is an important development since the detection of early stage disease increases the chance for R0 resection and potential cure for colorectal cancer. The incidence of Stage II and III disease are respectively 27% and 24%, while Stage IV disease is present in 19% of patients in the United States. A remarkable observation is that older patients are diagnosed more frequently at an early stage (Stage 0 and 1) and diagnosed three times less frequently with stage IV disease than younger patients. A possibility is that younger patients feel less at risk and ignore symptoms for a longer period of time and are therefore diagnosed at a later stage<sup>[12]</sup>.

The relative 5-year survival rates in the United States show that when the disease is detected early, at a localized stage, survival rates for Stage I colon and rectal cancer are 93% and 92%, respectively. At Stage II disease the 5-year survival rates are between 72%-85% for colon cancer and between 56%-73% for rectal cancer. The fluctuations in Stage II survival rates are due to the fact that Stage II disease includes both T3 (Stage II A) and T4 (Stage II B) tumors. For more advanced disease at diagnosis, the survival rates drop significantly. At Stage III, the 5-year survival rates for colon and rectal cancer vary from 44%-83% and 30%-67% respectively. Again the wide range of survival rates reflects the fact that Stage III disease is further categorized into the following sub-categories, Stage III A (T1-2, N1, M0), Stage III B (T3-T4, N1, M0) and Stage III C (any T, N2, M0) disease. For Stage IV colorectal disease the 5-year survival rate may be as low as 8%<sup>[13,14]</sup>.

## SURGICAL TREATMENT

Surgical management is the primary treatment of potentially curable colorectal cancer. In most cases, this involves resection of the primary tumor and regional lymph nodes. However, treatment of curable colorectal cancer patients may vary from endoscopic polypectomy for malignant polyps or local excision in carefully selected patients with limited rectal carcinomas to multimodality management for locally advanced rectal cancers or cancers invading adjacent organs. The objective in all cases is to maximize both oncologic and functional results. Due to the improvements in

surgical techniques, as well as better screening and new developments in adjuvant therapy, the ratio of people with potentially curable disease has increased over the past decades. This evolution has included the development of total mesorectal excision, the introduction of laparoscopic surgery, the sentinel lymph node technique, curative resections of liver and lung metastasis and improvements in adjuvant therapies such as chemotherapy and radiotherapy.

En bloc resection and the no-touch technique were first described in 1967 by Trumbell *et al* and remain valid and important<sup>[15]</sup>. The best prevention strategy of potential tumor cell distribution is maintained by the surgeon strictly using the principles of en-bloc resection. As the likelihood of lymph node involvement increases with depth of tumor invasion (5.6% for pT<sub>1</sub>, 10% for pT<sub>2</sub>, 36.7% for pT<sub>3</sub>, and 77.7% for pT<sub>4</sub> colon carcinoma)<sup>[16]</sup>, invasive adenocarcinomas require ligation and resection of the lymphovascular pedicle directly draining the intestinal segment containing the tumor. When the lesion is equidistant between two pedicles, then both should be encompassed in the resection. Another surgical option is the no-touch isolation technique with primary ligation of the corresponding vessels, and dissection of the lymph nodes<sup>[17-20]</sup>. The concept of this technique is to avoid tumor manipulation during surgery so that shedding of tumor cells into the lymphatic or vascular circulation is kept to a minimum. The presence of free cancer cells within the lymphatic, vascular circulation or in the peritoneal cavity can be detected by mRNA coding using qPCR and is associated with a poorer prognosis for patients undergoing curative colorectal cancer surgery<sup>[21,22]</sup>. However, there seems to be limited benefit in the no-touch isolation group; the morbidity and mortality rates after a 5-year follow-up of patients were equal<sup>[20]</sup>. More recently, a study by Hayashi *et al* has suggested that the no-touch isolation technique may be useful to prevent cancer cells from being shed into the portal vein during surgical manipulation<sup>[23]</sup>. Today, en-bloc resection without the primary ligation of corresponding vessels is still the more common technique.

Other advances in surgical techniques have resulted in less tumor recurrence and given patients with advanced colorectal disease the opportunity to undergo surgery with curative intent. In 1982 Heald developed an important surgical technique for the treatment of rectal cancer. The concept of total mesorectal excision (TME) was introduced in conjunction with low anterior resection (LAR) as a means of procuring all perirectal fat while facilitating sphincter preservation<sup>[24]</sup>. Recent studies have reported local recurrence rates of around 10% in various TME series<sup>[25-30]</sup>. In the study by Moore *et al*, local recurrence rates of less than 5% have been reported with a distal margin of 1 cm, provided that the mesorectum can be excised as a complete lymphovascular package<sup>[31]</sup>. Today TME has been successfully taught as a standardized procedure and translated to other colorectal surgical environments with reproducible cancer-specific outcomes<sup>[32]</sup>.

Advances in laparoscopic equipment and technique have revolutionized the surgical approach to many diseases. Laparoscopic surgery for colorectal cancer is currently considered an acceptable alternative to open resection for colorectal cancer. There are small but measurable short term benefits such as decreased post-operative narcotic use, earlier return of bowel function, shorter length of stay and better cosmetic results. Today there is no question that laparoscopic surgery can be performed safely and effectively by experienced surgeons. There is enough evidence that survival rates are not compromised by the laparoscopic approach<sup>[33,34]</sup>.

Although the first laparoscopic colon resection was reported in 1991<sup>[35]</sup>, the adoption of minimally invasive colon resection has been impeded by several factors. First, laparoscopic colon surgery is technically demanding. Second, and more importantly, there has been historical concern about whether minimally invasive surgery for colonic malignancies would achieve adequate oncologic resection. The most recent studies, including retrospective and prospective registries, as well as comparative studies clearly demonstrate that oncologic principles are not compromised by laparoscopic techniques, and the yield of lymph nodes, surgical margins (proximal, distal and radial), and length of bowel resected were comparable to open cancer surgery<sup>[17,36,37]</sup>. Another problem that has been discussed controversially is the issue of whether laparoscopic surgery is associated with an increased hematogenous and intraperitoneal tumor cell distribution. Some studies have shown that there is a higher incidence of intraperitoneal tumor cell dissemination during laparoscopic resection for colorectal cancer when compared to open surgery for colorectal cancer<sup>[38,39]</sup>. On the other hand, other studies have contradicted this observation<sup>[40,41]</sup>. Finally, another concern has been the incidence of port-site metastasis after laparoscopic resection<sup>[16,42-45]</sup>. Although port-site metastases have not been restricted to laparoscopic surgery for colorectal cancer, the major impact of this phenomenon has been in this field. A closer look at the literature reveals most reports with a high incidence rate were small series published in the early 1990s<sup>[44,45]</sup>. Within the last ten years, three large trials of laparoscopic surgery for colorectal cancer have been published that clearly demonstrated a low incidence of wound recurrence not statistically significantly increased compared to wound recurrence after open laparotomy sites<sup>[33,34,46]</sup>. However, it should be cautioned that these trials were not adequately powered to fully address the question. Moreover, in the COST-trial<sup>[33]</sup>, wound recurrence rates demonstrated an incidence of port site recurrence that, although both low and not statistically significantly different from that after open surgery, was nevertheless more than twice the rate of wound implantation seen after open surgery (0.5% incidence in the laparoscopic arm vs 0.2% in the open surgical arm of the trial). Although the question therefore still has not been completely addressed, it appears that the incidence is quite low and within acceptable clinical range, and it seems dubious that a randomized trial of sufficient

size ever will be conducted to settle conclusively this issue. It seems likely that insufficient technical skills and experience at the beginning of the laparoscopic era contributed to the early reports that described considerably higher rates of port site recurrence.

Many cancers, including colorectal cancer, spread first to the lymph nodes before reaching other parts of the body. Lymph node status remains one of the most important prognostic factors in the management of colorectal cancer. In patients without nodal disease, recurrent tumors still develop in about 15% to 20% of cases within 5 years of diagnosis<sup>[47]</sup>. The reasons for this are unclear, but may depend upon the quality of surgical resection and conventional pathologic review. Node-negative patients are usually not treated with adjuvant chemotherapy outside a clinical trial because of the lack of definitive evidence of survival benefit. Patients with nodal disease, on the other hand, should be treated with adjuvant chemotherapy because of potential reduction of mortality up to 33%<sup>[48]</sup>. Therefore, it is critical to avoid pathological understaging of the specimen. Standard pathologic evaluation may overlook low volume nodal metastasis, thereby failing to identify nodes imperative to accurate staging. Inconsistencies in number of nodes harvested at time of pathologic processing impact significantly colon cancer staging accuracy. This nodal sampling error serves as the basis for guidelines establishing a 12 node minimum for adequate staging utilizing conventional techniques<sup>[49]</sup>. Up to 78% of metastases are identified in subcentimeter nodes that may be overlooked during standard gross pathologic dissection of resected specimens<sup>[49-51]</sup>. Microscopic examination of 1 or 2 hematoxylin and eosin-stained sections of a 5-mm node limits pathologic assessment to < 1% of the entire node, making identification of small tumor cell aggregates challenging. In a study by Saha *et al*, sentinel lymph node mapping appears superior to conventional pathologic review and may therefore be a useful method to avoid understaging<sup>[47]</sup>. Nodal positivity was 48% for the group assigned sentinel-lymph-node mapping, compared with 35% for the group assigned conventional staging ( $P < 0.001$ ). In this study, sentinel lymphatic mapping accounts for the upstaging of 13% of colon cancer. In other studies, sentinel lymph node mapping accounts for the upstaging of 19%-24% of patients<sup>[52-57]</sup>. The consequence of upstaging is that these patients now become candidates for adjuvant chemotherapy. After a minimum of 2 years follow-up, patients assigned nodal mapping ( $n = 153$ ) had an overall recurrence of 7%, compared with 25% ( $n = 162$ ) for the patients assigned conventional staging ( $P = 0.001$ )<sup>[47,58]</sup>. As the sentinel lymph node technique has developed, some investigators describe over 90% success in identifying the sentinel node and accuracy rates of approximately 90%<sup>[47,53-56]</sup>.

In patients with colorectal metastases, advances in surgical techniques have made it possible that the goal of surgery is no longer palliative but of curative intent. Therefore, a complication such as wound recurrence may have grave clinical consequences for patients that

are operated on with curative intent. It thus becomes increasingly important that surgeons minimize tumor spill into the peritoneal cavity or into the lymphatic/vascular systems during surgical procedures for colorectal cancer. If not, tumors may recur and compromise a potentially curative resection.

The liver is the most common site for colorectal metastasis, since the venous outflow of the gut first reaches the liver through the portal system before flowing back into the systemic circulation. Approximately one-third of patients diagnosed with colorectal cancer will develop synchronous or metachronous metastases to the liver. The incidence of synchronous metastasis has ranged from 23.0% to 46.8%<sup>[59-62]</sup> and the 5-year survival rate after hepatic resection has been reported to be 14% to 40% in studies with more than 100 or more patients<sup>[60,61]</sup>. Several studies have suggested that careful selection of patients for hepatic resection of colorectal metastases can result in favourable survival<sup>[63-65]</sup>. A recent study by Rees *et al* found 7 risk factors (Basingstoke Prediction Index) that were found to be independent predictors of poor survival in a multivariate analysis<sup>[66]</sup>. The 7 risk factors were number of hepatic metastases > 3, node positive primary, poorly differentiated primary, extrahepatic disease, tumor diameter ≥ 5 cm, carcinoembryonic antigen level > 60 ng/mL, and positive resection margin. The first 6 of these criteria were used in a preoperative scoring system and the last 6 in the postoperative setting. Patients with the worst postoperative prognostic criteria had an expected median cancer-specific survival of 0.7 years and a 5-year cancer-specific survival of 2%. Conversely, patients with the best prognostic postoperative criteria had an expected median cancer-specific survival of 7.4 years and a 5-year cancer-specific survival of 64%<sup>[66]</sup>. It is therefore very important to preoperatively assess if resection is achievable, most preferably with a 1 cm margin<sup>[67,68]</sup>. It is often difficult to measure a 1 cm distance to the tumor edge on the specimen because of dissection or cautery artifact. Other times a surgical margin of 1 cm cannot be obtained because of the relation of the tumor to the hepatic veins, portal veins, or vena cava. When tumor is left behind after surgery, meaning that RO resection is not achieved, the survival is not different than that in the nonresected group.

While surgical resection remains the gold standard of therapy, only a few patients are suitable candidates for curative surgical resection because of the presence of liver malignancy in unresectable locations, the number of and anatomic distribution of tumor lesions, or the presence of extrahepatic disease or poor liver function. An alternative treatment to control and potentially cure liver disease has been developed for use in patients with malignant liver tumors. Radiofrequency ablation (RFA), also known as “radiofrequency thermal ablation”, is a recently developed thermoablative technique. It induces temperature changes by using high-frequency alternating current applied *via* electrodes placed within the tissue to generate areas of coagulative necrosis and tissue desiccation<sup>[69,70]</sup>. Overall recurrence for colorectal cancer

was most common after RFA (84% *vs* 64% RFA and resection *vs* 52% resection only,  $P = 0.001$ )<sup>[71]</sup>. Thus, RFA has been reserved as an adjunctive tool to resection, when complete resection is not possible, either alone or in combination with resection<sup>[72-74]</sup>. The study by Abdalla *et al* demonstrates that RFA alone or in combination with resection for unresectable patients does not provide survival comparable to resection, and provides survival only slightly superior to nonsurgical treatment<sup>[71]</sup>.

After colorectal metastases to the liver, the lungs are the second most common site of metastasis. The number of possibly resectable cases of lung metastasis after primary surgery for colorectal cancer has increased considerably over the past 20 years. The typical pattern of lung metastasis is single or multiple nodules rather than miliary tumors or lymphangitis carcinomatosa. No effective chemotherapy regimen has been found for metastatic disease. Hence, a surgical procedure to eliminate pulmonary metastases is generally accepted as the only potentially curative treatment. In favor of surgery is the recent trend toward earlier detection of pulmonary metastases as small peripheral densities with increasingly common use of screening with spiral or high-resolution computed tomography. The reported 5-year survival rates for lung metastectomy surgery were 24% to 63%, and most were around 40%<sup>[75-90]</sup>. The criteria for resection of pulmonary metastases from colorectal carcinoma included unilateral or bilateral excisable lung lesions per preoperative chest radiography, no local recurrence of primary lesions, and no extrapulmonary lesions with the exception of associated prior or simultaneous resectable hepatic metastases. Elevated CEA level and the number of metastasis are the most significant prognostic factors for overall survival after resection of lung metastases from colorectal cancer<sup>[91]</sup>.

## ADJUVANT THERAPY

For the treatment of rectal cancer, adjuvant radiotherapy has become a standard procedure. The following two schedules of treatment have been explored over the last decades: short term treatment that delivers 25 Gy in 5 fractions during 1 wk, followed immediately by surgery, and conventional schedules that deliver 40 Gy to 50 Gy in 20-25 fractions during 4 to 5 wk, followed by surgery 3-6 wk later. Regardless of the schedule, preoperative radiotherapy decreases local recurrence rates by 50%-60% when compared to surgery alone<sup>[92,93]</sup>. The conventional schedules are delivered in combination with chemotherapy to patients with locally advanced rectum cancer (T3-T4 tumors and N+ disease). The radiobiological dose delivered in a short term treatment schedule is too low for adequate response in locally advanced rectal tumors. In 2001, a study by Marijnen *et al* demonstrated that short term treatment with 25 Gy during 1 wk did not achieve tumor down staging for T1-T3 tumors within a period of 10 d<sup>[94]</sup>. However, a schedule of 50.4 Gy given over a period of 6 wk in combination with 5-FU and leucovorin did cause

down staging of locally advanced rectum tumors<sup>[95]</sup>. Today the standard treatment for locally advanced rectum carcinoma is pre-operative radiotherapy in combination with 5-FU and leucovorin<sup>[96]</sup>. In addition, it has been demonstrated that timing of chemoradiation for locally advanced tumors is important, since less toxicity and better local control may be achieved when chemoradiation is given pre-operatively instead of post-operatively<sup>[97]</sup>. Thus far, there has been no conclusive demonstration of a gain in overall survival for patients with locally advanced rectal tumors treated with adjuvant chemoradiation<sup>[98]</sup>.

During the past years, various phase I and II trials have been performed with capecitabine<sup>[98,99]</sup>. It is a form of chemotherapy that is administered orally and is a tumor activated fluoropyrimidine carbonate. During the last of three enzymatic processes, thymidine phosphorylase converts capecitabine to 5-FU. The enzyme thymidine phosphorylase is found in high concentrations in rectal tumors and it is therefore less likely that healthy tissue within the radiation field is subjected to 5-FU. The advantages of this form of therapy are less toxicity, oral administration, and less chance of infections since a venous port access catheter is no longer necessary. Although the phase II trials with capecitabine in combination with radiotherapy for locally advanced rectum tumors show promising results, there are currently no phase III trials that give information about local recurrence during a long term follow-up period. However, the National Surgical Breast and Bowel Project trial in the United States is planning on performing such a study in the near future. If the results from this study show acceptable local recurrence rates, then capecitabine may replace the 5-FU/leucovorin schedule. A study by Kim *et al* suggests that the addition of leucovorin to capecitabine does not work synergistically but actually seems more toxic<sup>[100]</sup>. For this reason a combined capecitabine and leucovorin schedule does not seem desirable. Other phase I and II studies have tried to combine capecitabine with oxaliplatin<sup>[101-103]</sup>. The results seem promising as well, and grade III/IV toxicity does not seem greater than the capecitabine and 5-FU/leucovorin schedules. Although there are many new developments, 5-FU and leucovorin in combination with radiotherapy remains the standard of neo-adjuvant treatment in most countries for patients with locally advanced rectum carcinoma.

Although research efforts continue to be directed at deriving new cytotoxic and antiproliferative agents directed specifically at cancer cells, the concept of targeting the angiogenic support of tumors has recently become of interest, and angiogenesis inhibitors have also been introduced for treatment of cancer. Bevacizumab is an anti-VEGF antibody. When combined with conventional chemotherapy, this agent has been reported to prolong survival in patients with advanced colorectal cancer treated in a palliative setting<sup>[104,105]</sup>. Additionally, recent trials with neo-adjuvant chemotherapy suggest that irresectable liver metastases can be downstaged with this agent. Thus, an increasing number of patients

with colorectal metastases to the liver may now become candidates for liver resection. Indeed, in such patients preoperative treatment with bevacizumab and chemotherapy may be associated with less blood loss compared to chemotherapy alone<sup>[106]</sup>. If bevacizumab and chemotherapy are discontinued at least 8 wk before hepatic resection, the addition of bevacizumab to preoperative irinotecan and oxaliplatin does not increase morbidity after hepatic resection<sup>[106,107]</sup>. Unfortunately, there are no studies yet that compare whether the combination of bevacizumab and chemotherapy allows for better downstaging of liver metastases than conventional chemotherapy alone. This will be an important subject for future study, as will the long term outcomes of patients downstaged with these agents and then subjected to liver resection of the remaining obvious metastases.

Despite all of these advances in surgical techniques and adjuvant therapies, colorectal tumor recurrence remains a problem. Manfredi *et al* described a 5-year cumulative rate of local recurrence of 12.8% and a 25.6% per cent rate of distant metastases<sup>[108]</sup>. During surgery it is important that tumor free margins of the resected specimen are achieved and that tumor spill is avoided. Unfortunately, some tumor spill occurs in approximately half of patients that are operated for colorectal cancer<sup>[109]</sup>. Many research studies have provided evidence for direct implantation of the port site or surgical wound by exfoliated cancer cells, hematogenous seeding, tissue manipulation, sterilization by pneumoperitoneum, patient's positioning and immune dysfunction as potentially etiologic factors. Approximately 0.2%-1% of patients will eventually develop wound recurrence<sup>[33,110]</sup>. Many of these patients also exhibit more diffuse peritoneal recurrence, although approximately half exhibit isolated wound recurrence. Either phenomenon has a negative impact on survival for those patients that are operated with curative intent. Since more than 80% of patients with colorectal disease are initially operated with curative intent, a complication such as wound or peritoneal recurrence may drastically influence their 5-year survival rate in a negative manner.

## TUMOR CELL ADHESION

The contrast between the high rates of tumor cell spillage and circulating tumor cells and the much lower rates of clinical tumor metastasis or implantation after surgery suggests that tumor implantation may be regulated in some way. The mechanisms that determine which tumor cells adhere to target organs and tissues are poorly understood. Normally, if a cell is unable to attach to the extracellular matrix, it dies through induction of the cell suicide program known as apoptosis. Cancer cells, however, develop a means to avoid death in this situation. Cells that have suffered irreparable DNA damage activate specific proteases and nucleases that destroy the proteins and DNA of the cell, thereby effectively limiting the spread of potentially deleterious mutations. Cancer cells often exhibit mutations in genes

involved in regulating this pathway.

Since not all cancer cells that are shed into the peritoneal cavity undergo apoptosis, there is always a possibility that these cells will eventually cause wound metastasis. Tumor implantation begins with the adhesion of tumor cells to the matrix proteins in the wound. The extracellular matrix consists chiefly of type I and IV collagens, laminins, heparin sulfate proteoglycan, fibronectin, and other noncollagenous glycoproteins<sup>[111]</sup>. Cell adhesion to extracellular matrix proteins is mediated by diverse receptors, most notably by members of the integrin family. Integrins, heterodimeric transmembrane proteins, are composed of noncovalently associated alpha and beta subunits that define the integrin-ligand specificity<sup>[112]</sup>, and their pattern of expression is likely to promote specific cellular adhesions. Both the physiologic status of the cell<sup>[113]</sup> and divalent extracellular divalent cation concentrations<sup>[114]</sup> can influence the affinity between integrins and their ligands. After adhesion of the cell, proliferation and angiogenesis are then required to support tumor growth, invasion and subsequent metastasis.

#### **Treatments to prevent wound or peritoneal metastasis**

During the past years not much progress has been booked in reducing wound recurrence in patients with curable colorectal cancer. The application of topical ointments<sup>[115,116]</sup>, abdominal irrigation<sup>[117]</sup> and port-site resection of wounds<sup>[118]</sup> have had limited success. The most promising results to date are probably the studies that investigate the anticancer effect of COX-2 inhibitors. Various studies have shown that COX-2 inhibitors have both antiangiogenic<sup>[119,120]</sup> and apoptotic effects<sup>[121,122]</sup> on human colon cancer cells. A more recent study demonstrated that COX-2 inhibitors down-regulated  $\beta 1$ -integrin expression, with consequent impairment of the ability of colon cancer cells to adhere to and migrate on extracellular matrix in an *in vitro* study<sup>[115]</sup>. It is therefore possible that these drugs may reduce wound recurrence since they may interfere with the adhesion of the cell to extracellular matrix. However, to date, the mechanisms of drug action and interaction are still far from clear, and their roles within the clinical setting are yet to be observed. It is therefore important to further investigate factors that may be of significance during wound implantation and eventual tumor formation.

#### **Extracellular influences on colon cancer cell adhesion**

Interaction between cells and the extracellular matrix are in large part mediated by integrins in divalent cation-dependent processes. This means that extracellular processes that alter divalent cation concentrations may also influence colon cancer cell adhesion to the extracellular matrix. Local shifts in the concentrations of extracellular Mg<sup>2+</sup> and Ca<sup>2+</sup> occur during wound healing, impacting the function of divalent cation-dependent cell surface molecules responsible for cell-cell and cell-extracellular matrix interactions<sup>[123]</sup>. Early in the process, when cell migration into the wound is

initiated, Mg<sup>2+</sup> is elevated and Ca<sup>2+</sup> is reduced. As wound healing progresses, wound concentrations of Mg<sup>2+</sup> and Ca<sup>2+</sup> return to normal plasma levels. Ebert *et al* reported that Mn<sup>2+</sup> and Mg<sup>2+</sup> stimulate binding of HT-29 colon cancer cells to extracellular matrix proteins<sup>[124]</sup>, and similar effects have been described in SW 620 and Caco-2 human colon cancer cells<sup>[125]</sup>. However, calcium inhibits adhesion of SW 620 and Caco-2 human colon cancer cells to collagen I, which is the dominant collagen of the interstitial matrix<sup>[125]</sup>. Furthermore, Mg<sup>2+</sup> and Mg<sup>2+</sup> potentiate cancer cell adhesion to murine surgical wounds and subsequent tumor development. In contrast, Ca<sup>2+</sup> inhibits cancer cell adhesion to murine surgical wounds and subsequent tumor development<sup>[126]</sup>. The biological and chemotherapeutic response characterization of transplantable mouse colon tumors suggests that they are reasonable models for colon cancer in humans<sup>[127]</sup>. Although more studies are required, these results raise the possibility that in the future, manipulation of divalent cation concentrations in irrigation of the surgical site may diminish perioperative tumor implantation.

#### **Effects of physical forces on colon cancer cell adhesion**

Cancer cells are subjected to pressure during surgical manipulation and passage through the venous and lymphatic system. Cells that are shed into the peritoneal cavity postoperatively are also subjected to increased pressure from postoperative edema. Surgical manipulation during either laparoscopic or open procedures is likely to result in the direct application of much higher pressures to tumors or lymphatic channels containing malignant cells. For instance, during laparoscopic colectomy for cancer, intra-abdominal pressure is often increased by 15 mmHg as the abdominal cavity is expanded to provide room to operate. The pressure engendered by a surgical forceps grasping tissue may be as high as 1500 mmHg<sup>[128]</sup>. Although pressure by the surgeon's hand during tumor dissection has not been quantified to our knowledge, parallel studies suggest that intraocular pressures may exceed 50 mmHg during ocular manipulation during enucleation<sup>[129]</sup>. Normal portal venous pressures may be as high as 10 mmHg, and this may increase substantially in portal hypertension. Mesenteric venous pressures may exceed this under normal circumstances to generate portal flow and might be accentuated by intra-abdominal pressure generated by ascites, Valsalva maneuvers, or bowel edema after surgery. Mesenteric lymphatic pressures in the setting of tumor infiltration into the lymphatics are unclear but might also be expected to be of similar orders of magnitude. Tumor cells in the systemic arterial circulation, of course, are exposed to substantially higher pressures.

Physical forces such as shear stress, and pressure have been reported to affect colon cancer cells<sup>[130]</sup>. Increasing ambient pressure and the application of shear stress increased cell adhesion of several colon cancer cell lines and primary human colon cancer cells isolated directly from surgical specimens<sup>[130,131]</sup>. Indeed, an increase of

15 mmHg above ambient pressure had a maximum effect on colon cancer cell line adhesion *in vitro*<sup>[130]</sup>. An interesting observation is that during colorectal cancer surgery cells are shed into the abdominal cavity and subjected to increases in shear during irrigation and increased pressure during and after surgical procedures. Such increases in pressure may enhance the adhesion of shed cells to surgical sites. Although these original studies were performed *in vitro*, 30 min exposure to 15 mmHg increased pressure has more recently been demonstrated to increase cancer cell adhesion to murine surgical wounds<sup>[132]</sup> and to adversely affect survival in a murine transplantable tumor model<sup>[133]</sup>. There are obviously manifest differences between transplantable tumors in mice and the pathophysiology of human colon cancers, but as these same signal events have also been described in primary human colon cancer cells<sup>[130,131]</sup>, the animal data are suggestive that the same pathway might affect the development of metastatic tumors in humans.

#### **The effect of pressure on focal adhesion-associated proteins**

If pressure and shear stimulate the adhesion of cancer cells<sup>[125,130]</sup>, it may then be important to unravel the intracellular mechanisms that mediate this effect so that interventions can ultimately be targeted to prevent cancer cell adhesion. In many cells, the focal adhesion kinase FAK transduces signals after adhesion through association with the cytoplasmic domains of integrin subunits<sup>[134]</sup>. However, “inside-out signaling” by which intracellular events modulate integrin function is less well understood. A study by Cooke *et al* suggests that mechanical stimulation of enterochromaffin-derived BON cells directly or indirectly stimulates a G protein-coupled receptor that activates Gαq, mobilizes intracellular calcium, and causes 5-HT release<sup>[135]</sup>. Although this study did not portray increased adhesion due to mechanotransduction, it did show that shear stress on carcinoid cells activate an intracellular cascade that releases 5-HT. Consistent with such force-activated intracellular signaling, Thamilselvan *et al* demonstrated that extracellular pressure may increase integrin affinity and promote colon cancer adhesion *in vitro* via actin-dependent inside-out FAK and Src signals<sup>[136]</sup>. Indeed, it is likely that the intracellular cascade involved in colon cancer cell adhesion to extracellular matrix is very complex. Recently, the activation of PI 3-kinase/Akt signaling pathway has been correlated with prostatic metastasis<sup>[137]</sup>, colon cancer cell invasion<sup>[138]</sup> and post-operative growth<sup>[139]</sup>. The overexpression of the PI 3-kinase/Akt pathway has also been described in human cancers including ovarian and colonic carcinomas<sup>[140,141]</sup>. Recent studies suggest that the PI 3-kinase/Akt pathway may also be required for pressure-stimulated cancer cell adhesion<sup>[142]</sup>, acting specifically via Akt-1<sup>[143]</sup>.

Several key structural proteins also seem to be involved in the mechanotransduction pathway, including cytoskeletal elements<sup>[144]</sup>, the adapter proteins paxillin<sup>[145,146]</sup> and alpha actinin-1<sup>[147]</sup>. Both paxillin and alpha actinin-1 facilitate focal adhesion formation

and physically link integrin-associated focal adhesion complexes with the cytoskeleton. These focal adhesion associated proteins are often abnormally expressed or mutated in cancer cells<sup>[148-150]</sup>. Therefore, they may be important in tumor biology in general. Although these focal adhesion associated proteins are not kinases themselves, these proteins facilitate the interaction of various kinases and other proteins required for this pathway to function. This makes them a promising target to uncouple the pathway required for force-activated adhesion without actually inhibiting cellular kinases, possibly leading to fewer side effects. Indeed, in a preliminary proof of principle, knockout of alpha actinin-1 has been shown to abolish the effect of pressure on tumor-free survival in a murine transplantable tumor model<sup>[133]</sup>.

#### **CONCLUSION**

Over the past decades, screening for colorectal neoplasm has shown to be critical for prevention, early diagnosis, downstaging, and improved survival. Beyond intensified screening programs, surgical techniques have evolved over the past years. Total mesorectal excision has improved survival rates for rectal cancer<sup>[92]</sup>. Other major advances have included liver and lung resections for patients with colorectal metastasis, so that at least some of these patients are no longer candidates for palliative treatment but instead can be treated with curative intent. Besides improvements in surgical techniques, adjuvant therapies such as radiotherapy and chemotherapy also have undergone improvement. At this moment, sentinel lymph node mapping is a technique that lies on the frontier, as does the proper role of anti-angiogenesis agents. Some studies suggest that the sentinel lymph node technique may upstage a significant number of patients who then become candidates for chemotherapy, while anti-angiogenic therapy may downstage patients who then become candidates for surgical resection of known metastases. However, before conclusions are made on these points, further follow-up of patient cohorts will be necessary. The cellular biochemistry involved in metastasis currently lies beyond the frontier. Unfortunately, little is known about the intracellular and extracellular cascades that may influence colorectal cancer cell adhesion and metastasis. Several studies have suggested that increased pressure and shear stress activate cancer cell adhesion. Further studies of the pathways that regulate integrin-driven cancer cell adhesion may identify ways to disrupt these signals or block integrin-mediated adhesion so that perioperative adhesion and eventual metastasis can be prevented in the future, adding yet another strategy to combat colorectal malignancy.

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## TOPIC HIGHLIGHT

**Yusuf Bayraktar, Professor, Series Editor**

# Gaucher disease: New developments in treatment and etiology

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## INTRODUCTION

Gaucher disease (GD) is a storage disease in which macrophage sphingolipidosis accumulation occurs. This progressive disease results from deficiency of glucocerebrosidase (acid- $\beta$ -glucosidase) in lysosomes. This enzyme is responsible for cleaving  $\beta$ -glycoside into  $\beta$ -glucose and ceramide subunits<sup>[1]</sup>. GD is inherited in an autosomal recessive fashion. The OMIM (Online Mendelian Inheritance in Man: <http://www.ncbi.nlm.nih.gov/Omim>) code for adult type GD is 230800.

It has 2 major forms: non-neuropathic (type 1, most commonly observed type in adulthood) and neuropathic (type 2 and 3). The disease is characterized by massive splenomegaly due to excessive accumulation of glucosylceramide in splenic macrophages. Other than spleen, so called "Gaucher cells" are the lipid-laden macrophages and can be observed in liver and bone marrow. Therefore GD causes organ damage where macrophages are densely present. Clinical alterations in bone, liver and spleen resulting in splenomegaly, hepatomegaly, hematological changes and orthopedic complications are the most predominant ones. Rarely kidney, skin, heart and central nervous system may be involved.

Recent advances in genetic technology have made it possible to manage the GD patients with enzyme replacement treatment (ERT). Substrate reduction treatment (SRT) is also currently available. In this review, we will discuss new developments in adult type GD disease in terms of its etiology and treatment from a gastroenterologists' point of view.

## ETIOLOGY AND PATHOGENESIS

The gene coding for the enzyme responsible for GD is located on chromosome 21. In this single gene, about 200 mutations are defined up to date<sup>[1]</sup>. Most commonly observed mutations are N370S, L444P, RecNciI, 84GG, R463C, recTL and 84 GG is a null mutation in which there is no capacity to synthesize enzyme. However, N370S mutation is almost always related with type 1

## Abstract

Gaucher disease (GD) is an autosomal recessive disease which if undiagnosed or diagnosed late results in devastating complications. Because of the heterozygous nature of GD, there is a wide spectrum of clinical presentation. Clinicians should be aware of this rare but potentially treatable disease in patients who present with unexplained organomegaly, anemia, massive splenomegaly, ascites and even cirrhosis of unknown origin. The treatment options for adult type GD include enzyme replacement treatment (ERT) and substrate reduction treatment (SRT) depending on the status of the patient. Future treatment options are gene therapy and "smart molecules" which provide specific cure and additional treatment options. In this review, we present the key issues about GD and new developments that gastroenterologists should be aware of.

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**Key words:** Gaucher disease; Enzyme replacement treatment; Substrate reduction treatment; Gene therapy; Liver fibrosis

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**Table 1** Worldwide most common mutations in GD

Population	Most frequently observed mutations	Frequency of mutations (%)
Turkish <sup>[3]</sup>	N370S	61.80
	L444P	
Japanese <sup>[4]</sup>	L444P	55
	F213I	
Taiwanese <sup>[5]</sup>	L444P	78.50
	RecNciI	
Czech and Slovak <sup>[6]</sup>	N370S	76
	L444P	
Ashkenazi Jews <sup>[7]</sup>	RecNciI	
	N370S,	93
	c.84-85insG IVS2 + 1G->A L444P	
Hungarian <sup>[8]</sup>	N370S	70.30
	L444P	
Spanish <sup>[9]</sup>	RecNciI	
	N370S	68.70
	L444P	

disease and milder forms of disease. Very rarely, deficiency of sphingolipid activator protein (Gaucher factor, SAP-2, saposin C) may result in GD. This rare condition is due to congenital absence of carrier protein involved in sphingolipid catabolism<sup>[2]</sup>.

Worldwide differences in genetic mutations are shown in Table 1. Therefore, genetic heterogeneity results in phenotypic heterogeneity. The phenotypic presentation of the same mutation may vary in a wide spectrum. This difference is most possibly related with ethnicity, genetic background, environmental and nutritional factors. But most important of all, the exact pathophysiological mechanisms of GD are not clear yet. Since the total accumulation of glycosphingolipid in a massively enlarged spleen constitutes only about 2%, there must be other unknown pathways that result in splenic proliferation, activation and enlargement<sup>[10]</sup>. One of the most important changes occurs in macrophages. The accumulation of glycosphingolipid in the lysosomes of macrophages results in some expected consequences by unknown mechanisms. One important pathway is the activation of macrophages. The great majority of evidence of macrophage activation stems from the fact that patients with GD have increased levels of macrophage-derived inflammation related molecules such as interleukin-1 $\beta$ , interleukin-6, interleukin-10 and TNF- $\alpha$ <sup>[11,12]</sup>. With regard to macrophage activation, these cytokines might be responsible for some consequences like increased osteoclastic activity in bones resulting in osteopenia, osteoporosis and fractures.

Activated macrophages also secrete some other molecules that can be used as a marker of disease activity and surveillance. The enzyme chitotriosidase (CT) and CC chemokine ligand 18 (CCL8) are examples of these markers. CT is originally a chitinase which is responsible for degradation of chitin. In humans, CT plays a role during the remodeling phase of the tissue healing process and in immune-chemotaxis<sup>[13,14]</sup>. Previous studies reported that screening of increased CT activity can be used as a GD marker<sup>[15,16]</sup>. But the null allele in

the general population, which is expressed at a mean frequency of 4%, causes deficiency of the enzyme<sup>[17]</sup>. The levels of the enzyme may also increase in elderly people<sup>[18]</sup> and in other granulomatous diseases such as sarcoidosis<sup>[19]</sup>. In addition, other macrophage markers such as macrophage inflammatory protein (MIP)-1 $\alpha$ , MIP-1 $\beta$ <sup>[20]</sup> and soluble CD163<sup>[21]</sup> can also be used for surveillance of the disease.

## CLINICAL MANIFESTATIONS

In general, GD patients present initially with hematological abnormalities due to hypersplenism. Gastroenterologists can be consulted because of anemia, massive splenomegaly or hepatomegaly of unknown cause. Although rare, patients presenting with hepatosplenomegaly and ascites are reported<sup>[22]</sup>. As mentioned previously, GD involvement changes in severity according to the density of macrophages in the particular organ and partially depends on the phenotypically heterozygous nature of the disease itself.

The most common mutation, N370S, could result in subtle symptoms and silent disease due to presence of some degree of enzymatic activity resulting in delays in diagnosis. The delays in diagnosis eventually results in irreversible complications. A recent study by Mistry *et al* showed that in adult patients with type 1 disease the average time from first appearance of symptoms of GD to final diagnosis was 48 mo. In addition, hematology-oncology specialists who were managing two-thirds of the patients considered diagnosis of GD only in 20% for the classical symptoms (bone pain, organomegaly and low blood counts)<sup>[23]</sup>.

The diagnosis of GD is straightforward. The demonstration of genetically miscoded or absent enzyme levels is diagnostic. For this purpose, direct analysis of glucocerebrosidase in peripheral blood leukocytes is utilized. The activity of glucocerebrosidase is variable in white cell type<sup>[24]</sup>, enzyme activity usually increases from granulocytes to lymphocytes to monocytes. Type 1 GD patients have 10 to 15 percent of the normal enzyme activity whereas type 2 and 3 patients usually have lower activity. Due to considerable overlap of enzymatic activity between heterozygote carriers and normal individuals, enzyme analysis cannot distinguish GD carriers from non-GD-carriers. In order to differentiate the carrier state, the genetic analysis which was mentioned previously should be applied for definitive diagnosis.

After diagnosis, GD patients should be classified according to clinical severity scores. The modified scoring system developed by Zimran (Zimran severity score index, ZSSI)<sup>[25]</sup> should be used during initial evaluation and during follow up of patients taking treatment (Table 2).

### Gastrointestinal system involvement in GD

In the type 1 GD, gastrointestinal complications such as hepatomegaly, splenomegaly, cirrhosis, ascites, and esophageal variceal hemorrhage predominate and

are well known. However, other associations such as increased risk of hepatic carcinogenesis and cholelithiasis are not taken into consideration.

In the literature, there are reports of increased risk GD associated hepatocellular carcinoma (HCC)<sup>[26-28]</sup>. In a recent survey, the risk of development of HCC in GD patients is found to be 141 times more than normal controls<sup>[29]</sup>. The overall risk of malignancy (especially hematological malignancies such as multiple myeloma) development is known to be increased. However, some studies suggest that this risk is not different from the normal population in early and middle age<sup>[30]</sup>.

Patients with GD, especially females over age of forty are found to be increased in risk of cholelithiasis. This increased risk is usually attributed to factors like anemia, prior splenectomy, hepatic involvement and an increased biliary excretion of glucosylceramide<sup>[31,32]</sup>. The management of cholelithiasis is not different from non-GD patients.

The portal hypertension observed in GD has 2 causes. The first is the overflow in the portal system secondary to splenomegaly which usually resolves after splenectomy. The second is observed in patients who already had a splenectomy. In these patients, massive infiltration of Gaucher cells in the liver parenchyma results in intrahepatic portal hypertension. The accumulation of excessive sphingolipid in liver macrophages (Kupffer cells) might result in activation of some unknown mechanisms or cell-to-cell interactions might ensue which eventually results in hepatic fibrosis. Hepatic fibrosis and eventual cirrhosis is the most feared complication of GD for liver involvement<sup>[33,34]</sup>. Although ERT results in reversal of hepatomegaly to normal, hepatic fibrosis still remains a challenge. The exact pathophysiological pathways of progressive hepatic fibrosis are unknown, but progressive fibrosis is observed mostly in pediatric patients. In initial presentation, hepatic transaminase levels are found to be elevated in nearly 50% of patients<sup>[25]</sup>.

Other non-specific findings of GD involvement of the liver are hepatomegaly (detected in 100% of patients with splenomegaly but detectable only in 87% of patients with splenectomy), non-specific mass like lesions in liver, peri-portal and retroperitoneal lymph nodes<sup>[35]</sup>.

Liver transplantation is rarely necessary compared to a few decades ago because of the introduction of ERT. However, development of features of progressive liver fibrosis such as elevated liver transaminase levels and no normalization in hepatomegaly despite adequate enzyme treatment should alert for rapidly developing fibrosis in a given patient. Liver transplantation should always be an option in patients with GD. Theoretically, there is a risk of recurrence of liver disease due to excessive burden of glycosphingolipid influx more than transplanted liver can handle. This risk is higher in patients with a spleen; therefore a splenectomy (if not performed until time of transplantation) should also be performed when transplantation is decided.

Despite its rarity, GD may involve other sites of the

**Table 2 Zimran severity score index, 1992 (SSI scores of 0-10: Mild disease; 11-25: Moderate disease; > 25: Severe disease)**

Feature	Detail	Score
Cytopenia	Non-splenectomized	1
	Splenectomized	
	Leukopenia	1
	Anemia	1
Splenomegaly	Thrombocytopenia	1
	None	0
	Mild	1
	Moderate	2
Splenectomy	Massive	3
		3
		3
		3
Hepatomegaly	None	0
	Mild	1
	Moderate	2
	Massive	3
Liver enzymes (aspartate aminotransferase, alkaline phosphatase, gamma-glutamyltransferase, lactate dehydrogenase)	Normal	0
	Some abnormal	1
	All abnormal	2
Signs of clinical liver disease		4
		20
CNS involvement		4
		4
Other organ involvement (kidney, lungs or any other)		
Bone disease-objective findings	No signs	0
	X-ray or nuclear scan abnormality	1
Bone disease-objective findings	No pain	0
	Mild pain	2
	Chronic pain unrelated with fractures	3
Bone fractures	No fracture	0
	Post-traumatic fracture	1
	Pathologic fracture or aseptic necrosis	5

gastrointestinal system other than the liver and spleen. GD may result in "Gaucher cell pseudotumors" in the abdomen resulting in mass like lesions<sup>[36,37]</sup>. This phenomenon is very important because GD is related with increased incidence of cancer but currently, GD pseudotumors are rare due to ERT. Association of GD with colonic infiltration of "Gaucher cells"<sup>[38]</sup>, massive gastrointestinal bleeding due to ileal involvement<sup>[39]</sup> and Menetrier disease of the stomach<sup>[40]</sup> are also reported.

## TREATMENT STRATEGIES IN GD

### Pre-treatment evaluation in GD

Since adult GD patients come to attention after development of symptomatic anemia, organomegaly or complications such as fractures, indications for specific treatment of GD are beyond the presence of these symptoms. The available ERT and SRT options are expensive (annual cost of ERT is around 1 billion US dollars<sup>[41]</sup>) and individual responses for these treatments are highly variable so that some patients may report no improvement. Therefore, with parallel to variability in phenotypic presentation, decision for treatment

must be individualized. In our personal practice, severe organomegaly, high degree of cytopenia, event of minor bleeding due to thrombocytopenia, bone disease, liver enzyme elevations with severe organomegaly and presence of any organ involvement other than liver-spleen-bone triad are indications of treatment. The aims of treatment are reversal of organomegaly, prevention of complications and increase in quality of life.

### **ERT**

Alglucerase (extracted from human placenta) is the first enzyme treatment modality for GD patients to provide specific treatment. Major limitations of this product were its low degree of reproducibility (because of low number of available placenta), risk of blood borne infections, very short half-life (about 4.7 min in blood and 2 h in visceral organs including liver and spleen)<sup>[42,43]</sup>. These limitations had resulted in new research and development of a recombinant enzyme; imiglucerase. Imiglucerase is obtained from genetically manipulated ovary cells of Chinese hamsters. This enzyme has a longer half life and lack of blood borne infection risk. Despite the clinical results, imiglucerase looks excellent; there is only one randomized controlled trial with imiglucerase up to date. In this study by Schiffmann *et al*<sup>[44]</sup>, the primary end-point was the improvement in bone mineral density of the lumbar spine. ERT (combination of alglucerase and imiglucerase) was compared with vitamin D and calcitriol in three patient arms. The total number of randomized patients was 29. The authors concluded that "ERT alone, or in combination with calcitriol, cannot repair the bone composition in splenectomized adult Gaucher patients. They also stated that the ERT significantly improved hemoglobin, platelet counts, and liver volume. There are two other randomized trials. The first one was designed to compare the effectiveness of imiglucerase vs. alglucerase (15 patients per treatment arm) which found no significant difference in the two molecules<sup>[45]</sup>. The second randomized controlled trial is a recent study by deFost *et al*<sup>[46]</sup> who showed that "low frequency administration of ERT in adult Gaucher type I patients maintains stable disease in most patients".

Although the number of randomized controlled trials in ERT does not satisfy clinicians as compared with other conditions, this currently available treatment option remains unique. The other option of treatment is substrate reduction therapy (SRT) and has a limited field of clinical use.

### **SRT**

The idea underlying SRT is the inhibition of formation of sphingolipids that accumulate during GD. N-alkylated imino sugars are the prototype of this treatment. N-butyl-deoxynojirimycin (NB-DNJ) or miglustat is used on GD to reduce the formation of glucosylceramide by inhibiting the glucosylceramide synthase enzyme<sup>[47]</sup>. There are various studies related to efficacy of SRT, which showed improved blood counts, decrease in volumes of liver and spleen and increased quality of life<sup>[48,49]</sup>. One recent study showed

comparable results with ERT<sup>[50]</sup>. The most important advantages of SRT are its oral administration (ERT is given as intravenous administration over several hours and must be repeated in 2-3 wk intervals), low costs and availability. Major drawbacks of SRT are safety issues. Theoretically, long term inhibition of glycosphingolipid inhibition may result in detrimental results but no major adverse reaction is reported up-to-date. Minor adverse reactions are weight loss, mild tremor, diarrhea, and gastrointestinal upset. In patients who received previous ERT, switch to SRT is well tolerated<sup>[51]</sup>. SRT is currently limited only to adult type 1 patients who can not tolerate ERT.

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## **"THE FUTURE" OF GD THERAPY**

### **Gene therapy**

The administration and incorporation of a healthy genome replacing a deficient genome appears to be the cure for GD. There are a few studies in the literature investigating this possibility. In early animal models of gene therapy the vectors responsible for "infecting" a healthy genome were viruses (adeno-associated virus, lentivirus and retrovirus). These studies showed promising results for the future<sup>[52-54]</sup>. Neither ERT nor SRT have achieved excellent results in terms of neurological and pulmonary involvement due to problems such as inability to pass through the blood-brain barrier. The advantage of gene therapy is its widespread infection of specific targeted cells in the body substantially increasing the enzyme levels to a considerable level.

### **Chaperone treatment**

The ability of imino sugars to increase the strength of the target enzyme (glucocerebrosidase) is shown previously by Sawkar *et al* in a study<sup>[55]</sup>. These chemicals increase the half life of the enzyme by inhibiting its degradation and by providing stabilization. Although this option currently does not seem to offer a monotherapy option, chaperones might be an option for combination treatment strategies.

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## **CONCLUSION**

Adult type GD is a heterozygous disease. Because of vulnerability to delayed diagnosis, timely diagnosis and early initiation of appropriate therapy are crucial and prevent detrimental complications and stops the progression of disease to some extent. In the future, it might be possible to "cure" this genetic disease by gene-vaccines. Therefore, we require more investment on investigations for gene therapy. ERT and SRT have undeniable therapeutic effects, but there seems to be need for more evidence before putting them into "gold-standard" therapy for all patients.

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## TOPIC HIGHLIGHT

**Yusuf Bayraktar, Professor, Series Editor**

# Primary sclerosing cholangitis - What is the difference between east and west?

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## INTRODUCTION

Primary sclerosing cholangitis (PSC) is a rare cholestatic liver disease characterized by chronic inflammation and fibrotic obliteration of the hepatic biliary tree, resulting in bile stasis and hepatic fibrosis. Ultimately cirrhosis, end-stage liver disease and death ensue. It is commonly associated with inflammatory bowel disease (IBD).

PSC is more commonly a disease of adults, with patients typically presenting during the 4th and 5th decades<sup>[1-3]</sup>, although it has been reported to occur in the pediatric age group<sup>[1]</sup>. Patients typically present with pruritus and fatigue at the early stages of the disease, although patients with incidental elevated liver enzymes may be diagnosed earlier. As the cholestatic picture progresses with further obliteration of the bile ducts and increased fibrosis, patients develop jaundice and signs of advanced liver disease. Rare presentations include variceal bleeding and cholangiocellular carcinoma (CCC)<sup>[4,5]</sup>. Due to the diverse clinical picture, PSC patients are generally categorized based on the presence of symptoms, involvement of small *versus* large bile ducts, and its association with IBD or other autoimmune diseases (Table 1).

The cause of PSC is unknown; however, it is not a typical autoimmune disease especially since sex predominance varies with geography. In this review we attempted to establish any differences regarding epidemiology, natural history and management of PSC between different geographical locations, as well as providing insight into recent developments regarding the pathogenesis and treatment of the disease.

## Abstract

Primary sclerosing cholangitis (PSC) is a chronic, progressive, cholestatic liver disease characterized by inflammation and fibrotic obliteration of the hepatic biliary tree. It is commonly associated with inflammatory bowel disease (IBD). A number of complications can occur which require special consideration, the most important of which is the development of cholangiocellular carcinoma (CCC). Unfortunately, no medical therapy is currently available for the underlying liver disease. Liver transplantation is an effective, life-extending option for patients with advanced PSC. Geographical variations between East and West include a second peak for age with a lower association with IBD in a Japanese population and female predominance in a lone study from Turkey. The clinical and biochemical Mayo criteria may not be universally applicable, as different patients show variations regarding the initial presentation and natural course of the disease. Directing research towards explaining these geographical differences and understanding the pathogenesis of PSC is required in order to develop better therapies for this devastating disease.

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**Key words:** Primary sclerosing cholangitis; Endoscopic retrograde cholangiopancreatography; Magnetic resonance cholangiopancreatography; Cholangiocellular carcinoma; Ursodeoxycholic acid

## EPIDEMIOLOGY AND SYMPTOMATOLOGY

Takikawa *et al*<sup>[2]</sup> have been pioneers in establishing the

**Table 1 Mayo criteria for the diagnosis of PSC<sup>[27]</sup>**

<b>Mayo criteria</b>	
Typical cholangiographic abnormalities involving any part of the biliary tree	Multifocal stricturing and beading, usually involving both the intrahepatic and extrahepatic biliary system
Compatible clinical and biochemical findings (for longer than 6 mo)	Cholestatic symptoms, history of inflammatory bowel disease Twofold to threefold increase in serum alkaline phosphatase levels
Exclusion of identifiable causes of secondary sclerosing cholangitis	AIDS cholangiopathy Bile duct neoplasm (unless diagnosis of PSC previously established) Biliary tract surgery, trauma Choledocholithiasis Congenital abnormalities of biliary tract Caustic sclerosing cholangitis Ischemic stricturing of bile ducts Toxicity or stricturing of bile ducts related to intra-arterial infusion of floxuridine

characteristics of PSC in Japanese patients. In an early analysis of 192 patients<sup>[2]</sup>, they discovered two peaks for age distribution, a characteristic which they reported as “unique”, with the disease being associated more often with IBD in the younger age group. In a more recent study<sup>[3]</sup>, the same group performed a nationwide survey, comparing the results with their previous study. They reported on male predominance (59%) with a mean age of 47 years at diagnosis. Although most patients were asymptomatic at the time of diagnosis, jaundice (28%) and pruritus (16%) were the most commonly encountered presenting symptoms.

Ponsioen *et al*<sup>[4]</sup> evaluated the natural history of PSC in a Dutch population. Of the 174 patients included in this study, 60% were male, with a mean age of 40.4 years<sup>[4]</sup>. In a similar study by Broome *et al* on 305 Swedish PSC patients, 64% were male and the median age at the time of diagnosis was 39 years (range 5-80)<sup>[5]</sup>. Of the symptomatic 171 (56%) patients, abdominal pain (37%), jaundice (30%), pruritus (30%), and fever (17%) were most commonly reported complaints. In a more recent multicenter study on 273 German patients, again male predominance was established at 71.4%, with a mean age at time of diagnosis of 32.4 years (range 9-72 years)<sup>[6]</sup>. Slightly more than half of these patients were symptomatic initially, with right upper quadrant abdominal pain once being again the most prevalent symptom (34.4%).

On the other side of the Atlantic, in an early study, Weisner *et al*<sup>[7]</sup> evaluated the natural history of PSC in 174 patients; 37 were asymptomatic and 137/174 (79%) had symptoms related to underlying liver disease. At the time of diagnosis, the mean age was 39.9 years, and 66% of the patients were male. Long-term follow-up (mean: 6.0 years; range: 2.7-15.5 years) was available in all patients. In a more recent population based study in Minnesota by the Mayo clinic, it was projected that approximately 29 000 cases of PSC exist in the white USA population. In this study, the median age at diagnosis was 40 years, and 68% of the patients were men. Although asymptomatic patients with incidental

**Table 2 Comparison of characteristics of PSC patients: European and Japanese studies**

<b>Parameter</b>	<b>Takikawa (n = 388)</b>	<b>Broome (n = 305)</b>	<b>Ponsioen (n = 174)</b>	<b>Tischendorf (n = 273)</b>
Age (yr)	47	39	40.4	32.4
Male (%)	59	64	60	71
Symptoms				
Jaundice	28	30	NA	NA
Pruritus	16	30	NA	NA
IBD	37	81	66	63
CD	2.4	7	13.2	10.6
UC	29	72	47.7	51.7
Laboratory				
ALP	88	91.5	NA	NA
Bilirubin	39	41	NA	40
pANCA (+)	13	NA	NA	69
Diagnostic procedures				
ERC	80	87.2	76.4	100
MRC	32	NA	NA	NA
Liver biopsy	78	83.2	NA	100
BD involvement				
SD	1.6	0	NA	3.3
Only EHBD	4.8	6	NA	3.7
Only IHBD	27	27	NA	24.9
IHBD+EHBD	68	67	NA	68.1
Treatment				
UDCA	78	NA	NA	NA
Steroid	31	NA	NA	NA
Endoscopy	14	NA	NA	NA
Liver transplantation	10	NA	8	39.6

abnormal liver tests was not an infrequent clinical scenario, most patients presented with symptoms of advanced stages of the disease, including jaundice, pruritus, fever, or manifestations of portal hypertension.

In an earlier study from Turkey, the “crossroad between east and west”, by Bayraktar *et al*<sup>[8]</sup> evaluating the association between PSC and IBD, the median age of presentation for patients with PSC was 35 years (range 19-48 years). The most intriguing feature was that 15 of the 16 patients with PSC were females, a predominance that had never been previously reported. Although results of two subsequent studies originating from Turkey<sup>[9,10]</sup> were found to be consistent with those from the West, the reasons behind the findings in the Bayraktar study remain elusive.

It would seem that PSC patients worldwide share the same characteristics regarding sex, age and symptoms on presentation (Table 2). The only differences of note would be the second peak for age of presentation observed in the Japanese population, as well as of course the overwhelming female predominance reported in the Turkish study.

## ASSOCIATION WITH IBDS

The intimate relationship between PSC and ulcerative colitis (UC), and to a lesser extent Crohn’s disease (CD), is no secret. One of the most attractive hypotheses linking the two entities is that disruption of intestinal mucosa due inflammation results in increased permeability and eventual translocation of bacteria into

the portal system, whose cell products may trigger an immune response in genetically susceptible individuals resulting in peribiliary fibrosis. Indeed, Grant *et al*<sup>[11,12]</sup> managed to demonstrate the enterohepatic circulation of lymphocytes where memory cells originating from the intestines may actually stimulate hepatic inflammation due to the liver and the intestines sharing the same homing receptors. This may help explain why the course of PSC is usually dependent on the severity and extent of bowel involvement.

In the Dutch study<sup>[4]</sup>, a total of 114/174 (66%) patients were known to have concurrent IBD, of which 73% had UC while 25% had CD. Tichendorf *et al* reported similar results in a German population, with 172/273 (63%) having concomitant IBD (82% UC, 17% CD, and 1.2% indeterminate colitis). A study in a Swedish population<sup>[5]</sup> reported a slightly higher association of IBD with PSC of 81% (249/305), of which 88% had UC, and 8% had CD. The situation in the US was no different, with an association of 71% between IBD and PSC, mainly UC<sup>[7]</sup>.

In Japan, however, a much lower prevalence of IBD has been reported by Takikawa *et al*<sup>[3]</sup>, with only 125 of the 388 patients (32%) having an established diagnosis of IBD, 79% of which had UC, while 6.4% were diagnosed with CD. They also discovered that most patients who were afflicted with IBD were mainly adolescents or young adults. The author's went so far as to suggest that this low prevalence deemed some of the Mayo diagnostic criteria to be inapplicable for Japanese patients.

The association with IBD seems to show variability depending on geographical location, with higher rates in the European and American population, and a significantly lower association in Japanese patients (Table 2).

## PATHOGENESIS OF PSC

The etiology and pathogenesis of PSC are not yet well understood. However, it is widely believed that immune dysregulation plays a key role in the development of the disease.

### Immunogenetics

Most studies on the immunogenetics of PSC were concentrated in the late eighties to the early nineties. Earlier reports by Schrumpf *et al*<sup>[13]</sup> and Chapman *et al*<sup>[14]</sup> demonstrated associations of HLA-B8 and -DR3 of up to 60% in patients with PSC associated with ulcerative colitis. In DR3 negative patients, a second association of up to 55% was reported for DR2<sup>[15]</sup>. The most striking result was reported by Prochazka *et al*<sup>[16]</sup>, where they found HLA-DRw52, encoded by the gene locus DRB3, in 100% PSC patients evaluated for liver transplantation, with a relative risk of 109 when compared to a control group. Subsequent studies failed to demonstrate a similar correlation<sup>[17]</sup>, which raised the suspicion that HLA-DRw52 may perhaps be associated with more severe disease, thus suggesting the need for liver transplantation in such patients. Clinical significance lies in the fact that

DR3 positive patients seem to have an earlier age of onset when compared to DR3 negative, DR2 positive patients. Similarly, DRB380101 positivity, coding for DR52, was associated with reduced survival rate<sup>[15]</sup>.

*MICA* and *MICB* genes, found in the class I region between HLA-B and DRB, express MICA, which is responsible for the activation of T-cells in the gastrointestinal tract. Although initially identified in association with IBD, their contribution to genetic susceptibility to develop PSC has been investigated. PSC was found to be associated to the extended B8-MICA5.1-MICB24-DR3 haplotype<sup>[18]</sup>. In another study, a previously unreported protective association with the DRB1\*0701-DQB1\*0303 haplotype was also demonstrated<sup>[19]</sup>. Other reported associations include significantly increased TNFA2 allele frequency PSC patients, particularly the homozygous genotype in a southern European population<sup>[20]</sup>.

Intercellular adhesion molecule-1 (ICAM-1, CD54) gene polymorphisms have been implicated in the susceptibility to IBD. ICAM-1 is expressed on proliferating and interlobular bile ducts and elevated serum levels of soluble ICAMs have also been detected. Surprisingly, the E469E homozygote status for ICAM-1 was found to be associated with protection against PSC<sup>[21]</sup>.

Studies regarding the immunogenetics of PSC were inconclusive in establishing a difference between East and West, due to a lack of extensive population based research.

### Autoantibodies

There is no specific autoantibody for PSC, although ANCA positivity has been known to occur in up to 88% of patients, while ANA positivity has been observed in a substantial portion (53%) of PSC patients<sup>[22]</sup>. More notably, anticardiolipin positivity, reported in up to two-thirds of patients, was found to be associated with more prominent histological changes and disease severity<sup>[23]</sup>. In the report by Moritoki *et al*<sup>[23]</sup>, it was concluded that autoimmunity plays a more important role in autoimmune hepatitis and primary biliary cirrhosis rather than PSC, a notion that is supported by the fact that PSC does not respond to immunosuppressive treatment. Some degree of association has also been reported with *H pylori* IgG<sup>[24]</sup>.

Data in the pertinent literature was insufficient to help establish any geographical differences in immunogenetics.

## DIAGNOSIS OF PSC-LABORATORY, ENDOSCOPY, HISTOLOGY AND RADIOGRAPHY

In the Japanese study<sup>[3]</sup>, ALP levels were elevated in 65% of patients, while 39% had eosinophilia. ANA were positive in 36% of patients. The majority of the patients (80%) were diagnosed with endoscopic retrograde cholangiography (ERC), while for 32% magnetic



**Figure 1** Classical ERCP findings for PSC: Multifocal stricturing (thin arrows) and beading (thick arrow) of the intrahepatic and extrahepatic biliary ducts.

resonance cholangiography (MRC) was utilized, by which more than two-thirds of the patients were observed to have involvement of both intrahepatic bile ducts (IHBD) and extrahepatic bile ducts (EHBD). Isolated involvement of the IHBD and EHBD was encountered in 27% and 4.8% of patients, respectively, while small duct (SD) PSC was observed only in 1.6% of cases. Sixty-nine percent of the patients had histologically proven bile duct damage, with cholestasis apparent in 46% of the 284 patients who underwent a liver biopsy.

Tischendorf *et al*<sup>[6]</sup> also reported a similar rate of simultaneous involvement of IHBD and EHBD (68%). While 24.9% and 3.7% of patients had either IHBD or EHBD involvement, respectively, SD involvement was encountered in 3.3% of patients. In this study, all patients had undergone ERC evaluation, with no mention of MRC. The Swedish population<sup>[5]</sup> wasn't far different with 67% dual involvement, 27% IHBD involvement only, and 6% had only extrahepatic PSC. Interestingly, none of the patients in this study had SD PSC.

ERC and liver biopsy are still the most widely used modalities for the diagnosis of PSC, although use of MRC is on the rise.

#### ERC vs MRC for PSC

PSC was first described by Delbet in 1924<sup>[25]</sup>. The advent of the widespread use of endoscopic retrograde cholangiopancreatography (ERCP) in the mid-1970s led to further recognition of what was previously thought to be a very rare disease. In an excellent report, MacCarty *et al* described what are now known as the classical ERCP findings (Figure 1) in PSC<sup>[26]</sup>, which later formed the backbone for the updated Mayo Clinic diagnostic criteria of 1984<sup>[27]</sup>.

ERC remains the current standard for imaging of the biliary tract in patients with suspected PSC. However, being less invasive, MRC has gained popularity in recent years. Although promising, many authors have had reservations regarding the sensitivity and specificity of MRC for diagnosing and defining the extent and the severity of PSC. Two recent reports compared these two modalities head-to-head. Berstad *et al*<sup>[28]</sup> thought that the diagnostic accuracy of ERC and MRC were comparable,

despite MRC providing a slightly poorer depiction than ERC of extrahepatic and intrahepatic ducts. They reported independent reader sensitivity and specificity rates of 80% and 87%, with an accuracy of 83% for MRC, compared to 89%, 80% and 85 % for ERC. They concluded that MRC and ERC performed equally well in the diagnosis of PSC when used blinded to clinical information. In a separate study by Moff *et al*<sup>[29]</sup>, EHBD and IHBD visualization was excellent in 64% and 66% of MRCs, and 86% and 74% of ERCS. MRC had sensitivity ranging from 81%-91%, and specificity 85%-96% for diagnosis of PSC. Interobserver agreement for the diagnosis of PSC and for identifying the presence of IHD strictures was good for both modalities, but once again only ERC was good for the presence and the severity of EHD strictures. Similarly, for the assessment of disease severity patients with PSC, interobserver agreement was very poor for both MRC and ERC. They concluded that ERC and MRC were comparable for diagnosing PSC, with very good interobserver agreement for the diagnosis of PSC and IHD strictures. Only ERC had good agreement for EHD strictures. Interobserver agreement was very poor for both MRC and ERC when disease severity of PSC was assessed.

## COMPLICATIONS OF PSC

### ***Cholelithiasis, choledocholithiasis, and biliary strictures***

Chronic cholestasis predisposes to the formation of cholesterol gallstones and bile stasis with bacterial cholangitis leads to the formation of pigment stones of the bile ducts, which are known to occur in a third of PSC patients. Continuing inflammation eventually results in the development of benign biliary strictures, usually of the EHBD, and they have been reported in up to 7% of patients within 10 years<sup>[30-32]</sup>. Patients usually present increased jaundice, pruritus or relapsing bacterial cholangitis. Progression of these symptoms warrants cholangiographic examination. Endoscopic intervention, with balloon dilatation for biliary strictures, remains the preferred treatment modality. Some authors have advocated the use of short-term biliary stenting to help improve prognosis<sup>[33]</sup>. Surgical intervention should be avoided where possible, as it may predispose to recurrent bacterial cholangitis, while at the same time making future attempts at liver transplantation more challenging.

### **CCC**

CCC is the most feared complication among patients with PSC, occurring in 7% to 15% of patients with PSC<sup>[34,35]</sup>. Chronic inflammation of the bile ducts and cholestasis predispose the development of CCC in PSC patients, although a correlation between severity of disease and incidence of CCC has yet to be established. The difficulty in establishing a diagnosis of CCC lies in the fact that they may not be easily distinguished from benign biliary strictures. The usual serum marker CA 19-9 is not useful in this setting, as PSC itself may result in marked elevations, and secondary bacterial cholangitis

has also been reported to result in increases in CA 19-9 levels<sup>[36,37]</sup>. Nevertheless, in a patient with PSC, sudden and unexpected clinical deterioration, which is associated with progressive elevation of alkaline phosphatase and serum CA 19-9 (> 100 U/mL), in the absence of bacterial cholangitis indicates probable development of CCC.

Novel diagnostic methods include digital image analysis (DIA) and fluorescence *in situ* hybridization (FISH) performed on bile duct brushings collected at the time of ERC. DIA allows deoxyribonucleic acid (DNA) content quantification, assessment of chromatin distribution and nuclear morphology, while FISH offers promise to evaluate bile duct lesions for cellular aneuploidy and chromosomal aberrations<sup>[37]</sup>. A number of studies have demonstrated higher sensitivity of both modalities when compared to standard cytological examination, with comparable specificities<sup>[36,38,39]</sup>.

The diagnosis of CCC requires a meticulous and careful combination of a clinical exam, biochemical results, and imaging procedures (ERC, MRC), especially in patients who present with sudden clinical deterioration. Early diagnosis of CCC in PSC can be treated by liver transplantation in selected medical centers.

## SPECIAL CONSIDERATIONS

### **Pruritus**

Pruritus in PSC is a rather disabling symptom, resulting in a diminished quality of life. The mechanism behind pruritus associated with cholestasis is unknown. Ursodeoxycholic acid (UDCA), cholestyramine, and antihistaminics opiate receptor antagonists have been used to treat patients with cholestatic pruritus<sup>[40]</sup>.

### **Fat-soluble vitamin deficiency**

Fat-soluble, A, D and E, vitamin deficiencies have been recorded to occur in 2%-40% of patients with PSC, especially in those with advanced disease<sup>[41]</sup>. Recommended treatment doses for established or suspected deficiencies are 25-50 000 units two to three times per week orally for Vitamins A and D and 100 U/d for Vitamin E. Vitamin E deficiency is the most difficult to correct, with poor responses to replacement therapy. Vitamin K deficiency, although rare, is treatable with intravenous replacement.

### **Metabolic bone disease**

Metabolic bone disease, usually caused by osteoporosis, rather than osteomalacia, is relatively common and an important complication among patients with PSC<sup>[42]</sup>. It is a rather unfortunate complication, with no proven therapy. Calcitonin and bisphosphonates have been tested on patients with primary biliary cirrhosis with mixed results<sup>[43,44]</sup>, but data on their benefit on PSC is still lacking.

For patients who are on steroid therapy for PSC associated with IBD or AIH, recommendations include close monitoring of bone mineral density with initiation

of vitamin D and calcium supplementation at the first signs of osteopenia.

## MEDICAL TREATMENT OF PSC

### **UDCA**

A hydrophilic dihydroxy bile acid, UDCA has its roots in ancient Chinese medicine. Its ability to dissolve gallstones contributed to its newly found worldwide fame in the eighties. It was then that its benefit for the cholestatic syndromes was established. Described mechanisms of action include stimulation of hepatobiliary secretion, inhibition of apoptosis and the protection of bile epithelial cells from the toxic effects of hydrophobic bile acids<sup>[45]</sup>.

The use of UDCA was first explored after the earlier success with primary biliary cirrhosis. Although the three major studies all showed decreases in liver enzyme levels with UDCA, they failed to demonstrate any improvement in symptoms or liver histology<sup>[46-48]</sup>. In a meta-analysis by Chen<sup>[49]</sup>, no difference was observed between UDCA and placebo regarding overall survival and disease progression with the development of complications, requiring transplantation. UDCA also did not prevent deterioration of histological or cholangiographic findings. However, patients included in these studies had advanced disease, making them less responsive.

The general belief is that although UDCA is widely recommended in the treatment of PSC, there is a desperate need for new therapies which may hopefully prevent disease progression.

### **Immuno modulators/suppressants**

Steroid therapy has been the mainstay for the treatment of autoimmune liver diseases; however, its role in the treatment remains controversial. Good response rates have been observed on patients showing histologic signs of both PSC and AIH, otherwise known as autoimmune cholangitis, an overlap syndrome<sup>[50]</sup>. This group of patients show more characteristic signs of autoimmune disease, such as female predominance, which may account for this response.

In classical PSC, however, the situation is rather bleak. No study has conclusively demonstrated the benefit of systemic steroids in preventing disease progression<sup>[51,52]</sup>. Endoscopic application of topical corticosteroid failed to impress, but in fact resulted in more frequent episodes of bacterial cholangitis<sup>[53]</sup>. The results of these studies have left many clinicians baffled, but an interesting study by Tjandra *et al*<sup>[54]</sup> offered an explanation. They demonstrated a reduction in steroid receptors on hepatic T lymphocytes in a rat model of cholangitis, making them less responsive to steroid treatment. Many authors firmly believe that corticosteroids only help to augment the risks commonly associated with classical PSC, such as metabolic bone disease (osteoporosis) and increased susceptibility to infections.

Several studies on other agents like methotrexate, colchicine, D-penicillamine, pentoxifylline and

tacrolimus<sup>[55,56]</sup>, failed to show any added benefit in the treatment of PSC.

## LIVER TRANSPLANTATION FOR PSC

Liver transplantation is the only option that can reverse or correct end-stage liver disease seen in advanced PSC. Controversy lies in the most appropriate timing for surgery, since transplantation after the development of CCC is associated with a poorer outcome<sup>[57]</sup>. The classical indications still apply, including complicated cirrhosis, intractable itch and fatigue, jaundice refractory to endoscopic or medical treatment or the development of hepatocellular or CCC<sup>[58]</sup>. The MELD system is used in the United States for all patients with end stage liver diseases, regardless of etiology.

Survival rates after transplantation for PSC have improved throughout the years, rates as high as 84%<sup>[59]</sup>. Post-transplantation survival has been found to be dependent on a number of pretransplantation factors, such as compromised renal function and the presence of hepatobiliary malignancy at the time of surgery, with recurrence of the original disease being a particular problem<sup>[60-62]</sup>.

## CONCLUSION

PSC is a chronic slowly progressive cholestatic liver disease of unknown etiology. A number of complications can occur, which require special consideration, the most important of which is the development of CCC. Unfortunately, no medical therapy is currently available for the underlying liver disease. Liver transplantation is an effective, life-extending option for patients with advanced PSC.

Geographical variations include a second peak for age with a lower association with IBD in a Japanese population, female predominance in a lone study from Turkey. The clinical and biochemical Mayo criteria may not be universally applicable, as different patients have shown variations regarding the initial presentation and natural course of the disease.

Directing research towards explaining these geographical differences and understanding the pathogenesis of PSC is required in order to develop better therapies for this devastating disease.

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## ESOPHAGEAL CANCER

# Lithium inhibits proliferation of human esophageal cancer cell line Eca-109 by inducing a G<sub>2</sub>/M cell cycle arrest

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## Abstract

**AIM:** To investigate the effect of lithium on proliferation of esophageal cancer (EC) cells and its preliminary mechanisms.

**METHODS:** Eca-109 cells were treated with lithium chloride, a highly selective inhibitor of glycogen synthase kinase 3β (GSK-3β), at different concentrations (2–30 mmol/L) and time points (0, 2, 4, 6 and 24 h). Cell proliferative ability was evaluated by 3-(4,5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, and cell cycle distribution was examined by flow cytometry. Expressions of p-GSK-3β, β-catenin, cyclin B1, cdc2 and cyclin D1 protein were detected by Western blotting, and the subcellular localization of β-catenin was determined by immunofluorescence. The mRNA level of cyclin B1 was detected by reverse transcription polymerase chain reaction (RT-PCR).

**RESULTS:** Lithium could inhibit the proliferation of Eca-109 cells. Lithium at a concentration of 20 mmol/L lithium for 24 h produced obvious changes in the distribution of cell cycle, and increased the number of cells in G<sub>2</sub>/M phase ( $P < 0.05$  vs control group). Western blotting showed that lithium inhibited GSK-3β

by Ser-9 phosphorylation and stabilized free β-catenin in the cytoplasm. Immunofluorescence further confirmed that free β-catenin actively translocated to the nucleus. Moreover, lithium slightly elevated cyclin D1 protein expression, whereas lowered the cyclin B1 expression after 24 h lithium exposure and no obvious change was observed for cdc2 protein.

**CONCLUSION:** Lithium can inhibit the proliferation of human esophageal cancer cell line Eca-109 by inducing a G<sub>2</sub>/M cell cycle arrest, which is mainly mediated through the inhibition of lithium-sensitive molecule, GSK-3β, and reduction of cyclin B1 expression.

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**Key words:** Lithium; Esophageal cancer; Cell cycle; Glycogen synthase kinase 3β

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## INTRODUCTION

Esophageal cancer (EC) is prevalent in some regions of the world, and occurs at a very high frequency in certain parts of China and the mortality rate ranked the fourth among cancer-related death<sup>[1,2]</sup>. However, the molecular basis of esophageal carcinogenesis remains poorly understood.

Glycogen synthase kinase-3β (GSK-3β) is a serine/threonine kinase that controls cell survival and cell fate through its involvement in multiple signaling pathways<sup>[3]</sup>. Recent studies in colorectal cancer, pancreatic cancer, hepatocellular carcinoma and ovarian cancer<sup>[4-7]</sup> demonstrate that GSK-3β is involved in the process of tumorigenesis. Inhibition of the expression and activity of GSK-3β attenuates cell proliferation and causes apoptosis in colorectal, pancreatic and ovarian cancer cells<sup>[4,5,7]</sup>.

Lithium has been shown to be a specific and noncompetitive inhibitor of GSK-3 $\beta$  activity *in vitro*<sup>[8,9]</sup> and *in vivo*<sup>[10]</sup>. It has been proved that lithium could promote<sup>[11-14]</sup> or inhibit<sup>[7,15-17]</sup> cell cycle transition and proliferation of some primary cultures or cell lines by inhibiting GSK-3 $\beta$ , depending on the cell type. However, whether lithium influences the growth and proliferation of EC cells remains unknown to date.

In this report, we chose Eca-109 cells as a model, and observed the potential role of lithium in cell cycle progression and growth of EC cells and investigated its preliminary mechanisms.

## MATERIALS AND METHODS

### Cell culture and treatment

Human esophageal squamous cell carcinoma cell line Eca-109 was obtained from Institute of Biochemistry and Cell Biology, Shanghai, China and maintained in RPMI 1640 medium (Gibco Biocult, Paisley, UK) supplemented with 10% calf bovine serum (Sijiqing Biotechnology, China), 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin at 37°C in a water-saturated atmosphere of 5% CO<sub>2</sub> in air. For G<sub>0</sub>/G<sub>1</sub> synchronization, when Eca-109 cells grew to 70% confluence, the routine medium was removed and replaced by free-serum medium for 24 h. Then these cells were cultured in the free-serum medium supplemented with 2-30 mmol/L lithium (Alexis, USA) for indicated times.

### 3-(4,5-dimethylthiazole-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT) assay

The IC<sub>50</sub> of lithium on Eca-109 cells was measured by MTT assay, which was conducted as described before<sup>[18]</sup>, and was calculated by Logit method. Briefly, one thousand Eca-109 cells ( $5 \times 10^3$ /mL) were seeded in 96-well plates and cultured for 12 h. When they were adhesive, these Eca-109 cells were exposed to a range of concentrations of lithium from 2 to 30 mmol/L for 72 h, respectively. The Eca-109 cells treated with routine medium served as negative control. All exposures were performed in six wells. At the end of exposure, 20  $\mu$ L of MTT (Sigma, USA) stock solution (5 mg/mL) was added to 200  $\mu$ L of medium in each well and plates were incubated for 4 h at 37°C, and subsequently 150  $\mu$ L of dimethyl sulfoxide (DMSO) was added to each well. The plates were incubated about 10 min at room temperature and read with enzyme-linked immunosorbent assay (490 nm) to determine absorbance values ( $A$ ). The rate of inhibition was calculated by the following equation:

$$\text{Rate of growth inhibition (\%)} = (1 - A_{\text{treated}}/A_{\text{control}}) \times 100\%.$$

### Flow cytometry

Before the analysis was conducted by FCM, Eca-109 cells were exposed to lithium at a concentration of 20 mmol/L for 0, 2, 4, 6 and 24 h, respectively. According to the routine method,  $1.0 \times 10^6$  Eca-109 cells from the control and treated groups were harvested by

trypsinization and centrifugation, washed twice with ice-cold PBS and resuspended in PBS containing 10 mg/L propidium iodide (Sigma, USA) and 100 mg/L RNase A (Huamei Biotechnology, China) and then incubated at 25°C in the dark for at least 30 min. The percentage of cell population in each phase of the cell cycle was measured using FACStar and the results were analyzed with the software CELLQUEST. All measurements were carried out with the same instrument under the same experimental conditions.

### Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from specimens using Trizol reagent (Invitrogen, USA) and treated with DNase I (Tiangen Biotechnology, China). cDNA was synthesized from 2  $\mu$ g of total RNA according to the manufacturer's instruction (Fermentas, USA), and negative control reactions were run without reverse transcriptase. An equal volume of product was subjected to PCR. The levels of gene transcripts were quantified as the ratio of the intensity of the target gene to the intensity of  $\beta$ -actin. The PCR primers used in this study were as follows: forward primer 5'-CCATTATTGATCGGTTCATGCAGA-3' and reverse primer 5'-CTAGTGCAGAATTCAAGCTGTG GTA-3' for cyclin B1 (585 bp)<sup>[19]</sup>.  $\beta$ -actin was amplified as internal control using the following primers: forward primer 5'-AGTTGCGTTACACCCTTTCTTG-3' and reverse primer 5'-TCACCTTCACCGTTCCAGTT-3', with a 150 bp fragment product. The amplification conditions were 30 cycles of 94°C for 50 s, 55°C for 40 s, 72°C for 40 s for cyclin D1, and 30 cycles of 94°C for 50 s, 59°C for 40 s, 72°C for 40 s for cyclin B1 and  $\beta$ -actin. PCR products were run on 1.6% agarose gel and results were analyzed using Image Tool 3.0.

### Extraction of nuclear protein

Cells were lysed in 400  $\mu$ L of ice-cold buffer A [10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 0.1 mmol/L ethylene diamine tetraacetic acid (EDTA), 0.1 mmol/L EGTA, 1 mmol/L DTT, 0.5 mmol/L PMSF] by gentle pipetting. The cells were allowed to swell on ice for 15 min, then 40  $\mu$ L of a 10% solution of Nonidet P-40 was added and the tube was vigorously vortexed for 10 s. The homogenate was centrifuged for 30 s in a microcentrifuge. The supernatant was transferred and the nuclear pellet was lysed with 50  $\mu$ L of buffer B [20 mmol/L HEPES (pH 7.9), 0.42 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L PMSF] and the tube was vigorously rocked at 4°C for 15 min on a shaking platform. The nuclear extract was centrifuged for 5 min in a microcentrifuge at 4°C. The supernatant containing nuclear protein was stored at -70°C.

### Western blotting

Cells were washed twice with ice-cold PBS, collected by adding 0.25% trypsin and lysed in buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L ethylene diamine tetraacetic acid (EDTA), 0.25%

sodium deoxycholate, 1% TritonX-100, 0.1% sodium dodecyl sulfate (SDS), 1 mmol/L NaF, 1 mmol/L Na<sub>3</sub>VO<sub>4</sub>], protease inhibitors (10 mg/L aprotinin and 1 mmol/L phenylmethylsulfonyl fluoride) were added to obtain whole cell protein. Equal amounts of cell protein, quantified by BCA protein assay kit (Pierce Biotechnology, Rockford, IL), were subjected to 10% SDS-polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride membrane. The membranes were blocked with 5% non-fat milk in TBST [50 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 0.1% Tween 20] for 2 h at room temperature, and subsequently incubated with primary antibody (anti-p-GSK3β, 1:400, anti-cyclin D1, 1:200, anti-cyclin B1, 1:400, anti-cdc2, 1:500, β-actin, 1:2000, were all purchased from Sant Cruz Biotechnology, USA, and anti-β-catenin, 1:400, was purchased from Chemicon Biotech, USA) in blocking buffer at 4°C overnight. Following a wash with TBST, the membranes were incubated with horseradish peroxidase conjugated rabbit anti-mouse secondary antibody (1:1000, Dako, Denmark) for 2 h at room temperature. The membranes were washed with TBST and protein bands were visualized by enhanced chemiluminescence according to the manufacturer's instructions (KPL, USA). The β-actin bands were taken as loading control. The protein quantity was analyzed by UTHSCSA Image Tool 3.0. The target protein expression was evaluated by the relative intensity ratio of target protein/loading control.

### Immunofluorescence

Cells were plated on the coverslips which had been put into the six-well plates in advance. After being treated with 20 mmol/L lithium for the indicated time as described above, cells were washed with ice-cold phosphate-buffered saline (PBS) prior to fixing with 4% paraformaldehyde for 15 min at room temperature. Next, cells were treated with 1 g/L TritonX-100 for 30 min and subsequently incubated with a blocking solution (10% normal goat serum in PBS) for 20 min, anti-β-catenin monoclonal antibody in PBS (1:100, Chemicon, USA) overnight at 4°C and followed by fluorescein isothiocyanate-labeled goat anti-mouse IgG (Dako Biotechnology, Denmark) for 1 h at room temperature. Finally, the cells were visualized and photographed with an Olympus fluorescence microscope.

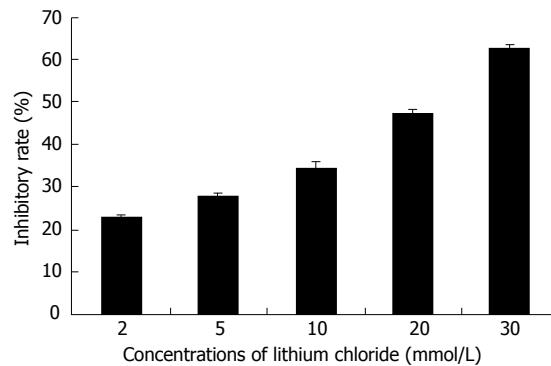
### Statistical analysis

The data were obtained from at least three repeated experiments and expressed as mean ± SD. In order to compare the data between the treated groups and the control group, statistical significance was analyzed through analysis of variance (ANOVA) and values of  $P < 0.05$  were considered significant.

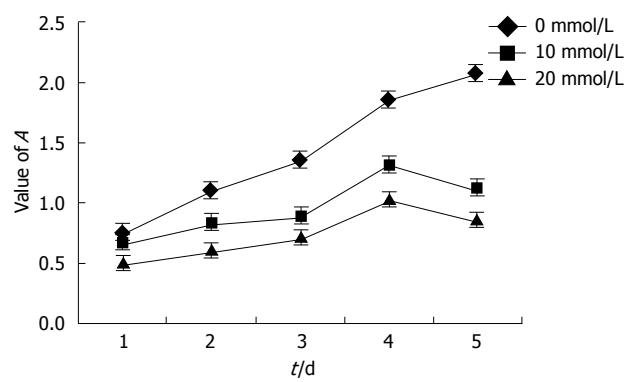
## RESULTS

### Lithium inhibits proliferation of Eca-109 cells

The mean IC<sub>50</sub> of lithium was  $21.7 \pm 0.5$  mmol/L. The effect of inhibition of lithium on Eca-109 cells could be



**Figure 1** Lithium could impose inhibition on Eca-109 cells in day 3. With the increased concentration of lithium, the inhibitory effect was enhanced. Each bar came from six wells of 96-well plate and represented mean ± SD.



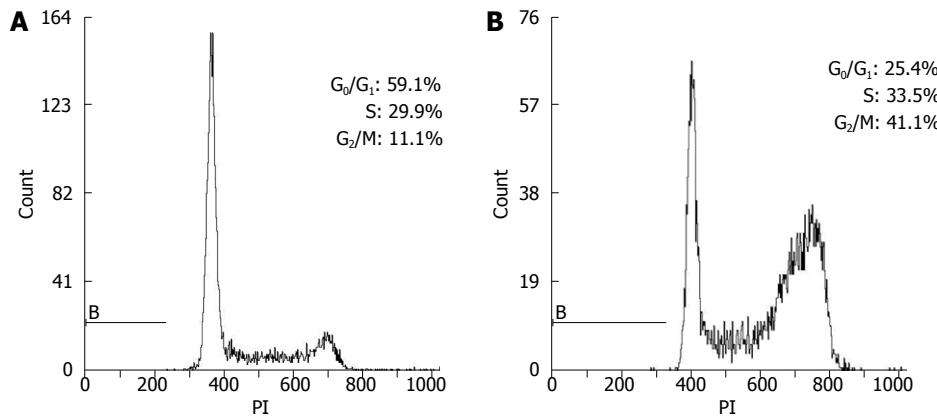
**Figure 2** Growth curves of Eca-109 cells plotted by MTT assay. Each point was the mean ± SD from six independent experiments.

observed at a concentration of 2 mmol/L. From 2 to 30 mmol/L, the inhibitory effect was enhanced along with the increased concentration of lithium. The exhibition of dose-response relationship could be observed (Figure 1).

The growth curves of Eca-109 cells within 5 d after treatment with lithium were also plotted according to the data obtained from the MTT assay (Figure 2). The Eca-109 cells treated with different concentrations of lithium showed much slower growth than the untreated cells. The results indicated that the proliferation of Eca-109 cells *in vitro* was inhibited compared to that of the untreated cells.

### Lithium induces a G<sub>2</sub>/M cell cycle arrest in Eca-109 cells

To determine whether the lithium-induced inhibition of proliferation of Eca-109 cells was due to altered cell cycle regulation, Eca-109 cells were treated with lithium for various times, and cell cycle profiles were monitored by flow cytometric analysis of DNA content (Figure 3 and Table 1). The percentage of cells in G<sub>0</sub>/G<sub>1</sub> phase decreased with the length of treatment from 59.6% at 0 h to 22.1% at 24 h, and the percentage of cells in S phase increased accordingly from 28.5% at 0 h to 42.5% at 6 h. At 24 h, while the cells were entering from S phase to G<sub>2</sub>/M phase, the percentage of cells in S phase decreased from 42.5% at 6 h to 31.5% at 24 h, but the percentage of cells in G<sub>2</sub>/M phase was increased markedly. The distribution in the phases of



**Figure 3** Lithium inhibited the proliferation of Eca-109 cells by an arrest in G<sub>2</sub>/M phase. Eca-109 cells were treated with lithium (20 mmol/L) for 0 h (A) and 24 h (B). Eca-109 cells were harvested and cell cycle profiles were obtained by staining with propidium iodide (PI).

**Table 1** Lithium affects cell cycle progression in Eca-109 cells

Groups	Distribution in cell cycle (%) <sup>1</sup>		
	G <sub>0</sub> /G <sub>1</sub>	S	G <sub>2</sub> /M
0 h	59.6	28.5	11.9
2 h	53.6	33.6	12.8
4 h	47.4 <sup>a</sup>	39.4 <sup>a</sup>	13.2
6 h	43.1 <sup>a</sup>	42.5 <sup>a</sup>	14.5
24 h	22.1 <sup>a</sup>	31.5	46.4 <sup>a</sup>

<sup>1</sup>Lithium reduced the G<sub>0</sub>/G<sub>1</sub> phase population sharply and increased the population in G<sub>2</sub>/M phase accordingly. <sup>a</sup>P < 0.05 vs the control group.

cell cycle indicated that lithium could promote Eca-109 cells entering into S phase and then G<sub>2</sub>/M, in which the population was increased by approximate 4 folds compared with that of the untreated group at 24 h. These results suggested that the growth-inhibitory effect of lithium on Eca-109 cells might be partly due to its ability to induce G<sub>2</sub>/M cell cycle arrest.

#### Lithium induces a G<sub>2</sub>/M arrest by decreased cyclin B1 expression

Having established that lithium induces a G<sub>2</sub>/M cell cycle arrest, we attempted to characterize, at the molecular level, the mechanisms by which this effect is achieved. Since the cyclin B1/cdc2 complex is a master intracellular regulator entry into mitosis<sup>[20]</sup>, we therefore investigated the effects of lithium in Eca-109 cells on key regulators of the G<sub>2</sub> to M phase transition, cdc2 and cyclin B1 using Western blotting. Treatment for 24 h with lithium reduced the protein expression of cyclin B1 (Figure 4A). Moreover, down-regulation of cyclin B1 expression at 24 h was also confirmed at the mRNA levels by RT-PCR (Figure 4B). Similar to the untreated cells, the total levels of cdc2 protein remained stable upon lithium treatment (Figure 4C). These findings suggested that the lithium-induced G<sub>2</sub>/M arrest was probably due to decreased expression of cyclin B1, and had nothing to do with the expression of cdc2 protein.

#### Lithium induces β-catenin stabilization via inhibition of GSK-3β

Inhibition of GSK-3β (GSK-3β Ser-9 phosphorylation)

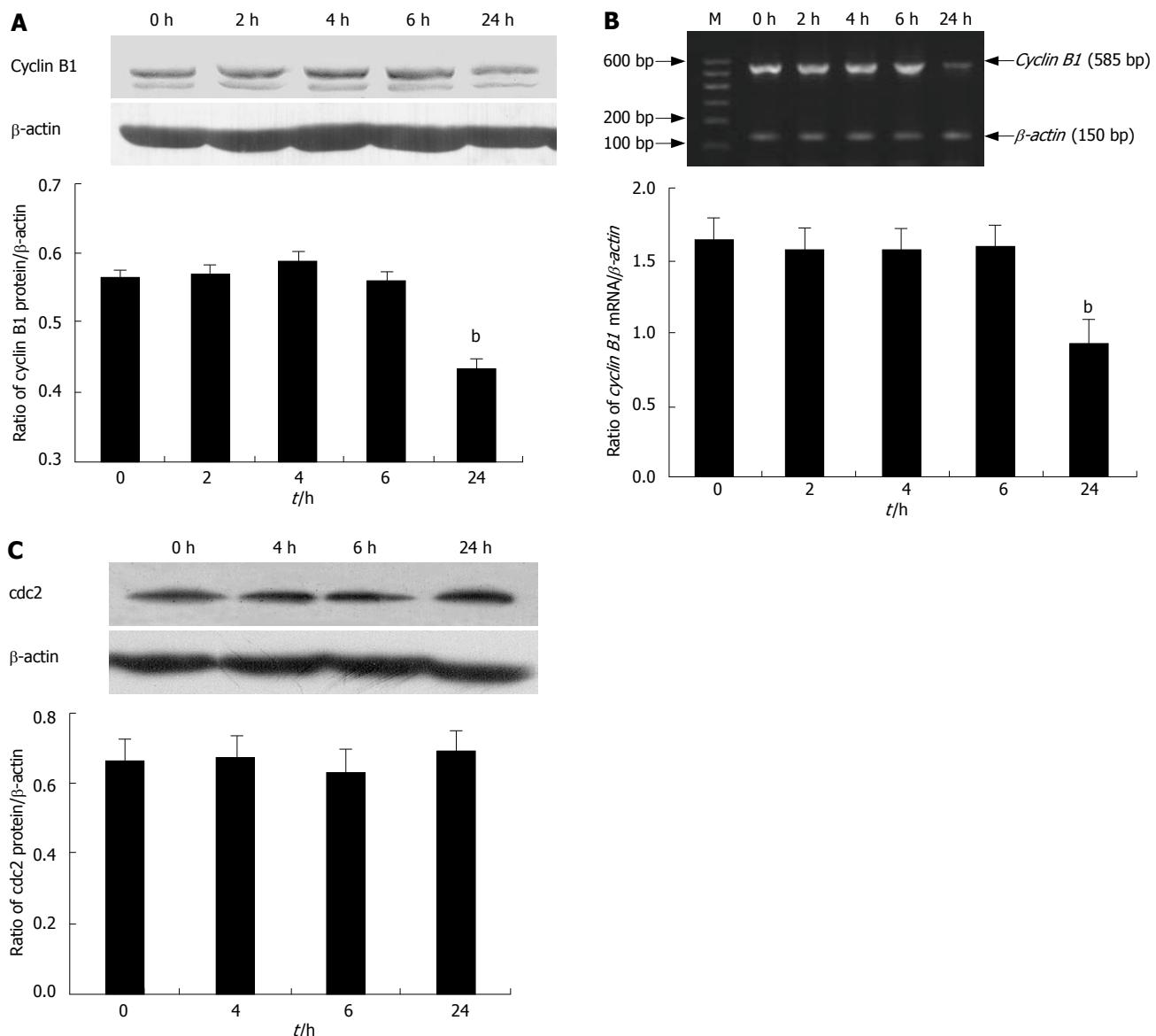
was assessed by Western blotting using a phospho-Ser-9-specific antibody. As shown in Figure 5A, 20 mmol/L lithium increased the phosphorylated inactive form of GSK-3β in Eca-109 cells. Moreover, at a range of 2-30 mmol/L, this effect of lithium was dose-dependent (data not shown). β-catenin, a signaling molecule in the Wnt/β-catenin signaling pathway, is a target of GSK-3β. Inactivation of GSK-3β by Wnt signaling or by lithium leads to stabilization and nuclear translocation of β-catenin<sup>[21]</sup>. Here, we investigated the expression of β-catenin by Western blotting of total and nuclear extracts from Eca-109 cells treated with lithium. Results indicated that the intracellular total protein concentrations of β-catenin were increased following stimulation with 20 mmol/L lithium (Figure 5A). An increase of β-catenin nuclear pool was also observed in Eca-109 cells, exhibiting the similar trend (Figure 5B). In contrast to the untreated cells, in which β-catenin was primarily located in the cytoplasm, β-catenin was predominantly located in the nuclear of the lithium-treated Eca-109 cells (Figure 5C). These results showed that lithium could induce β-catenin nuclear translocation by inhibition of GSK-3β activity.

#### Lithium up-regulates intranuclear cyclin D1 levels

GSK-3β has been shown to regulate cyclin D1 proteolysis by directly phosphorylating cyclin D1 at Thr-286<sup>[22,23]</sup>. We hypothesized that inhibition of GSK-3β by lithium would be associated with an increase in cyclin D1 protein. As shown in Figure 6, lithium treatment induced a slight increase in the cyclin D1 protein levels by 1.3-fold at 4 h and 1.8-fold at 6 h of lithium treatment as compared with control cells, and the cyclin D1 protein levels diminished to the normal level at 24 h, which may be attributed to the decrease of cells in G<sub>0</sub>/G<sub>1</sub> phase.

## DISCUSSION

In this report, we treated EC cell line Eca-109 cells with different concentrations of lithium and in different time points, and observed the effect of lithium on EC cells. Results indicated that lithium inhibited the proliferation of Eca-109 cells (Figures 1 and 2). We performed flow cytometry to assess cell cycle progression of Eca-109 cells treated with

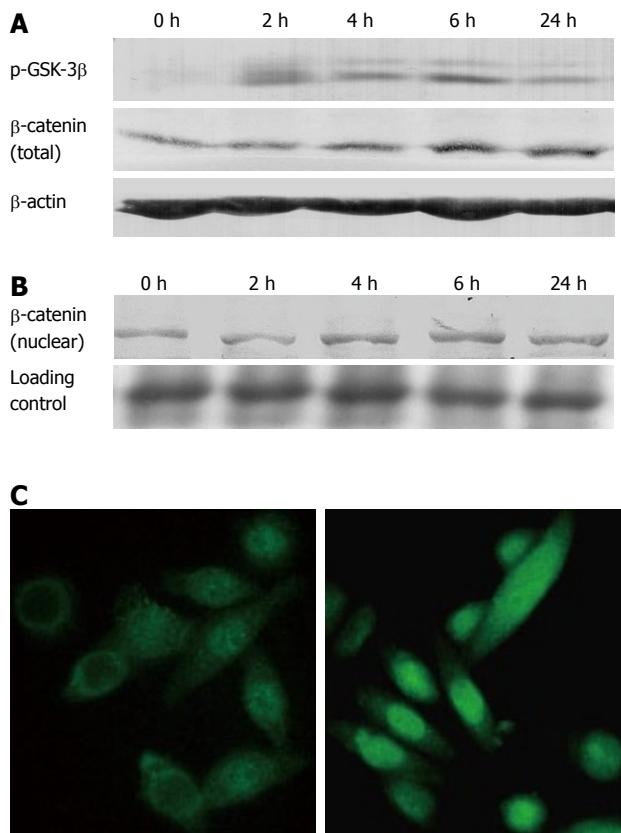


**Figure 4** Effects of lithium on cell cycle regulatory molecules. **A:** Western blotting analysis for cyclin B1 protein levels after treatment with 20 mmol/L lithium; **B:** RT-PCR analysis for quantification of the relative mRNA abundance of *cyclin B1* after treatment with 20 mmol/L lithium; **C:** Western blotting analysis for cdc2 protein levels after treatment with 20 mmol/L lithium. <sup>b</sup>P < 0.01 vs the control group.

lithium. Analysis of cell cycle distribution showing an increase of cells in G<sub>2</sub>/M phase and a decrease in G<sub>0</sub>/G<sub>1</sub> phase indicated that lithium could lead to a G<sub>2</sub>/M cell cycle arrest (Figure 3 and Table 1), which is the common characteristic of cell cycle arrest induced by lithium. However, different cell types have their own doses of sensitivity<sup>[15,17]</sup>. In the report of Mao *et al*<sup>[17]</sup>, 5 mmol/L lithium was enough to induce a G<sub>2</sub>/M phase arrest of bovine aortic endothelial cells. In our study, the mean IC50 of lithium on Eca-109 cells growth was 21.7 ± 0.5 mmol/L by MTT assay (Figure 1). Therefore, 20 mmol/L lithium was used to treat EC cells for indicated times for correlation detections.

Inhibition of cell proliferation can occur through activation of several possible checkpoints during cell cycle progression. Lithium is a highly selective inhibitor of GSK-3β, a multifunctional serine/threonine kinase which has a variety of putative substrates including cyclin

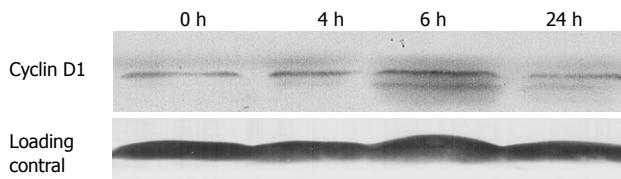
D1, p21<sup>Waf1/Cip1</sup>, and transcription factors like c-myc, c-jun and β-catenin<sup>[24]</sup>, which are implicated in the regulation of cell proliferation. It is well known that intracellular β-catenin levels are regulated through GSK-3β mediated phosphorylations on its serine and threonine residues (at Ser-33, Ser-37 and Thr-41)<sup>[25]</sup>. Inhibition of GSK-3β activity by lithium can result in β-catenin stabilization and its accumulation in the nucleus<sup>[26]</sup>. Our Immunoblot results showed that in Eca-109 cells lithium inhibited GSK-3β by Ser-9 phosphorylation and stabilized free β-catenin in the cytoplasm (Figure 5A). An increase of β-catenin nuclear pool was also observed (Figure 5B). Immunofluorescence studies further confirmed that free β-catenin translocated to the nucleus where β-catenin was transcriptionally active (Figure 5C). Although inhibition of cell proliferation by β-catenin signaling has not been described to date, β-catenin may be the signal for cell cycle arrest. Orford *et al*<sup>[27]</sup> showed that the nuclear



**Figure 5** Lithium induced  $\beta$ -catenin stabilization via inhibition of GSK-3 $\beta$  in vitro. **A:** Eca-109 cells were incubated with 20 mmol/L lithium for 0-24 h. Expressions of p-GSK-3 $\beta$  and total  $\beta$ -catenin protein were analyzed by Western blotting. The untreated Eca-109 cells (0 h) served as negative control; **B:** Western blotting analysis for  $\beta$ -catenin protein level of nuclear lysates; **C:** Immunofluorescence demonstrated a predominant cytoplasm localization of  $\beta$ -catenin in the untreated cells (left) whereas after incubation with lithium (20 mmol/L) for 6 h,  $\beta$ -catenin was predominantly located in the nucleus of Eca-109 cells (right).

localization of  $\beta$ -catenin was cell cycle regulated in the epithelial Madin-Darby canine kidney cells with a peak during the S phase. Interestingly, with the percentage of cells in S phase increased,  $\beta$ -catenin expression increased accordingly in our experiments (Table 1 and Figure 5). Damalas *et al*<sup>[28]</sup> reported that the accumulation of p53 in mouse fibroblasts NIH3T3 overexpressed a stable form of  $\beta$ -catenin (S37A). Stabilization of p21<sup>Waf1/Cip1</sup> and induction of G<sub>2</sub>/M cell cycle arrest by lithium was demonstrated in bovine aortic endothelial cells, where lithium up-regulated p21<sup>Waf1/Cip1</sup> protein level through activation and stabilization of p53<sup>[17]</sup>, an effect of lithium possibly associated with GSK3 $\beta$ , as p53 was recently shown to be a substrate of this kinase<sup>[29]</sup>. Furthermore, lithium was shown to stabilize p21<sup>Waf1/Cip1</sup> by inhibiting GSK-3 $\beta$  activity, and induce G<sub>2</sub>/M cell cycle arrest in human umbilical vein endothelial cells<sup>[30]</sup>. It is thus possible that a sustained retention of  $\beta$ -catenin in the nucleus during G<sub>2</sub> or G<sub>2</sub>/M transition can be a signal for p53 induction and cell cycle arrest.

The cyclin B1/cdc2 complex is a master intracellular regulator entry into mitosis<sup>[20]</sup>, G<sub>2</sub>/M cell cycle arrest may be associated with decreased expression or activity



**Figure 6** Lithium induced cyclin D1 stabilization via inhibition of GSK-3 $\beta$  in vitro. Eca-109 cells were incubated with 20 mmol/L lithium for 0-24 h. Expression of cyclin D1 was analyzed by Western blotting. The untreated Eca-109 cells (0 h) served as negative control.

of this complex. Recently, Smits *et al*<sup>[15]</sup> observed a G<sub>2</sub>/M cell cycle arrest in various transformed (P19 embryonal carcinoma, U2OS osteosarcoma, and SK-N-MC neuroepithelioma) or immortalized (NIH3T3) cell lines after lithium treatment. They found that the activity of the cyclin B1/cdc2 complex was impaired after lithium treatment due to sustained phosphorylation of cdc2 on tyrosine residue 15, and the reduction in cyclin B1/cdc2 kinase activity was not caused by the reduction of cyclin B1 expression, since cyclin B1 protein levels were not influenced by lithium treatment. We detected the expression of cyclin B1 and cdc2 protein, and found that lithium lowered protein levels of cyclin B1 (Figure 3A), which is also a signal for cell cycle arrest in G<sub>2</sub>/M. The mRNA levels of *cyclin B1* exhibited the similar results (Figure 3B). The above results suggested that lithium could influence the expression of cyclin B1 at the levels of both transcription and translation in Eca-109 cells. Chen *et al*<sup>[31]</sup> observed that lithium treatment caused a cell cycle arrest at G<sub>2</sub>/M phase in pig airway epithelial cells, thus inhibiting proliferation of the cells. However, the increased expression of cyclin B1 was observed, which may be attributed to the accumulation of cells in G<sub>2</sub> phase. Our results indicated that G<sub>2</sub>/M cell cycle arrest was partly due to the reduction of cyclin B1 expression. However, further research is needed to clarify the mechanisms of cyclin B1 expression induced by lithium. Moreover, lithium treatment produced no obvious influences on cdc2 protein (Figure 3C). As we did not investigate the activity of the cyclin B1/cdc2 kinase, we could not know the change of cyclin B1/cdc2 kinase activity.

Previously, it was reported that phosphorylation of cyclin D1 at Thr-286 by GSK-3 $\beta$  regulates positively proteasomal degradation of cyclin D1<sup>[22]</sup>. Therefore, inactivation of GSK-3 $\beta$  was expected to lead to an increase of cyclin D1 protein. Our results agreed well with this rationale. Lithium treatment resulted in the inactivation of GSK-3 $\beta$  and induced a slight increase of cyclin D1 protein at 4 and 6 h (Figure 6). These results indicated that cyclin D1 might be involved in the transition of cell cycle from G<sub>1</sub> to S phase in Eca-109 cells induced by lithium. Chen *et al*<sup>[31]</sup> observed that lithium treatment increased cyclin D1 expression and induced a G<sub>2</sub>/M cell cycle arrest in pig airway epithelial cells, which was consistent with our results. However, they failed to find evidence of G<sub>1</sub>/S transition. Mao *et al*<sup>[17]</sup> also observed a slight increase of cyclin

D1 and G<sub>2</sub>/M cell cycle arrest in lithium-treated bovine aortic endothelial cells. On the contrary, a recent investigation on NIH3T3 cells indicated that the change of GSK-3β activity by lithium had no effect on cyclin D1 expression<sup>[32]</sup>. The causes of the discrepancy from these data might lie in the different experimental methods and different cell lines as well.

In conclusion, our present study demonstrated, for the first time, that lithium can arrest the growth of EC cells and induce a G<sub>2</sub>/M cell cycle arrest, which is mainly mediated through the inhibition of lithium-sensitive molecule, GSK-3β, and reduction of cyclin B1 expression. However, further studies are needed to determine the precise mechanisms that contribute to the regulation.

## COMMENTS

### Background

Esophageal squamous cell carcinoma (ESCC) is one of the leading causes of cancer-related death in certain parts of China, and the molecular basis of esophageal carcinogenesis remains poorly understood. It is necessary to develop effective new strategies for the treatment of esophageal cancer (EC).

### Research frontiers

Recent researches have proved that lithium could promote or inhibit cell cycle transition and proliferation of certain primary cultures or cell lines by inhibiting GSK-3β, depending on the cell type. However, whether lithium influences the growth and proliferation of EC cells remains unknown to date.

### Innovations and breakthroughs

This study demonstrated for the first time that lithium can inhibit the proliferation of esophageal squamous cell carcinoma cells (Eca-109), which is mainly mediated by the inhibition of GSK-3β and reduction of cyclin B1 expression.

### Applications

This study indicates the possibility for the treatment of the esophageal squamous cell carcinoma with lithium chloride.

### Terminology

Lithium is an inhibitor of GSK-3β activity. Lithium has been reported to reduce GSK-3β activity in two ways, both directly and by increasing the inhibitory phosphorylation of GSK-3β. These dual effects can act in concert to magnify the influence of lithium on crucial GSK-3β-regulated functions (gene expression, cell structure and survival).

### Peer review

The purpose of this study is reasonable and results are clearly demonstrated, however, further studies are needed to determine the precise mechanisms that contribute to the regulation.

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## ESOPHAGEAL CANCER

# Antitumor effect and mechanism of *Gecko* on human esophageal carcinoma cell lines *in vitro* and xenografted sarcoma 180 in Kunming mice

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growth factor (VEGF) and basic fibroblast growth factor (bFGF) were detected by immunohistochemistry. The cell apoptotic rate was detected by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay.

**RESULTS:** The *A* value in each group treated with *Gecko* after 72 h was reduced significantly in EC9706 and in EC1. The tumor weight in each group of *Gecko* was decreased significantly ( $1.087 \pm 0.249$  vs  $2.167 \pm 0.592$ ;  $1.021 \pm 0.288$  vs  $2.167 \pm 0.592$ ;  $1.234 \pm 0.331$  vs  $2.167 \pm 0.592$ ;  $P < 0.01$ , respectively). However, the thymus index and Spleen index of mice in *Gecko* groups had no significant difference compared with the NS group. The immunoreactive score of VEGF and bFGF protein expression of each *Gecko* group by immunohistochemical staining were lowered significantly. The apoptosis index (AI) of each group was increased progressively with increase of dose of *Gecko* by TUNEL.

**CONCLUSION:** *Gecko* has anti-tumor effects *in vitro* and *in vivo*; induction of tumor cell apoptosis and the down-regulation of protein expression of VEGF and bFGF may be contributed to anti-tumor effects of *Gecko*.

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**Key words:** Chinese medicine *Gecko*; Human esophageal carcinoma cells; S180 sarcoma of mouse; Vascular endothelin growth factor; Basic fibroblast growth factor; Apoptosis

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## INTRODUCTION

Malignant tumor is always a great menace to human health. The incidence and mortality of tumor keep ascending all over the world. Anti-tumor therapies contain surgery, radiotherapy and chemotherapy, and it depends on synthetic agents to a great extent in chemotherapy. Although chemical anti-neoplastics have definite effect; they cause severe adverse effects. Moreover, many chemotherapy drugs bring about multiple drug resistance. Great efforts have been made to develop new anti-cancer pharmaceuticals from Chinese herbal medicine<sup>[1-4]</sup>.

*Gecko* is a traditional Chinese medicine found to restrain inflammation and allergic response, detumescence and alimentation. *Gecko* is also called *Tian Long*, *Shou Gong*. It contains *Gekko swinhonis* Gunther, *Gekko japonicus*, etc.. It is cold in nature and flavor. The dosage forms are pill, powder and mastic<sup>[5]</sup>. Although the *Gecko* was not recorded in a medical dictionary, there have been reports that *Gecko* and its compound prescriptions could treat malignant tumors, tuberculosis, osteomyelitis and syrinx. In clinical practice, it has definite effect against malignant tumors, especially digestive system tumors, such as esophagus cancer, gastric cancer and liver cancer<sup>[6-8]</sup>. Research on *Gecko* mainly focused on the anti-tumor compound prescriptions, but there have been few pharmacological studies of *Gecko* and its mechanisms of anti-tumor action.

The present study was to observe the anti-tumor activities of *Gecko* *in vivo* and *in vitro* using the transplanted tumor model of the mouse S180 sarcoma on mice and the human esophageal carcinoma cells and investigate the effects of *Gecko* on cell apoptosis and the expression of (vascular endothelin growth factor) VEGF and (basic fibroblast growth factor) bFGF of S180 mice.

## MATERIALS AND METHODS

### Materials

Chinese medicine *Gecko* was purchased from Anhui Bozhou Yonggang Co. Ltd. They were identified *Gekko japonicus*. The whole dry *Gecko* were ground to fine powder and diluted in suspension using 0.2% carboxymethyl cellulose (CMC). Cytoxin (CTX) was purchased from Jiangsu Hengrui Medicine Co. Ltd. (Batch No. H32020857). Fifty Kunming mice and ten SD rats were provided by the Medical Experimental Animal Center, Henan Province. All were female. The code number of the animals was 0001350. The human esophageal carcinoma EC9706 cell line was of esophageal carcinoma of fungating type which is well-differentiated squamous cell carcinoma, EC1 cell line was isolated from esophageal carcinoma cell line EC9706 and one subline, and the S180 mouse sarcoma cell line was kindly provided by the Medical College, Zhengzhou University.

### Cell culture and establishment of S180 model

The cells were grown in a monolayer culture containing

humidified 5% CO<sub>2</sub> in air at 37°C. They were cultured in RPMI-1640 (Sigma, USA) medium supplemented with 10% fetal calf serum, 100 U/mL and penicillin and 100 mg/L streptomycin. The S180 model was established by subcutaneous injection as previously described<sup>[9]</sup>. Briefly, the S180 mouse sarcoma cells with ascites were harvested, diluted with sterilized saline at a ratio of 1:8 (cell concentration was adjusted to 1.0 × 10<sup>6</sup>/mL), and inoculated subcutaneously into the right armpit region of Kunming mice.

### Preparation of the serum with medicine

Ten SD rats (aged 10-12 wk and weighing 280 ± 30 g) were randomly divided into five groups: the negative group (NS group), the positive group (CTX group) and three *Gecko* groups. The CTX group received 50 mg/kg intraperitoneally once a day. Three *Gecko* groups were treated respectively with oral administration of *Gecko* at a dose of 13.5 g/kg, 9 g/kg and 4.5 g/kg, twice a day. The NS group received the equivalent amounts of normal saline in the same way. After 1 wk of treatment, according to the method principle of Li Yikui<sup>[10,11]</sup>, blood was collected aseptically and serum was collected by centrifugation. It was deactivated at 56°C for 30 min and filtrated for sterilization. Medical serum was stored at -20°C for use.

### 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell growth was measured by a modified MTT assay<sup>[12,13]</sup>. The logarithmic cells (EC9706, EC1) were dispersed with 0.02% (w/v) EDTA to prepare 1 × 10<sup>4</sup>/mL cell suspension, and partitioned into 96-well plates at 180 μL/well for 24 h culture in a 5% CO<sub>2</sub> incubator under 37°C. Five groups were divided, the negative group, the CTX group and the three *Gecko* groups. Each group had 6 wells. Each well was treated respectively with 20 μL medical serum prepared as above. Cells were incubated for 24, 48 and 72 h. Then 20 μL MTT (Sigma, USA) was added to each well and the cells were further incubated at 37°C for 4 h. The supernatant was removed and 200 μL DMSO (Sigma, USA) was added into each well to solubilize the formazan product. The absorbance also known as *A* value at wavelength of 470 nm was measured by a microplate reader (Sigma, USA). Triplicate experiments were performed in a parallel manner for each concentration point and the results were presented as mean ± SD. Cell inhibitory rate was calculated by the following formula: inhibitory rate (%) = (1-*A* treated/*A* control) × 100%.

### Viable cell number counting and growth curve

EC9706, EC1 cells (1 × 10<sup>4</sup>/mL cell suspension) were plated onto 96-well plates at 180 μL/well. Each well was treated respectively with 20 μL medical serum prepared as above and cultured for 7 d. Viable cells were counted under the inverted light microscope by trypan blue dye (Sigma, USA) exclusion method and growth curves were drawn.

**Table 1** Inhibition effect of *Gecko*-contained serum on EC9706 by MTT assay (mean  $\pm$  SD, n = 6)

Groups	24 h		48 h		72 h	
	A	Inhibition rate (%)	A	Inhibition rate (%)	A	Inhibition rate (%)
NS	0.472 $\pm$ 0.05		0.97 $\pm$ 0.118		1.63 $\pm$ 0.224	
CTX	0.286 $\pm$ 0.06 <sup>a</sup>	39.1	0.55 $\pm$ 0.09 <sup>a</sup>	42	0.82 $\pm$ 0.163 <sup>a</sup>	49.6
Gecko 1	0.355 $\pm$ 0.04 <sup>a</sup>	24.8	0.65 $\pm$ 0.136 <sup>a</sup>	32.5	0.99 $\pm$ 0.154 <sup>a</sup>	39.1
Gecko 2	0.372 $\pm$ 0.07 <sup>a</sup>	21	0.721 $\pm$ 0.09 <sup>a</sup>	25.1	1.15 $\pm$ 0.925 <sup>a</sup>	29.8
Gecko 3	0.393 $\pm$ 0.03 <sup>a</sup>	16.4	0.756 $\pm$ 0.07 <sup>a</sup>	22.4	1.27 $\pm$ 0.07 <sup>a</sup>	22.3

<sup>a</sup>P < 0.05 vs control.

### Inhibitory effect of *Gecko* on S180 in vivo

Twenty-four hours after establishment of S180 model, the fifty transplanted mice (aged 8-10 wk and weighing 26  $\pm$  2 g) were randomly divided into five groups: the NS group, the CTX group (CTX was proved to have definite effect against mouse S180 sarcoma and frequently used in experiment), and the three *Gecko* groups. They were treated respectively with oral administration of saline once a day, intraperitoneal injection of CTX 100 mg/kg only once, and oral administration of *Gecko* at a dose of 13.5 g/kg, 9 g/kg and 4.5 g/kg once a day. After 2 wk of treatment, the anti-tumor activity was evaluated by tumor tissue weighing. The following formula was used: Tumor inhibitory rate (%) = (1-average tumor weighing of administration team/average tumor weighing of the control)  $\times$  100%<sup>[9]</sup>.

### Immune function

According to the above-mentioned methods, at the 14th day, 5% chicken-red cells were injected intraperitoneally into each group. After 12 h, the mice were killed and 1 mL peritoneal fluid was drawn for glass slide. After incubated for 30 min, peritoneal fluid was fixed with the mixture of acetone/methanol (1:1, v/v) and dyed with 4% Giemsa stain. Peritoneal macrophages were counted under microscope. The effect of *Gecko* on phagocytosis of enterocoelia macrophage was evaluated by the chicken-red cell phagocytic index and phagocytic rate<sup>[14]</sup>. At the same time, thymus and spleen were taken from mice. The impact on immune organ was evaluated based on the thymus index and spleen index<sup>[15]</sup>.

### Immunohistochemistry

The tumor tissues were fixed with 10% neutral formalin at room temperature for 24 h. The paraffin-embedded specimens were cut into sections with a thickness of 5  $\mu$ m. The detection procedure was done as described in Kit protocol (Wuhan Boster Biological Technology Co. Ltd). PBS instead of the first antibodies was used in the negative control. The VEGF and bFGF positive cells were defined when there was an aggregation of brown particles in the cytoplasm of the tumor cells. The immunoreactive score was determined by the sum of extension and intensity as reported previously<sup>[16]</sup>. The intensity of staining was scored on a scale of 0 to 3 (0 = negative staining, 1 = weakly positive staining, 2 = moderately positive staining, and 3 = strongly positive staining). The extent of positivity ("extent of

distribution" of positive cells) was estimated on a scale of 0 to 4 (0 = negative, 1 = positive staining in 1%-25% of cells, 2 = positive staining in 26%-50%; 3 = positive staining in 51%-75%; and 4 = positive staining in 76%-100%). The combined staining score (extension + intensity) was considered as positive staining.

### Detection of apoptosis by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL)

Apoptotic cells were detected *in situ* using TUNEL method according to the manufacturer's instructions<sup>[17]</sup>. Slices were treated with proteinase K and 0.3% H<sub>2</sub>O<sub>2</sub>, labeled with fluorescein dUTP (Wuhan Boster Biological Technology Co.Ltd) in a humid box for 1 h at 37°C, then combined with POD-horseradish peroxidase, stained with DAB and counterstained with methyl green. Controls received the same management except the labeling fluorescein dUTP. Dark brown nucleus represented the positive apoptotic cells. In each section, 5 high -power fields were chosen and the apoptosis index (AI), the percentage of positive cells in the total cells, was calculated. The AI was calculated as follows: AI = number of apoptotic cells/total number  $\times$  100%.

### Statistical analysis

Data were presented as mean  $\pm$  SD and analyzed with SPSS 11.0 software. The one-way ANOVA or Student's *t* test was used to analyze data. All comparisons were made with untreated controls and significance of difference is indicated as P < 0.05 and P < 0.01.

## RESULTS

### Effects of *Gecko* on cell proliferation

As shown in Tables 1 and 2, compared with control group, the growth of cells treated with serum of different concentrations of mice with medicine *Gecko* was inhibited significantly in a concentration and time-dependent manner. Seventy-two h after treatment, the inhibitory rates were 22.3%-39.1% (EC9706) and 18%-34.1% (EC1). Compared with the NS group, the differences were significant among the groups of *Gecko* (P < 0.05).

### Growth curves of EC9706 and EC1

To investigate anti-tumor activity of the *Gecko* *in vitro*, tumor cells treated with serum medicine were cultured

**Table 2** Inhibition effect of *Gecko*-contained serum on EC1 by MTT assay (mean  $\pm$  SD, n = 6)

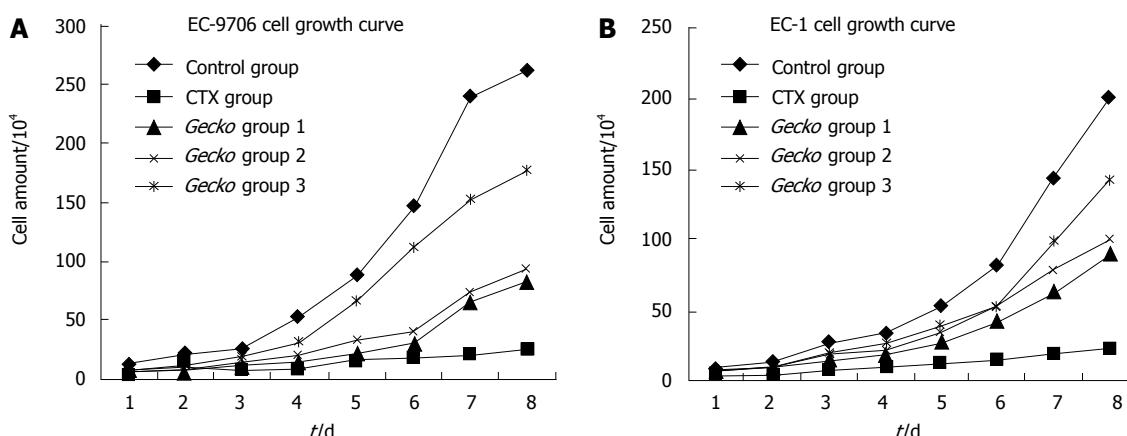
Experiment	24 h		48 h		72 h	
	A	Inhibition rate (%)	A	Inhibition rate (%)	A	Inhibition rate (%)
NS	0.282 $\pm$ 0.09		0.506 $\pm$ 0.05		1.329 $\pm$ 0.73	
CTX	0.19 $\pm$ 0.03 <sup>a</sup>	32.6	0.298 $\pm$ 0.124 <sup>a</sup>	41.1	0.673 $\pm$ 0.08 <sup>a</sup>	48.7
Gecko 1	0.215 $\pm$ 0.08 <sup>a</sup>	24.8	0.368 $\pm$ 0.03 <sup>a</sup>	27.2	0.865 $\pm$ 0.08 <sup>a</sup>	34.1
Gecko 2	0.234 $\pm$ 0.04 <sup>a</sup>	16.6	0.378 $\pm$ 0.03 <sup>a</sup>	25.1	0.94 $\pm$ 0.112 <sup>a</sup>	30.5
Gecko 3	0.255 $\pm$ 0.05 <sup>a</sup>	9.5	0.426 $\pm$ 0.08 <sup>a</sup>	15.8	1.075 $\pm$ 0.132 <sup>a</sup>	18

<sup>a</sup>P < 0.05 vs control.**Table 3** Inhibitory effects of *Gecko* on transplanted sarcoma 180 in mice (mean  $\pm$  SD, n = 10)

Groups	Dose (g/kg)	Weight (g)		Tumor weight (g)	Inhibitory rate (%)
		Pre-treatment	Post-treatment		
NS		26.1 $\pm$ 2.57	28.1 $\pm$ 2.64	2.167 $\pm$ 0.592	
CTX	0.1	26.2 $\pm$ 2.13	24.3 $\pm$ 2.91 <sup>a</sup>	0.548 $\pm$ 0.135 <sup>b</sup>	74.6
Gecko 1	13.5	26.5 $\pm$ 2.44	28.8 $\pm$ 3.01	1.087 $\pm$ 0.249 <sup>b</sup>	49.8
Gecko 2	9.0	26.1 $\pm$ 2.11	26.6 $\pm$ 2.18	1.021 $\pm$ 0.288 <sup>b</sup>	52.8
Gecko 3	4.5	25.4 $\pm$ 1.90	27.8 $\pm$ 3.74	1.234 $\pm$ 0.331 <sup>b</sup>	43.1

<sup>a</sup>P < 0.05, <sup>b</sup>P < 0.01 vs control.**Table 4** Influence of *Gecko* on immune organs of transplanted sarcoma 180 in mice (mean  $\pm$  SD, n = 10)

Groups	Dose (g/kg)	Thymus index ( $\times 10^3$ )	Spleen index ( $\times 10^3$ )	Phagocytic rate(%)	Phagocytic index
NS		2.662 $\pm$ 0.131	8.143 $\pm$ 0.294	30.2	0.414
CTX	0.1	1.939 $\pm$ 0.981 <sup>a</sup>	5.376 $\pm$ 0.570 <sup>a</sup>	7.0 <sup>b</sup>	0.085 <sup>b</sup>
Gecko 1	13.5	2.260 $\pm$ 0.092	6.943 $\pm$ 0.306	16.8 <sup>a</sup>	0.206 <sup>a</sup>
Gecko 2	9.0	2.680 $\pm$ 0.064	7.418 $\pm$ 0.209	19.0 <sup>a</sup>	0.218 <sup>a</sup>
Gecko 3	4.5	2.376 $\pm$ 0.051	7.354 $\pm$ 0.236	20.5 <sup>a</sup>	0.231 <sup>a</sup>

<sup>a</sup>P < 0.05, <sup>b</sup>P < 0.01 vs control.**Figure 1** Growth curves of EC9706 (A) and EC1 (B) in all groups.

for 7 d, and then the cell growth curve was drawn. Under the inverted light microscope, obvious difference was observed in the cell morphology among the five groups of cells. Compared with the control group, growth curves of the three *Gecko* groups gradually moved down in a dose-dependent manner (Figure 1). These results indicated that the serum with medicine *Gecko* could inhibit EC9706 and EC1 growth and proliferation *in vitro*.

#### Anti-tumor effects of *Gecko* on S180 mice

As shown in Table 3, compared with the NS group, the tumors of the *Gecko* and CTX groups shrank significantly (P < 0.01), but the tumor weight of three *Gecko* groups had no difference statistically compared with the CTX group (P > 0.05). The tumor inhibitory rates of the CTX group and *Gecko* groups were 74.6%, 49.8%, 52.8% and 43.1%, respectively. After experiment, there no significant

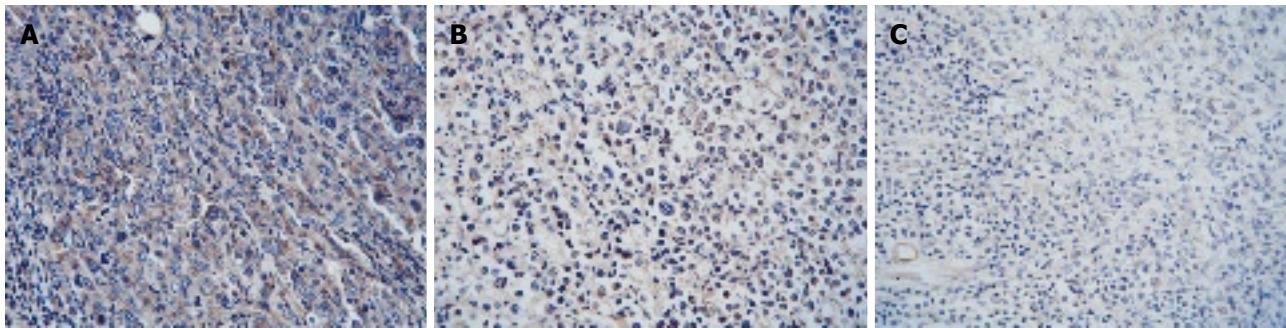
difference was found in mouse weight between the *Gecko* groups and the NS group (P > 0.05). Compared with the NS groups, the weight of mice in the CTX group decreased significantly (P < 0.05).

#### Influence of *Gecko* on immune organs

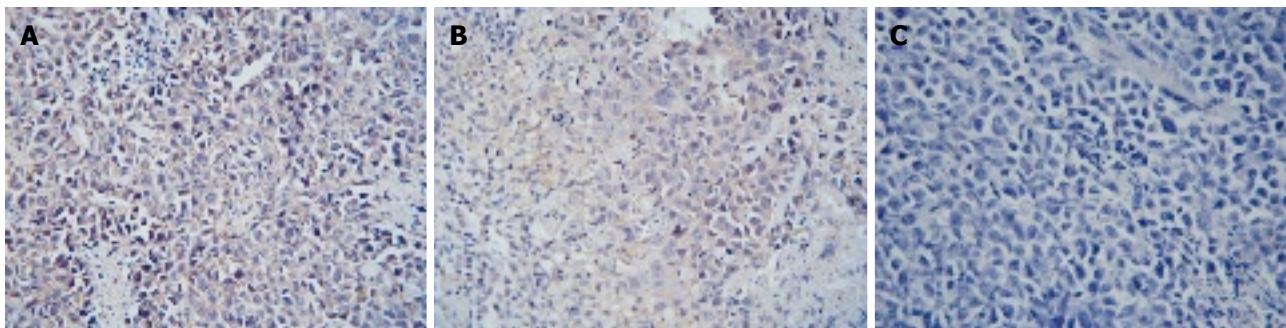
As shown in Table 4, compared with the NS group, the thymus index and Spleen index of mice in the CTX group decreased significantly (P < 0.05). However, there was no significant difference between the *Gecko* groups and the NS group; CTX could decrease the phagocytic index and phagocytic rate very significantly (P < 0.01); *Gecko* could decrease the phagocytic index and phagocytic rate significantly (P < 0.05). These results indicated that *Gecko* could not enhance the immune functions.

#### Effects of *Gecko* on VEGF and bFGF expressions

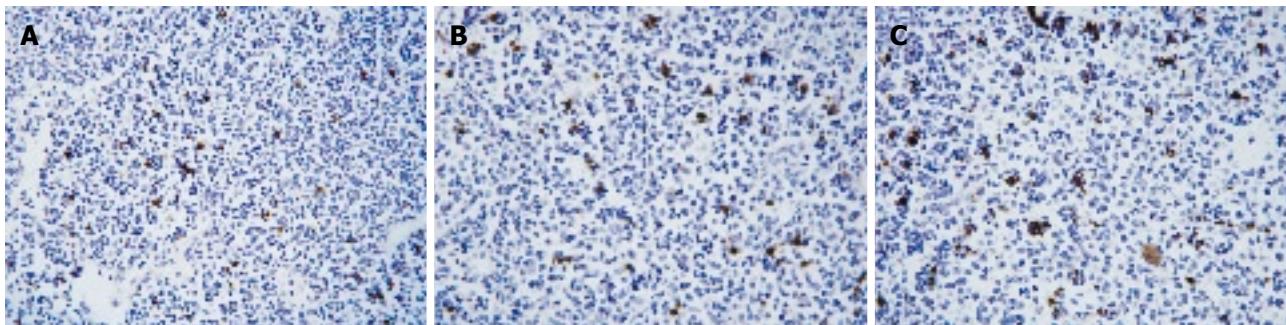
As shown in Figures 2 and 3, the VEGF and bFGF



**Figure 2** Expression of VEGF on S180 tissues in mice (DAB,  $\times 400$ ). NS group (**A**), Gecko group (**B**), CTX group (**C**).



**Figure 3** Expression of bFGF on S180 tissues in mice (DAB,  $\times 400$ ). NS group (**A**), Gecko group (**B**), CTX group (**C**).



**Figure 4** Apoptotic cells on S180 the tissue in mice (DAB,  $\times 400$ ). NS group (**A**), Gecko group (**B**), CTX group (**C**).

positive cells were defined when there was an aggregation of brown particles in the cytoplasm of the tumor cells. The proteins of VEGF and bFGF had high expression in the tissue of transplanted sarcoma 180 in mice. Compared with the NS group, the immunoreactive score of expression VEGF and bFGF expression of CTX group and *Gecko* groups decreased significantly (*P* < 0.05). It indicated *Gecko* could decrease VEGF and bFGF protein expression in the tissue of transplanted sarcoma 180 in mice.

#### Effects of *Gecko* on AI

As shown in Figure 4, dark brown nucleus represented the positive apoptotic cells on the tissue of transplanted sarcoma 180 in mice. As shown in Table 5, compared with the NS group, AI of the CTX group and the *Gecko* groups increased very significantly (*P* < 0.01), indicating that *Gecko* could increase AI in the tissue of transplanted sarcoma 180 in mice.

**Table 5 Expressions of VEGF, bFGF and AI on S180 tissues in mice (mean  $\pm$  SD,  $n = 10$ )**

Groups	Dose (g/kg)	AI (%)	Immunoreactive score	
			VEGF	bFGF
NS		3.58 $\pm$ 0.87	5.80 $\pm$ 0.73	4.81 $\pm$ 0.82
CTX	0.1	17.95 $\pm$ 2.14 <sup>b</sup>	4.00 $\pm$ 0.38 <sup>a</sup>	3.00 $\pm$ 0.63 <sup>a</sup>
<i>Gecko</i> 1	13.5	11.28 $\pm$ 1.03 <sup>b</sup>	4.62 $\pm$ 0.52 <sup>a</sup>	3.34 $\pm$ 0.77 <sup>a</sup>
<i>Gecko</i> 2	9.0	13.56 $\pm$ 2.13 <sup>b</sup>	4.75 $\pm$ 0.68 <sup>a</sup>	3.46 $\pm$ 0.56 <sup>a</sup>
<i>Gecko</i> 3	4.5	11.05 $\pm$ 1.69 <sup>b</sup>	4.83 $\pm$ 0.72 <sup>a</sup>	3.52 $\pm$ 0.62 <sup>a</sup>

<sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 vs control.

#### DISCUSSION

The processing of *Gecko* introduced in the “Holy Benevolent Prescription” is to “grind it into powder”, while according to “The Medical Science Outline”, the processing is “drying by baking”<sup>[18]</sup>. Basically two

processing methods, living and drying, are used in clinical practice. For fresh *Gecko*, there are a few studies describing the mechanisms of lyophilized powder of fresh *Gecko* in inhibiting H22 hepatocarcinoma and C6 glioma cells<sup>[19-22]</sup>, but there has been no experimental study on anti-tumor of drying *Gecko*. We therefore, selected drying *Gecko* as a study object.

Serum pharmacological method was first introduced by Japanese authors. It is a good approach to study traditional Chinese medicine(TCM). The serum pharmacology points out that the serum collected from animals treated with traditional Chinese medicine can be used in experiments *in vitro*. This kind of experimental methods can expel various interference of traditional Chinese medicine and they are close to the true process of the pharmacological effect inside the body<sup>[10-11,23]</sup>. So we used the method of serum pharmacology *in vitro* experiments and observed the effect of *Gecko*-contained serum on growth of the tumor cells. In this study, MTT showed the growth of EC9706, EC1 cells treated with different concentrations of the *Gecko*-contained serum was inhibited significantly in a concentration and time-dependent manner. Growth curves of the three *Gecko* groups gradually moved down, being obviously dose dependent. These results indicated that the *Gecko*-contained serum could inhibit the growth and proliferation of human esophageal carcinoma cells. *In vivo*, the transplanted tumor model of the mouse S180 sarcoma was established. In the *Gecko* groups at dosage of 13.5, 9 and 4.5 g/kg, the inhibitory rate was 49.8%, 52.8% and 43.1%, respectively. The differences of the groups of *Gecko* were very significant from the control group ( $P < 0.01$ ). These results indicated that *Gecko* could inhibit growth of solid tumor of S180 mice.

The effect on anti-tumor of TCM is related to pathways and targets. Most studies on anti-tumor mechanisms of TCM showed that TCM could inhibit tumors though supporting the healthy energy and strengthening the body resistance<sup>[1,2]</sup>. In our study, the thymus index, Spleen index, phagocytic index and phagocytic rate showed that *Gecko* could not reinforce immunity of organism. These results indicated the anti-tumor mechanism of TCM might not be related to reinforcement of immune organs.

With the development of modern molecular technology, the anti-angiogenic effect and inducing apoptosis by TCM has become a new area for the research and development of TCM. Apoptosis is a fundamental cellular activity to maintain the physiological balance of the organism and plays a necessary role as a protective mechanism against carcinogenesis by eliminating damaged cells or cells that proliferate excessively<sup>[24]</sup>. Furthermore, the apoptotic cell death plays an important role in the regulation of pathological conditions as well, such as development and progression of malignant tumors<sup>[25,26]</sup>. Among the methods that are used to identify cells undergoing the apoptotic process, the TUNEL technique is one of the most successfully utilized<sup>[26]</sup>. Angiogenesis, the growth of new blood vessels from pre-existing capillaries, is necessary for solid tumor growth and metastasis. Angiogenesis is initiated

by the release of certain angiogenic factors from tumor cells. VEGF and bFGF which have been shown to be the most potent angiogenic factors are associated with tumor-induced angiogenesis<sup>[27,28]</sup>. To investigate the mechanisms of anti-tumor action of *Gecko*, we detected the protein expression of VEGF and bFGF using immunohistochemical method and detected apoptotic index by the TUNEL. The results indicate *Gecko* can decrease VEGF and bFGF protein expression in tumor tissues and induce tumor cell apoptosis.

In conclusion, this study has demonstrated that traditional Chinese medicine *Gecko* has anti-tumor activity *in vitro* and *in vivo*; its mechanism might be related to the induction of tumor cell apoptosis and down-regulation of protein expression of VEGF and bFGF.

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## COMMENTS

### Background

Malignant tumor is always a great menace to human health. Chemical anti-neoplastics have definite effect, but they cause much severe adverse effects. *Gecko* is a traditional Chinese medicine. It could treat malignant tumors, especially in the tumor of digestive tract. Research on *Gecko* mainly focuses on anti-tumor compound prescriptions, but the pharmacological studies of *Gecko* and its mechanisms of anti-tumor action are rare.

### Research frontiers

Basically, two processing methods of *Gecko*, living and drying, are used in clinical practice. Questions still remain to be answered such as what is the difference of anti-tumor effect between fresh *Gecko* and dry *Gecko*? What kind of tumors can *Gecko* treat? What is the mechanism of *Gecko* in treating tumors?

### Innovations and breakthroughs

This article describes the effect of the *Gecko* on esophageal carcinoma cell lines (EC9706 and EC1) *in vitro* and xenografted sarcoma 180 *in vivo*. Serum pharmacological method *in vitro* was used to study the anti-tumor mechanism of *Gecko* in the aspect of the anti-angiogenic and apoptosis inducing effect and investigate if *Gecko* had impact on immune organs.

### Applications

The incidence and mortality of tumors keep ascending all over the world. The study on anti tumor effect and mechanisms of anti-tumor action of *Gecko* can provide the theoretical evidence in clinical practice. Furthermore, the results may lay a foundation for further research in *Gecko*'s effective constituent.

### Terminology

Serum pharmacological method is a good approach to study traditional Chinese medicine *in vitro*. It can expel various interference of traditional Chinese medicine as it is close to the true process of the pharmacological effect inside the body

### Peer review

This is a well-conducted study. This manuscript describes the effect of the Chinese medicine *Gecko* on esophageal carcinoma cell lines (EC9706 and EC1) and in xenografted sarcoma 180 Kunming mice. The studies have provided data that support the conclusions of the authors. This is an interesting study.

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# Characteristics of liver on magnetic resonance diffusion-weighted imaging: Dynamic and image pathological investigation in rabbit liver VX-2 tumor model

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## Abstract

**AIM:** To investigate dynamical and image pathological characteristics of the liver on magnetic resonance (MR) diffusion-weighted imaging (DWI) in the rabbit VX-2 tumor model.

**METHODS:** Forty New Zealand rabbits were included in the study and VX-2 tumor piece was implanted intrahepatically. Fifteen animals received two intrahepatic implantations while 25 had one intrahepatic implantation. DWI, T1- and T2-weighted of magnetic resonance imaging (MRI) were carried out on the 7th and the 14th d after implantation and DWI was conducted, respectively on the 21th d. Ten VX-2 tumor samples were studied pathologically.

**RESULTS:** The rate of lump detected by DWI, T1WI and T2WI was 78.7%, 10.7% and 53.5% ( $\chi^2 = 32.61$ ,

$P < 0.001$ ) on the 7th d after implantation and 95.8%, 54.3% and 82.9% ( $\chi^2 = 21.50$ ,  $P < 0.001$ ) on the 14th d. The signal of most VX-2 tumors on DWI was uniform and it was equal on the map of apparent diffusion coefficient (ADC). The signal of VX tumors did not decrease on the 7th d after implantation, most of them slowly growing during the week following implantation without significant cell dying within the tumor. VX-2 tumors grew increasingly within 14 d after implantation but the signal of most VX-2 tumors on DWI or on the map of ADC was uniform or uneven and ADC of VX tumors decreased obscurely or slightly because tumor necrosis was still not obvious. On the 21th d after implantation, the signal of most VX-2 tumors on DWI or on the map of ADC was uneven because tumor necrosis was evident and ADC of VX-2 tumor necrotic areas decreased. The areas of viable cells in VX-2 tumors manifested a high signal on DWI and a low signal on the map of ADC. The areas of dead cells or necrosis in VX-2 tumors manifested low signals on DWI and low, equal or high signals on the map of ADC but they manifested high signals on DWI and on the map of ADC at the same time when the areas of necrotic tumor became liquefied or cystic. The border of tumors on DWI appeared gradually distinct and internal signals of tumor became progressively uneven.

**CONCLUSION:** The manifestations of viable, necrotic and liquefied or cystic areas in VX-2 tumors on DWI are typical and DWI is of significant and potential values in clinical application in both the early detection and diagnosis of liver tumors.

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**Key words:** Liver; VX-2 tumor; Diffusion-weighted imaging; Apparent diffusion coefficient; Rabbit

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## INTRODUCTION

Magnetic resonance (MR) diffusion-weighted imaging (DWI) is a new functional imaging technology developed in recent years<sup>[1-5]</sup>, which is able to reflect non-woundingly water molecule diffusion *in vivo*. It is generally accepted that DWI is valuable in the qualitative and quantitative diagnosis of cerebral ischemia during the hyper-inchoate period<sup>[6-9]</sup>. In the past, DWI has not been extensively used in the diagnosis and evaluation of progression and prognosis of hepatic tumors, mainly because of its poor image quality. However, with the recent development of MR software and scanning technology, especially for echo planar imaging (EPI) series, the DWI poor imaging quality and slow scanning speed have been overcome<sup>[10-14]</sup>. This has led to the employment of DWI on the monitoring of focal and diffuse hepatic lesions, such as cysts, hemangiomas, hepatocellular carcinomas, metastases and liver cirrhosis. As reported by Ichikawa *et al*<sup>[15-16]</sup>, Yamashita *et al*<sup>[17]</sup>, Taouli *et al*<sup>[18]</sup>, and Sun *et al*<sup>[19]</sup>, the apparent diffusion coefficient (ADC) of such lesions grew gradually on DWI, the ADC of cysts being the biggest, followed by benign tumors and malignant tumors. Colagrande *et al*<sup>[20]</sup> demonstrated that the coagulation necrosis manifested a low signal when compared to the normal parenchyma. Kamel *et al*<sup>[21]</sup> confirmed in 8 patients with hepatocellular carcinoma that the apparent diffusion coefficient (ADC) becomes higher with the extent of tumor necrosis and that the signals of 6 tumors were higher than normal parenchyma on DWI. The signals of liquor puris in abscesses were moderate or high on DWI but they were lower than the signals of cysts and cystic metastatic tumors because of their high consistency, and the signals of abscess walls were equal to those of normal parenchyma around<sup>[22-26]</sup>.

From what has been discussed above, DWI, especially ADC, has potential values in reflecting characteristics of hepatic pathological changes and differentiating benign tumors from malignant tumors<sup>[27-31]</sup>. However, to date, no dynamic and image pathological investigation on the characteristics of hepatocellular carcinomas on DWI has been reported. Rabbit liver VX-2 tumor is the most valuable animal model of hepatocellular carcinoma in imaging investigation. The purpose of our experiment was to investigate the dynamic characteristics and the pathological mechanisms underlying the signals on DWI in rabbit VX-2 tumor models after implantation and to evaluate the superiority of DWI in detecting, diagnosing and differentiating tumors.

## MATERIALS AND METHODS

### Animals

Animal studies were carried out under the supervision

of a veterinarian according to the Guidelines of Chinese Ministry of Health for the Use of Laboratory Animals. All animals were provided by the Laboratory Animal Center of the Second Xiangya Hospital and all protocols were approved by the Animal Use and Care Committee of the Second Xiangya Hospital.

Forty New Zealand normal white rabbits were employed (22 males and 18 females), weighing 1.7-2.5 kg and aged 5-6 mo.

### Establishment of VX-2 tumor model

The rabbit VX-2 tumor strain was provided by the Fourth Military Medical University of China.

### Procedure of implantation

The VX-2 tumor donors were anesthetized by injecting 3% soluble pentobarbitone into auriborder vein or abdominal cavity at a dose of 1 mL/kg. The site of implantation was disinfected by Iodine, the skin was incised to expose one cauliflower of the subcutaneous tumor which was implanted before experiment and then one cauliflower was excised from it. The tumor parenchyma was exposed and quickly put into saline solution containing 40 000 unit gentamycin per 100 mL. The necrotic tissue and blood clot were discarded and the viable tumor tissue was divided into 1-2 mm<sup>2</sup> microblocks.

The VX-2 tumor strain was subsequently implanted into the liver parenchyma of rabbits as follows. The animals were anesthetized by injecting 3% soluble pentobarbitone and the skin of abdomen was disinfected. The liver lobe chosen for implantation was exposed through incising the skin and vagina musculi recti abdominis. The tube containing the VX-2 tumor strain was implanted into the lobe by rotating and pushing it into the liver parenchyma. Then a gelatin sponge was inserted to prevent bleeding. Afterward, the peritoneum, musculus, and skin were sutured, respectively. Implantation was performed in one lobe in 25 rabbits and in two lobes in 15 rabbits. After implantation, 200 000 unit penicillin was administered by intramuscular injection for 4 d while the animal room was kept dry and ventilated.

### MRI protocol

The animals were anesthetized with 3% soluble pentobarbitone injected into auriborder vein at different doses based on the animal status to make sure that the breathing was slow and stable. T1-weighted imaging (T1WI), T2-weighted imaging (T2WI) and DWI were performed on a 1.5-Tesla Signa Twinspeed MR scanner (General Electron Medical Systems, USA), using a small diameter cylindrical brain radiofrequency coil. DWI (axial) and MRI (T1WI and T2WI, axial) were carried out on the 7th d and the 14th d after implantation and DWI (axial) was performed also on the 21th d after implantation. The scanning parameters of DWI included spin echo echo planar imaging (SE-EPI) series, *b*-value 100 and 300 s/mm<sup>2</sup>, repetition time (TR) 6000 ms, echo time (TE) 45 ms, 20 cm × 15 cm field of view (FOV),

8NEX, 2 mm thick layer, 0.5 mm space,  $128 \times 128$  matrix, etc. The scanning parameters of common MRI included fast reverse, fast spin echo (FRFSE) series, T1WI (TR 400/TE 12.3 ms), T2WI (TR3000/TE80 ms), 20 cm  $\times$  15 cm FOV, 4 NEX, 5 mm thick layer, 0 mm space,  $256 \times 192$  (T1WI) and  $320 \times 256$  (T2WI) matrix.

### Pathological protocol

Twelve samples of VX-2 tumors were processed. Ten samples were taken on the 21th d after implantation, one on the 7th d and the other one on the 14th d. The animals were euthanized with an overdose of 3% soluble pentobarbitone into auriborder vein. The VX-2 lump surrounded by normal liver parenchyma was taken under aseptic conditions. Then the lump was cut open and sliced (Figure 1). After 24 h of fixation in formaldehyde solution, all samples were embedded in mineral wax.

### Statistical analysis

The distinction of tumor detection between DWI, T1WI and T2WI on the 7th d and the 14th d after implantation was respectively assessed. The statistical significance was calculated by  $\chi^2$  analysis using SPSS12.0 software.

## RESULTS

### DWI, T1- and T2-weighted imaging

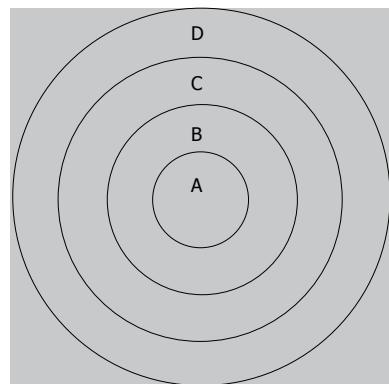
Thirty-eight of 40 rabbits were still alive 21 d after implantation, while 2 rabbits died from overdose of anesthesia. Forty-seven VX-2 tumors were detected in 47 hepatic lobes of 35 rabbits.

On the 7th d after implantation, 37 lumps, 3 lumps and 15 lumps were respectively detected by DWI, T1- and T2-weighted imaging and the detection rates were 78.7% (37/47), 10.7% (3/28) and 53.8% (15/28), respectively. The difference of detection was significant among DWI, T1- and T2 weighted imaging, between DWI and T2 weighted imaging and between T1- and T2 weighted imaging ( $\chi^2 = 32.61$ ,  $P < 0.001$ ;  $\chi^2 = 5.22$ ,  $P = 0.022$ ;  $\chi^2 = 11.79$ ,  $P < 0.001$ ). On the 14th d after implantation, 45 lumps, 19 lumps and 29 lumps were detected by DWI, T1- and T2-weighted imaging and the detection rates were 95.87% (45/47), 54.3% (19/35) and 82.9% (29/35), respectively. The difference of detection was significant among DWI, T1- and T2-weighted imaging, between DWI and T2 weighted imaging and between T1- and T2 weighted imaging ( $\chi^2 = 21.50$ ,  $P < 0.001$ ;  $\chi^2 = 3.78$ ,  $P > 0.05$ ;  $\chi^2 = 6.63$ ,  $P = 0.01$ ). On the 14th d after implantation, all 47 tumors were detected by DWI ( $b = 100$  or  $b = 300$  s/mm $^2$ ).

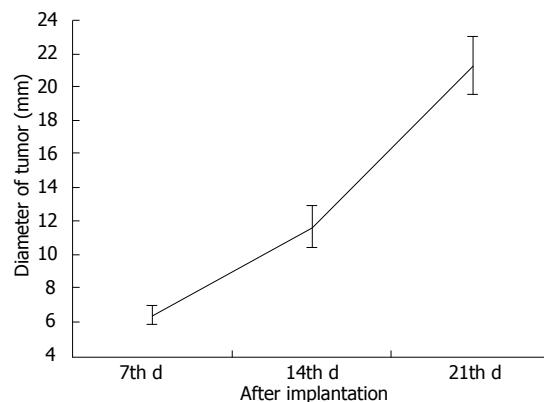
The average diameter of VX-2 tumors detected by DWI, T1- and T2 weighted imaging was 6.49 (3.00-12.90) mm, 11.78 (5.00-25.10) mm and 21.44 (7.50-36.70) mm respectively on the 7th, the 14th d and the 21th d after implantation (Figure 2).

### Image manifestations of hepatic VX-2 tumor

The signals of most VX-2 tumors were low and the



**Figure 1** A: The area of VX-2 tumor center; B: The area of VX-2 tumor periphery; C: The area of VX-2 tumor outer layer; D: The normal liver parenchyma area around tumor when the values of ADC and signals were measured on DWI and samples were investigated pathologically.

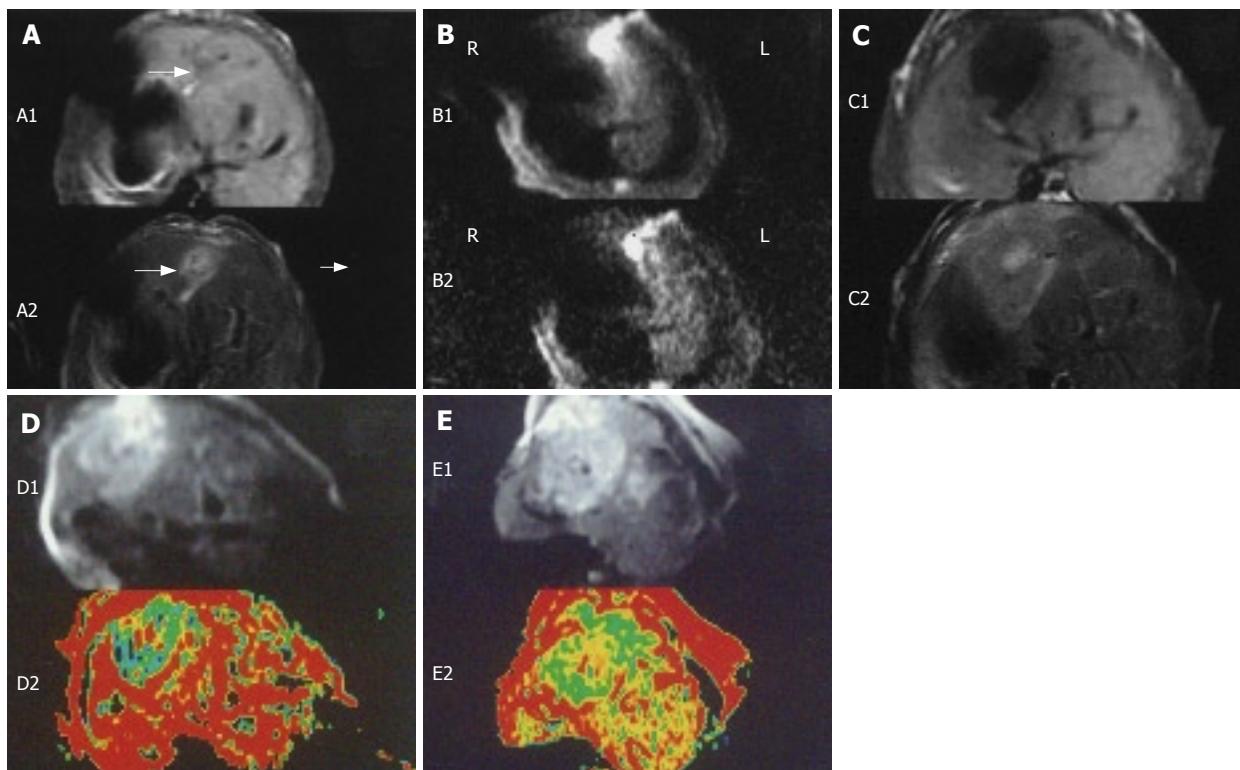


**Figure 2** The average diameter of VX-2 tumors on the 7th, the 14th and the 21th d after implantation detected by DWI, T1- and T2 weighted imaging.

borders were unclear on T1-weighted imaging (5/15), whereas the signals were high and the borders were clear on T2-weighted imaging (14/15) seven days after implantation (Figure 3). The signals of VX-2 tumors were high with unclear borders on T1-weighted imaging (18/19), while they were high with clear borders on T2-weighted imaging (14/29) two weeks following implantation (Figure 3). DWI appearance of hepatic VX-2 tumors is summarized in Tables 1 and 2.

### Manifestations of VX-2 tumor dissection and pathology

On the 7th and 14th d after implantation, intrahepatic VX-2 tumors appeared pale without identifiable margin or envelope. No lumps were detected in the hepatic peritumoral parenchyma, whereas residuum of gelation sponge in or around VX-2 tumors was detected. On the 21th d after implantation, many encroachments and metastatic tumors were found in dissection, including abdominal wall encroachment in 15 cases, abdominal dropsy in 16, mesenterium encroachment in 15, lung metastases in 19, pleural effusion in 12 and diaphragm encroachment in 2. The extrahepatic surface was uneven and VX-2 tumors were pale, with a clear distinction between tumoral tissues and normal surrounding parenchyma. The tumors were hard and there was no clear amiculas. Cavitates of unequal size were found in the lumps of some cases because of kermesinus liquid running off after the tumors were cut open and there was some residuum of gelation sponge in or around the lumps of some tumors (Figure 4).



**Figure 3** Image manifestations of hepatic VX-2 tumor on DWI, ADC map, T1WI or T2WI. **A:** A1, a low signal and obscure margin of VX-2 tumor on T1WI and A2, a slightly high signal on T2WI on the 7th day after implantation; **B:** a high signal and distinct margin of VX-2 tumor on DWI when  $b$  value was 100 (B1) or 300 (B2)  $\text{s/mm}^2$  and low SNR when  $b$  value was 300  $\text{s/mm}^2$  on the 7th day after implantation; **C:** C1, a low signal of VX-2 tumor on T1WI and C2, a slightly high signal of it on T2WI and a lamellar high signal in the tumor center on T2WI on the 14th day after implantation; **D:** A high signal and distinct margin of VX-2 tumor on DWI when  $b$  value was 100  $\text{s/mm}^2$  (D1) and low signal of VX-2 tumor and a lamellar high signal in its center on the map of ADC on the 14th d after implantation (D2); **E:** A high and uneven signal of VX-2 tumor on DWI when  $b$  value was 100  $\text{s/mm}^2$  (E1) and low signal of VX-2 tumor while a high signal in the center tumor on the map of ADC on the 21th d after implantation (E2).

**Table 1 DWI manifestations of 47 hepatic VX-2 tumor models ( $b = 100 \text{ s/mm}^2$ )**

Tumors detected	Border		Signal		Signal of center			ADC map	
	Clear	Unclear	Even	Uneven	High	Equal	Low	Equal	Low
A	37	5	32	30	7	1	32	4	29
B	45	28	17	25	20	2	32	11	20
C	47	45	2	9	38	5	14	28	4
									43

A: 7th d after implantation; B: 14th d after implantation; C: 21th d after implantation.

**Table 2 DWI manifestations of 47 hepatic VX-2 tumor models ( $b = 300 \text{ s/mm}^2$ )**

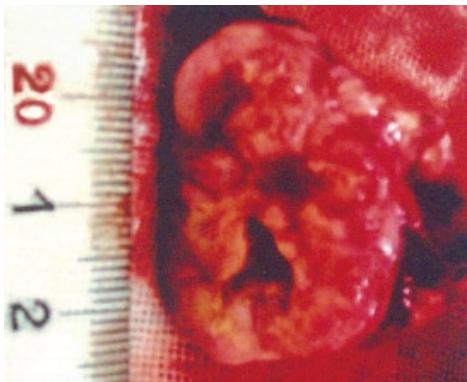
Tumors detected	Border		Signal		Signal of center			ADC map	
	Clear	Unclear	Even	Uneven	High	Equal	Low	Equal	Low
A	37	5	32	29	8	0	33	4	33
B	45	30	15	25	20	2	32	11	19
C	47	46	1	9	38	3	16	28	3
									44

A: 7th d after implantation; B: 14th d after implantation; C: 21th d after implantation.

Macroscopically, the surface of the peritumoral hepatic parenchyma was brown-grey; 11 small lumps, observed on the liver surface, proved to be metastatic by pathology. The texture of normal hepatic parenchyma was soft after being cut open. Microscopically ( $\times 100$ ), the peritumoral architecture and cell morphology were normal and necrotic zones were not observed. However, there were some different thickening blood

vessels and inflammatory cells as well as areas of edema or ballooning degeneration. At higher magnification ( $\times 400$ ), the size of cell was equal on the whole and there was abundant endochylema in cells. Abnormal caryocinesia was not observed in cell nucleuses.

The outer layer areas and the periphery areas in VX-2 tumors appeared grey, fish and hard without distinct borders and amiculas between the area of



**Figure 4** Cavitas was observed in the tumor center of some samples on the 21th d after implantation.

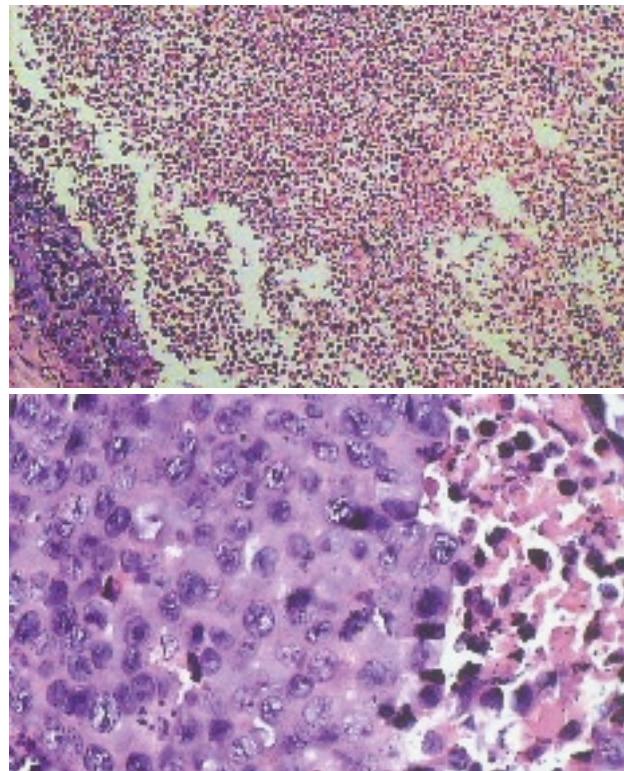
normal hepatic parenchyma and the area of VX-2 tumor outer layer (Figure 4). The distribution of necrotic areas differed between the cancer center and its periphery, being prevalent in the tumor core and significantly less at its periphery or its outer layer. Tumor necrotic tissue looked like tofukasu and tumor viable tissue looked like fish (Figure 4).

At low magnification ( $\times 100$ ), a plenty of tumor nests in lumps were observed and they showed inequality in size, and round or ellipse in shape. There were more blood capillaries and little connective tissue in lumps. Calcification changes and residuum of gelation sponge could be observed in some cases. There were unequal necroses in the area of most VX-2 tumor center but there was little necrosis in the area of tumor periphery (Figure 5).

At higher magnification ( $\times 400$ ), the size of most tumor cells was not equal and tumor nests differed in size, and round or ellipse in shape. There was little intercellular substance in tumors and some cell membranes appeared poorly defined. The size of cell nucleuses was uneven and the number of cell nucleuses was not equal so that the karyoplasmic ratio of cells was altered. Most of cell nucleuses were stained deeply and some of them had obvious caryocinesia. Some apoptotic cells were also observed.

## DISCUSSION

Diffusion is caused by water molecular random motion, so-called “Brownian motion”<sup>[24-27]</sup>. By adding a powerful polar and quick switching gradient radiofrequency (RF) pulse, it is possible to amplify these phase changes in order to detect water molecule diffusion motion, known as diffusion-weighted imaging (DWI)<sup>[16,18,20,25]</sup>. By using such an approach, the signals of hepatocellular carcinoma, metastases and hemangiomas were higher than that of the surrounding hepatic parenchyma, as reported by Ichikawa *et al*<sup>[15,16]</sup>, Taouli *et al*<sup>[18]</sup> and Yang *et al*<sup>[32]</sup>. This phenomenon can be explained by differences in terms of water molecular motions, which are limited in hepatocellular carcinoma, metastases and hemangiomas when compared to normal hepatic tissues. Changes of their phase position in the magnetic



**Figure 5** Cell nest of VX-2 tumor and wide zone of necrosis in the tumor were observed under microscope (up  $\times 100$ , down  $\times 400$ ).

field were therefore smaller and signal deamplification is reduced. Although the limitation of water molecular motion in the hemangioma is smaller than that of hepatocellular carcinoma or metastasis, the signal of the former was higher than that of the latter because there were more water molecules in the hemangioma.

As VX-2 tumor is a solid tumor and its body mainly consists of tumor cells, tumor nests and other cells, its water molecular diffusion motion is restricted. The signals of VX-2 tumor on DWI are higher than that of normal hepatic parenchyma because the signal deamplification of VX-2 tumor is small. On the 7th, the 14th d and the 21th d after implantation in our experiment, the signals of most VX-2 tumors were higher than that of normal parenchyma, and they looked like “lamp bulbs” (Figure 3). Their margins were usually distinct and their ADC values were lower than that of normal parenchyma, confirming the published data.

On the 7th d after implantation, the ability of detecting tumors by DWI was significantly higher than that of T1-weighted imaging (78.7% vs 10.7%) or T2-weighted imaging (78.7% vs 53.5%) and the signal contrast on DWI between VX-2 tumor and normal parenchyma was more evident when compared to T1-weighted or T2-weighted imaging (Figure 3A and B). DWI has important and potential clinical value in detecting tumors earlier because a powerful polar and quick switching gradient RF pulse besides MRI routine RF is added, molecular phase changes are amplified, which has not been reported in literatures before. The signals of most VX-2 tumors on DWI were high and uniform, including the central area of tumor on the

7th d after implantation; the signals on the map of ADC were equal even if most borders were unclear (Figure 3), probably because, at this time, the blood provision of VX-2 tumors is sufficient and there is no coagulative necrosis or colliquation.

On the 14th d after implantation, the signals of DWI in 31% (b-value 100 s/mm<sup>2</sup>) and 38% (b-value 300 s/mm<sup>2</sup>) of all tumor models were uneven and the borders were relatively distinct. The low signals detected in the central areas of 11 cases proved by pathology to be caused by unabsorbed gelatin sponge. At this time, no coagulative necrosis or colliquation were observed. Inflammation and cellular edema tended to decrease at d 14 after implantation in comparison with day 7.

On the 21th d after implantation, the signals of VX-2 tumors were high and 95% of margins were distinct on DWI while the signals on the map of ADC were low, with equally distinct borders. However, the histopathological examination did not show amiculas or distinct borders between VX-2 tumors and normal parenchyma ( $\times 100$  and  $\times 400$ ). A possible explanation of the high signals and distinct borders of tumors on DWI is related to the obvious limitations of water molecular motion in VX-2 tumors. The VX-2 tumor periphery area manifested mostly uniform, producing a high signal on DWI and low signal on the map of ADC. Samples in the area of VX-2 tumor periphery looked like grey fish macroscopically (Figure 4), containing viable tumor cells which showed inequality in size, and round or ellipse tumor nests under microscope (Figure 5). The intra-tumor signals were uneven on DWI with low and unequal areas in 28 lumps (Figure 3), where coagulative necrosis was confirmed by pathology (Figure 5). Because VX-2 tumors grow quickly, blood provision becomes insufficient causing local or lamellar coagulation necroses in the tumor. This result confirms the published data by Colagrande *et al*<sup>[20]</sup>, Kamel *et al*<sup>[21]</sup> and Geschwind *et al*<sup>[22]</sup>. The limitation of water molecule diffusion is inversely proportional to the extent of the tumor necrosis, because intact cell membranes can restrict the diffusion of water molecules when tumor cells are viable. The necrotic areas generated unequal signals (Figure 3D and E), being significantly smaller than the areas of viable tumor. Histologic analysis demonstrated unequal size, endochylema disproportion and pathological caryocinesis of most viable tumor cells. Areas of high signal on DWI were observed intratumorally in 5 cases (b-value 100 s/mm<sup>2</sup>) and 3 cases (b-value 300 s/mm<sup>2</sup>) (Figure 3D). Histopathological analysis showed that these areas were due to colliquation after necrosis. The signals of constitution on DWI were affected not only by diffusion of water molecules, but also by its T2-value contribution. When the T2-value of this constitution is long (value long<sup>2</sup>) and the b-value is small in diffusion-weighted imaging scanning, the signal of constitution will be affected significantly by the long T2-value contribution, the so called "shine-through". As reported by Yamashita *et al*<sup>[17]</sup>, Taouli *et al*<sup>[18]</sup> and Yang *et al*<sup>[32]</sup>, the limitation of water molecular motion in hepatic cysts was lower than that of the normal parenchyma; however,

the signals of the cysts were higher on DWI because the number of water molecules in hepatic cysts is higher than the hepatic parenchyma. The great amount of extracellular water molecules within the necrotic region caused by cell lysis allows free diffusion to take place, justifying the resultant high signals on DWI.

As shown in Figure 2, the growth velocity of VX-2 tumor from day 7 to 14 after implantation was lower than that from d 14 to 21. At the same time, several infiltrating and metastatic tumors were found by dissection on the 21th d after implantation. According to our experience, it is a period of quick growing for VX-2 tumor from the 14th d to the 21th d after implantation and it is suitable for rabbit VX-2 tumor models to be treated interventional or by other therapies.

Overall, the appearance of viable, necrotic and liquefied or cystic areas of VX-2 tumor on DWI is typical. Therefore, DWI could be applied in clinics for the early detection and diagnosis of liver tumors. The areas of viable cells in VX-2 tumors are associated with high signals, distinct borders, so called "lamp bulb" on DWI and low signals on the map of ADC. On the contrary, the necrotic areas in VX-2 tumors show low signals on DWI and equal or low signals on the map of ADC. Finally, high signals on DWI and on the map of ADC are produced when the areas of necrotic tumor are liquefied or have become cystic.

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## COMMENTS

### Background

Magnetic resonance (MR) diffusion-weighted imaging (DWI) is a new functional imaging technology developed in recent years, which is able to reflect non-woundingly water molecule diffusion *in vivo* and is valuable in the qualitative and quantitative diagnosis of cerebral ischemia during the hyper-inchoate period. Ichikawa *et al*, Yamashita *et al*, Taouli *et al*, Sun *et al* and Yang *et al* have reported the characteristics of hepatic lesions on DWI, such as cysts, hemangiomas, hepatocellular carcinomas, metastases and liver cirrhosis. However, because of its poor image quality, DWI has not been extensively used in the diagnosis and evaluation of progression and prognosis of hepatic tumors in the past. Moreover, to date, a dynamic and image pathological investigation in the characteristics of hepatocellular carcinomas on DWI has never been reported. We believe that DWI has potential values in reflecting characteristics of hepatic pathological changes and differentiating benign tumors from malignant tumors with the recent development of MR software and scanning technology, especially for echo planar imaging (EPI) series.

### Research frontiers

Many studies of hepatic pathological changes on DWI have been reported. ADC values of benign lesions, such as hepatic cysts and hemangiomas, were higher than those of malignant lesions, such as hepatocellular carcinomas and metastases on DWI and many studies also indicated that the signals of tumor coagulative necrotic areas were lower than that of tumor viable areas. Moreover, Colagrande *et al* demonstrated that the coagulation necrosis manifested a low signal when compared to the normal parenchyma. There has been no dynamical and image pathological investigation on the characteristics of the liver on DWI in the rabbit VX-2 tumor model.

### Innovations and breakthroughs

This study clearly demonstrates that the areas of viable cells in VX-2 tumors manifested a high signal on DWI and a low signal on the map of apparent diffusion coefficient (ADC), the areas of dead cells or necrosis in VX-2 tumors manifested a low signal on DWI and a low, equal or high signal on the map of ADC, but they manifested high signals on DWI and on the map of ADC at the same time when the areas of necrotic tumors had become liquefied or cystic. The manifestations of viable, necrotic and liquefied or cystic areas in VX-2 tumors on DWI were typical. The rate of lump detected by DWI was much higher than that by T1WI or T2WI after implantation. DWI has significant and potential clinical application values in detecting and differentiating viable tumors from necrotic tumors and in the early detection and diagnosis of liver tumors.

### Applications

Physicians can apply this knowledge to evaluate obviously progressive hepatic tumors and differentiate accurately the areas and degrees of necrotic tumors from that of viable tumors.

### Peer review

This is an interesting, well designed and written study on the clinical significance of the liver on diffusion-weighted imaging and the manuscript contained important information on the manifestations of viable, necrotic and liquefied or cystic areas in tumors and clinical application values in the early detection and diagnosis of liver tumors.

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# Value of contrast-enhanced intraoperative ultrasound for cirrhotic patients with hepatocellular carcinoma: A report of 20 cases

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removed surgically if they were close to the main lesion or followed by examinations of alpha-fetoprotein (AFP) level and ultrasound and/or CT/MR every 3 mo.

**RESULTS:** IOUS found 41 nodules in total, among which 17 (41.46%) were newly detected compared to preoperative imaging. Thirty-three nodules were diagnosed malignant by CE-IOUS, including one missed by IOUS. The sensitivity and specificity of CE-IOUS on detecting HCC nodules are 100% (33/33) and 100% (9/9), respectively. Nine nodules were considered benign by CE-IOUS, four was confirmed at histology and five by follow-up. CE-IOUS changed the surgical strategy in 35% (7/20) of patients and avoid unnecessary intervention in 30% (6/20) of patients.

**CONCLUSION:** CE-IOUS is a useful means to characterize the nodules detected by IOUS in cirrhotic liver, to find isoechoic HCC nodules which can not be shown on IOUS and to improve the accuracy of conventional IOUS, thus it can be used as an essential tool in the surgical treatment of cirrhotic patients with HCC.

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**Key words:** Cirrhosis; Liver neoplasms; Intraoperative ultrasound; Microbubble contrast agent; Hepatectomy

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Lu Q, Luo Y, Yuan CX, Zeng Y, Wu H, Lei Z, Zhong Y, Fan YT, Wang HH, Luo Y. Value of contrast-enhanced intraoperative ultrasound for cirrhotic patients with hepatocellular carcinoma: A report of 20 cases. *World J Gastroenterol* 2008; 14(25): 4005-4010 Available from: URL: <http://www.wjnet.com/1007-9327/14/4005.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4005>

## INTRODUCTION

Intraoperative ultrasound (IOUS) is an important tool for surgical treatment of liver tumors<sup>[1,2]</sup>, and particularly for hepatocellular carcinoma (HCC). It has been shown

that IOUS is the most accurate diagnostic technique for detecting focal liver lesions (FLL) and has a great impact on the surgical approach to liver tumors<sup>[3-7]</sup>. However, in cirrhotic patients with HCC, not all nodules detected by IOUS are neoplastic<sup>[8]</sup>. Compared with CT and magnetic resonance (MR) imaging, IOUS has better spatial resolution with high frequency probe<sup>[9]</sup>, however, it can not provide information about tumor vascularity and tissue microcirculation. How to differentiate small HCC from the nodules detected by IOUS poses a big challenge both for surgeons and ultrasonic doctors. The application of intravenous ultrasound contrast agents during transcutaneous ultrasonography of the liver has shown to improve nodule characterization in comparison with unenhanced ultrasound<sup>[10-14]</sup>. Therefore, we investigated if the application of contrast-enhanced ultrasound examination intraoperatively could solve the aforementioned deficiencies of IOUS during liver exploration.

## MATERIALS AND METHODS

This study was conducted under the approval of the Committee of Ethics of West China Hospital and informed consents of all the patients were obtained. From January 2007 to September 2007, a total of 20 consecutive cirrhotic patients with HCC scheduled to undergo partial liver resection were studied. They included 15 men and 5 women, aged 28-68 (mean 50) years. Preoperative contrast enhanced CT and/or MR scans were performed within 1-2 wk of operation. Contrast enhanced CT scans were obtained with multi-detector CT scanners (Somatom Sensation 64, Siemens Medical Solutions, Erlangen, Germany). The patients received 120-150 mL iopromide (Ultravist 300 or 370; Schering, Berlin, Germany) at a rate of 3 mL/s. CT was performed during the arterial phase using a 25-35 s delay, portal venous phase using a 70-75 s delay, and equilibrium phase using a 3 min delay after i.v. administration with 7-mm section thickness. Contrast-enhanced MRI examinations were performed with a 1.5 T imaging system (Gyroscan Intera, Philips Medical Systems Best, Netherlands), using a breathhold 3D gradient echo sequence with fat saturation sequence, following an iv bolus of 0.1 mmol gadobenate dimeglumine (MultiHance, Bracco SpA, Milan, Italy) per kg of body weight at a rate of 2 mL/s. Data was acquired in the hepatic arterial, portal venous, and equilibrium phases. CT was performed in 15 patients and MRI in two and both in three patients. The number of the lesions was recorded.

### Intraoperative imaging techniques

We used VIVID4 unit (GE, USA) with an I-shaped 10-4 MHz intraoperative probe for IOUS scans. After mobilization of the liver, IOUS was performed to search for nodules and suspected lesions were counted and mapped. CE-IOUS was carried out both for lesion characterization and new nodule detection. Considering no specific intraoperative probe is available for contrast

**Table 1 HCC nodules detected at preoperative imaging, IOUS and CE-IOUS**

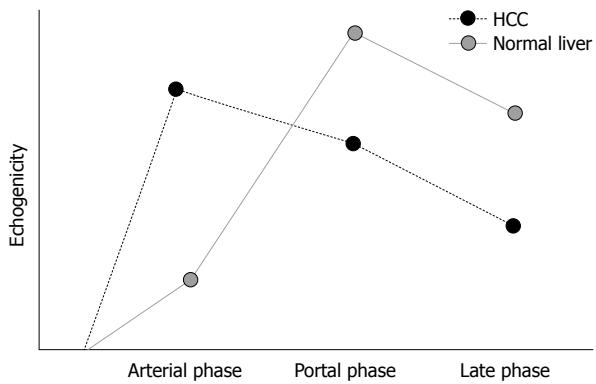
Patient no.	Number of HCC nodules		
	Preoperative imaging	IOUS	CE-IOUS
1	1	3	1
2	2	4	2
3	1	3	2
4	2	2	2
5	1	1	1
6	1	1	1
7	1	1	1
8	1	2	1
9	1	3	3
10	1	2	1
11	1	1	1
12	1	1	1
13	1	1	1
14	2	2	2
15	1	2	2
16	2	5	4
17	1	1	1
18	1	3	2
19	1	2	2
20	1	1	2
Total	24	41	33

study, we used IU22 unit (Philips, USA) equipped with a 5-2 MHz convex transducer and a 9-3 MHz linear transducer instead. Both of the probes have the capacity for contrast enhanced ultrasound studies. The contrast agent was SonoVue (Bracco Imaging, Milan, Italy) which consists of sulphur hexafluoride microbubbles stabilized by a phospholipid shell; 4.8 mL of SonoVue per exploration was injected intravenously through a peripheral vein. A low mechanical index (MI < 0.1) mode was used. All phases of contrast enhancement, including arterial (10-20 s to 25-35 s after injection), portal (30-45 s to 120 s) and late parenchymal (> 120 s) phases were recorded and analyzed<sup>[13]</sup>. HCC is characterized by arterial phase hyper-enhancing and wash out of microbubbles during the portal and late phase, while benign solid lesions are characterized by persistence of contrast enhancement during the portal and late phase<sup>[15]</sup>.

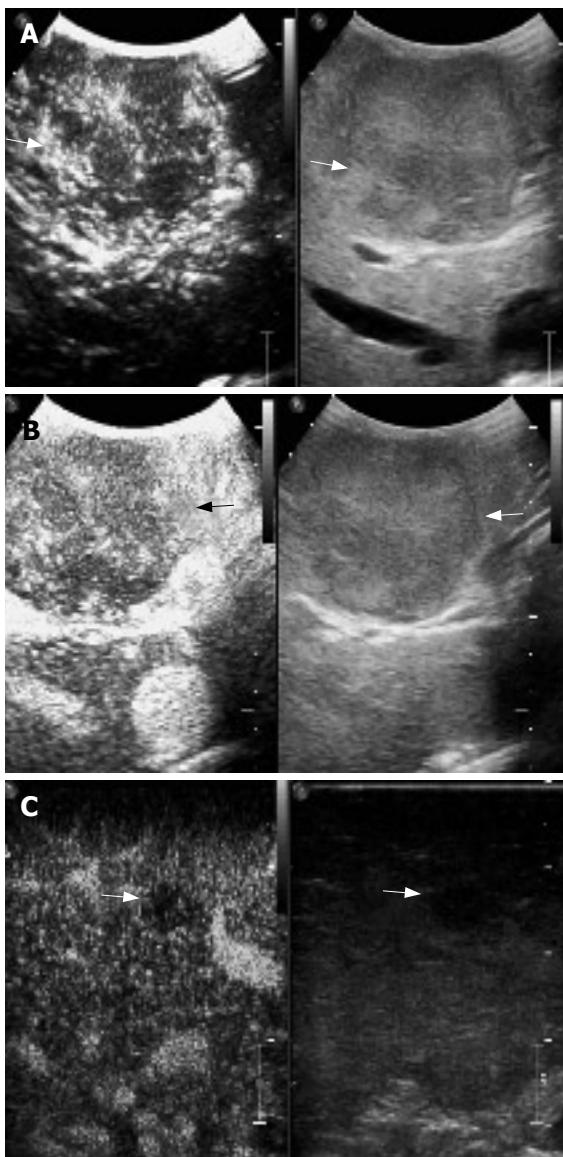
Nodules showing arterial hyper-enhancement and/or hypo-enhancement in late parenchymal phase were removed surgically. Ultrasound-guided biopsy and ethanol ablation would be an alteration if the nodule can not be removed surgically. Nodules depicting isoenhancement both in arterial and late parenchymal phases were considered benign and removed only in cases located close to the main lesion and the others were followed by examinations of alpha-fetoprotein (AFP) level and ultrasound and/or spiral CT every 3 mo.

## RESULTS

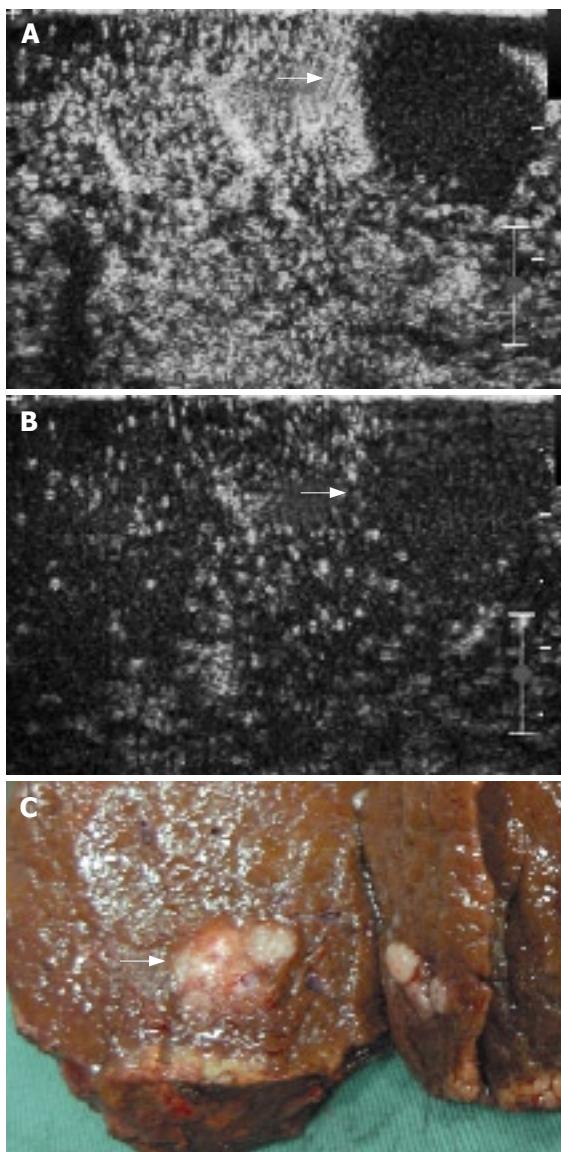
The number of nodules detected by different imaging methods is summarized in Table 1. The sensitivity and specificity of CE-IOUS in detecting HCC nodules is 100% (33/33) and 100% (9/9), respectively. IOUS and CE-IOUS confirmed the existence of all the 24



**Figure 1** It illustrates the enhancing pattern of HCC and normal liver parenchyma. With more arterial supply, HCC appears hyperechoic in the arterial phase and the lesion is slightly and then clearly hypoechoic, and relative to the surrounding parenchyma in the portal and late phases with the washout of contrast agents.

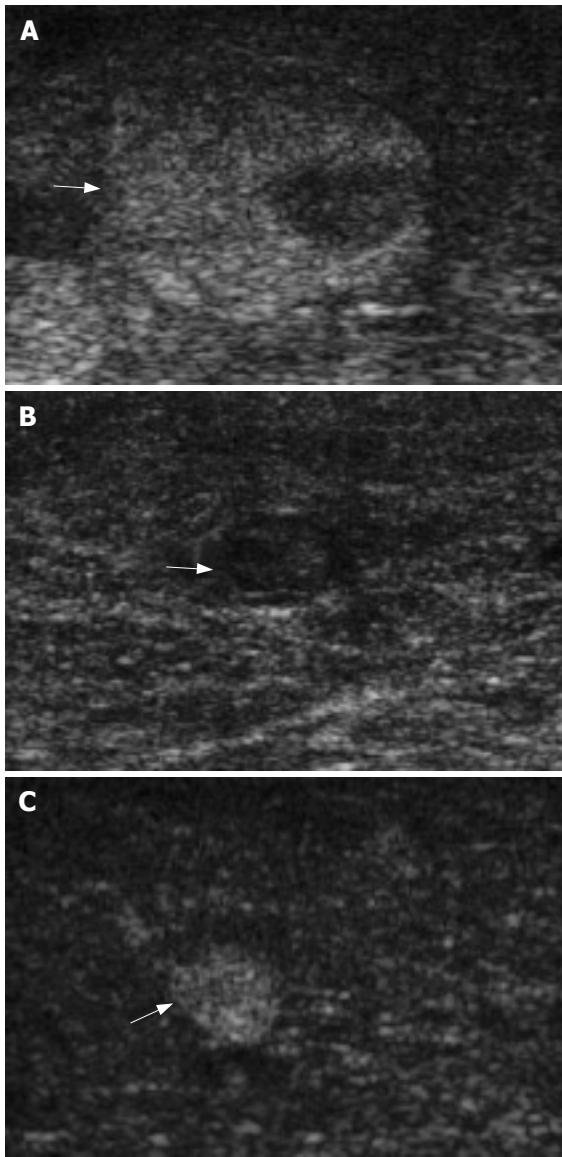


**Figure 2** A mosaic nodule in a cirrhotic liver at IOUS. **A:** At arterial phase, the nodule shows early enhancement (arrows); **B:** The nodule demonstrates contrast agent wash-out in late phase: it was a typical appearance of HCC and proved malignant at histology (arrows); **C:** Another hypoechoic nodule at IOUS was hypoenhanced in late phase: it was considered intrahepatic metastasis and confirmed malignant at histology (arrows).



**Figure 3** A mosaic nodule in a cirrhotic liver at IOUS. **A:** The nodule shows no contrast agent uptake in arterial phase (arrows); **B:** The nodule shows no contrast agent uptake in late parenchymal phase (arrows); **C:** Specimen of the mass after resection proved to be HCC at histology.

nodules found preoperatively. Among the 24 lesions, which were proved to be HCC at histology, 23 nodules demonstrated arterial phase hyper-enhancement and late phase hypo-enhancement (Figures 1 and 2). One nodule had no contrast agent uptake both in arterial and late parenchymal phases (Figure 3). Seventeen nodules were newly detected by IOUS (Figure 4). Among the 17 IOUS newly detected nodules, 8 were hyper-enhanced in arterial phase and hypo-enhanced in late phase on CE-IOS and proved to be malignant at histology. The other 9 nodules illustrated isoenhancement in both arterial and late parenchymal phases (Figure 5). Four of them were removed because they were located close to the main lesions and these lesions were dysplastic nodules in histology. Five nodules were not removed and follow-up ultrasound and/or CT showed no sign of malignancy with normal AFP level after 6–15 mo. One isoechoic HCC nodule



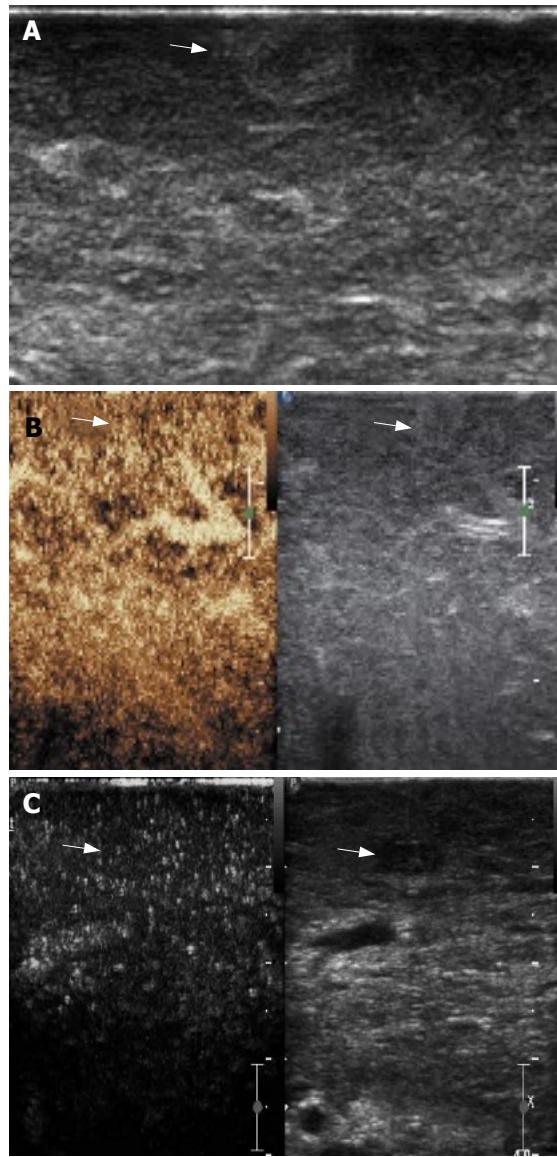
**Figure 4** Appearance of nodules in a cirrhotic liver at IOUS. **A:** Mosaic pattern nodule (arrows); **B:** Hypoechoic nodule (arrows); **C:** Hyperechoic nodule (arrows).

was missed by IOUS, but showed a typical contrast agent washout pattern on CE-Ious late parenchymal phase (Figure 6).

CE-Ious changed the surgical strategy in 35% (7/20) of patients, including additional tumor enucleation in five and ultrasound guided ethanol injection in two patients. CE-Ious also avoided unnecessary intervention in 30% (6/20) of patients who otherwise need additional nodule enucleation or extended hepatectomy according to the IOUS results.

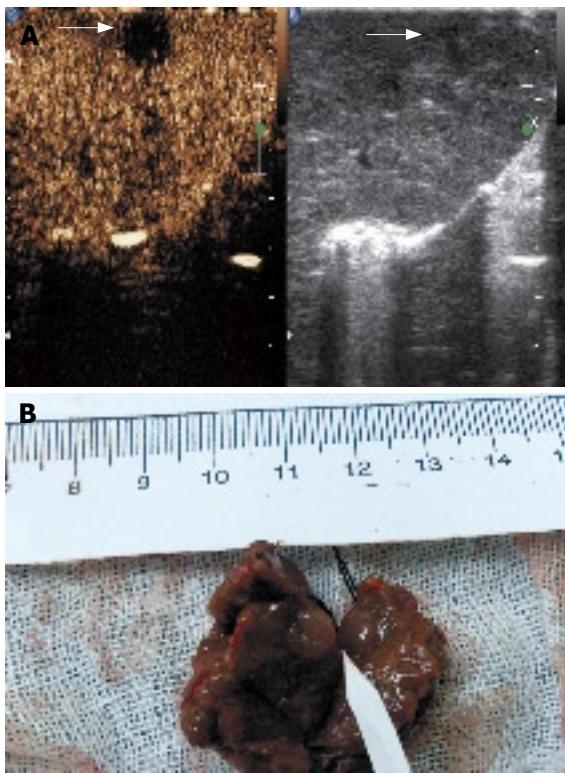
## DISCUSSION

Radicality and preservation as much remnant liver parenchyma as possible are a goal for hepatic resection for cirrhotic patients with HCC. Since it is not uncommon that multiple nodules exist in cirrhotic liver with HCC, differential diagnosis of such nodules is critical, because the operation strategy may have to be changed if



**Figure 5** **A:** A hyperechoic nodule detected at IOUS in cirrhotic liver; **B:** The nodule shows no arterial enhancement and late parenchymal phase wash-out; **C:** A hypoechoic nodule detected at IOUS in cirrhotic liver, and it shows the same enhancing pattern with the surrounding parenchyma. These two nodules were considered dysplastic and the diagnosis was confirmed by pathologic examination.

another HCC lesion is found. Utilizing high frequency probe, compared with CT and MRI, IOUS has better spatial resolution and it is regarded as the most accurate diagnostic method for detecting FLL. In our study, besides the 24 nodules which were already detected preoperatively, IOUS found 17 new nodules in 50% (10/20) of patients. However, new nodules lack specific IOUS findings for HCC. Some studies showed that nodules with a mosaic ultrasound pattern are malignant in 84% of cases; about 24%-30% of hypoechoic nodules, and 0%-18% of hyperechoic nodules are malignant<sup>[16]</sup>. In our study, only 47.06% (8/17) of the IOUS newly detected nodules were confirmed to be HCC at histology. Patients would be at higher risk for postoperative complications if all the nodules detected by IOUS were removed. So it is urgent that new ways are developed to increase the accuracy of IOUS.



**Figure 6** A: An isoechoic nodule measuring 7 mm was missed at IOUS but appeared clearly at late phase of CE-IOUS with contrast agent wash-out; B: Specimen of the nodule after resection proved to be HCC at histology.

Studies suggest that liver ultrasound performed with microbubble contrast agents has led to improved characterization and detection of FLL since it provides information about tumor vascularity and tissue microcirculation comparable to that provided by contrast-enhanced CT and MR imaging, with the added benefit of real-time imaging<sup>[17,18]</sup>. Different enhancing patterns to differentiate FLL have been described<sup>[19,20]</sup>, for example, typical HCC shows arterial phase hyperenhancement and wash-out of contrast agent in portal and late parenchymal phase. The late phase is useful to determine the benign or malignant nature of a lesion while the arterial phase helps predict its histology<sup>[21-25]</sup>. About 86%-93% of benign lesions retain the contrast in the late phase, while 78%-98% of the malignant ones demonstrate wash-out of the contrast<sup>[21,22]</sup>.

In our study, CE-IOUS confirmed the malignancy of the 24 HCC nodules detected preoperatively and showed the vascularity and microcirculation of the IOUS newly detected nodules. In total, 96.97% (32/33) of the HCC nodules depicted arterial phase hyperenhancement and 100% (33/33) showed late phase hypoenhancement, while 100% (9/9) of dysplastic nodules illustrated isoenhancement in both arterial phase and late phase.

CE-IOUS can also help find isoechoic HCC nodules. An isoechoic nodule measuring 7 mm which was missed at IOUS showed a typical contrast agent wash-out in late phase at CE-IOUS. Its malignancy was confirmed at histology. This phenomenon highlights the importance of scanning of the whole cirrhotic liver in late phase of CE-IOUS to detect occult lesions at IOUS.

It is known that IOUS affects surgical strategy in 20%-43.8% of patients with liver tumors<sup>[26-28]</sup>. CE-IOUS findings further demonstrate the benign or malignant nature of the lesions detected by IOUS. Since the duration of late parenchymal phase is much longer than arterial phase, a comprehensive assessment of the whole liver parenchyma which can not be accomplished in arterial phase is required in late phase. Generally, resection of the hypo-enhanced nodules in late phase on CE-IOUS is recommended. In our study, CE-IOUS changed the surgical strategy in 35% (7/20) of patients and avoided unnecessary intervention in 30% (6/20) of patients. That is to say CE-IOUS makes IOUS more accurate, thus enhancing the impact of this technique on operative decision-making for liver tumors.

There is still atypical enhancing mode of HCC. One nodule with a diameter of 2 cm which was enhanced in preoperative CT, mosaic at IOUS and hard on palpation showed no contrast agent uptake in both arterial and late phases. It was proved to be HCC at histology. This atypical pattern of enhancement of HCC nodule at CE-IOUS, when compared with that at spiral CT, supposes that some tumor vascular architecture may permit the spread of CT contrast agent while prevent that of ultrasound contrast agent<sup>[29]</sup>. However, this needs further studies to find any pathological correlations.

In our experience, high frequency probe (L9-3) is more suitable for CE-IOUS study of superficial lesions because of its high resolution in the near field. But for lesions deeper than 3 cm, lower frequency probe (C5-2) is recommended for better penetration and echo-enhancement. The respiratory tract pressure increased during general anesthesia and the scanning is performed directly on the liver surface, bubble destruction thus increased<sup>[30]</sup>. Therefore 4.8 mL SonoVue per exploration is recommended for CE-IOUS.

In conclusion, though only preliminary experience is available, CE-IOUS appears to be an exciting and promising new application. It helps differentiate nodules detected by IOUS in cirrhotic liver and find isoechoic nodules which can not be shown on IOUS, and then improves the accuracy of conventional IOUS. Considering the small number of patients, more studies are needed for further evaluation of this new technique.

## COMMENTS

### Background

Intraoperative ultrasound (IOUS) is an important tool for liver tumors, but it has some drawbacks such as lack of specificity to differentiate cirrhotic nodules from small malignant nodules. Contrast-enhanced ultrasound was shown with improved nodule characterization by recent studies. Therefore, authors investigated if the application of contrast-enhanced ultrasound intraoperatively could solve the aforementioned deficiencies of IOUS.

### Innovations and breakthroughs

The application of CE-IOUS improves nodule characterization. It helps surgeons to eradicate malignant nodules and avoid unnecessary intervention of benign ones.

### Applications

CE-IOUS raised the accuracy of conventional IOUS, thus improving the management of nodules detected in liver surgeries.

### Terminology

To apply ultrasound examination during surgeries, IOUS is considered an

essential tool for detecting focal liver lesions (FLL) and has a great impact on the surgical approach to liver tumors. Besides the merits of IOUS, CE-Ious can display the tumor vascularity and tissue microcirculation using intravenous ultrasound contrast agents, thus helping differentiate malignant nodules from benign ones.

#### Peer review

The research is quite interesting and the results are relevant to improve the quality of hepatic surgery, mainly in cirrhotic patients. The role of contrast-enhanced ultrasound in the study of hepatocellular carcinoma has already been studied in the preoperative setting. However few studies have focused on its intraoperative role, and thus this report is quite innovative.

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CLINICAL RESEARCH

# How good is endoscopic ultrasound for TNM staging of gastric cancers? A meta-analysis and systematic review

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a pooled sensitivity of 99.2% (95% CI: 97.1-99.9). For nodal staging, the pooled sensitivity for N1 was 58.2% (95% CI: 53.5-62.8) and N2 was 64.9% (95% CI: 60.8-68.8). Pooled sensitivity to diagnose distant metastasis was 73.2% (95% CI: 63.2-81.7). The *P* for chi-squared heterogeneity for all the pooled accuracy estimates was > 0.10.

**CONCLUSION:** EUS results are more accurate with advanced disease than early disease. If EUS diagnoses advanced disease, such as T4 disease, the patient is 500 times more likely to have true anatomic stage of T4 disease.

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**Key words:** Gastric cancer; Staging; Meta-analysis; Endoscopic ultrasound

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## Abstract

**AIM:** To evaluate the accuracy of endoscopic ultrasound (EUS) for staging of gastric cancers.

**METHODS:** Only EUS studies confirmed by surgery were selected. Only studies from which a  $2 \times 2$  table could be constructed for true positive, false negative, false positive and true negative values were included. Articles were searched in Medline, Pubmed, Ovid journals, Cumulative index for nursing & allied health literature, International pharmaceutical abstracts, old Medline, Medline nonindexed citations, and Cochrane control trial registry. Two reviewers independently searched and extracted data. The differences were resolved by mutual agreement.  $2 \times 2$  tables were constructed with the data extracted from each study. Meta-analysis for the accuracy of EUS was analyzed by calculating pooled estimates of sensitivity, specificity, likelihood ratios, and diagnostic odds ratio. Pooling was conducted by both the Mantel-Haenszel method (fixed effects model) and DerSimonian Laird method (random effects model). The heterogeneity of studies was tested using Cochran's *Q* test based upon inverse variance weights.

**RESULTS:** Initial search identified 1620 reference articles and of these, 376 relevant articles were selected and reviewed. Twenty-two studies ( $n = 1896$ ) which met the inclusion criteria were included in this analysis. Pooled sensitivity of T1 was 88.1% (95% CI: 84.5-91.1) and T2 was 82.3% (95% CI: 78.2-86.0). For T3, pooled sensitivity was 89.7% (95% CI: 87.1-92.0). T4 had

## INTRODUCTION

Gastric cancer is one of the most common cancers worldwide. Despite the decreasing incidence and mortality, gastric cancer remains the world's second leading cause of cancer-related deaths in the world<sup>[1]</sup>. A treatment option for patients with gastric cancer depends on an accurate evaluation of the stage of the cancer. The prognosis of patients with gastric cancer is determined by the tumor extent and includes both nodal involvement and direct tumor extension beyond the gastric wall<sup>[2,3]</sup>.

Accurate staging of gastric cancers is essential for well-informed decisions on patient management. Accurate gastric cancer staging needs to address two critical questions: which patients qualify for curative therapy and which patients qualify for palliative therapy? This is becoming increasingly important with improvements in non-surgical treatment regimens. Surgery is the

main stay of curative therapy for gastric cancers. While patients with early localized disease clearly benefit from complete surgical resection, increasing evidence exists that multimodal treatment including chemoradiotherapy is superior to surgery alone for patients with resectable gastric cancer<sup>[4]</sup>. Accurate local cancer staging provides the information necessary for such important decisions to be made while not denying patients potentially curative surgical resection, with or without neoadjuvant therapy. The development of other non-surgical techniques at both ends of the disease spectrum has also reinforced the need for accurate cancer staging. Endoscopic ultrasound (EUS) in conjunction with endoscopic mucosal resection has become an appropriate alternative to surgery for superficial non-invasive cancers<sup>[5]</sup>.

The 5-year survival of patients with gastric cancer ranges from 5% to 95% depending on the tumor stage<sup>[6]</sup>. Early gastric cancer or superficial spreading carcinoma is defined as adenocarcinoma limited to the gastric mucosa or submucosa. Patients with early gastric cancer have a favorable prognosis<sup>[7]</sup> and the survival is > 90% after surgical resection<sup>[8-11]</sup>. Therefore, a diagnostic tool that helps diagnose the depth of tumor invasion in the gastric mucosa is essential. In those patients considered unfit for curative surgery, accurate staging is essential to allow an informed decision to be made regarding the most appropriate method of palliation. If comparisons of the outcomes of available and future treatment protocols are to be made, comparable input data-specific to the stage of disease should be available from all patients. This is particularly important if the patient does not undergo primary surgical resection, due to the consequent loss of pathological confirmation, as then the stage of the cancer can only be assessed from the best imaging modality or modalities.

Imaging studies like computerized topographic scan have the advantage of being widely available and noninvasive, but is not very accurate for assessing the depth of invasion or the presence of lymph node involvement<sup>[12,13]</sup>. EUS has emerged as one of the tests for preoperative staging of upper gastrointestinal cancers. The advantage of EUS is the ability to differentiate the layers of gastric mucosa. The accuracy of EUS in staging gastric cancers has been varied, with reports that EUS understages the depth of invasion and overstages the nodal invasion because of inflammation around the tumor or in the lymph nodes<sup>[14]</sup>. The goal of this meta-analysis and systematic review is to evaluate the accuracy of EUS in staging gastric cancers. Due to multiple studies published that looked at EUS in staging gastric cancers and no published meta-analysis in this area, this meta-analysis was performed in an attempt to answer this very important clinical question.

The EUS criteria for depth of tumor invasion and nodal metastasis have changed over the past two decades. Also, the technology of EUS has changed over this period of time. It is not clear if this change in EUS criteria and technology has had an impact on gastric cancer staging. In this meta-analysis and systematic review, we pooled the available studies to evaluate if

changing EUS criteria or technology affects the accuracy of EUS to stage gastric cancers<sup>[15]</sup>.

This meta-analysis and systematic review was written in accordance with the proposal for reporting by the QUOROM (Quality of Reporting of Meta-analyses) statement<sup>[16]</sup>. Since this manuscript looks at diagnostic accuracy of a test, the study design for this meta-analysis and systematic review conformed to the guidelines of Standards for Reporting of Diagnostic Accuracy (STARD) initiative<sup>[17]</sup>.

## MATERIALS AND METHODS

### **Study selection criteria**

Only EUS studies confirmed by surgery were selected. EUS criteria used for T staging were: T1- the tumor invades the lamina propria or submucosa but does not invade the muscularis propria, T2- the tumor invades but does not extend beyond the muscularis propria, T3- tumor penetrates serosa (i.e. visceral peritoneum) without invasion of adjacent structures, and T4- the tumor invades adjacent structures. The criteria used for nodal metastasis were: larger than 1 cm or hypoechoic or round instead of elliptical. Distal metastasis was defined as metastasis to peritoneum or liver. Only studies from which a 2 × 2 table could be constructed for true positive, false negative, false positive and true negative values were included.

### **Data collection and extraction**

Articles were searched in Medline, Pubmed, Ovid journals, Cumulative Index for Nursing & Allied Health Literature, ACP journal club, DARE, International Pharmaceutical Abstracts, old Medline, Medline nonindexed citations, OVID Healthstar, and Cochrane Control Trial Registry. The search terms used were endoscopic ultrasound, EUS, ultrasound, gastric cancer, nodal invasion, staging, surgery, sensitivity, specificity, positive predictive value, and negative predictive value. 2 × 2 tables were constructed with the data extracted from each study. Two authors (SP and JR) independently searched and extracted the data into an abstraction form. Any differences were resolved by mutual agreement.

### **Quality of studies**

Clinical trial with a control arm can be assessed for the quality of the study. A number of criteria have been used to assess this quality of a study (e.g. randomization, selection bias of the arms in the study, concealment of allocation, and blinding of outcome)<sup>[18,19]</sup>. There is no consensus on assessing studies without a control arm and also, these criteria do not apply to studies without control arm<sup>[19]</sup>. Therefore, for this meta-analysis and systematic review, studies were selected based on completeness of data and studies that met the inclusion criteria.

### **Statistical analysis**

Meta-analysis for the accuracy of EUS in staging gastric cancers was performed by calculating pooled estimates

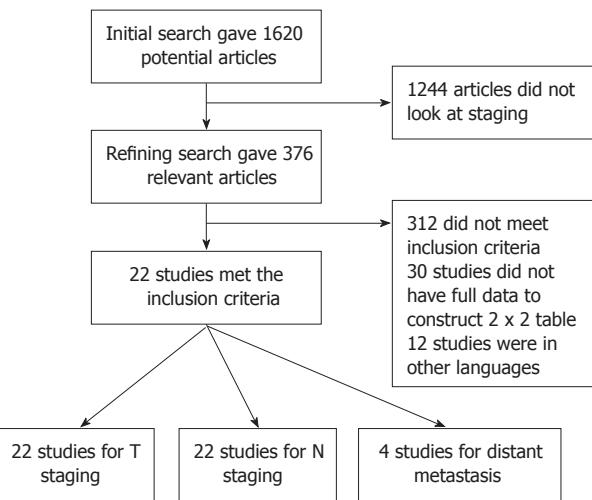
**Table 1 Characteristics of studies included in this meta-analysis for calculating diagnostic accuracy of EUS for gastric cancer staging**

No.	Author	Year of publication	No. of patients	Type of study	Confirmatory procedure
1	Grimm et al <sup>[28]</sup>	1993	147	Prospective	Surgery
2	Francois et al <sup>[29]</sup>	1996	29	Consecutive	Surgery
3	Schimizu et al <sup>[30]</sup>	1994	125	Consecutive	Surgery
4	Dittler et al <sup>[31]</sup>	1993	254	Consecutive	Surgery
5	Ziegler et al <sup>[32]</sup>	1993	118	Prospective	Surgery
6	Botet et al <sup>[33]</sup>	1991	50	Prospective	Surgery
7	Xi et al <sup>[34]</sup>	2003	35	Prospective	Surgery
8	Caletti et al <sup>[35]</sup>	1991	42	Prospective	Surgery
9	Akahoshi et al <sup>[36]</sup>	1989	74	Prospective	Surgery
10	Tio et al <sup>[37]</sup>	1989	72	Prospective	Surgery
11	Massari et al <sup>[38]</sup>	1996	99	Prospective	Surgery
12	Saito et al <sup>[39]</sup>	1991	110	Prospective	Surgery
13	Murata et al <sup>[40]</sup>	1988	146	Prospective	Surgery
14	Hunerbein et al <sup>[41]</sup>	1996	19	Consecutive	Surgery
15	Perng et al <sup>[42]</sup>	1996	69	Consecutive	Surgery
16	Tio et al <sup>[43]</sup>	1989	75	Prospective	Surgery
17	Tio et al <sup>[44]</sup>	1986	36	Prospective	Surgery
18	Shimoyama et al <sup>[45]</sup>	2004	45	Consecutive	Surgery
19	Willis et al <sup>[46]</sup>	2000	116	Consecutive	Surgery
20	Rosch et al <sup>[47]</sup>	1992	41	Consecutive	Surgery
21	Javaid et al <sup>[48]</sup>	2003	112	Consecutive	Surgery
22	Potrc et al	2006	82	Prospective	Surgery

of sensitivity, specificity, likelihood ratios, and diagnostic odds ratio. EUS studies were grouped into periods of time to standardize the change in EUS technology and also to standardize the change in EUS criteria for lymph node involvement. These periods of time were 1986 to 1994, 1995 to 1999, and 2000 to 2006. Pooling was conducted by both Mantel-Haenszel method (fixed effects model) and DerSimonian Laird method (random effects model). The confidence intervals were calculated using the F distribution method<sup>[20]</sup>. For 0 value cells, a 0.5 was added as described by Cox<sup>[21]</sup>. The point estimates in the Forrest plots are proportional to the weight or size of the individual study. The heterogeneity of the sensitivities and specificities were tested by applying the likelihood ratio test<sup>[22]</sup>. The heterogeneity of likelihood ratios and diagnostic odds ratios were tested using Cochran's *Q* test based upon inverse variance weights<sup>[23]</sup>. Heterogeneity among studies was also tested by using summary receiver operating characteristic (SROC) curves. SROC curves were used to calculate area under the curve (AUC). The effect of publication and selection bias on the summary estimates was tested by Egger bias indicator<sup>[24]</sup> and Begg-Mazumdar indicator<sup>[25]</sup>. Also, funnel plots were drawn using the standard error and diagnostic odds ratio to look at bias<sup>[26,27]</sup>.

## RESULTS

Initial search using the search terms identified 1620 reference articles. Among these, 376 relevant articles were selected and reviewed by two authors independently. Twenty-two studies ( $n = 1896$ ) which met the inclusion criteria were included in this analysis and data was extracted from these studies<sup>[28-49]</sup>. All the selected 22



**Figure 1** Flow sheet shows search results.

studies were published as full-text articles in peer review journals. Figure 1 shows the search results and Table 1 shows the details of the included studies. The pooled estimates given here are estimates calculated by the fixed effect model.

### T stage

Pooled sensitivity and specificity of T1 was 88.1% (95% CI: 84.5-91.1) and 100.0% (95% CI: 99.7-100.0) respectively. Figure 2A shows the Forrest plot of sensitivity and specificity of various EUS studies in staging T1 gastric cancers. For T2, the sensitivity was 82.3% (95% CI: 78.2-86.0) and specificity was 95.6% (95% CI: 94.4-96.6). Forest plot in Figure 2B depicts the sensitivity and specificity of EUS in staging T2 cancers. Pooled sensitivity for T3 was 89.7% (95% CI: 87.1-92.0) and specificity was 94.7% (95% CI: 93.3-95.9). Figure 2C depicts sensitivity and specificity of EUS to stage T3 cancers. T4 had a pooled sensitivity of 99.2% (95% CI: 97.1-99.9) and specificity of 96.7% (95% CI: 95.7-97.6). The sensitivity and specificity of EUS to stage T4 in various studies is Figure 2D as a Forrest plot. Pooled likelihood ratios and diagnostic odds ratios for various T stages are shown in Table 2. The pooled estimates of sensitivity, specificity, likelihood ratios, and diagnostic odds ratio computed by random effect model were similar to the fixed effect model. The *P* for chi-squared heterogeneity for all the pooled accuracy estimates was  $> 0.10$ .

### N stage

The pooled sensitivity and specificity for N1 was 58.2% (95% CI: 53.5-62.8) and 87.2% (95% CI: 84.4-89.7) respectively. N2 had a pooled sensitivity of 64.9% (95% CI: 60.8-68.8) and specificity of 92.4% (95% CI: 89.9-94.4). Pooled likelihood ratios and diagnostic odds ratios for various N stages are shown in Table 2. All the pooled estimates calculated by random effect model were similar to fixed effect model. The chi-squared heterogeneity for all the pooled accuracy estimates showed a *P*  $> 0.10$ .

**Table 2 Accuracy of EUS with confidence intervals to diagnose T and N stages in gastric cancer patients**

	<b>Pooled sensitivity</b>	<b>Pooled specificity</b>	<b>Pooled LR +</b>	<b>Pooled LR-</b>	<b>Pooled DOR</b>
T1	88.1% (84.5-91.1)	100.0% (99.7-100.0)	90.1 (48.9-165.7)	0.17 (0.10-0.28)	605.6 (296.8-1235.6)
T2	82.3% (78.2-86.0)	95.6% (94.4-96.6)	17.3 (10.9-27.5)	0.23 (0.17-0.290)	108.6 (56.6-208.1)
T3	89.7% (87.1-92.0)	94.7% (93.3-95.9)	14.3 (10.3-19.8)	0.13 (0.08-0.19)	144.4 (95.4-218.7)
T4	99.2% (97.1-99.9)	96.7% (95.7-97.6)	19.6 (14.1-27.2)	0.07 (0.04-0.12)	507.8 (247.5-1042.1)
N1	58.2% (53.5-62.8)	87.2% (84.4-89.7)	4.1 (2.4-7.1)	0.49 (0.41-0.58)	9.5 (5.3-16.9)
N2	64.9% (60.8-68.8)	92.4% (89.9-94.4)	6.7 (4.1-10.9)	0.39 (0.31-0.49)	26.6 (13.9-50.7)

LR+: Positive likelihood Ratio, LR-: Negative likelihood Ratio, DOR: Diagnostic odds ratio.

**Table 3 Accuracy of EUS with confidence intervals to stage gastric cancers over the past two decades**

	<b>No. of studies</b>	<b>Pooled sensitivity</b>	<b>Pooled specificity</b>	<b>Pooled LR +</b>	<b>Pooled LR-</b>	<b>Pooled DOR</b>	
T1	1986 to 1994	12	56.3 % (49.7-62.6)	89.1% (85.5-92.1)	4.6 (1.6-13.8)	0.51 (0.38-0.69)	9.3 (2.9-30.1)
	1995 to 1999	4	82.2 % (67.9-92.0)	100.0 % (97.9-100.0)	72.9 (18.2-288.8)	0.13 (0.03-0.68)	562.5 (92.0-3438.4)
	2000 to 2006	5	84.8% (71.1-93.7)	100.0% (98.9-100.0)	88.9 (25.3-312.4)	0.22 (0.13-0.38)	428.9 (96.6-1904.9)
T2	1986 to 1994	12	84.9% (79.8-89.2)	96.7% (95.4-97.8)	20.5 (14.8-28.4)	0.19 (0.13-0.30)	207.0 (109.5-391.6)
	1995 to 1999	4	74.4% (57.9-87.0)	90.9% (85.4-94.8)	6.7 (2.5-18.1)	0.33 (0.19-0.55)	23.7 (5.3-106.2)
	2000 to 2006	5	79.5% (70.8-86.5)	94.6 % (91.3-96.9)	16.8 (5.3-53.8)	0.22 (0.12-0.38)	79.6 (22.9-277.2)
T3	1986 to 1994	12	89.6% (86.3-92.3)	95.3% (93.6-96.7)	15.5 (11.4-21.1)	0.12 (0.07-0.22)	160.2 (95.5-268.7)
	1995 to 1999	4	90.3% (80.1-96.4)	91.3% (85.8-95.2)	11.1 (3.5-35.4)	0.12 (0.06-0.25)	93.3 (30.6-284.6)
	2000 to 2006	5	89.8% (83.3-94.5)	94.8% (91.3-97.2)	13.9 (7.7-25.1)	0.12 (0.04-0.37)	154.7 (55.7-429.5)
T4	1986 to 1994	12	98.9% (95.9-99.9)	97.1% (95.9-98.0)	23.3 (14.6-37.4)	0.07 (0.04-0.14)	626.4 (256.1-1532.5)
	1995 to 1999	4	100% (92.5-100.0)	95.8% (91.5-98.3)	14.3 (7.6-26.9)	0.05 (0.01-0.18)	441.7 (85.6-2279.1)
	2000 to 2006	3	100.0% (87.2-100.0)	95.7% (91.9-98.0)	16.4 (7.9-33.9)	0.07 (0.02-0.33)	260.1 (43.7-1547.4)
N1	1986 to 1994	10	56.3% (49-62.6)	89.1% (85.5-92.1)	4.6 (1.6-13.6)	0.5 (0.4-0.7)	9.3 (2.9-30.1)
	1995 to 1999	4	64.6% (53.3-74.9)	83.5% (74.9-90.1)	3.6 (2.3-5.6)	0.5 (0.4-0.6)	8.8 (4.3-18.1)
	2000 to 2006	5	57.8% (49.0-66.20)	85.5% (79.3-90.4)	4.4 (2.9-6.6)	0.5 (0.3-0.7)	10.2 (4.8-21.8)
N2	1986 to 1994	10	70.6% (65.4-75.5)	94.7% (91.7-96.8)	11.0 (4.6-26.6)	0.3 (0.2-0.4)	63.9 (30.9-132.3)
	1995 to 1999	4	70.2% (59.9-79.2)	83.2% (74.1-90.1)	3.9 (2.5-6.1)	0.4 (0.3-0.5)	11.7 (5.6-24.1)
	2000 to 2006	4	49.0% (40.7-57.30)	93.0% (87.5-96.6)	6.4 (1.7-24.1)	0.6 (0.4-0.7)	12.7 (2.9-56.2)

### M stage

Data for EUS accuracy to diagnose distant metastasis was available in four studies<sup>[29,35,36,41]</sup>. The pooled sensitivity to diagnose distal metastasis was 73.2% (95% CI: 63.2-81.7). EUS specificity was 88.6% (84.8-91.7). The positive likelihood ratio to diagnose distal metastasis was 17.2 (2.8-106.3) and the negative likelihood ratio was 0.4 (95% CI: 0.2-0.7). The diagnostic odds ratio of EUS to correctly diagnose distal metastasis was 60.9 (95% CI: 8.2-463.7). All the pooled estimates calculated by random effect model were similar. The *P* for chi-squared heterogeneity for all the pooled accuracy estimates was > 0.10. The SROC curve showed an AUC of 0.98 with a standard error (SE) of 0.005. This curve showed a *Q* value of 0.94 with a SE of 0.01, as shown in Figure 3.

### Affect of technology

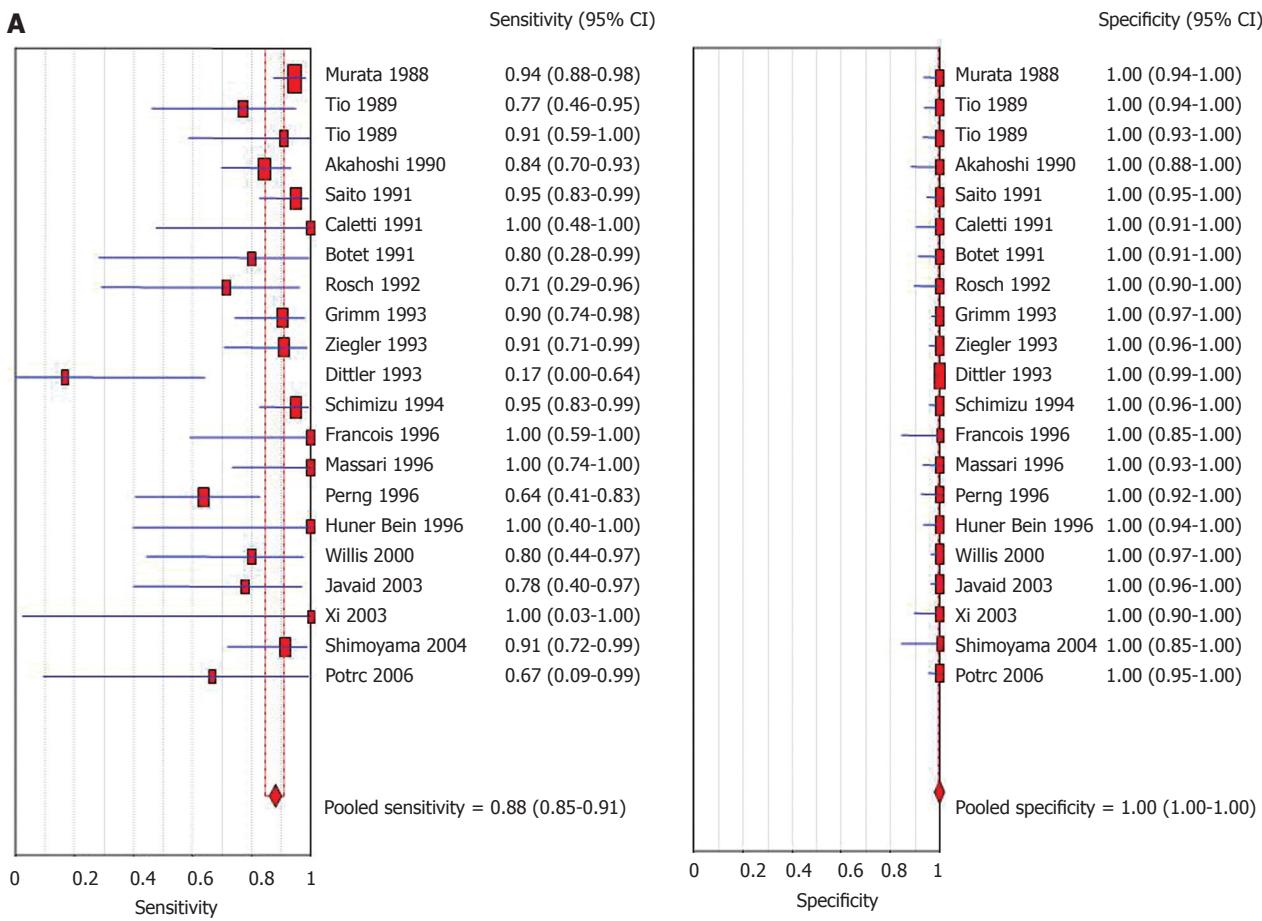
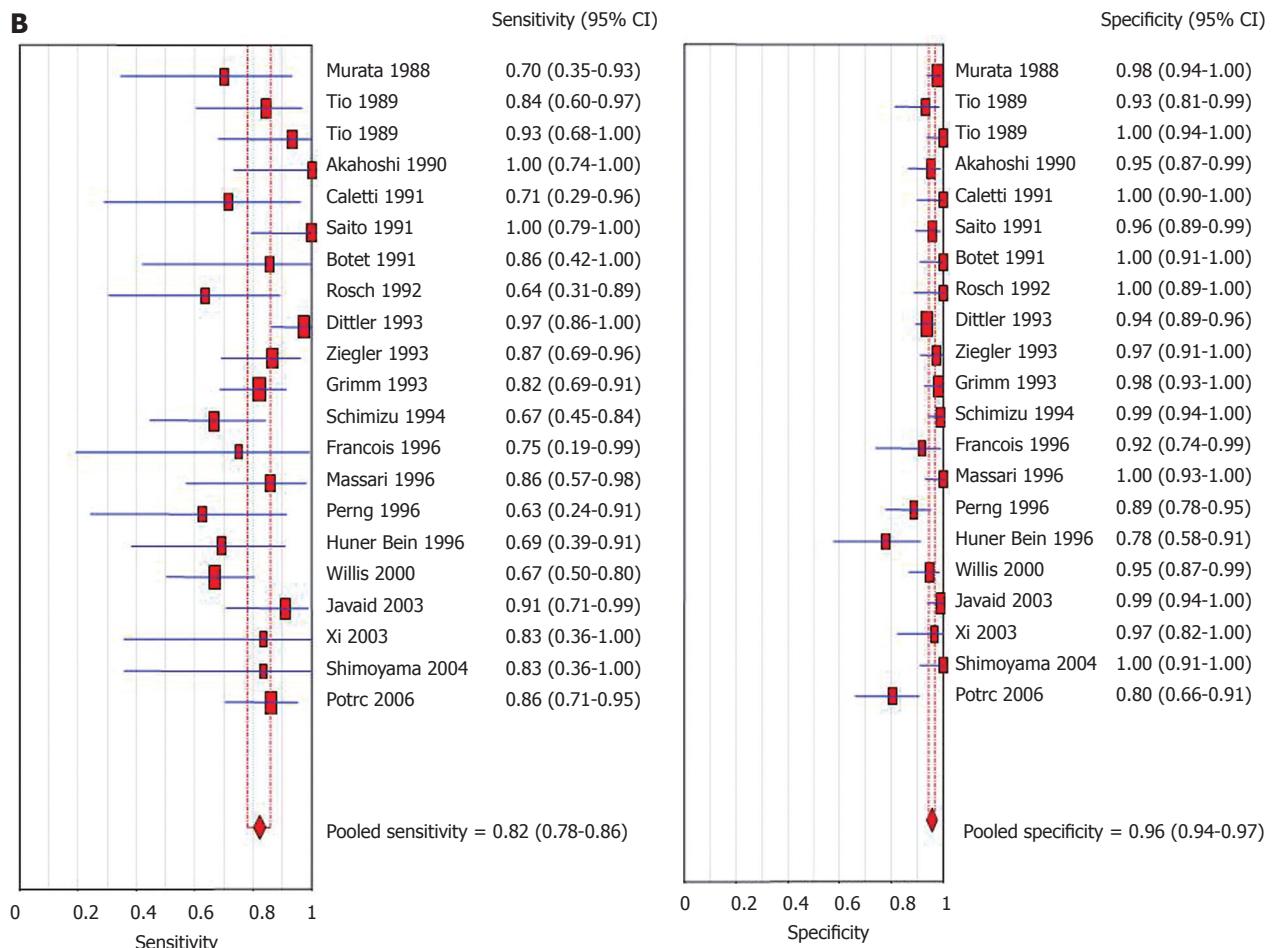
EUS studies were grouped into three periods of time to standardize the change in EUS technology and also to standardize the change in EUS criteria for tumor staging. These periods of time were 1986 to 1994, 1995 to 1999, and 2000 to 2006. The pooled estimates of studies during these periods of time are shown in Table 3. The *P* for chi-squared heterogeneity for all the pooled accuracy estimates was > 0.10. The bias calculations using Egger's bias indicator gave a value of 0.97 (95% CI = -0.77 to 2.71, *P* = 0.26). The bias calculated by Begg-Mazumdar indica-

tor gave a Kendall's tau value of 0.19, *P* = 0.24. The funnel plot for bias is shown in Figure 4.

## DISCUSSION

Correct staging of patients with gastric cancer helps to direct precise therapy and predict prognosis<sup>[7-9]</sup>. Majority of patients in all the included studies had adenocarcinomas. Patient with other types of gastric cancers couldn't be excluded from the analysis. Gastric cancer staging aids in determining non-operative versus operative management, pre-operative adjuvant chemoradiation, and local excision or endoscopic therapy versus wide resection<sup>[50]</sup>. Imaging modalities such as trans-abdominal B-mode ultrasonography, computed tomography, or magnetic resonance imaging lack the ability to differentiate layers of the gastric mucosa<sup>[51,52]</sup>.

The studies included in this analysis used established criteria for gastric cancer staging<sup>[53]</sup>. The definition for T staging given in the methodology was used in the included studies. This meta-analysis and systematic review shows that the pooled sensitivity of EUS for tumor invasion (T stage) is high and it is higher for advanced disease when compared to early disease. The pooled specificity for depth of tumor invasion is very high for all the T stages. Diagnostic odds ratio is defined as the odds of having a positive test in patients with

**A****B**

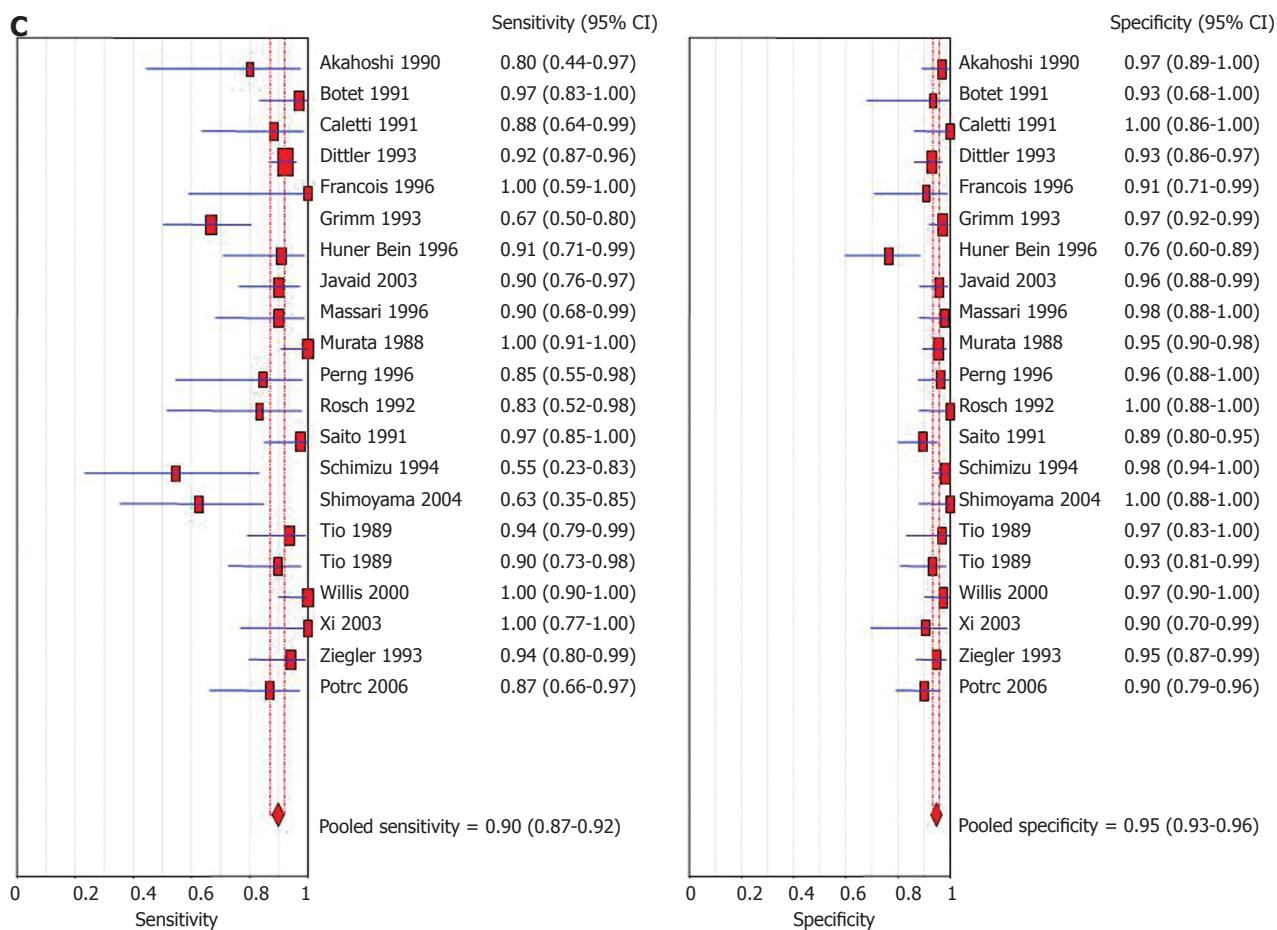
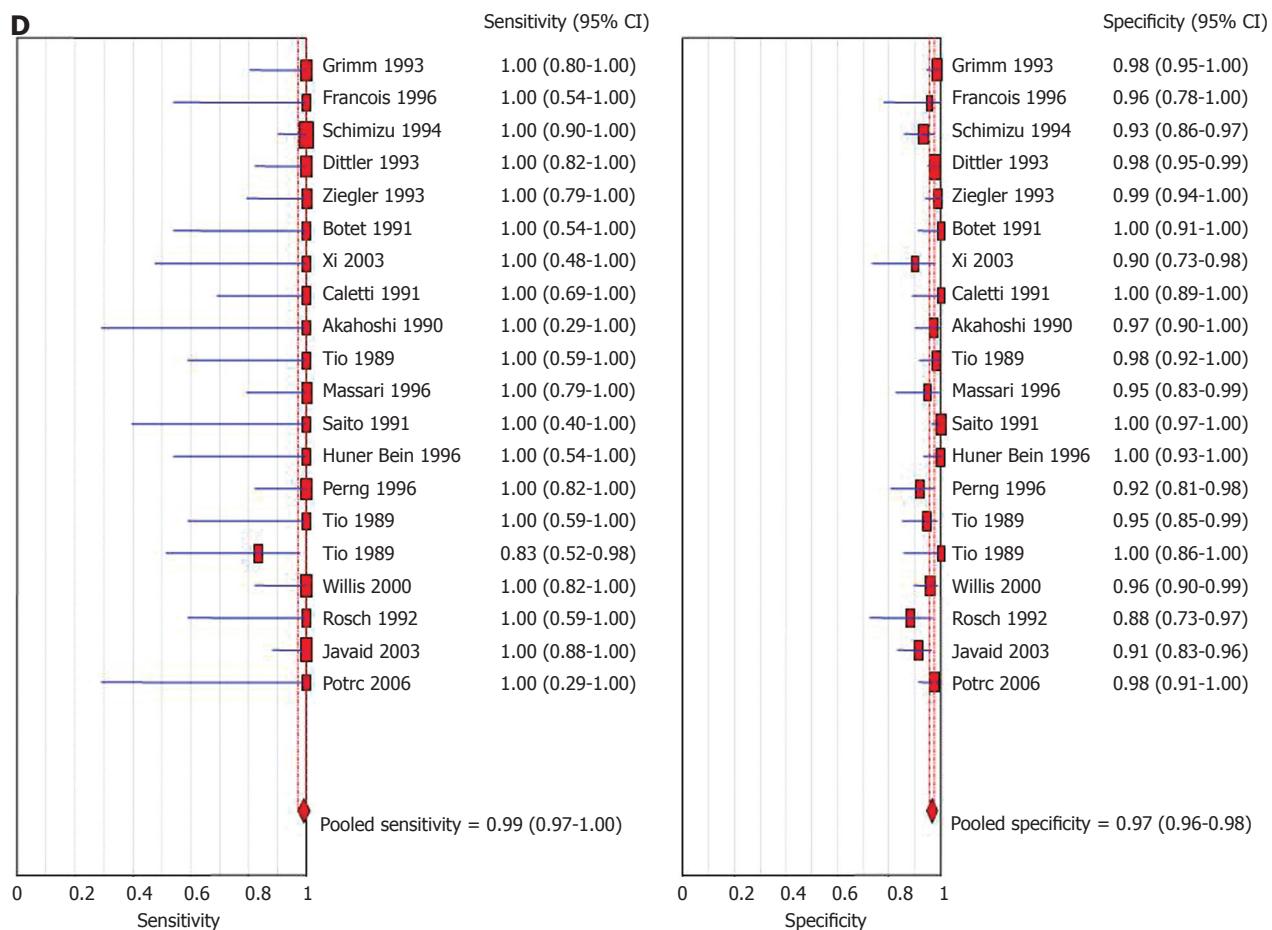
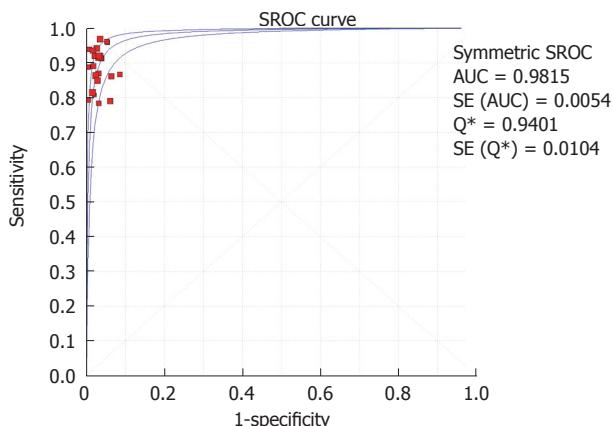
**C****D**

Figure 2 Forrest plot showing sensitivity and specificity of EUS in diagnosing T stage of gastric cancers. A: T1 Stage; B: T2 Stage; C: T3 Stage; D: T4 Stage.

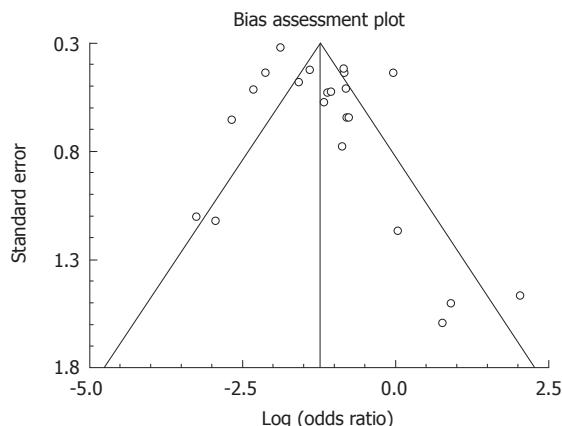


**Figure 3** Summary receiver operator curve showing AUC.

true anatomic stage of the disease when compared to patients who don't have the disease. EUS has a very high diagnostic odds ratio for T staging. For example, if EUS demonstrates a patient has T1 disease, the patient has odds of 605 times to have the correct anatomic stage of T1 disease. This helps physicians offer curative therapy either surgical or endoscopic with confidence to patients with early disease<sup>[10]</sup>. Another perspective is: if a small lesion is found with the tissue diagnosis of gastric cancer, EUS would be an excellent test to examine the depth of tumor invasion due to a very high sensitivity and specificity.

The negative likelihood ratio of a test is a measure of how well the test performs in excluding a disease state. Lower the negative likelihood ratio, the better the test performs in excluding a disease. The positive likelihood ratio is a measure of how well the same test identifies a disease state. For T staging, EUS has a low negative likelihood ratio for T4 disease when compared to T1 disease and a high positive likelihood ratio for all T stages. This indicates that EUS performs better in excluding T4 disease than T1 disease. Another perspective of looking at it is: if EUS diagnoses T2 disease then the patient might still have anatomic T1 disease but if EUS diagnoses T1 disease then the patient probably truly has anatomic T1 disease and can have curative therapy. This helps physicians offer endoscopic treatments such as EMR or ESD with confidence for T1 gastric cancer as alternatives to curative surgery<sup>[54-58]</sup>.

According to our meta-analysis, EUS is more accurate to diagnose advanced than early gastric cancer. If EUS diagnoses advanced disease, such as T4 disease, the patient is 500 times more likely to have true anatomic T4 stage of disease and will benefit from palliative therapy. This helps physicians to offer with confidence surgical and non-surgical palliative therapies such as placement of self-expanding metal stents for obstruction<sup>[59]</sup>. For nodal staging of gastric cancers, all the pooled accuracy estimates of EUS are higher for N2 (advanced disease) when compared to N1. The accuracy of EUS in diagnosing N stage is not high. Also, it is not clear if using all the three criteria together or in combination for nodal involvement improves the diagnostic accuracy. The



**Figure 4** Funnel plot showing bias assessment.

role of FNA in nodal staging of gastric cancers could not be evaluated because of lack of substantial number of studies with the data. Obtaining tissue, however, is expected to improve accuracy of EUS/FNA but more studies are needed before this is concluded.

Over the last two decades, the sensitivity of EUS for T staging improved, especially for early disease (T1). Thus EUS is vital in answering the question which patients qualify for curative therapy. EUS, however, as an imaging modality did not improve for N staging. The specificity for both T and N staging remained high during the past two decades. For distant metastasis, though the number of studies with data was smaller, the pooled specificity is high but the sensitivity is not as high. EUS as a diagnostic tool is not designed to look at distant metastasis.

Heterogeneity among different studies was determined by drawing SROC curves and finding the AUC, since different studies might use slightly different criteria for staging. An AUC of 1 for any test indicates that the test is excellent. SROC curves for EUS showed that the value for AUC was very close to 1, indicating that EUS is an excellent diagnostic test for staging gastric cancers.

Publication bias and selection bias can affect the summary estimates. Studies with statistically significant results tend to be published and cited. Smaller studies can show larger treatment effects due to fewer case-mix differences (e.g. patient with only early or late disease) than larger trials. This bias can be estimated by bias indicators and by drawing funnel plots. Bias among studies can affect the shape of the funnel plot. In this meta-analysis and systematic review, bias calculations using Egger bias indicator<sup>[24]</sup> and Begg-Mazumdar indicator<sup>[25]</sup> showed no statistically significant bias. Funnel plot also showed no significant bias.

In conclusion, EUS is an accurate and minimally invasive diagnostic tool to evaluate T stage of a patient with gastric cancer. EUS results are more accurate with advanced disease than early disease. If EUS diagnoses advanced disease, such as T4 disease, the patient is 500 times more likely to have true anatomic T4 stage of disease. Considering that new curative and palliative endoscopic therapies are emerging as an alternative

to surgery in gastric cancer, EUS should be strongly considered for staging. Our meta-analysis supports the use of EUS in determining if curative or palliative therapies are the most appropriate approach for a particular stage of the disease. Further studies are needed to improve accuracy of EUS in detecting early nodal invasion.

## COMMENTS

### Background

Gastric cancer is one of the most common cancers worldwide. The prognosis of patients with gastric cancer is determined by the tumor extent and includes both nodal involvement and direct tumor extension beyond the gastric wall. Endoscopic ultrasound (EUS) has emerged as one of the tests for preoperative staging of upper gastrointestinal cancers. The goal of this meta-analysis and systematic review by Puli *et al* was to evaluate the accuracy of EUS in staging gastric cancers.

### Research frontiers

The accuracy of EUS in staging gastric cancers has been varied, with reports that EUS understages the depth of invasion and overstages the nodal invasion because of inflammation around the tumor or in the lymph nodes.

### Innovations and breakthroughs

Due to multiple studies published that looked at EUS in staging gastric cancers but no published meta-analysis in this area, this meta-analysis was performed in an attempt to answer this very important clinical question of how good EUS is in TNM staging of gastric cancers.

### Applications

EUS is an accurate and minimally invasive diagnostic tool to evaluate the T stage of a patient with gastric cancer. EUS results are more accurate with advanced disease than early disease. If EUS diagnoses advanced disease, such as T4 disease, the patient is 500 times more likely to have true anatomic T4 stage of disease. Considering that new curative and palliative endoscopic therapies are emerging as an alternative to surgery in gastric cancer, EUS should be strongly considered for staging. Our meta-analysis supports the use of EUS in determining if curative or palliative therapies are the most appropriate approach for a particular stage of the disease. Further studies are needed to improve accuracy of EUS in detecting early nodal invasion.

### Terminology

Meta-analysis for the accuracy of EUS in staging gastric cancers was performed by calculating pooled estimates of sensitivity, specificity, likelihood ratios, and diagnostic odds ratio. Pooled was conducted by both Mantel-Haenszel method (fixed effects model) and DerSimonian Laird method (random effects model).

### Peer review

This is an interesting study; also it is a comprehensive review article, comparing the results of some authors' studies. The effectiveness of endoscopic ultrasound use for TNM staging of gastric cancers was discussed.

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CLINICAL RESEARCH

# Is the pain in chronic pancreatitis of neuropathic origin? Support from EEG studies during experimental pain

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## Abstract

**AIM:** To prove the hypothesis that patients with chronic pancreatitis would show increased theta activity during painful visceral stimulation.

**METHODS:** Eight patients and 12 healthy controls underwent an experiment where the esophagus was electrically stimulated at the pain threshold using a nasal endoscope. The electroencephalogram (EEG) was recorded from 64 surface electrodes and "topographic matching pursuit" was used to extract the EEG information in the early brain activation after stimulation.

**RESULTS:** A major difference between controls and patients were seen in delta and theta bands, whereas there were only minor differences in other frequency bands. In the theta band, the patients showed higher activity than controls persisting throughout the 450 ms of analysis with synchronous brain activation between

the channels. The main theta components oscillated with 4.4 Hz in the patients and 5.5 Hz in the controls. The energy in the delta (0.5-3.5 Hz) band was higher in the controls, whereas the patients only showed scattered activity in this band.

**CONCLUSION:** The differences in the theta band indicate that neuropathic pain mechanisms are involved in chronic pancreatitis. This has important implications for the understanding and treatment of pain in these patients, which should be directed against drugs with effects on neuropathic pain disorders.

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**Key words:** Chronic Pancreatitis; Neuropathic pain; Esophagus; Thalamocortical system; Electroencephalography

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## INTRODUCTION

Chronic pancreatitis (CP) is defined as a disease causing inflammation of the pancreas resulting in progressive and irreversible morphological and functional derangements with end-stage exocrine/endocrine failure. The most important symptom is abdominal pain that is present in 80%-90% of patients along evolution of the disease<sup>[1]</sup>. Pancreatic pain presents characteristically with severe dull epigastric pain, eventually radiating to the back. The pain is often recurrent, intense and long-lasting, and may be associated with malnutrition, narcotic addiction and major socio-economical problems. The pain mechanisms are

incompletely understood and perhaps multifactorial. So far, the following causes have been suggested: (1) increased intrapancreatic pressure within the pancreatic duct or parenchyma ensuing tissue ischemia; (2) inflammation in the pancreas; (3) extrapancreatic causes of pain such as bile duct and duodenal stenosis due to extensive pancreatic fibrosis and inflammation, and (4) alterations in pancreatic nerves including an increase in nerve fibers and neurogenic inflammation<sup>[2]</sup>. Histological findings in the pancreas in patients with pain due to CP have revealed an increase in the number and diameter of pancreatic nerve fibers and in the amount of neurotransmitters. These findings are also seen after neuronal lesions in other tissues and have supported the theory that neurogenic inflammation plays a key role in the pain pathogenesis<sup>[3-5]</sup>. Supporting the neurogenic pain theory, the pain in CP is often described as shooting and lancinating. Therefore, it may clinically resemble that of neuropathic pain typically presenting as stimulus-independent shooting or burning pain together with stimulus-evoked hyperalgesia<sup>[6]</sup>.

Due to the difficulties to access the pancreas and potential harmful complications during the procedure, it is not possible directly to investigate whether, for example, allodynia or other characteristic features of neuropathic pain are present during manipulation of the organ. However, visceral pain is typically diffuse and difficult to localize, which to a large extend can be explained by the widespread termination of visceral afferents into 2nd order neurons in multiple segments of the spinal cord<sup>[7]</sup>. Due to this overlap in central termination of visceral afferents, disease mechanisms of the pancreas are typically also reflected during pain stimulation of the esophagus<sup>[8]</sup>. Hence, experimental models where the esophagus is involved will typically reflect any abnormal pain mechanisms relating to the pancreas. Previous studies have given evidence for neuroplastic changes and reorganization of the brain in patients with chronic and neuropathic pain<sup>[9]</sup>, and in recent experiments done by our group such changes were also seen during pain stimulation of the esophagus in patients with CP<sup>[10]</sup>. An approach to detect characteristics of neuropathic pain in CP can, therefore, be related to analysis of the electroencephalographic (EEG) during pain stimulation of the esophagus. It has been proposed that power in the theta (3.5-7.5 Hz) EEG band may be a hallmark of patients with different kinds of neuropathic pain<sup>[11-13]</sup>. Recently, Sarnthein and coworkers showed that patients with neuropathic pain disorders exhibited higher spectral power of the resting EEG especially in the theta range<sup>[14]</sup>. After treatment with a therapeutic lesion in the thalamus, the pain resolved together with normalization of the EEG abnormalities. Hence, EEG analysis may be a tool to diagnose neurogenic pain. Among the methods used for brain imaging EEG (and magnetoencephalography) has the highest temporal resolution reflecting the functions of the brain directly. However, the scalp EEG is a composite of oscillatory activity from deep neuronal sources reaching the scalp by volume conduction with major distortion of the signal. Furthermore, the overall signal is composed by infinity of electrical sources resulting in complex dynamic

changes over short time periods. Therefore, mathematical models are needed to analyze the EEG. Power analysis using, for example, Fast Fourier Transform is a commonly used method, but it does not give reliable information about the frequency content in the very short time window of less than 0.5 s representing the exogenous pain component<sup>[15]</sup>. A relatively new approach for the analysis of biomedical signals is represented by adaptive methods<sup>[16,17]</sup>. Recently, a new method called Topographic Matching Pursuit (TMP) was developed to analyze specific components of the EEG<sup>[18]</sup>. The method decomposes the multichannel EEG signal into a relatively small number of components (called atoms) chosen from a very big and redundant set (called dictionary). Such atoms are able to describe specific spatio-temporal evolution of EEG components in adaptive and very short time windows.

In the current study, TMP analysis was used to evaluate the EEG after experimental pain in the esophagus in patients with chronic pancreatitis and in healthy controls. We hypothesized that there would be differences in the theta band due to the neurogenic component involved in CP.

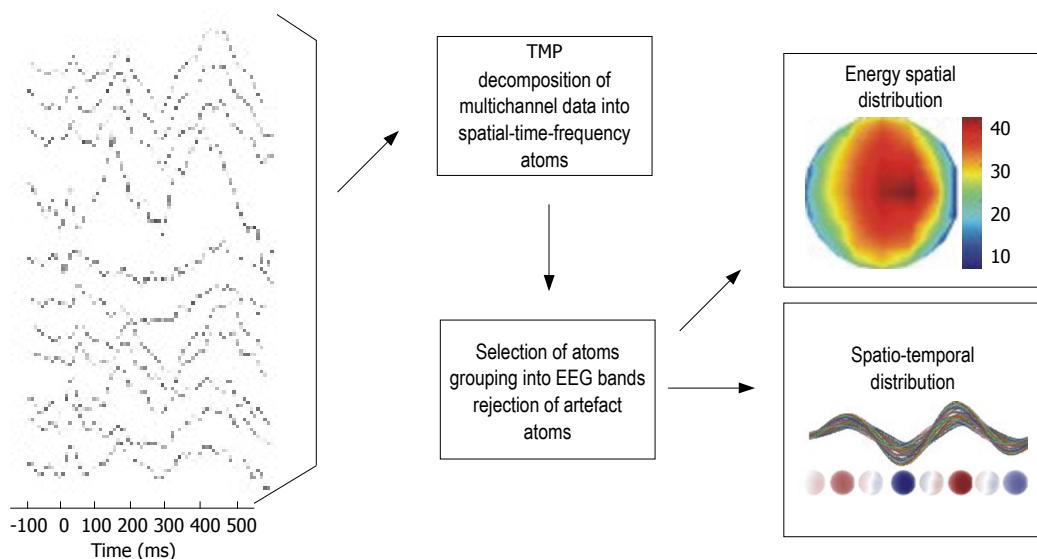
## MATERIALS AND METHODS

### Subjects

Eight patients, six men and two women, mean age 55 years (range, 53-62 years) with CP fulfilling the Marseille-Rome/Cambridge diagnostic criteria<sup>[19]</sup> were enrolled in the study. They all had intermittent pain attacks characteristic for CP and were only included if they had no present alcohol abuse or chronic intake of painkillers. The duration of the pancreatitis was mean 6.1 (range, 1-10) years and the mean frequency of the pain was 5.0 times a week (range, 2-7) with a mean duration of 3.9 h (range, 2-9). None of the patients had any other diseases associated with pain or any history of neurological diseases. Twelve volunteers (ten men and two women, median age 37.5 years (23-49) served as controls. They were all healthy without any gastrointestinal symptoms or disorders associated with pain. The study was conducted according to the Helsinki II Declaration and approved by the Ethical Committee of North Jutland (No. VN 2003/120 mch).

### Experimental protocol and gut stimulation

The subjects were fasting 8 h before the experiment. The same method as previously described was used<sup>[20]</sup>. Briefly, intubation of the esophagus was done through the nose with a 6 mm endoscope [Ultra Slim Gastroscope (Pentax EG-1840)]. The device for bipolar electrical stimulation was inserted through the biopsy channel in the endoscope. The stimulator was positioned 5 cm above the gastro-esophageal junction. A constant current electrical stimulator (Noxitest, Aalborg, Denmark) was used to deliver the visceral stimuli. The intensity of the current was limited to 80 mA and the voltage to 200 V. Before all stimulations the inter-electrode impedance was measured to be < 2 kΩ, securing optimal electrode position throughout the experiment. The intensity of the current was gradually increased in steps of 0.1-0.5 mA until the



**Figure 1** The analysis scheme used for the data investigation. The EEG data were decomposed into a set of TMP atoms, which parameterized the data. Next, the atoms were grouped into bands according to their frequency parameter. This allowed convenient analysis of time and spatial properties of oscillations in all frequency bands.

sensation changed from unpleasantness to pain (pain detection threshold). During the experiment the subject rested quietly and relaxed with the eyes open and was asked to minimize blinking and focus on a fixed point. A single stimulus consisted of 5 fast rectangular constant current pulses at 200 Hz with duration of 1 ms<sup>[21]</sup>. Thirty-five single stimuli were applied with intensity at the pain threshold with an inter-stimulus interval of 5 s. During the stimulations the subjects were observed by a physician, and the electrocardiogram was continuously monitored.

### Recordings

The EEG was recorded from 64 surface electrodes using a standard EEG cap (Quick-Cap International, Neuroscan, USA) following the extended international 10-20 system. The impedance of the electrodes was below 2 kΩ. In addition, two electrodes were placed at the right upper brow and the left external canthus to monitor eye movements. A linked-ears reference was used. EEG signals were recorded and sampled at 1000 Hz, and bandpass filtered between 0.05 Hz and 70 Hz (SynAmps, Neuroscan, USA).

### EEG analysis using TMP

Following the recording, thirty-five single trials were visually examined for artifacts. The contaminated epochs were removed off-line. The data were then baseline corrected and corrected for linear trends (SynAmps, Neuroscan, USA). The artifact free epochs of 450 ms were then collapsed into a single average file. The averaged data from the patients and controls were submitted to TMP analysis. In summary, the TMP is an adaptive and iterative algorithm. It creates spatio-temporal data approximations using a relatively small number of simple components (called atoms) chosen from a very big and redundant set (called dictionary). The TMP method is based on the Matching Pursuit algorithm<sup>[22]</sup>. However, the standard "Gabor atoms" used in the Matching Pursuit describes only the temporal evolution of EEG components in a single channel or channel-averages. The TMP uses atoms, which are distributed in time and space

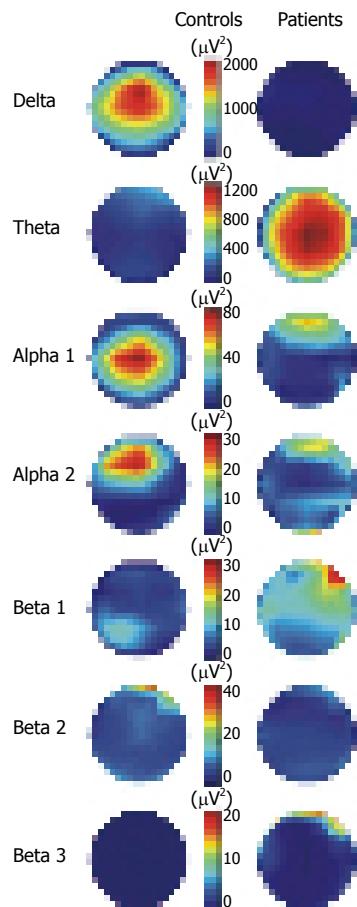
enabling the analysis of multichannel data. The TMP atoms are very well localizable in time, frequency and space domains and are described by only a few parameters. This approach simplifies analysis of multichannel data and enables identification and extraction of spatio-temporal components with biological meaning as well as identification of artifacts.

Figure 1 shows the analysis scheme used for the data in this study. First, the EEG data were decomposed into TMP atoms. Twenty atoms per subject were used. The computed atoms were grouped into frequency bands according to their modulation (frequency) parameter. This is an equivalent of a band-pass filtering into delta (0.5-3.5 Hz), theta (3.5-7.5 Hz), alpha1 (7.5-10 Hz), alpha2 (10-12.5 Hz), beta1 (12.5-18 Hz), beta2 (18-24 Hz) and beta3 (24-30 Hz). Typically, each frequency band was represented by one to three atoms. For each atom or group of atoms from a specific band, a spatial distribution of signal's energy as well as spatio-temporal patterns was examined.

## RESULTS

All subjects reported painful sensations to the electrical stimulation of the esophagus. The mean current intensities (at the pain thresholds) for the controls and patients were  $12.8 \pm 3.9$  mA and  $10.0 \pm 1.4$  mA, respectively ( $P = 0.08$ ).

The grand mean averages of the spatial distributions of signal energy during the 450 ms analysis after the evoked esophageal pain for patients and controls are shown in Figure 2. The spatial distributions are shown separately for the delta, theta, alpha and beta bands. Clearly major differences between controls and patients were seen in the delta and theta bands, whereas there were only minor differences in the other frequency bands. In Figure 3, the temporal evolution together with the spatial distribution maps of energy for the delta and theta activities in the analyzed time period are shown in the two groups. The butterfly plots showing the individual channels show that the mean amplitude (energy) in the delta band was higher in the controls. The patients only showed scattered activity in this band without any



**Figure 2** The spatial distribution of energy divided into frequency bands for patients and controls throughout the 450 ms of analysis. Note that a different color scaling for each frequency band was used.

consistency between the individual channels. In the theta band, the patients showed higher activity than controls and the theta activity persisted throughout the 450 ms of analysis, whereas in controls it was strongly attenuated after 250 ms. Finally, the theta activity had a lower frequency in the patients. The main theta components oscillated with about 4.4 Hz in the patients and with about 5.5 Hz in the controls. When the mean activity in the patients was subtracted from that in controls (difference in the Figure 3), there were major differences in energy. The delta activity in controls and theta activity in patients had similar amplitudes and topographic distributions. However, the delta oscillations in controls were slightly stronger in the frontal lobe, whereas the theta activity in patients was stronger in the parietal lobe (Figure 3B1 and 3B4).

In Figure 4, the topography relating to the time evolution in the delta and theta bands are shown. The time evolution of the spatial distribution showed differences for patients and controls. The oscillations in the delta band for controls were synchronous with a stable monopolar spatial distribution. This means that there were only minor and constant phase differences between channels. In contrast, the patients exhibit slightly larger phase differences between different channels which desynchronized the oscillations (see arrows in Figure 4). The amplitude of the oscillations was also smaller for patients in the delta band. However, in the theta band the patients exhibit synchronous brain activation with almost constant and relatively small phase differences between the channels. The theta activity for controls, however, had inconstant phase differences between channels. The

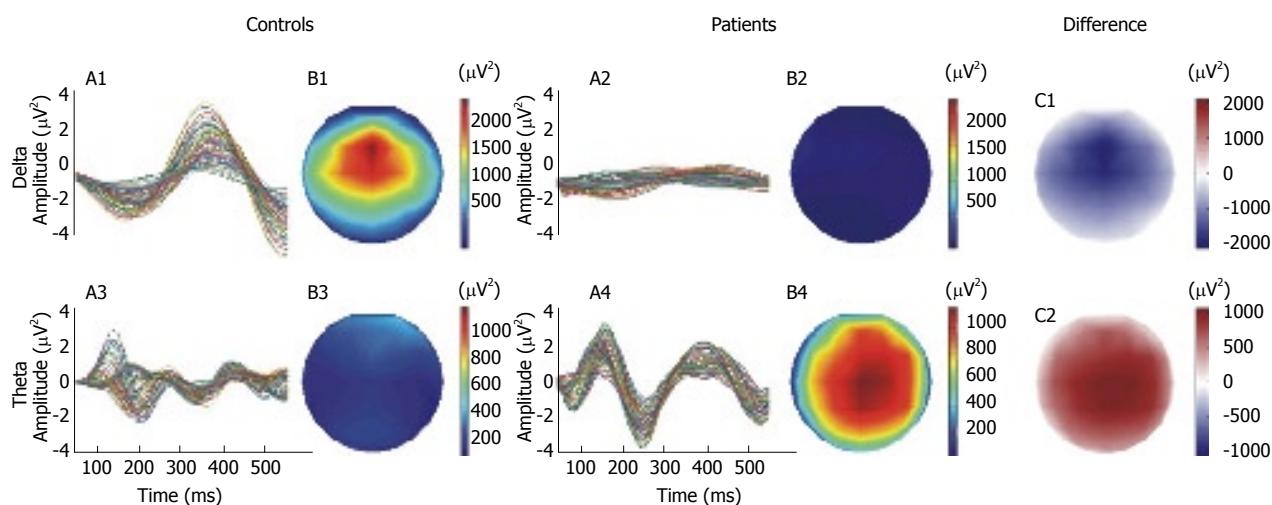
topographical EEG data evolution of the theta activity was rather consistent in all patients as shown in Figure 5.

## DISCUSSION

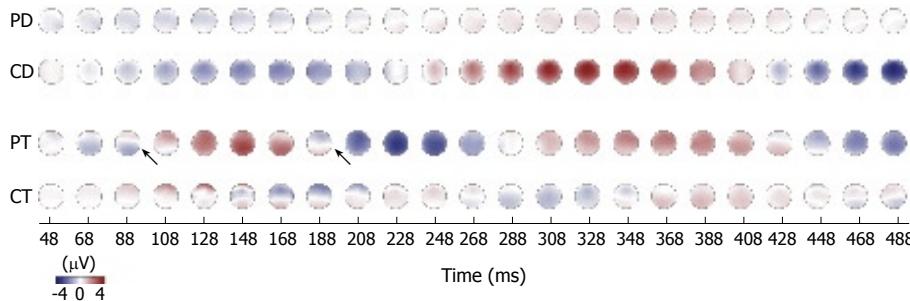
The topographic matching pursuit method allowed multichannel signal decomposition of EEG data and showed that during evoked visceral pain there are major differences in the delta and theta band between patients with chronic pancreatitis and healthy controls. The differences in the theta band were the most consistent and indicate, together with previous observations, that neuropathic pain mechanisms may be involved in chronic pancreatitis.

In the classical description of EEG, time and frequency distribution is computed using frequency analysis, but as the specific brain processing of phasic exogenous pain stimuli is very short lasting (about 100-200 ms)<sup>[15]</sup> ordinary signal analysis cannot be used (see below). To encompass the problems with time-resolution, some researchers have used a tonic pain stimulus<sup>[23]</sup>. However, tonic pain with the same intensity is difficult to evoke consistently in GI tract where, for example, long-lasting stimulation typically creates a waxing or waning response<sup>[24-26]</sup>. Furthermore, nausea and vomiting is often evoked interfering with the cortical response<sup>[27]</sup>. Hence, in experimental pain experiments of the gut phasic stimuli such as electrical stimulation of the mucosa are often used. Another way to overcome the problems with analysis of short time segments is to give a series of stimuli and compute the evoked brain response based on summation of the stimulus sequences<sup>[28]</sup>. However, such averaged evoked brain responses carry the risk for discarding much of the dynamic information contained in the original data<sup>[20]</sup>. To obtain detailed information of the EEG in individual channels, other methods where the signal is analyzed must, therefore, be added.

Common analysis schemes of multichannel bioelectromagnetic data involve analysis of selected single channels in the time or time-frequency domain. Typically, methods such as Wavelet Transform are used to extract the time-frequency information from the data. These represent signals by correlations with all their basis functions. This may result in "blurred" representations of data when some simple signal components are represented in terms of many basis functions and interpretation can be difficult. Further, they are only stable for rather long EEG segments and discrete changes relating to the exact time and duration of individual waveforms cannot be measured<sup>[18]</sup>. The TMP method represents a completely different approach to signal analysis. It is able to decompose the data under analysis into a relatively small number of simple components. Such a concise description of the data simplifies the analysis and interpretation of the results. The parameterization of signal components allows convenient further analysis. It enables identification and separation of signals with biological origin or removal of artifacts<sup>[18]</sup>. Each signal component is represented by a TMP atom that can be inspected individually in the time, frequency and space domains, and a topographic distribution of amplitude and phase differences between



**Figure 3** The temporal evolution and the spatial distribution maps of energy of the delta and theta activities for the 450 ms of analysis. In the right panel, the difference maps of energy were computed by subtracting the energy maps of controls from the patients.



**Figure 4** The time evolution of amplitude distribution for delta and theta bands in patients and controls. Note the stable, monopolar spatial distribution of delta activity in controls (CD). This is due to constant and small phase differences between measurement channels. The theta activity in patients has slight changes in phase and, therefore, the spatial distribution is not always stable (see arrows). PD: Patients delta; PT: Patients theta; CD: Controls delta; CT: Controls theta.

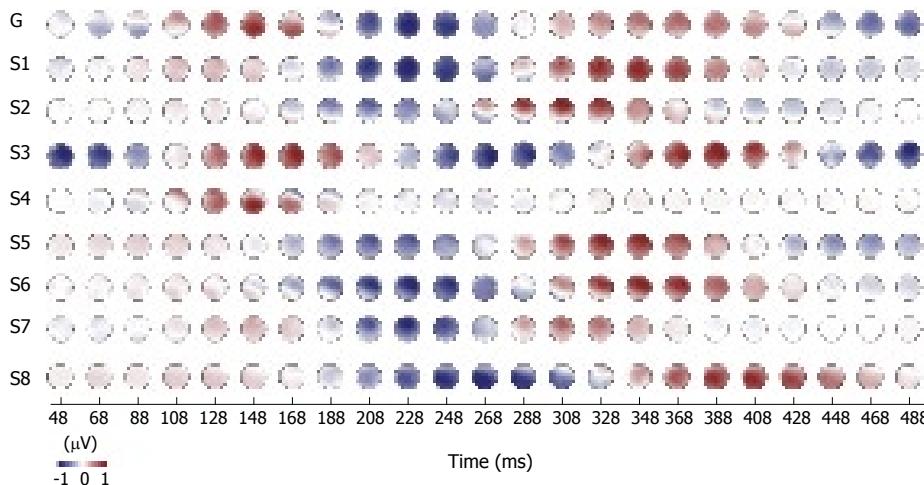
channels can be plotted. Additionally, temporal changes of amplitude topography can be analyzed. This allows identification of patterns and components in the time-frequency-space domain. The main disadvantage of the TMP algorithm is its high computation cost.

We used electrical stimulation of the esophagus to mimic the visceral pain response. Although the pain was not evoked in the pancreas per second, the visceral afferents terminate rather diffuse in the spinal cord affecting many neurons at different segmental levels<sup>[7]</sup>. Hence, the esophagus innervates neurons spreading from C5-L2 explaining the diffuse pain seen in the clinic<sup>[29]</sup>. The visceral afferents from different organs terminate often in the same functional neuron pool, resulting in viscero-visceral convergence (e.g. pain from one visceral can cause a change in the sensory response in another<sup>[30]</sup>). In accordance with the neurophysiologic findings we have previously shown that patients with pancreatitis have hypoalgesia to distension in both the esophagus and duodenum<sup>[8]</sup>. Furthermore, that the latency of the evoked brain potentials to electrical stimulation of both the esophagus and duodenum was decreased in these patients<sup>[10]</sup>. Thus it is most likely that any neuroplastic changes and abnormal mechanisms in the pain processing caused by the intermittent pancreatic pain will also affect visceral pain evoked in the nearby organs.

Neuropathic pain is typically described as stimulus-independent shooting, lancinating or burning pain<sup>[6]</sup> resembling the pain in CP. Recently, evidence for a neuropathic component of the pain in CP was provided

by histological findings demonstrating an increase in the number and diameter of pancreatic nerves. Furthermore intensification of neurotransmitters, such as substance P, calcium gene-related peptide and interleukin 8, being markers of neuronal plasticity were found<sup>[5]</sup>. The growth-associated protein-43 is increased after neuronal lesions and has been found in the majority of pancreatic nerve fibers with a correlation with the pain scores<sup>[31]</sup>. As increased activity in the theta band seems to be a marker of neuropathic pain<sup>[14]</sup>, the current findings strengthen this neuropathic pain hypothesis in CP. The increased theta activity may reflect a thalamocortical dysrhythmia, as shown with coherence between direct single-unit thalamic recordings in patients suffering from ongoing neuropathic pain and other neurological disorders<sup>[12,13]</sup>. In these studies, it was hypothesized that the theta activity reflects a disturbance in the thalamocortical interplay needed for higher cognitive functions such as those engaged in pain. The normalization of the EEG after therapeutic lesions in the thalamus (and a 95% reduction of the pain), further support this hypothesis<sup>[14]</sup>. Our patients all suffered from intermittent pain and hence we studied evoked gut pain and not the resting EEG. Besides a demonstration of increased theta activity we were able to show that the activity persisted throughout the 450 ms of analysis, whereas it faded out in controls. Furthermore, the theta pattern was consistent in all patients.

The low activity in the delta band was consistent in all patients. However, there was a major spread between subjects and the features were more difficult to



**Figure 5** Normalized time evolution of amplitude distributions for theta bands in patients with chronic pancreatitis No. 1-8. G: Grand mean of all patients.

interpret. Increase in delta power, especially in the frontal derivations has been shown in some previous studies using experimental tonic pain<sup>[23]</sup>, but the findings were not consistent<sup>[32-34]</sup>. Recently, we used independent component analysis of the EEG following esophageal stimulation in healthy subjects<sup>[20]</sup>. In this experiment, electrical pain evoked dominant synchronous activity in the delta band in several brain regions, especially in the components with dipoles in the thalamus and cingulate cortex. Whether the difference between controls and patients seen in the current study reflects the expected delta power increase during experimental pain in healthy volunteers, or whether it is a feature of chronic pain remains to be investigated in further detail.

The findings of the current study may have clinical implications. Treatment of chronic pancreatitis is difficult and it often becomes necessary to use strong analgesics such as opioids<sup>[11]</sup>. However, drugs shown to be effective in neuropathic pain also seem to be efficient. Hence, Wilder-Smith *et al* showed that tramadol, a weak opioid drug with several non-opioid effects in the central nervous system, in high doses was superior to traditional opioids in the treatment of pain in CP<sup>[35]</sup>. Tricyclic antidepressants and pregabalin, which are also effective in neuropathic pain, have in our experience been of value as adjuvant therapy in difficult patients. Obviously, it could be interesting to use the current method before and after effective therapy of pain in patients with CP.

The increased theta activity during experimental visceral pain in patients with chronic pancreatitis gives further evidence that the pain mechanisms in this disease share many features with those seen in neuropathic pain. This has important implications for the clinical understanding and treatment of pain in chronic pancreatitis, which may be directed against drugs with effects on neuropathic pain disorders.

## COMMENTS

### Background

The pain mechanisms in chronic pancreatitis are incompletely understood, but it has been suggested that neuropathic mechanisms (like in phantom pain) are important. The energy in the theta (3.5-7.5 Hz) band of the electroencephalogram may be a hallmark of patients with neuropathic pain, and we compared the energy in the theta band during painful stimulation of the esophagus in patients

with chronic pancreatitis and in healthy controls.

### Research frontiers

Histological findings in the pancreas in patients with pain due to chronic pancreatitis have mimicked those seen in patients with neurogenic inflammation. The neurogenic theory is also supported by clinical and experimental findings.

### Innovations and breakthroughs

To assess the neurophysiological mechanisms standardization of the pain stimulus in experimental settings is necessary. During short-lasting experimental pain stimulation of the esophagus, as used in the paper, it is difficult to compute discrete electrophysiological signals using conventional methods. We, therefore, used a new method called "Topographic Matching Pursuit" and found a selective increase of the theta energy in the patients during the evoked esophageal pain.

### Applications

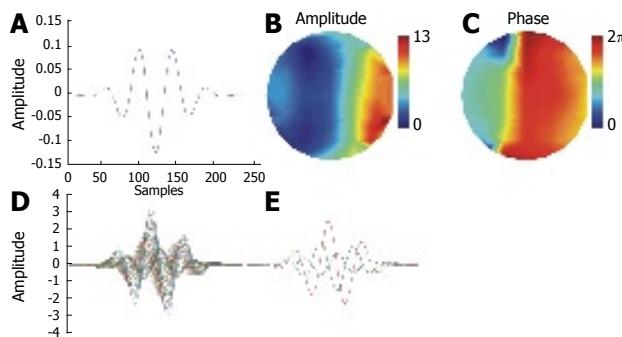
The increased theta activity during experimental pain gives further evidence that the pain mechanisms in chronic pancreatitis share many features with neuropathic pain. This has important implications for the clinical understanding and treatment of pain in chronic pancreatitis.

### Peer review

The differences in the theta band indicate that neuropathic pain mechanisms are involved in chronic pancreatitis. This has important implications for the understanding and treatment of pain in these patients, which should be directed against drugs with effects on neuropathic pain disorders.

## APPENDIX

Topographic Matching Pursuit (TMP) is an adaptive method for sparse decomposition of multichannel signals. It is based on the Matching Pursuit (MP) algorithm<sup>[36-39]</sup>. It iteratively creates approximations of signals using relatively small number of simple components chosen from a very big and redundant set. The components are called atoms. A set of atoms is called dictionary. Typical atoms are scaled and modulated as translated Gaussian functions and are called Gabor atoms. The advantage of Gabor atoms is their optimal time-frequency resolution. MP has very interesting and useful properties. However, it can be directly used only for decomposition of single-channel data, because Gabor atoms are distributed only in time. Biomedical signals are typically measured with multiple sensors. The TMP algorithm is searching the standard MP dictionary to find a Gabor atom, which has the biggest similarity simultaneously to all measurement channels. A multichannel extension of the MP algorithm was discussed by Gribonval and applied to EEG data by Durka *et al*<sup>[40]</sup>. In the TMP version of multichannel MP the amplitude and the phase shift of the Gabor atom is optimized for each channel in order to obtain the



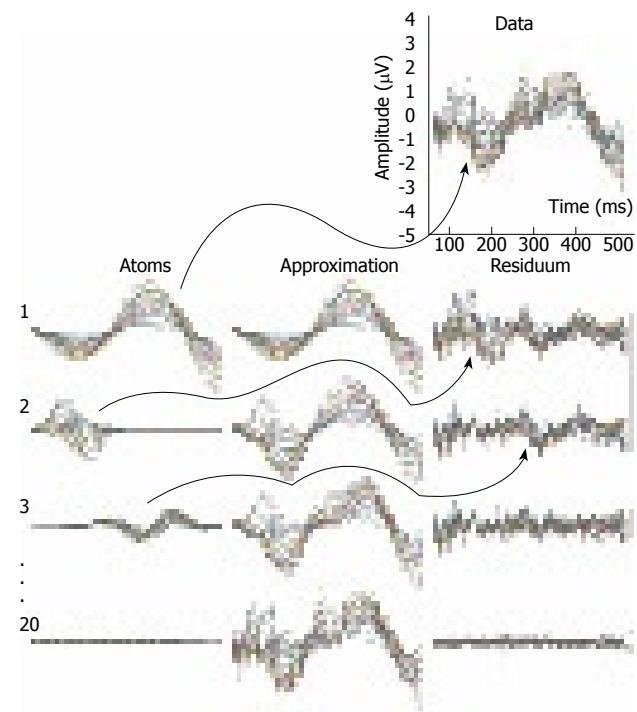
**Figure Appendix1** Example of a TMP atom for a 61 channel EEG system. **A:** Base Gabor atom; **B:** Spatial distribution of amplitude; **C:** Spatial distribution of phase; Overlay plots of the TMP atom: All 61 channels (**D**), channels 1, 10 and 30 (**E**).

highest correlation of the Gabor atom with the respective channel. The Gabor atom with the highest value of the sum of squared correlation coefficients with all measurement channels is chosen for the approximation in the first iteration: with  $\text{Fi-i-th}$  data channel,  $g_{\gamma}$ -atom from the dictionary with index  $\gamma$ .

The correlation coefficients define a topographic distribution of amplitude over measurement channels. One can describe a TMP atom as a Gabor atom with an additional list of amplitudes and phase shifts for all channels (Figure Appendix1). A high amplitude value for a particular channel indicates strong presence of the component in this channel. In contrast, a low amplitude value denotes that the component is weakly present in this channel. Next, the TMP atom which has the biggest similarity with the multichannel data under analysis (as described above) is subtracted from the data. This creates the first order spatio-temporal residuum of the data. The data under analysis can be treated as the 0th order residuum. The further steps of the TMP algorithm can be summarized in the three iterative points: (1) Search the dictionary for the atom, which has the biggest similarity (correlation) with the previously computed spatio-temporal residuum. (2) Create the next order residuum by subtracting the chosen atom from the previously computed residuum. (3) Repeat steps 1 to 2 until the current residuum is sufficiently low.

An example of decomposition of EEG data is presented in Figure Appendix2. The EEG data is presented in the top right corner. Each row in the figure represents one iteration of the TMP algorithm. The chosen atoms and computed approximations with their respective residues for four different iterations are presented. The approximations are computed by summing atoms from all previous and the present iteration. With increasing number of iterations, one can observe the convergence of the approximation to the EEG data. Simultaneously, the residuum, which is the difference between the data and the approximation, is vanishing.

The result of the TMP algorithm is a set of TMP atoms. The sum of the atoms approximates the data under analysis. The TMP atoms are localized very well in the time, frequency and space domains. They have six



**Figure Appendix2** Example of decomposition of multichannel EEG data. The data is presented in the top-right corner (61 channels overlay plot). In the following rows chosen atoms, approximations and residues for the 1st, 2nd, 3rd, and 20th iteration are shown. In each iteration the chosen atom is the most similar one with the previously computed residuum (see arrows). The residuum is created by subtracting the chosen atom from the residuum computed in the previous iteration. The signal itself can be treated as the 0th order residuum. The approximation in each iteration is the sum of all atoms chosen in the present and previous iterations. Hence, in the first iteration the chosen atom is simultaneously the first approximation of the data. Note that with the increasing number of iterations the approximation is approaching the data and the residuum is vanishing. All time plots of atoms, approximations and residues are 61-channel overlay plots shown in the same scale.

parameters: scale (time length), modulation (frequency), translation (time localization), and relating to the different channels-a spatial distribution of phase and amplitude. Thus, the choice of the atoms for the approximation parameterizes the multichannel data under analysis. This allows convenient analysis and processing of such parameterized data. For example it enables identification and separation of signal components with biological origin or removal of artifacts. Each signal component represented by a TMP atom can be inspected individually in the time, frequency and space domains. A topographic distribution of amplitude and phase differences between channels can be plotted. Additionally, temporal changes of amplitude topography can be analyzed. This allows identification of patterns and components in the time-frequency-space domain.

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BASIC RESEARCH

# Inflammatory bowel disease in rats: Bacterial and chemical interaction

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nued treatment groups: the animals showed slow rate of increase in body weight, diarrhea, bloody stools, high colonic ulcer score, as well as histological alterations characteristic of UC, with an extensive inflammatory reaction. During the course of the experiment, the MPO activity was consistently elevated and the TNF- $\alpha$  gene expression was upregulated compared to the control animals.

**CONCLUSION:** The experimental ulcerative colitis model used in the present study resembles, to a great extent, the human disease. It is reproducible with characteristics indicative of chronicity.

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**Key words:** Colitis; *Escherichia coli*; Iodoacetamide; Inflammatory bowel disease model; Gastrointestinal inflammation

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## Abstract

**AIM:** To develop a novel model of colitis in rats, using a combination of iodoacetamide and enteropathogenic *E. coli* (EPEC), and to elucidate the pathophysiological processes implicated in the development of ulcerative colitis (UC).

**METHODS:** Male Sprague-Dawley rats ( $n = 158$ ) were inoculated intrarectally on a weekly basis with 4 different combinations: (a) 1% methylcellulose (MC), (b) 100  $\mu$ L of 6% iodoacetamide (IA) in 1% MC, (c) 200  $\mu$ L containing  $4 \times 10^8$  colony factor units (CFU) of EPEC, and (d) combined treatment of (IA) followed by bacteria (B) after 2 d. Thirty days post treatment, each of the four groups was divided into two subgroups; the inoculation was stopped for one subgroup and the other subgroup continued with biweekly inoculation until the end of the experiment. Colitis was evaluated by the clinical course of the disease, the macroscopic and microscopic alterations, activity of myeloperoxidase (MPO), and by TNF- $\alpha$  gene expression.

**RESULTS:** Findings indicative of UC were seen in the combined treatment (IA + B) as well as the IA conti-

## INTRODUCTION

It is well established that ulcerative colitis (UC) is an inflammatory condition of the gastrointestinal tract (GIT), resulting from interrelated genetic and environmental factors, especially bacteria which cause disruption of the mucosal barrier thus exposing the mucosal immune system to luminal bacteria and bacterial products<sup>[1]</sup>.

For several years, researchers have been addressing the question as to whether a specific pathogen could cause inflammatory bowel disease (IBD)<sup>[2]</sup>. Attempts made, so far, to find a causative bacterial strain for IBD, and particularly for UC, have been unsuccessful. Over the years, evidence has accumulated implicating endogenous luminal bacteria in the pathogenesis of

UC, especially since the highest bacterial concentration and diversity are found in the colon. Much attention has been given to the role of *E. coli* in the onset of UC, since this organism is the predominant facultative anaerobic gram negative species of the normal intestinal flora. *E. coli* play an important role in promoting the stability of the intestinal microbial flora and in maintaining the normal intestinal physiology<sup>[3]</sup>. Besides commensal strains, certain clones of *E. coli*, possess virulent properties that cause disease in humans. More recently, it has been suggested that a particular subtype of *E. coli* may play a pathogenic role in UC<sup>[4,5]</sup>. Studies on mucosal adhesion of pathogenic bacteria in UC revealed an enhanced adhesion of *E. coli* isolates (obtained from stool specimens and rectal biopsies of UC patients) to buccal epithelial cells causing mucosal damage similar to that seen with enteropathogenic *E. coli* (EPEC)<sup>[4-7]</sup>. Adherence of EPEC strains on the intestinal mucosa is a complicated process and produces dramatic effects on the ultrastructure of the cells resulting in reduced tight junction density and leaky epithelial barrier<sup>[7-9]</sup>.

More than 60 different experimental models of IBD have been developed in the past two decades<sup>[10,11]</sup>. These studies confirmed the need for the presence of normal enteric flora for the development of experimental colitis<sup>[12-15]</sup>. However, none of the models mimic precisely the human disease. Therefore, more studies are required to further develop this area of research. The iodoacetamide-induced acute UC model in rat developed by Satoh *et al*<sup>[16]</sup> has been extensively studied<sup>[17,18]</sup>. In this model, an array of morphological and functional alterations have been described, however, the model lacked features of chronicity<sup>[17-20]</sup>.

The present study reports the successful development of a chronic UC model, using the combined effects of iodoacetamide, a sulphydryl group blocker, and enteropathogenic *E. coli* (EPEC), a strain with adhesion properties, instilled repetitively into the descending colon of rats.

## MATERIALS AND METHODS

### Animals

A total of 158 adult male Sprague-Dawley rats, weight range  $200 \pm 25$  g, were used in this experiment in accordance with the criteria of the Institutional Animal Care and Use Committee for the care and use of animals. The study was conducted at the American University of Beirut. Animals were housed in rack mounted cages, with a maximum of 10 rats per cage, and kept on a 12 h light/dark cycle in a controlled temperature and humidity room. Standard laboratory pelleted formula and tap water were provided ad libitum.

### Induction of experimental colitis

The rats were randomized into 4 groups: (1) the methylcellulose treated control group ( $n = 37$ ), the animals were inoculated intrarectally on a weekly basis with 100  $\mu$ L of 1% methylcellulose (MC), the vehicle (Sigma, M-0512, USA); (2) the iodoacetamide-treated group ( $n = 42$ ), in

this group the rats were inoculated with 100  $\mu$ L of 6% iodoacetamide (IA) (Sigma, I-6125, USA) dissolved in methylcellulose according to the previously described study by Satoh *et al*<sup>[16]</sup>; (3) The bacteria-treated (B) group ( $n = 37$ ), this group was inoculated with 200  $\mu$ L suspension containing  $4 \times 10^8$  colony factor units of EPEC; (4) The combined treatment group ( $n = 42$ ), received a combination of iodoacetamide and bacteria (IA + B); this group was inoculated intrarectally on a weekly basis with the same doses of IA followed by bacteria after 48 h. Experimental colitis was induced by regular weekly intracolonic inoculation, 7 cm proximal to the anal verge using a 2 mm diameter polyethylene tube. On day 30, each group was split into 2 subgroups. In one subgroup, the inoculation of different treatments continued bi-weekly while in the second subgroup the treatment was discontinued. Three rats from each group and later subgroup (after 30 d), were anesthetized by intraperitoneal injection of sodium pentobarbital (75 mg/kg body weight) and sacrificed on day 3, 7, 14, 21, 42, 56, and 70. A portion of the colon was fixed in 10% formalin while the remaining part was stored at -80°C.

### Clinical course assessment

The animals were observed on a daily basis and checked for diarrhea, loose stools, gross rectal bloody stools, or any other gross abnormalities. The weight of each animal was obtained on a weekly basis to check for weight loss after induction of colitis. These observations were reported as a numerical score (Table 1).

### Macroscopic assessment

The evaluation of inflammation was performed according to the modified criteria for colonic changes (Table 1)<sup>[17,21]</sup>. Parameters like diarrhea, hyperemia, adhesions, ulceration and megacolon were assessed to describe the inflammatory status. Each colon was assigned, in a double blind way, a score on a scale ranging from 0 (normal) to 15 (maximal activity of colitis) indicating ulcerations and severe inflammation of the colon.

### Microscopic assessment

The descending colon was removed and immersed in cold phosphate buffer (PBS) at pH 7.4. A 1.0 cm piece of the colon was removed proximal to the site of inoculation. It was immediately immersed in 10% buffered formalin and was processed for routine light microscopy according to standard procedures. Serial 5  $\mu$ m sections were cut and stained with hematoxylin and eosin (HE) and Periodic Acid Schiff (PAS) using standard methods. The microscopic alterations were assessed according to the criteria shown in Table 2<sup>[17-22]</sup>, and a numerical score of the colonic abnormalities was obtained. The histologic grades ranged from 0 (normal) to 18 (intense inflammatory reaction). The scoring was based on the findings of 2 independent observers obtained by examining six sections from each colon. Thus, the scores represented the average of 36 readings (2 observers, 6 sections per animal and 3 animals per time point). The histological abnormalities

**Table 1 Criteria for macroscopic grading of the experimental colitis**

Feature	Macroscopic grading			
	0	1	2	3
Stool	Normal	Loose stool	Diarrhea	Diarrhea with blood
Hyperemia	None	Focal	Focal and thickening of bowel wall	Extensive thickening of bowel wall
Adhesions	None	Mild	Moderate	Extensive
Megacolon	None	Mild	Moderate	Toxic megacolon
Ulcerations	None	Mild ulceration on one side < 1 cm	Moderate ulceration > 1 cm	Severe damage extending > 2 cm

**Table 2 Criteria for microscopic grading of experimental chronic colitis**

Feature	Histologic grading			
	0	1	2	3
Abnormalities of mucosal architecture	None (Normal)	Mild or focal, not exceeding lamina propria	Moderate, not exceeding the submucosa	Severe & diffuse, exceeding the submucosa
Crypt abnormalities	None	Mild atrophy	Moderate atrophy, Branched crypts	Severe atrophy, branched crypts, cryptitis, crypt abscess
Inflammatory cell infiltration	Normal	Scattered cells	Moderate or confluent cells	Massive infiltration of cells
Vascular dilatation	Normal blood vessels	Mild dilatation (localized)	Moderate dilatation of several blood vessels	Severe generalized dilatation of blood vessels
Edema	None	Low level limited to villi	In the submucosa	All over the section
Mast cells	Normal scattered cells	Three cells clustered in submucosa	Clusters of > 3 cells in the submucosa	Clusters in submucosa and serosa

were photographed and the findings were reported as numerical scores.

#### **Colonic inflammation assessment by myeloperoxidase activity (MPO)**

The assessment of MPO activity in the mucosal scrapings was carried out as a quantitative marker for granulocytic infiltration in the colonic tissue. Protein concentration and quantification were determined in the mucosal scrapings of the descending colon using the protein assay reagent kit (Bio-Rad). One unit of MPO activity was defined as the quantity of enzyme able to convert 1 μmol/min of hydrogen peroxide at 25°C as described by Krawisz *et al*<sup>[23]</sup>. The results were expressed as MPO units per gram of wet tissue.

#### **Analysis of TNF-α mRNA by reverse-transcriptase PCR (RT-PCR)**

Total RNA was extracted from colonic tissues by TRI Reagent (Invitrogen) reagent. RNA was resuspended in RNase free water, quantified and subjected to RT-PCR reaction using RT-PCR kit; Ready Mix Version (Abgene, Promega). RT-PCR was performed on the final volume of 25 μL containing 12.5 μL ready mix (optimize reaction buffer, dNTP mix, MgCl<sub>2</sub> and DNA polymerase), 0.5 μL of 25 pmol specific primers as described for TNF-α, β-actin, 0.5 μL of reverse transcriptase and 1 μg of RNA. Reverse transcription was performed at 48°C for 30 minutes, followed by heating for 2 min at 94°C. Then 45 cycles of PCR for TNF-α and 25 cycles for β-actin was performed using the following conditions: denaturation at 94°C for 30 s, annealing temp of 55°C for 60 s and extension

temperature of 68°C for 2 min, followed by a final extension step at 68°C for 7 min. The primers were TNF-α sense, 5'-AGAACT CCAGGCGGTGTCC-3'. TNF-α antisense, 3'-GATTCTGTGGGGACTCCC T-5' (484 bp). β-actin sense, 5'- AAC CCT AAG GCC AACCGTGAAAAG-3'; β-actin antisense, 3'-ATA CAACGGGATCTGAAGCTCG-5' (540 bp). The PCR products were separated in 1.5 % agarose gel electrophoresis and visualized by ethidium bromide staining (1 μg/mL). The DNA product sizes were estimated relative to 100 bp DNA ladder. A control reaction were run to rule out contamination of RNA with genomic DNA, in which reverse transcriptase was omitted from the reaction mixtures. Transcripts were normalized to the corresponding β-actin band and expressed as arbitrary density units.

#### **Analysis of TNF-α level by western blot**

Protein isolation was carried out by lysing colonic mucosal scrapings in 1.5 mL homogenization buffer (NaCl 11.7 g/L, MgCl<sub>2</sub>·6H<sub>2</sub>O 1 g/L, EDTA 0.76 g/L KCl 0.37 g/L, Tris 24.2 g/L, pH 7.4) using polytron homogenizer on ice. The homogenates were subjected to 30 s sonication and centrifugation at 12000 r/min for 10 min at 4°C. The protein content of the supernatant was quantified by Bio-Rad Reagents. The protein samples diluted in sample buffer 2X (10% glycerol, 5% betamercaptoethanol, 4% SDS, 125 mmol/L Tris-HCL 1 mol/L pH 6.8 and traces of bromophenol blue) were loaded as 40 μg/lane in 12% SDS-acrylamide gels, separated by electrophoresis, and electrotransferred to nitrocellulose membranes (Bio-Rad). To detect the specific protein, the TNF-α rabbit polyclonal anti-rat (Chemicon) antibody

was used at the concentrations recommended by the manufacturers. Equal loading of the protein samples was confirmed by parallel western blots for GAPDH. The intensities of the protein bands were quantitated by image scanning X-ray films and each band was measured by Image J software (NIH imaging software). Correction was performed by subtracting for level background and normalizing against GAPDH protein level.

### Statistical analysis

Statistical significance of differences between treatment and control groups was determined by the Student's *t* test. Where applicable, *P* values were reported for the 3 independent comparisons. Differences were considered statistically significant for *P* < 0.05. Values are presented as the mean  $\pm$  SD.

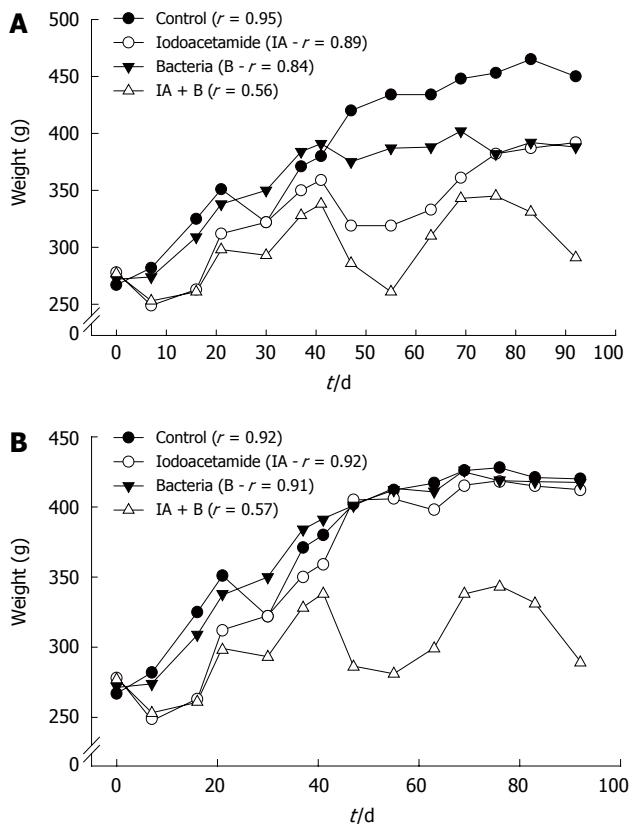
## RESULTS

The present study shows that the synergistic effect of repetitive intracolonic instillation of iodoacetamide and EPEC, resulted in chronic colitis, and the inflammatory process was sustained for the duration of the experiment: 100 d.

### Induction and clinical course

**Changes in animals' weight:** As shown in Figure 1, rats in the control, bacteria or iodoacetamide-treated groups had a trend towards increase in weight, which was significantly higher compared to rats treated with the combined treatment (iodoacetamide and bacteria), in both the continued and discontinued injection subgroups. Rats in the combined treatment group (IA + B) showed the lowest rate of increase in weight gain (*P* < 0.005), 0.15 g/d compared to 1.83 g/d for control, 1.14 g/d for iodoacetamide, and 1.48 g/d for the bacteria treated groups. Similar results were obtained in the discontinued injection subgroups: 0.13 g/d in the combined treatment (IA + B) compared to 1.5 g/d in the normal, 1.37 g/d in the IA and 1.45 g/d in the bacteria treated groups. The decrease in weight gain supports the diagnosis of chronic UC, a phenomenon not seen in the iodoacetamide-treated animals once the inoculations were discontinued after 30 d. Furthermore, two way ANOVA analysis for two experimental parameters (i.e. treatments and duration in days) detected the presence of significant differences among the different experimental groups (*P* < 0.005) as well as among the various treatment intervals (*P* < 0.005), with respect to the effect on body weight.

**Inspection of stools:** Throughout the duration of the study, the control animals passed normal beaded compact stools. Similarly, none of the animals injected with the bacterial suspension of EPEC alone demonstrated abnormal stools except in a few instances of loose stools, 1 to 2 d post inoculation. By contrast, the iodoacetamide-treated group developed diarrhea consistently for 2 to 3 d post instillation that changed later to loose stools. However, after 30 d when the animals were divided into the continued and discontinued subgroups:



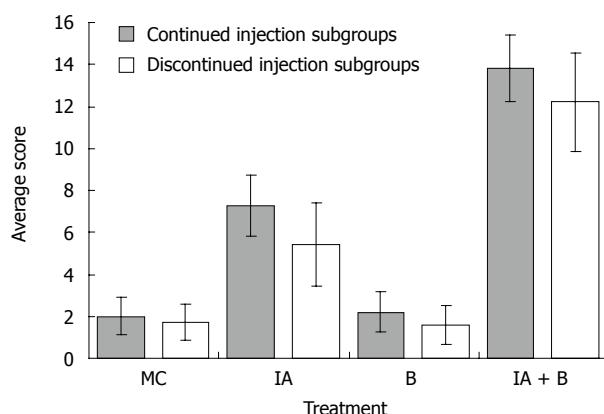
**Figure 1** Changes in the average rats' weight in various experimental groups, and in the continued and discontinued treatment subgroups. **A:** Continued injection subgroups; **B:** Discontinued injection subgroups. *r*: represents the correlation coefficient of weight change vs time. All readings were statistically significant (*P* < 0.005). Note the slow oscillating rate of increase in the two (IA + B) subgroups and in the continued injection IA subgroup.

the iodoacetamide-treated discontinued injection subgroup exhibited a pattern similar to the control group, starting 1 wk after the last IA injection and continuing for the duration of the experiment. The iodoacetamide-treated continued injection subgroup developed diarrhea for the first 2 to 3 d after the injection in nearly all animals, followed by loose stools and occasionally beaded compact stools for the remainder of the week until the next injection.

On the other hand, all animals in the combined treatment group (IA + B) both continued and discontinued subgroups, appeared sick and had severe diarrhea and bloody stools at some stage of the experiment, a feature rarely seen in the iodoacetamide-treated discontinued subgroup. These findings correlated with the lowest rate of weight increase observed in the combined treatment group (IA + B).

### Macroscopic findings

**MC-treated group (Control group):** The findings were considered as normal baseline. An average of three observations were made at each time point. Rats in the control group did not show any inflammation in the descending colon, however, a whitish discolouration at the site of the injection,  $\leq 0.5 \text{ cm}^2$  in diameter, was easily identified. The average score was  $2 \pm 0.9$  out of 15 indicating the absence of ulcer formation (Figure 2). These



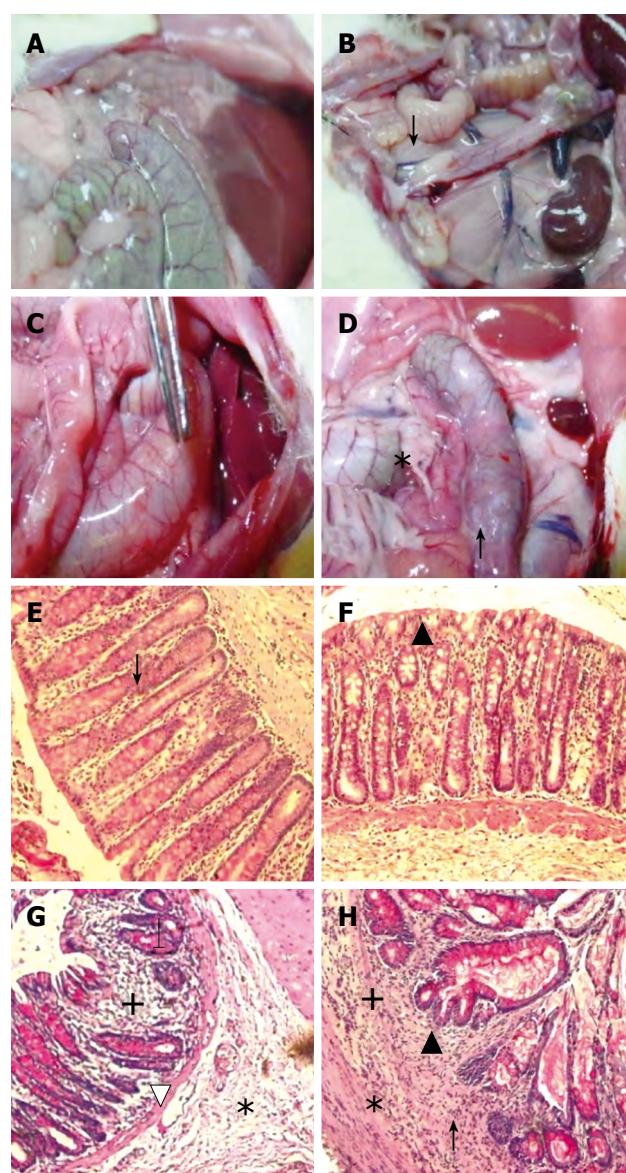
**Figure 2** Macroscopic assessment. The overall average colonic score at different time intervals of the various experimental groups both in the continued and discontinued injection subgroups (Readings represent the average of the scores on days 3, 7, 14, 28, 42, 56, and 70 in each group).

findings were observed although the experimental duration in the continued and discontinued subgroups was different (Figure 3A).

**B-treated group:** The findings in this group were similar to the control group except for the occasional findings of vasodilatation, and the presence of a white granulomatous area at the site of inoculation, with an overall size  $\leq 0.5 \text{ cm}^2$  (Figure 3B). The overall average score for the group was  $2.0 \pm 0.96$  out of 15, as illustrated in Figure 2.

**IA-treated group:** Perianal redness was sometimes noticed prior to sacrifice. The ascending and transverse colon were invariably normal. At most time points, inflammation was restricted to the descending colon including the occasional presence of an area of redness of the jejunum around the site of injection. There was dilatation of blood vessels and hyperemia around the site of the inoculation, along with the presence of adhesions (Figure 3C). The size of the inoculation site was 1 to  $1.5 \text{ cm}^2$ . The overall average score for the mucosal damage at the various time points in the continuous injection subgroup was  $7 \pm 0.9$  out of 15, while the average score in the discontinued injection subgroup was  $5.2 \pm 1.6$  out of 15. These findings correlated with the findings of less severe symptoms in treatment-discontinued subgroup compared to the treatment-continued subgroup (Figure 2).

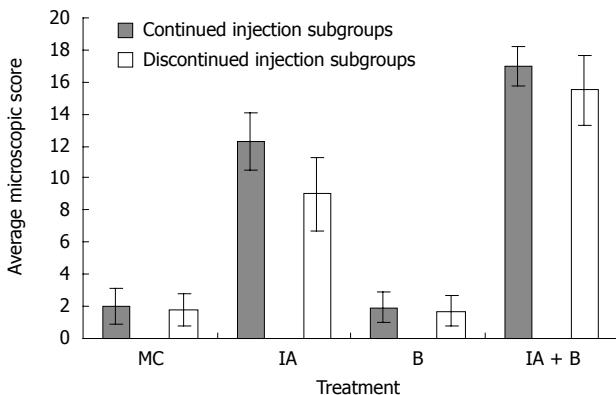
**IA + B-treated group:** Perianal redness was consistently observed, accompanied by staining with stools and blood. Protrusion of structures through the abdominal wall of the rat prior to surgery was clearly noticeable. The dilated descending colon was severely inflamed. The animals exhibited multiple abdominal adhesions over the descending colon with extensive generalized dilatations of the abdominal vessels (Figure 3D). Occasionally, megacolon was observed in this group and at times toxic megacolon was seen particularly in the continuous injection subgroup. It is important to note that the descending colon was hyperemic with vasodilatation starting from day 3 and persisting throughout the duration



**Figure 3** Representative photographs of macroscopic (upper row) and microscopic (lower row, HE stain  $\times 200$ ) findings on day 70 in treatment groups MC, B, IA and IA + B. **A and B:** Normal colon, note the whitish area at the site of multiple inoculation of the EPEC in colons of B-treated animals (↓); **C:** Note the vasodilatation and enlargement of the colon (forceps) in the IA treated rats; **D:** Note adhesions (\*), hyperemia and vasodilatation of an enlarged colon and a darkened site reflecting blackish mucosa (↑) in the IA + B treated animals; **E and F:** Normal microscopic appearance of the colon in the MC and B-treated groups. Well aligned parallel crypts (↓) continuous epithelial lining (▲) normal muscularis mucosa and normal lamina propria infiltration by cells; **G:** Shows cryptitis (↓), infiltration of inflammatory cells (+) and edema (\*) in the submucosal with thinning of the muscularis mucosa and vasodilatation (Δ); **H:** Crypt deformities and bifurcation (▲), cryptitis (↑), extensive inflammatory cells infiltrate (+) and edema (\*).

of the experiment (Figure 3D). The mucosa was always thickened, with a larger ulcer ( $2-3 \text{ cm}^2$ ). The overall average score was  $13.5 \pm 1.4$  for the continued and  $12 \pm 1.8$  for the discontinued injection subgroups (Figure 2).

In brief, the ulcer score in the control group was significantly lower than that in the iodoacetamide-treated group ( $P < 0.005$ ) and the combined treatment group ( $P < 0.005$ ) but not significantly different from the bacteria-treated group. Moreover, there was a significant



**Figure 4** Microscopic assessment. The overall average histological score in the different experimental groups both in the continued and discontinued injection subgroups (Readings represent average of day 3, 7, 14, 28, 42, 56, and 70 in each group).

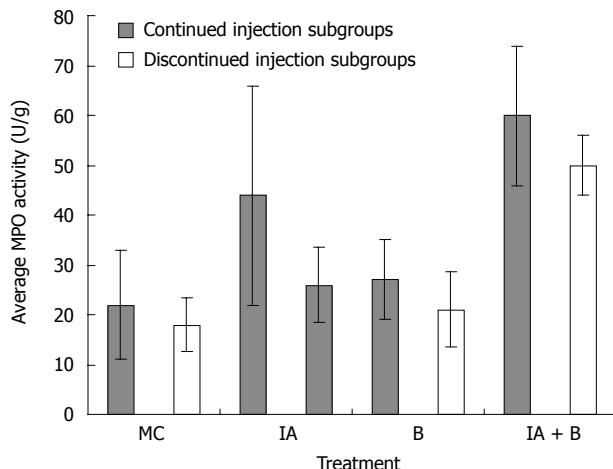
difference in the ulcer score between the iodoacetamide and the bacteria-treated groups ( $P < 0.005$ ) as well as between the combined treatment and the iodoacetamide groups ( $P < 0.05$ ). Furthermore, the score of the combined treatment group was significantly higher than that in the bacteria-treated animals ( $P < 0.005$ ).

#### **Microscopic findings**

The microscopic alterations focused mainly on the descending colon proximal to the site of the inoculation and its surroundings. The findings were compared with commonly reported colonic alterations seen in chronic ulcerative colitis. All layers of the colon were thoroughly studied and reference was made to the normal intestinal mucosa obtained from control animals.

**MC and B-treated groups:** The mucosal architecture of the MC group was considered as normal. There was no ulceration of the epithelial lining. The crypts, and the lamina propria inflammatory infiltrate were normal (Figure 3E). The overall average histologic score was  $2 \pm 1.2$  (Figure 4), with no significant difference between the continued and discontinued injection subgroups. Similar findings were observed in the B-treated group, with an average score of  $1.89 \pm 1.1$  (Figures 3F and 4).

**IA-treated group:** The iodoacetamide-treated continued injection subgroup showed focal mucosal ulceration and depletion of the epithelial lining; the inflammation involved the entire descending colon, sigmoid colon, rectum and anus (Figures 3G and 4). In addition, there was massive infiltration of inflammatory cells and an increase in the gut-associated lymphoid tissues. The crypts were partially destroyed, hypertrophied, and surrounded by inflammatory cells and edema (Figure 3G). The mucosa and submucosa contained several cell types including neutrophils, lymphocytes, macrophages, eosinophils, and clusters of mast cells. The overall average histologic score for IA-treated animals was  $12 \pm 1.35$ . However, when the treatment with IA was stopped, the tissues regained to a great extent their normal appearance, starting 1 wk after discontinuing the injections. The epithelial lin-



**Figure 5** Average MPO activity in the various experimental groups in the continued and discontinued injection subgroups (readings represent average of day 3, 7, 14, 28, 42, 56, and 70 in each group). The increase in the MPO activity in the IA+B subgroup was significantly greater ( $P < 0.005$ ) compared to all the other subgroups. The IA continued injection subgroup also showed a significant increase ( $P < 0.005$ ) compared to the control group.

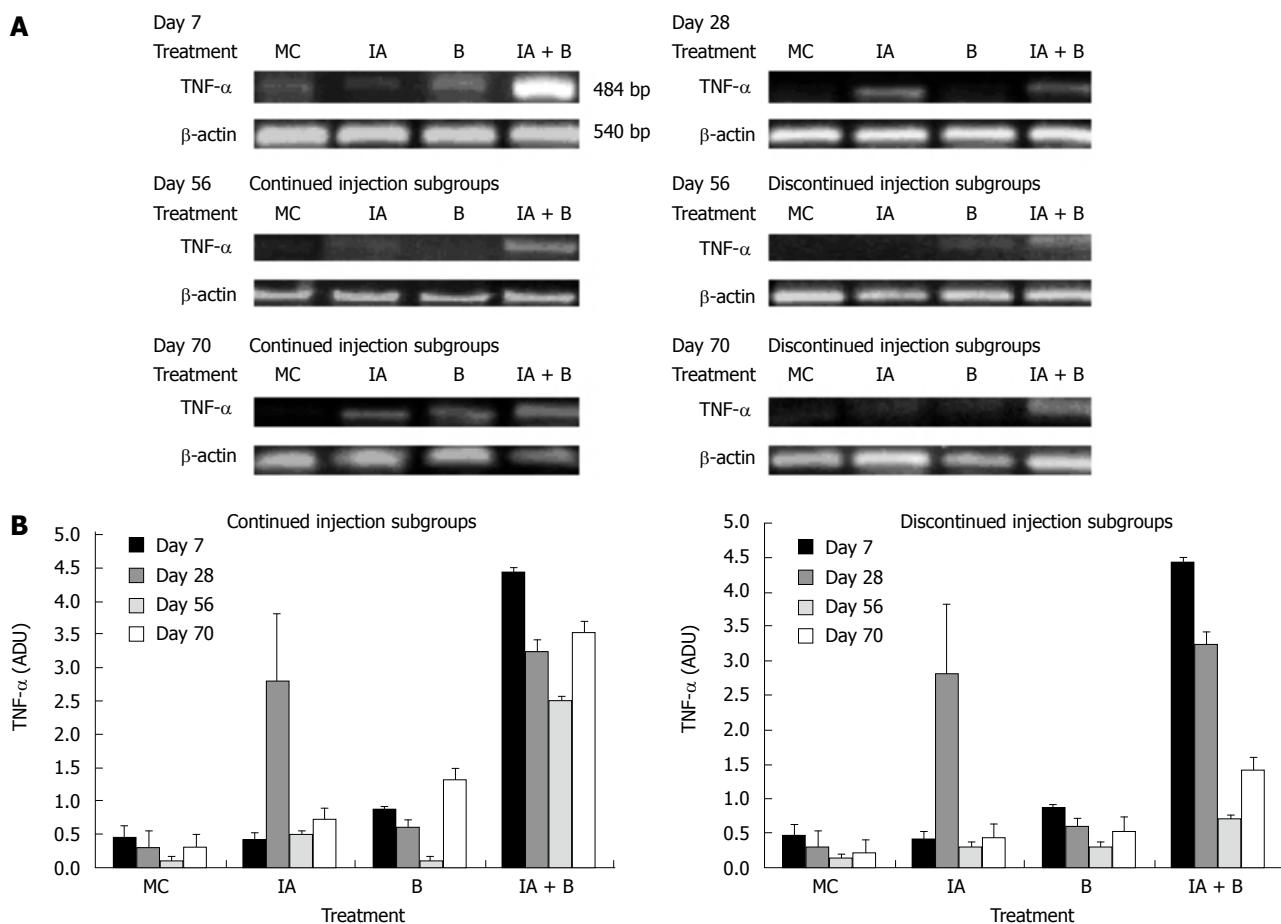
ing was almost normal, with little inflammatory infiltrate, scattered mast cells and no vasodilatation. The overall histologic score for IA-treated discontinued injection subgroup animals was  $9 \pm 1$ .

**Combined (IA + B)-treated group:** Severe inflammation was noted proximal to the site of inoculation with frequent involvement of all four layers of the colon. Histological examination revealed diffuse distortion of the mucosal architecture, with marked crypt atrophy and extensive infiltration by mononuclear cells (Figure 3H). The mucosal surface was irregular, villiform, and heavily infiltrated by various types of inflammatory cells exceeding that in the submucosa. The crypts showed loss of parallelism, with irregularity in crypt size, spacing and shape. In addition, cryptitis and crypt abscesses were present (Figure 3H). Mast cells were clustered in the submucosa. The sites affected by inflammation were edematous, ulcerated, and devoid of any glands. Furthermore, there was thinning or complete absence of the muscularis mucosa. Mucosal ulcers were covered with a large cellular debris (necrotized tissue and products of inflammation). These findings were observed throughout the duration of the experiment in both subgroups. The highest overall histologic score was seen in the continued injection subgroup, with an average score of  $16.2 \pm 0.9$ , compared to  $15.4 \pm 1.7$  for the discontinued injection subgroup (Figure 4).

#### **Myeloperoxidase (MPO) activity**

Assessment of MPO activity is considered a good estimate of the intestinal inflammatory status. MPO activity was consistently the highest in the combined (IA + B) treatment group receiving continuous inoculations, at all time points from day 7 to day 30. This high activity was maintained until the last day of the experiment (Figure 5).

One week following induction of inflammation, the MPO activity in the combined (IA + B) treatment group



**Figure 6** A: Representative photographs from three independent experiments of TNF- $\alpha$  mRNA expression in the descending colon in the various experimental groups at selected time points: day 7, day 28, day 56 continued injection subgroup, day 56 discontinued injection subgroup, day 70 continued injection subgroup, and day 70 discontinued injection subgroup. Band intensity was adjusted for the corresponding  $\beta$ -actin, and values were expressed as arbitrary density units (ADU); B: Expression of TNF- $\alpha$  mRNA in the descending colon in the various experimental groups both in the continued and discontinued injection subgroups, at all time points. Note the presence of significant difference in expression between the IA + B compared to the other groups ( $P < 0.005$ ).

reached an average of  $41 \pm 2.2$  U/g tissue which was significantly higher than the respective control values ( $11.7 \pm 2.1$ ,  $P < 0.005$ ) and almost twice the values of the bacteria-treated group ( $28.5 \pm 2.8$ ,  $P < 0.005$ ) and almost twice those of IA group ( $23.2 \pm 7.7$ ,  $P < 0.005$ ). The MPO activity in the IA ( $23.2 \pm 7.7$ ) and B ( $28.5 \pm 2.8$ ) treated animals were significantly higher than in the MC-treated group (Figure 5). A pattern similar to that observed on day 7 was also noted on day 14, 21, 28, 42, 56 and 70 (Figure 5).

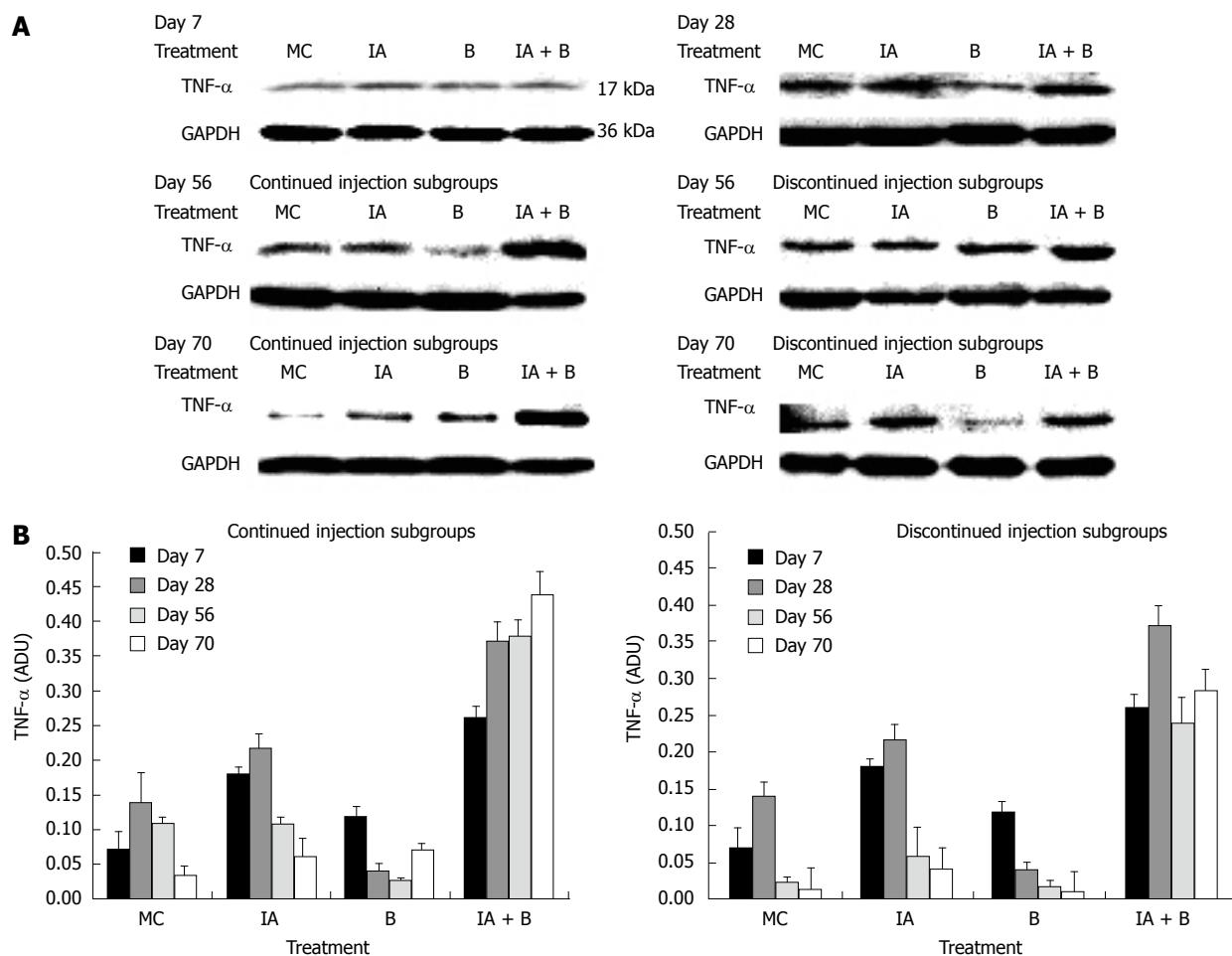
In general, in the continued injection experiment, the MPO score in the control group was significantly lower than that in all the other groups ( $P < 0.005$ ) but not significantly different from the bacteria-treated group. Furthermore, MPO level in the combined treatment group was significantly higher than that in the bacteria-treated animals ( $P < 0.005$ ). Even in the discontinued injection subgroups, there was a significant difference in MPO activity score between the control group and the combined treatment group ( $P < 0.005$ ). In brief, the combined treatment group had a significantly higher MPO activity score than the iodoacetamide-treated group ( $P < 0.05$ ).

#### Inflammatory signaling: TNF- $\alpha$ expression

TNF- $\alpha$  is a prototypical proinflammatory cytokine and a

key regulatory factor in various inflammatory processes involved in the pathogenesis of ulcerative colitis [24-27]. In this study, TNF- $\alpha$  mRNA expression levels were assessed as arbitrary density units (ADU) in relation to the endogenous level of  $\beta$ -actin. Our findings are similar to previous studies that reported an increase in TNF- $\alpha$  expression at both mRNA and protein levels in patients with IBD.

**TNF- $\alpha$  mRNA expression:** On day 7, TNF- $\alpha$  mRNA expression was increased 7-to-8 fold in the combined treatment (IA + B) group compared to the other groups (Figure 6A). On day 28, there was a significant rise in TNF- $\alpha$  expression in the IA-treated group reaching 7-fold higher than that in the B-group, slightly higher than that in the combined (IA + B) group which was slightly down regulated but still significantly higher than the other groups (Figure 6B). On day 56, there was a transient overall decline in the expression of the TNF- $\alpha$  mRNA in the IA-continued injection subgroup followed by an elevation in the levels on day 70. However, in the discontinued injection subgroup, there was a time-dependent decrease in the TNF- $\alpha$  levels following day 28 to day 70 post-treatment. On day 56, the TNF- $\alpha$  mRNA levels were reduced in all groups (Figure 6B).



**Figure 7** A: Representative photographs from three independent experiments of TNF- $\alpha$  protein expression in the descending colon in the various experimental groups at selected time points: day 7, day 28, day 56 continued injection subgroup, day 56 discontinued injection subgroup, day 70 continued injection subgroup and day 70 discontinued injection subgroup. Band intensity was adjusted for the corresponding GAPDH, and values were expressed as arbitrary density units (ADU); B: TNF- $\alpha$  protein expression in the descending colon in the various experimental groups both in the continued and discontinued injection subgroups, in response to treatment at all time points. Note the significant difference in expression between the IA + B and the other groups ( $P < 0.005$ ).

The combined treatment (IA + B) continued injection subgroup maintained a relatively higher level of the TNF- $\alpha$  mRNA compared to IA-treated or the B-treated and MC-treated control groups (Figure 6B). However, the discontinued injection subgroup exhibited mRNA expression below the detection limits (Figure 6B). During the course of the entire study, the increase in TNF- $\alpha$  mRNA reached its peak in the combined treatment (IA + B) group (both in the continued and discontinued injection subgroups) except for a transient rise in the iodoacetamide-treated group at day 28 (Figure 6B). However, it is important to note that after the inoculations were stopped, TNF- $\alpha$  mRNA expression decreased in the discontinued compared to the continued injection subgroup and this difference was observed at all time points, with greater expression in the combined treatment (IA + B) subgroup.

**TNF- $\alpha$  protein expression:** Western blot analysis of TNF- $\alpha$  protein expression showed that at 7 d post treatment, all groups exhibited close levels of TNF- $\alpha$  protein. In both MC and B-treated groups the protein levels were 2-fold lower than the protein levels in the

IA-treated and the combined treatment (IA + B) groups (Figure 7A). During the entire study, TNF- $\alpha$  protein levels were the highest in the combined treatment (IA + B) continued injection subgroup (Figure 7A), whereas in the discontinued injection subgroup all animals showed reduced expression following the cessation of the injections (Figure 7B). It is worth mentioning that the combined treatment (IA + B) discontinued injection subgroup maintained the highest values of TNF- $\alpha$  protein levels. In general, there was a good correlation between TNF- $\alpha$  protein and mRNA expression levels in all the experimental groups and subgroups, indicating the effect of treatment on both post-transcriptional and post-translational processes of TNF- $\alpha$ .

## DISCUSSION

Over the past three decades, several models of UC have been developed, with a variable range of clinical manifestations resembling those observed in human IBD, however, none of these closely mimic the clinical entity of human UC [10,11,16,17]. Using animal models, there is much indirect evidence to suggest an interaction between

luminal flora and the immune system, based mostly on the disruption of the immunoregulatory mechanisms<sup>[28-30]</sup>. The present study showed that a UC model, using an SH blocker and enteropathogenic *E. coli*, closely resembles the human situation and is reproducible with findings indicative of chronicity. The characteristics of the present model were colonic disease induced for nearly hundred days, the presence of clinical features such as weight loss (Figure 1), diarrhea and rectal bleeding, accompanied with macroscopic (Figure 2) and histological alterations (Figures 3 and 4) typical of chronic ulcerative colitis in humans<sup>[16,17,29,31]</sup>. Moreover, an analysis of colonic myeloperoxidase activity<sup>[32]</sup> showed a consistent elevation of activity in the combined IA + B treatment group, indicative of severe mucosal inflammation of the descending colon (Figure 5). Furthermore, upregulation of TNF- $\alpha$  mRNA and protein expression in the IA + B group supported the chronicity of the inflammatory process (Figures 6 and 7).

Analysis of body weight gain across the various experimental groups and subgroups revealed that rats treated with a combination of IA + B exhibited the lowest rate of weight gain in both the continued and the discontinued injected subgroups compared to MC, IA-treated discontinued injection subgroup and B-treated group. In addition, the IA continued injection subgroup behaved similar to the combined treatment continued injection subgroup, but with a higher overall growth rate. Once the injections in the IA-treated subgroup were discontinued (day 30), the growth rate increased again. Moreover, the combined treatment group showed an undulating course of increase and decrease in weight, which may indicate periods of relapse and remission of the disease. Such a decrease was observed consistently in both the continued and discontinued combined treatment subgroups as well as in the iodoacetamide-treated continued injection subgroup. On the other hand, the iodoacetamide-treated subgroup with discontinued treatment behaved like the controls after the injections were stopped (Figure 1B). The low weight gain (in the IA + B group) was due probably due to malabsorption secondary to a defective intestinal barrier. This barrier may not be restored appropriately because of persistent activation of mucosal inflammation<sup>[17]</sup>, with malabsorption in addition to diarrhea, affecting the physiology of other parts of the gut<sup>[33,34]</sup>. The changes in weight gain in the experimental groups (IA + B) were paralleled by the increased rates of diarrhea, loose and bloody stools, and sometimes swollen abdomen and megacolon.

Abdominal examination revealed tenderness over the descending colon as well as various degrees of megacolon in the combined treatment group. These findings provide further support to the validity of our animal model in inducing UC. Besides, the IA-treated animals in the continued injection subgroup showed similar findings but were not as consistent, particularly with regard to the development of megacolon. This feature was not encountered in the B-treated or MC control groups.

The validity of the combined treatment induced-UC was further supported by the high range of scores for macroscopic alterations. These included generalized va-

sodilatation, adhesions, enlargement of the descending colon and ulcer formation (Figure 2). These observations suggest that continuous stimulation by iodoacetamide is necessary for maintaining the inflammatory process. In this case, the score range in the iodoacetamide-treated and combined treatment groups showed moderate to severe inflammation in the descending colon (Figure 3C and D) and is consistent with the clinical findings seen in UC<sup>[14,18]</sup>. In general, the gross abnormalities observed in the combined treatment group were more consistent and more characteristic of chronic UC. There was diffuse hyperemia, with adhesions, megacolon, ulcerations, vasodilatation, and redness in the nearby segment of the jejunum. The overall score in the combined IA + B group with continued treatment ranged between 10-15, while the score ranged from 9-14 in the discontinued injection subgroup. Therefore, despite discontinuing the injections, the inflammatory process maintained its course, a point of special interest for further investigations.

In addition, the microscopic findings in the descending colon revealed histological abnormalities, particularly in the combined IA + B treatment group, followed by the IA-treated group with continuous injection (Figure 3). Such changes were seen mostly in the mucosa. These observations are in line with the findings seen in UC. However, in a few instances, the inflammation in the combined IA + B treatment group was very severe, involving all four layers of the colon, the mucosa, submucosa, muscular layer and serosa. Histological assessment of the colonic damage showed that during the entire duration of the experiment, the combined IA + B treatment group had the highest score, in both continued and discontinued subgroups (between 8 to 16). These changes were less pronounced in the iodoacetamide groups and were minimal or absent in the B-treated and MC-treated control groups (Figure 4). Therefore, changes in the microscopic structure of the combined treatment subgroups mimicked the changes commonly encountered in severe UC in humans<sup>[30,35,36]</sup>. These include extensive hyperemia and loss of epithelial lining, ulceration of the mucosa, severe depletion of goblet cells, loss of crypts, crypt abscesses, cryptitis, with dense inflammatory cell infiltration, severe dilatation of several blood vessels, loss or thinning of muscularis mucosa, and invasion by lymphoid cells<sup>[3,17,19]</sup>.

Assessment of MPO activity provides a reproducible and qualitative estimate of mucosal inflammation<sup>[23]</sup> and may serve as a quantitative index of disease severity. At the site of the mucosal injury, MPO content is a marker of the magnitude of neutrophil infiltration. The combined treatment group exhibited the highest MPO activity in the descending colon (Figure 5). It is therefore, clear that the degree of inflammation was the highest in the combined IA + B treatment group; in particular the subgroup with continued injection. The inflammatory activity was maintained at a much higher rate than in the control, B-treated or the IA-treated groups. However, it is important to note that once the injections were discontinued, the combined IA + B treatment subgroup maintained its highest activity compared to the other

groups. This observation provides further support to the notion that the inflammation persisted in the combined treatment group regardless of continued or discontinued injections. Once again, these findings are in line with the findings of chronic UC in humans<sup>[35,36]</sup>.

Further characterization of our animal model was carried out at the molecular level in order to evaluate the expression of inflammatory signaling. We analyzed the mRNA and protein expressions of TNF- $\alpha$ , a proinflammatory cytokine which can induce COX-2 and COX-2 protein expression. It should be noted that increased local expression of TNF- $\alpha$  is of prime importance in driving the chronic inflammatory reaction and the development of tissue injury<sup>[25,26]</sup>. There is significant correlation between the production of TNF- $\alpha$  and the severity of UC. High levels of proinflammatory cytokines in the mucosa lead to excessive production of matrix degrading enzymes by the gut fibroblasts, loss of mucosal integrity and ulceration. In UC, high levels of TNF- $\alpha$  have been documented in the lamina propria as well as increased TNF- $\alpha$  mRNA and protein expression in the mucosa associated with tissue injury<sup>[34,37]</sup>. TNF- $\alpha$  is the most important mediator of response to Gram-negative bacteria and also plays a critical role in the immune response to other micro-organisms<sup>[36]</sup>. There is much evidence to suggest that either enteric bacteria or immune dysfunction play a pivotal pathogenic role in IBD<sup>[1,2]</sup>. Endotoxins or lipopolysaccharides (LPS), derived from the outer membrane of Gram negative enteropathogenic *E. coli* interact with CD14 on the surface membrane of mononuclear cells, thus triggering a signal cascade that leads to the production and release of TNF- $\alpha$ , which is strongly involved in the pathogenesis of ulcerative colitis<sup>[38]</sup>. In this respect, both TNF- $\alpha$  and LPS may represent putative therapeutic targets for the treatment of UC.

The present study has demonstrated that mucosal scrapings of the colon contained significantly elevated levels of TNF- $\alpha$  mRNA and protein in both the combined treatment (IA + B) subgroups and at all time points compared to control animals (Figures 6 and 7). However, an occasional increase in TNF- $\alpha$  mRNA expression was noted in the IA-treated group on day 28 and 70.

The abnormalities resulting in chronic mucosal inflammation can be divided into two types; defects in immune regulation pathways and defects involved in the barrier function of the epithelium<sup>[5,8,9]</sup>. Models of disrupted barriers function have resulted in mucosal inflammation<sup>[5]</sup>. Chemical agents such as acetic acid, dextran sodium sulfate, and iodoacetamide, can injure the epithelium. In a normal animal, all means of chemical injuries result in a transient inflammatory process. Repair mechanisms step in and a stable mucosal barrier is reestablished. Indeed, a leading concept in the pathogenesis of chronic intestinal inflammation is the break in mucosal tolerance, an active process by which an injurious immune response is prevented, suppressed or shifted to a non-injuring class of immune reaction<sup>[39,40]</sup>. The intestine is in permanent contact with billions of bacteria belonging to the normal gut flora, food protein, and potentially

pathogenic bacteria and has to discriminate and define selective action towards pathogenic and non-pathogenic components. Mucosal tolerance exists in order to prevent an immune response against the body's "own" bacteria that would otherwise give rise to chronic intestinal inflammation. EPEC colonize the intestinal mucosa and, by subverting intestinal epithelial cell cytoskeleton function, produce a characteristic histopathological response. EPEC with invasive properties cause gross destruction of the epithelial architecture and tight junction proteins. Thus the presence of a chemical agent in addition to EPEC can create an optimal environment for the development and maintenance of chronic UC.

Experimental studies indicate that defects in intestinal permeability induced by iodoacetamide may facilitate the passage of LPS, derived from the Gram-negative enteropathogenic *E. coli* or other enteric bacterial flora, into the circulation. It is speculated that enhanced intestinal permeability, probably caused by the inflammation induced by iodoacetamide, could precede and prepare the ground for the development of UC in rats.

In conclusion, the development of animal models is critical in elucidating the molecular pathways by which human ulcerative colitis develops and progresses to an advanced stage. Indeed, identifying the molecular pathways involved in initiating this disorder may allow us to gain an insight into the strategy(ies) that can lead to IBD prevention. Furthermore, the present study may permit the characterization of molecular and morphologic effects of the disease that may enhance our ability to develop therapeutic agents with higher efficacy or with fewer side effects.

Furthermore, such a study may result in the development of agents that target molecular pathways known to play a role in the initiation of IBD, specifically in the subset of asymptomatic individuals at high-risk for the development of UC.

## COMMENTS

### Background

We employed the two-hit hypothesis, both chemical and bacterial, for our chronic ulcerative colitis (UC) model, based on several lines of indirect evidence implicating gut flora in the pathogenesis of inflammatory bowel disease in general, and ulcerative colitis in particular. Bacterial flora appears to be always involved in the development of ulcerative colitis, and a sulphydryl group blocker such as iodoacetamide may further increase the metabolic stress on the epithelial cells especially when it is instilled into the colon followed by enteropathogenic *E. coli*. The resident cells of the lamina propria, under such conditions, constitute an important target for endotoxins. The resulting secretion of cytokines causes the induction of a chronic colitis. This model requires a regular inoculation schedule to maintain the inflammatory process.

### Research frontiers

A chronic ulcerative colitis model which mimics to a great extent the human disease was developed. Our model is characterized by clinical, macroscopic, microscopic and molecular parameters that are similar to human UC.

### Innovations and breakthroughs

The present study confirmed one of the possible mechanisms of ulcerative colitis whereby a chemical reagent introduced into the colon induces colonic inflammation with morphological and clinical features suggestive of ulcerative colitis. The inflammatory condition was further provoked by inoculating *E. coli* bacteria at the same site allowing for an interaction between the bacteria, the mucosal barrier and the intestinal tissues, in particular the mucosal layer. Such

interactions aggravated the inflammatory reaction as evidenced by the upregulation of peroxidase, indicating an increase in the inflammatory cells, and the upregulation of TNF alpha, a prototypical proinflammatory cytokine secreted by the inflammatory cells and a key regulatory factor involved in ulcerative colitis. Such a phenomenon was maintained for the entire duration of the experiment.

### Applications

The present study provides a well characterized experimental model for ulcerative colitis essential for elucidating the mechanisms and molecular pathways involved. It also sheds light on the effect of the colonic microecology and the potential of manipulating the microbial environment in the intestines through the use of probiotics. This model may allow insight into strategies that can lead to the prevention of inflammatory bowel disease (IBD) and for the assessment of agents that can be used in the management of UC.

### Terminology

Inflammatory bowel disease (IBD): IBD is characterized by chronic, recurrent inflammation of the GI tract of unknown etiology. The disease varies in the extent and severity of the symptoms. IBD is known to develop between the ages of 15 to 35 years. Ulcerative colitis (UC): UC is an IBD characterized by passage of bloody diarrhea, which usually constitutes the earliest sign of the disease. Progression of UC may be associated with fever, abdominal pain and weight loss. UC is often termed left-sided colitis for it affects mainly the mucosa of the descending colon. The disease may extend to the proximal part of the large bowel; when the entire colon is affected, the disease is called pancolitis. Crohn's disease (CD): CD is an IBD that can involve any part of the digestive tract, from the oral cavity to the anus. It causes deep chronic ulcerations involving the mucosa and the deeper layers.

### Peer review

This is a solid and valuable piece of work. It is an extension of the authors' previous work on experimental colitis. It is definitely an original work of great scientific value. The experimental design is sound, results are well presented, and conclusions are documented by the findings of the study.

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RAPID COMMUNICATION

## Composition of inflammatory infiltrate and its correlation with HBV/HCV antigen expression

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### Abstract

**AIM:** To study the composition of liver inflammatory infiltrate in biopsy material from patients chronically infected with hepatotropic viruses and to evaluate the correlation of inflammatory infiltrate with hepatitis B virus (HBV) and hepatitis C virus (HCV) viral antigen expression in chronic B and C hepatitis.

**METHODS:** The phenotype of inflammatory cells was evaluated by the EnVision system, using a panel of monoclonal antibodies. HBV and HCV antigens were detected with the use of monoclonal anti-HBs, polyclonal anti-HBc and anti-HCV antibodies, respectively.

**RESULTS:** The cellular composition of liver inflammatory infiltrate was similar in the patients with B and C hepatitis: ~50%-60% of cells were T helper lymphocytes. Approximately 25% were T cytotoxic lymphocytes; B lymphocytes comprised 15% of inflammatory infiltrate; other cells, including NK, totalled 10%. Expression of HLA antigens paralleled inflammatory activity. Portal lymphadenoplasia was found more often in hepatitis C (54.5%) than in hepatitis B (30.6%). Expression of HBcAg was found more often in chronic B hepatitis of moderate or severe activity. Overall inflammatory activity in HBV-infected cases did not correlate with the intensity

of HBsAg expression in hepatocytes. Inflammatory infiltrates accompanied the focal expression of HCV antigens. A direct correlation between antigen expression and inflammatory reaction *in situ* was noted more often in hepatitis C than B.

**CONCLUSION:** Irrespective of the etiology and activity of hepatitis, components of the inflammatory infiltrate in liver were similar. Overall inflammatory activity did not correlate with the expression of HBsAg and HCVAg; HBcAg expression, however, accompanied chronic hepatitis B of moderate and severe activity.

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**Key words:** Chronic B and C hepatitis; Inflammatory infiltrate; Lymphoid follicle; Lymphadenoplasia

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### INTRODUCTION

A cytopathic effect is absent in hepatocytes during viral replication, which is a characteristic feature of infection with hepatotropic viruses. Necrosis of hepatocytes is considered to be a result of cellular immunity reactions directed against viral antigens on the surface of these cells<sup>[1,2]</sup>. It is assumed that immunological mechanisms, insufficient for the full eradication of viruses, are responsible for liver damage and extrahepatic manifestations of infection<sup>[3]</sup>. Extrahepatic manifestations of hepatitis B virus (HBV) and hepatitis C virus (HCV)

infection, i.e. glomerulonephritis and vasculitis caused by the deposition of immune complexes, are connected with the humoral immunity directed at viral antigens<sup>[4-6]</sup>.

Analysis of the etiopathogenic phenomena of chronic viral hepatitis takes into account two basic factors that influence the course and resolution of infection. The first, and of paramount importance, is a viral factor. It has been demonstrated that mutations in the HBV genome<sup>[7]</sup>, superinfection or coinfection with hepatitis D virus (HDV)<sup>[8]</sup>, infection with HCV 1b genotype, and appearance of HCV quasispecies worsen the disease prognosis<sup>[9]</sup>. However, the second factor of great importance is the host factor and virus to host interaction<sup>[10,11]</sup>.

Inflammatory infiltrate present in a needle biopsy specimen is evidence of immunological processes *in situ*, as a reaction to viral protein expression. Host attempts to eliminate viruses lead to damage and subsequent necrosis of hepatocytes<sup>[12-14]</sup>. Damage to infected hepatocytes is a result of immune reactions aimed at eliminating infection. Therefore, necrosis of hepatocytes and mononuclear cell reaction are key features of such a reaction<sup>[15]</sup>. The intensity of the immune reaction in liver tissue depends both on the immunological status of the host and immunogenicity of HBV or HCV proteins expressed in the liver.

The aim of this study was to evaluate the type of immune reaction (by immunophenotyping the inflammatory infiltrate components) and the relationship between viral antigen expression and inflammation. The pattern of immunological response may bring the insight into etiopathogenesis of chronic viral hepatitis and mechanisms of immune reactions *in situ*.

## MATERIALS AND METHODS

Liver biopsy taken from 217 patients of Hepatology Clinic, Institute of Infectious and Parasitic Diseases, chronically infected with HBV or HCV were evaluated histopathologically. All patients were diagnosed on the basis of plasma presence of HBsAg, anti-HBc, HBeAg, anti-HBc and anti-HCV by the immunoenzymatic method (Abbott, Chicago, USA) and presence of HBV DNA, HCV RNA by PCR and RT-PCR, respectively. Out of 217, there were 137 patients with chronic hepatitis C (mean age 41; range 9-73 years), 72 patients with chronic hepatitis B (mean age 29.7; range 7-79 years) and 8 patients with mixed etiology of hepatitis: HBV/HCV or HBV/hepatitis D viroid (HDV) (mean age 33.8; 21-44 years). Out of 217 liver biopsies, we chose at random 20 biopsies of chronic type B hepatitis and 20 biopsies of chronic type C hepatitis. The main criterion was the amount of frozen tissue and tissue in paraffin block.

### **Examination of needle liver biopsy specimens**

Specimens taken by a blind biopsy with 1.6 mm needle were received fresh on gauze rinsed with PBS. Tissue material > 15 mm in length was divided into 3 pieces with a sterile surgical blade. One fragment, ca. 5 mm in length, was frozen at -80°C in petroleum ether cooled with acetone and dry ice. The frozen tissue was stored

at -40°C for further use. The second fragment, 2 mm long, was frozen and stored at -65°C until homogenization, extraction of nucleic acids and testing by PCR or RT-PCR. The third fragment, at least 15 mm long, was fixed in 4% buffered formalin and routinely processed in paraffin. Serial slides 4 microns thick were stained with H&E, impregnated with silver by the Gomori method for reticulin fibres and stained by chromotrope 2R and aniline blue for collagen fibres.

Liver disease was diagnosed according to generally accepted criteria<sup>[16,17]</sup>. Examinations of inflammatory activity and the stage of fibrosis of chronic hepatitis were performed according to criteria proposed by international experts<sup>[18]</sup>. All histological features were finally scored using Histological Activity Index (HAI) using eighteen points scale to assess the grade of the disease (inflammatory activity in the lobules and portal tracts, piecemeal necrosis and bridging necrosis) as: minimal: 1-3 points; mild: 4-8 points; moderate: 9-12 points; severe: 13-18 points. The stage of the disease was assessed using five points scale as: 0-no fibrosis, normal connective tissue; 1-portal fibrosis, fibrous portal expansion; 2-periportal fibrosis, periportal or rare portal-portal septa; 3-septal fibrosis, fibrous septa with architectural distortion; 4-cirrhosis.

Analysis of the cellular composition of inflammatory infiltrate in liver tissue was performed on the frozen sections. A total of 40 tissue samples, five specimens each of minimal, mild, moderate and severe hepatitis B and C, were examined. The number of samples studied by immunohistochemistry was determined by the size of frozen material available for extensive evaluation. Five liver specimens diagnosed by histopathology as normal (liver biopsy performed for other reasons than hepatitis, e.g. Gilbert's syndrome), served as controls. The evaluation was performed not by calculating cells, but by approximation of proportion.

The phenotype of inflammatory cells was evaluated by the EnVision system (anti-mouse globulins, DAKO, DakoCytomation, 2600 Glostrup, Denmark) using monoclonal antibodies listed below: (1) Anti CD45RO (UCHL-1 clone)-activated T cells, memory cells of both CD4 and CD8 subpopulations; (2) Anti-CD45RO (OPD-4 clone)-CD4 memory cells; (3) Anti-CD8-cytotoxic CD8 lymphocytes; (4) Anti-CD45 RA (clone 4KB5)-most B lymphocytes and a small subpopulation of T naive lymphocytes; (5) Anti-CD20 (L26 clone)-B lymphocytes; (6) Anti-CD35 (follicular dendritic cells); (7) Anti-CD56 (part of NK cells); (8) Anti-CD68 (macrophages); (9) Anti-HLA (class I); anti-HLA DR (class II α-chain); anti-HLA DP, DQ, DR (class II β-chain).

Serial sections of frozen liver biopsy fragments were dried at 22°C and fixed in acetone, 5 min [for HBV and HDV, cluster of differentiation (CD) antigens, HLA class I & II molecules and adhesion molecules]; or in acetone, 5 min, followed by chloroform, 5 min (for HCV antigens).

The expression patterns of HBV, HCV and HDV antigens were investigated in order to prove the viral etiology of hepatitis. HBV antigens were detected in fro-

zen sections by the indirect immunoperoxidase method with the use of monoclonal anti-HBs antibodies (Dako) and rabbit polyclonal anti-HBc antibodies (immunization with HBcAg coding HBV fragment synthesized in *Escherichia coli*). HCV antigens were detected with the use of human FITC-labeled polyclonal anti-HCV antibodies<sup>[19,20]</sup>, followed by monoclonal anti-fluorescein antibodies and finally by anti-mouse globulin antibodies labeled with peroxidase (EnVision system, Dako). In cases in which HCVAg was not detected in the first attempt of staining, serial sections were examined. The immunomorphological search for viral antigens expression was performed on frozen, and additionally, if technically possible, on paraffin sections.

The specificity of the methods applied in this study was ascertained by negative staining results when a primary antibody was omitted, or replaced by animal or human sera that did not contain antibodies against antigens of the viruses examined.

## RESULTS

### Etiology

Chronic hepatitis C was confirmed histologically in 137 cases and chronic hepatitis B in 72 cases. There were also 8 patients with mixed etiology of HBV/HCV and HBV/HDV hepatitis.

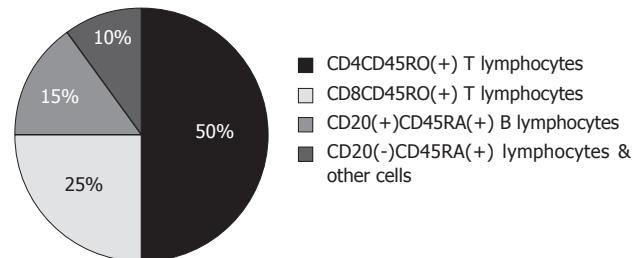
### The phenotype of cells in the inflammatory infiltrate

Cellular composition of the inflammatory infiltrate was the same both in patients with hepatitis B and hepatitis C (Figure 1). The same proportion was also found in the normal liver (a few mononuclear cells were scattered in sinusoids and single mononuclear cells in the portal tracts).

Lymphocytes of helper-inducer phenotype ( $CD4^+CD45RO^+$ ) made up 50%-60% of cells. CD4 lymphocytes were localized within lobules, as well as in portal tracts. They were the main component of the inflammatory infiltrate in areas of spotty necrosis within lobules, and in the foci of piecemeal necrosis. Lymphocytes of the CD8 phenotype constituted 20%-25% of the inflammatory infiltrate. In the normal liver they were present mainly in the sinusoids and as single cells in portal tracts. They made up a significant, but not the only, element of spotty and piecemeal necrosis. In some areas they constituted almost 100% of cells. In the portal tracts they were localized mainly in the peripheral part of this structure.

Altogether,  $CD45RO^+$  cells, i.e. activated T memory cells, constituted 75%-80% of the inflammatory infiltrate. In chronic hepatitis B or C of any activity,  $CD45RO^+$  T lymphocytes were found around foci of spotty necrosis within lobules, in portal tracts, and in sinusoids.

Taking into account the number of activated T lymphocytes ( $CD45RO^+$ ) (almost 80% of all the inflammatory cells) and percentage of T helper and T suppressor lymphocytes (~50% and ~25%, respectively), it may be



**Figure 1** Approximate composition (%) of inflammatory infiltrate in chronic hepatitis B and C.

presumed that about 5%-10% of T lymphocytes express both CD4 and CD8 phenotypes.

About 20% of the infiltrating cells consisted of  $CD45RA^+$  phenotype cells: B lymphocytes and a small subpopulation of virgin T lymphocytes. The number of  $CD45RA^+$  lymphocytes rose together with the increasing activity of hepatitis.  $CD20^+$  B lymphocytes constituted 5%-10% of the inflammatory infiltrate. In normal liver, single B cells were present only in the sinusoids. The number of B lymphocytes increased parallel to inflammatory activity, but the proportion of the cellular composition of inflammatory infiltrate remained constant.

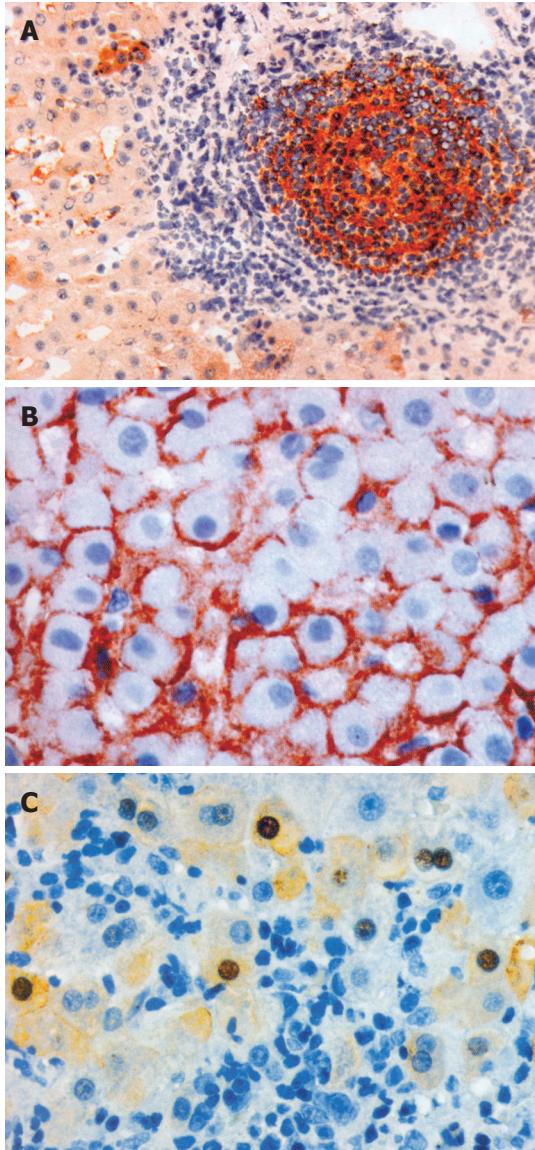
The relative composition of  $CD68^+$  macrophages and  $CD56^+$  NK cells in the infiltrate was also evaluated. In normal liver, macrophages were found mainly as single cells in sinusoids and in the portal tracts. The number of macrophages increased in sinusoids and portal tracts in chronic hepatitis B and C. Single  $CD68^+$  cells were found in lymphoid follicles (mainly secondary) and in germinal centers. Cells of the  $CD56^+$  phenotype made up only a small percentage (1%-2%) of the inflammatory infiltrate.

HLA class I antigens were detected in the form of tiny, granular deposits on sinusoidal cells of normal liver. They were present as tiny foci of a weak membranous pattern. In chronic hepatitis B and C the sinusoidal pattern of HLA class I molecule expression was also observed, irrespective of inflammatory activity. This was accompanied by the expression of these molecules on inflammatory and sinusoidal cells. The intensity of HLA class I expression in sinusoids was much stronger in comparison with the normal liver, not only because of their sinusoidal expression, but also because of their presence on inflammatory cells.

Expression of HLA class II molecules: DR  $\alpha$ -chain and DP, DQ, DR  $\beta$ -chain in normal liver was detected on sinusoidal cells and in the form of granular deposits of a sinusoidal pattern. Single mononuclear cells in sinusoids and portal tracts also showed  $\alpha$ - and  $\beta$ -chains of HLA class II expression. HLA class II molecule expression in chronic hepatitis B and C was detected as a sinusoidal pattern, and on most inflammatory cells in the portal tracts, in foci of piecemeal and spotty necrosis and in areas of bridging necrosis. Cells of lymphoid follicles in portal tracts showed a strong expression of these molecules. In most cases, HLA class II molecule expression on the surface of a hepatocyte was not observed.

**Table 1 Expression of viral antigens in the liver (total number of patients 217)**

Patients	HBsAg	HBcAg	HCVAg	HDVAg
Patients with chronic hepatitis B ( <i>n</i> = 72)	69 (95.83%)	43 (59.72%)	-	-
Patients with chronic hepatitis C ( <i>n</i> = 137)	-	-	67 (48.9%)	-
Patients with chronic hepatitis of mixed etiology ( <i>n</i> = 8)	8 (100%)	4 (50%)	4 (50%)	1 (12.5%)



**Figure 2** A: Expression of HCV antigens in the lymph follicle in the portal tract and in the peripheral hepatocyte (EnVision,  $\times 200$ ); B: Honeycomb pattern of HBsAg expression. No inflammatory reaction (EnVision,  $\times 400$ ); C: Inflammatory infiltrates around hepatocytes containing HBcAg: nuclear and cytoplasmatic expression of HBcAg (EnVision,  $\times 400$ ).

#### The formation of lymphoid follicles in chronic B and C hepatitis

Lymphoid follicles in portal tracts were present in both hepatitis B and C.

The dense clusters of mononuclear cells (follicles) were considered as primary (without germinal centers) or secondary (with germinal centers). Secondary follicles were quite rare—they were seen in 3 cases of chronic B and in 5 cases of chronic C hepatitis. Liver lymphade-

noplasia was seen more often in hepatitis C: 75 patients (54.7%) than in hepatitis B: 22 patients (30.6%); additionally, it was observed in 1 case of hepatitis B + D.

The occurrence of lymphoid follicles in chronic hepatitis B and C correlated with inflammatory activity. The phenotype of cells composing lymphoid follicles was also analyzed. Primary follicles were formed by B lymphocytes ( $CD20^+$ ), T lymphocytes ( $CD4CD45RA^+$  and  $CD4CD45RO^+$ ) of both naive and memory type, and  $CD8^+$  T lymphocytes. Macrophages ( $CD68$ ) and NK cells ( $CD56$ ) were scarce.

Secondary follicles contained germinal centers with B ( $CD20^+$ ) and with T ( $CD4CD45RO^+$ ) lymphocytes. Lymphoid follicles cells showed expression of histocompatibility antigens (HLA-II and HLA-I). We found also expression of HCV antigens inside germinal centers in the portal tract (Figure 2A). Outside follicles, there was a mixture of mononuclear cells, including T cells (also  $CD8^+$ ), macrophages, NK cells and scarce plasma cells.

#### The correlation of inflammatory infiltrate with HBV viral antigens expression in chronic B hepatitis

HBs antigen in liver tissue presented as cytoplasmic droplets, ‘festones’, cytoplasmic embeddings and as a honeycomb pattern. Although HBsAg expression usually did not correlate with the presence of inflammatory infiltrate (Figure 2B), in some cases an inflammatory response was present in the vicinity of hepatocytes with HBsAg expression. Intense and diffuse HBsAg expression was not accompanied by any inflammation, but the inflammatory reaction was related to focal and weak HBsAg expression. No strong correlation was noted between the pattern of HBsAg expression (droplets, festones, honeycomb pattern) and inflammatory activity. A negative correlation, however, was found between the intensity of HBsAg expression localized on membranes of hepatocytes (honeycomb pattern) and the activity of the inflammatory process.

In 43 cases (59.7%), HBcAg was detected in nuclei and/or cytoplasm of hepatocytes as well as on cellular membranes (Figure 2C). Table 1 presents expression of viral antigens in liver. HBcAg presence in relation to overall inflammatory activity was found in 2 patients (4.7%) with chronic hepatitis of minimal activity, 24 (55.8%) with hepatitis of mild activity, 12 patients (28%) with moderate activity, and in 5 patients (11.6%) with severe activity.

In 29 patients (40.3%) HBcAg was not detected. Among those 29 patients, in 15 (51.7%) there was minimal inflammatory activity, in 12 (41.4%) mild activity, while 1 case (3.5%) each of moderate and severe activity was found. To summarize, HBcAg was found predomi-

nantly in patients with chronic hepatitis of moderate and severe activity; only in 2 out of 19 such cases (10.5%) expression of HBcAg was not found. However, localization of HBcAg in the nuclei or cytoplasm of hepatocytes did not correlate directly with the inflammatory infiltrate at those sites. There were foci where inflammatory cells surrounded hepatocytes containing HBcAg, but there was no direct relation between the pattern of HBcAg expression and the presence or lack of inflammatory infiltrate. In addition, no correlation was found between the pattern of HBV antigen expression and the type of hepatocyte necrosis.

#### **The correlation of inflammatory infiltrate with HCV viral antigens expression in chronic C hepatitis**

HCV antigen (HCVAg) was detected in 67/137 patients (49%), and exclusively in the cytoplasm of hepatocytes as granules or amorphous deposits. There was a correlation between HCV antigen expression and inflammatory infiltrates within lobules and in areas of piecemeal necrosis. Furthermore, a positive correlation between the number of hepatocytes containing HCVAg deposits and inflammatory activity was found. A particularly strong expression of HCVAg was detected in hepatocellular carcinoma cells in a patient with liver cirrhosis.

A comparison was made between the inflammatory activity of chronic C hepatitis with or without HCVAg expression in the liver. It was observed that overall inflammatory activity did not correlate with the presence of HCVAg, partly because of the fact that detection of this antigen was dependent on the amount of sections investigated.

## **DISCUSSION**

The analysis of inflammatory infiltrate phenotype in this study showed that the proportions of mononuclear cell composition were similar, irrespective of the etiology and activity of chronic hepatitis. The most numerous were CD45RO<sup>+</sup> lymphocytes—they made up 75% of all inflammatory cells. B lymphocytes constituted 15% of inflammatory infiltrate and other cells (including NK cells) 10%. The CD4/CD8 ratio was > 1.5.

The results presented in this study are in accordance with those obtained by Volpes *et al* in 17 biopsy specimens from chronic hepatitis B and chronic hepatitis C and 5 specimens from acute hepatitis B and acute hepatitis C<sup>[21]</sup>. In all these cases, CD45RO<sup>+</sup> lymphocytes dominated in inflammatory infiltrate. Analysis of the relative composition of inflammatory infiltrate of chronic hepatitis C in children by Woźniakowska-Gesicka established that CD45RO<sup>+</sup> comprised 66%-75% and CD8<sup>+</sup> lymphocytes up to 33% of inflammatory cells. B lymphocytes (CD20<sup>+</sup>) made up 10%-33% of the infiltrate. The results of the present study correspond with those obtained as Woźniakowska-Gesicka *et al* reported<sup>[22]</sup>.

It is assumed that CD4<sup>+</sup> T lymphocytes recognize antigens that are presented by antigen-presenting cells, APC (macrophages, dendritic cells, B lymphocytes) with HLA class II molecules and thus take part in the local

inflammatory reaction<sup>[23]</sup>. The majority of liver infiltrating cells were of the CD45RO<sup>+</sup>CD4<sup>+</sup> phenotype. Furthermore, the proportion of T helper to T suppressor cells in liver tissue was ~2.0. This reflects the pathophysiological conditions of the inflammatory process in the liver. Considering the results of this study, a conclusion can be drawn that proportions of cells in normal and in hepatitis liver are fairly stable. Lukomska *et al* showed that cytotoxic lymphocytes in liver sinusoids derive from peripheral blood, and their number increases during inflammation due to *in situ* divisions<sup>[24]</sup>. This may suggest that the increase of other cellular elements during inflammation may reflect the same kind of divisions at inflammatory sites. CD4 effector cells coordinate the immune response by recognition of foreign antigens, but also by secretion of cytokines which act on other cells<sup>[25]</sup>.

The recognition of foreign antigens by effector cells is crucial for any attempts to eliminate viruses. We have compared the intensity of expression and localization of HLA class I and II molecules with different degrees of activity of hepatitis. HLA class I molecules appeared on the surface of hepatocytes both in the normal liver and in chronic hepatitis. The sinusoidal pattern and very delicate focal membranous expression on the surface of a few hepatocytes were seen in the normal liver. A more intensive expression was observed in cases of hepatitis. The simultaneous expression of viral antigens and HLA molecules on the surface of hepatocytes may certainly indicate the presence of viral antigens, but such expression was not always accompanied by inflammatory infiltrate. This may reflect the status of local tolerance to these antigens. Different intensities of HLA molecules were observed in serial staining of specimens, while the difference in specimen thickness did not exceed 0.5 μm. The other technical parameters, such as incubation time with antibodies or reaction time, did not differ by more than a few seconds. Altogether, localization of the molecule expression was repeatable, and this allowed us to draw conclusions.

A strong association was found between HLA class I expression and HBsAg presence on the surface of hepatocytes in the foci of piecemeal necrosis, with accompanying CD8<sup>+</sup> lymphocyte infiltrate. Senaldi *et al* studied isolated hepatocytes from the livers of children with suspected hepatitis and found HLA class I expression on the surface of 85%-100% hepatocytes<sup>[26]</sup>. Later, Fiore *et al* showed HLA class I expression on the hepatocytes of all chronic hepatitis C (CHC) cases studied, but found no association between the intensity of expression of these molecules and inflammatory activity<sup>[27]</sup>. Van den Oord *et al* proved that the pattern of expression of HLA class I molecules was independent of inflammatory activity, but in areas of spotty necrosis the expression had a focal membranous pattern<sup>[28]</sup>. The results of our study fit the data obtained by the last two authors. The expression patterns of HBV and HCV antigens and the activity of hepatitis showed only a partial association in the current study.

In this study, a granular pattern of HLA class II molecule expression on the surface of sinusoidal cells (macro-

phages, endothelial cells and most inflammatory cells) was found. HLA class II molecules were also found on cells comprising lymphoid aggregates in portal tracts and, less frequently, on the surface of follicular dendritic cell extensions. In a few cases, small groups of hepatocytes with the membranous type of HLA class II molecule expression were found. In most cases in this study, no expression of HLA-DR on hepatocytes was found, a finding that does not support the presumed role of hepatocytes as antigen presenting cells in the inflammatory process<sup>[28-30]</sup>. Using double immunohistochemical staining, Liang *et al* similarly did not find any association between HBcAg and HLA class II molecule expression<sup>[31]</sup>.

Infection with HBV and HCV caused inflammation in liver tissue and formation of lymphoid follicles in the liver. Lymphoid follicles were found more often in chronic hepatitis C (~55%) than in chronic hepatitis B (~31%).

Immunomorphological analysis of lymphoid follicles in chronic hepatitis B and C supports the hypothesis that they arise in portal tracts as a result of chronic local antigen stimulation. Taking into account the fact that lymphadenoplasia is considered as a humoral T-dependent B-cell reaction in chronic local antigenic stimulation, we may conclude that in many cases of chronic hepatitis a local humoral reaction takes place. This may influence the course of disease.

The degree of inflammatory activity did not correlate with the intensity of HBsAg expression in hepatocytes in this study. However, an inverse relationship between the degree of diffuse membranous HBsAg expression and inflammatory local response in chronic hepatitis B (CHB) of minimal and mild activity was established. Nuclear and/or cytoplasmic HBcAg expression in hepatocytes was frequently found in CHB of moderate and severe activity. There was no positive association between the degree of inflammation and the expression of HCV antigens in CHC. However, in comparison to CHB, focal expression of HCV antigens was frequently accompanied by inflammatory infiltrate. The lack of a close relationship between inflammatory cellular reaction and viral antigen expression may suggest that viral antigens are not the only components that take part in the inflammatory reactions *in situ*.

The mechanism of focal tolerance to viral antigens in the liver observed in this study-lack of cellular reaction in spite of intense and diffuse viral antigen expression-is unclear. A small number of CD56(+) NK cells may indicate the impairment of self *vs* non-self recognition, and may influence immunological response and elimination of the virus. In chronic inflammatory reaction, secondary immune response is more specific and therefore more efficient than natural immunity. The presence of lymphoid follicles in some portal tract reflects T-cell dependent B-cell reaction found in e.g. autoimmune inflammatory diseases, self perpetuating processes. In chronic viral hepatitis we observe both cellular and humoral reactions *in situ*. Detailed analysis of cellular reaction in the liver and of the topographic relations of inflammatory cells and viral antigen expression could

contribute significantly towards a better understanding of the immunopathogenesis of chronic hepatitis.

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## COMMENTS

### Background

Infection with hepatotropic viruses such as hepatitis C virus (HCV) and hepatitis B virus (HBV) can result in chronic infection, which leads to liver cirrhosis and hepatocellular carcinoma. Immunological mechanisms against viral infections are responsible for damage and necrosis of hepatocyte. Inflammatory infiltrate present in a needle biopsy specimen is evidence of immunological processes *in situ*, as a reaction to viral protein expression. The aim of this study was to evaluate the relationship between viral antigen expression and inflammation. The pattern of immunological response may bring the insight into etiopathogenesis of chronic viral hepatitis and mechanisms of immune reactions *in situ*.

### Research frontiers

The aim of this study was to determine the composition of liver inflammatory infiltrate in biopsy material from patients chronically infected with hepatotropic viruses and to evaluate the correlation of inflammatory infiltrate with HBV and HCV viral antigen expression in specimens of minimal, mild, moderate and severe chronic B and C hepatitis. Irrespective of the etiology and activity of hepatitis, components of the inflammatory infiltrate in liver were the same.

### Innovations and breakthroughs

The correlation between HBV antigens and HCVAg expression in liver and inflammatory infiltrate was investigated. Overall inflammatory activity did not correlate with the expression of HBsAg and HCVAg; however, HBcAg expression accompanied chronic hepatitis B of moderate and severe activity. The study was undertaken on a large amount of clinicopathological material consisting of 217 biopsy specimens (137 from hepatitis C, 72 from hepatitis B and 8 from mixed etiology). The innovative parts of this study were findings of HLA class I, HLA class II (connected with the antigen presentation), CD4 and CD8, and B lymphocytes in the vicinity of infected hepatocytes. T lymphocytes CD4 and CD8 were most often of CD 45 RO+, phenotype of the memory type.

### Application

The study offers precise immunohistochemical/ histopathological diagnosis of liver biopsy. It can bring along many mixed infections, detected not so often before. This offers the clinician the unique opportunity to compare the results of treatment with the previous picture.

### Peer review

This study described the composition of liver inflammatory infiltrate in biopsy materials and the correlation of inflammatory infiltrate with HBV and HCV viral antigen expression. The use of many liver biopsy samples is a strength point of the manuscript. The authors concluded that the components of inflammatory infiltrates in the liver were similar irrespective of the etiology and activity of hepatitis. This observation seems interesting for understanding the immunopathogenesis of viral hepatitis.

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RAPID COMMUNICATION

# Acute upper gastrointestinal bleeding in octogenarians: Clinical outcome and factors related to mortality

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of severe co-morbidity ( $P < 0.0001$ ) were related to mortality. In multivariate analysis, only the presence of severe co-morbidity was independently related to mortality ( $P = 0.032$ ).

**CONCLUSION:** While rebleeding and emergency surgery rates are relatively low in octogenarians with AUGIB, the presence of severe co-morbidity is the main factor of adverse outcome.

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**Key words:** Acute upper gastrointestinal bleeding; Octogenarians; Elderly; Co-morbidity; Mortality

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## Abstract

**AIM:** To evaluate the aetiology, clinical outcome and factors related to mortality of acute upper gastrointestinal bleeding (AUGIB) in octogenarians.

**METHODS:** We reviewed the records of all patients over 65 years old who were hospitalised with AUGIB in two hospitals from January 2006 to December of 2006. Patients were divided into two groups: Group A (65-80 years old) and Group B (> 80 years old).

**RESULTS:** Four hundred and sixteen patients over 65 years of age were hospitalized because of AUGIB. Group A included 269 patients and Group B 147 patients. Co-morbidity was more common in octogenarians ( $P = 0.04$ ). The main cause of bleeding was peptic ulcer in both groups. Rebleeding and emergency surgery were uncommon in octogenarians and not different from those in younger patients. In-hospital complications were more common in octogenarians ( $P = 0.05$ ) and more patients died in the group of octogenarians compared to the younger age group ( $P = 0.02$ ). Inability to perform endoscopic examination ( $P = 0.002$ ), presence of high risk for rebleeding stigmata ( $P = 0.004$ ), urea on admission ( $P = 0.036$ ), rebleeding ( $P = 0.004$ ) and presence

## INTRODUCTION

Despite considerable advances during the last decades, acute upper gastrointestinal bleeding (AUGIB) remains one of the most serious and potentially life-threatening medical cases that require hospitalization and careful monitoring of the patients. Bleeding may be caused by many different lesions of variable prognostic importance. Peptic ulcers and varices are the main and more significant causes of AUGIB. Approximately 45%-60% of the admissions for AUGIB worldwide are due to peptic ulcers<sup>[1-5]</sup>.

The percentage of older patients suffering from AUGIB has been increasing rapidly over the last years

in the Western World; the main reasons are the increase in life expectancy and the increased consumption of many drugs, such as non-steroidal anti-inflammatory drugs (NSAIDs) in this subgroup of patients. These drugs have characteristic side effects. They provoke ulcerogenesis and simultaneously increase the risk of peptic ulcer complications, mainly bleeding, in patients with a peptic ulcer history<sup>[6,7]</sup>.

Elderly patients constitute a subgroup with special characteristics who need careful handling during their hospitalization, because it is a population with considerable co-morbidity, higher medication use and greater risk for further complications. Age has been established as an independent significant risk factor for poor clinical outcome in patients with AUGIB. Mortality rates ranging from 12% to 35% for those aged over 60 years, compared with less than 10% for patients younger than 60 years of age, have been reported in previous studies<sup>[2,8,9]</sup>.

There has been limited information on the clinical outcome of the very elderly patients with AUGIB. The aim of this study was to evaluate the clinical outcome of very elderly patients being presented with AUGIB (those over 80 years old), and also to determine if there were differences in causes and clinical outcome of AUGIB in octogenarians in comparison with those younger than 80 years old. Finally, we aimed at examining factors related to mortality in octogenarians with AUGIB.

## MATERIALS AND METHODS

We retrospectively reviewed the records of all patients older than 65 years of age with AUGIB hospitalized in two hospitals from January to December of 2006. The patients were divided by age into two groups: Group A patients between 65 and 80 years old and Group B patients older than 80 years of age. We included both outpatients and bleeding episodes of the upper gastrointestinal (GI) tract that occurred in already hospitalized patients (inpatients). AUGIB was diagnosed when hematemesis, bloody nasogastric aspiration, or melena as well as other clinical or laboratory evidence of acute blood loss from the upper GI tract were present.

Bleeding patients were treated according to well established guidelines of the British Society of Gastroenterology Endoscopy Committee<sup>[10]</sup>. Management was in general the same and similar to that in the younger patients. The first steps, which are required, are clinical evaluation and resuscitation. According to the general practice, emergency endoscopy was performed in the 1st 24-hour of admission in the majority of patients, or immediately after resuscitation in patients with massive bleeding. In the majority of patients supplemental oxygen was provided during endoscopy. All patients were given topical pharyngeal anesthesia with 10% lidocaine spray. In most cases, sedation was avoided because of the risk of complications. When sedation was needed, titration of the doses was usually necessary.

Injection hemostasis with adrenaline diluted at 1:10.000 in saline 0.9% was the first line treatment in

patients with active peptic ulcer bleeding or ulcers with stigmata of recent bleeding. The use of endoclips and the application of thermal contact treatment, such as argon plasma coagulation (APC) were added to adrenaline injection in selected cases. In patients with esophageal variceal bleeding band ligation was widely used. Sclerotherapy with histoacryl injection was used for hemostasis in cases of fundic variceal bleeding. Medical treatment was the same for all patients, consisting of Proton Pump Inhibitors (PPIs) given intravenously in usual doses. Vasoactive drugs (somatostatin iv 250 mg bolus injection followed by 24-hour infusion of 250 µg/h for 5 d) were added to endoscopic treatment in patients with variceal bleeding.

Demographic and clinical characteristics of octogenarian patients were recorded and compared with those of younger patients. Each patient's age, gender, recent consumption of non-steroidal anti-inflammatory drugs (NSAIDs), oral anticoagulants and antiplatelet drugs, routine laboratory tests (hematocrit, urea, creatinine) and the presence of shock during admission, co-existing illnesses, the history of admissions in the past for gastric surgery or peptic ulcer complications, endoscopic diagnoses and clinical outcome have been registered in standardized database categories. Shock or hemodynamic instability was defined as systolic blood pressure less than 100 mmHg and a pulse rate of more than 100 beats/min.

Medical history of co-existing illnesses has been encountered as follows. The patients were separated into three categories, depending on the condition of the patient in the admittance. We categorized as stage 1 patients with no serious illnesses (hypertension, endocrine, orthopaedic diseases, etc.), in a rather good condition. As stage 2, we categorized patients with illnesses that need more careful monitoring than those in stage 1, as they ran the risk of destabilization (diabetes mellitus, cardiac problems, compensated cirrhosis, cancer in a stabilized condition), and finally we referred to stage 3 patients as those suffering from major health problems (such as recent myocardial infarction, decompensated cirrhosis, cancer with metastases)<sup>[11]</sup>.

Stigmata of active or recent bleeding were classified according to the Forrest Classification: Forrest Ia: active spurting bleeding; I b: active oozing bleeding; II a: non-bleeding visible vessel (NBVV); II b: adherent clot; II c: spots, a NBVV was defined as a raised red spot resistant to washing, III: no spots, ulcer with a clean base. Then we differentiated into two subgroups, depending on the need to administer endoscopic therapy or not: as high risk stigmata we named the peptic ulcers with Forrest Ia-Forrest II a, which are at greatest risk of rebleeding in comparison with the peptic ulcers with low risk stigmata Forrest II b-III.

The clinical outcome was analyzed according to the duration of hospitalization, the complications during hospitalization, the number of transfused blood units per patient, the rate of rebleeding, the need for emergency surgical hemostasis and mortality, defined as death within the hospitalization period and encountered

**Table 1** Clinicoepidemiological characteristics of patients with acute upper gastrointestinal bleeding

	Group A (65-80 yr)	Group B (> 80 yr)	P (95% CI)
Female, n (%)	83/269 (30.8)	65/147 (44.2)	0.009 (0.37-0.85)
Inpatients with AUGIB, n (%)	20/269 (7.4)	9/147 (6.1)	NS
History of ulcer disease/bleeding, n (%)	81/269 (30.1)	48/147 (32.6)	NS
Heart rate (on admission), (mean ± SD)	86.8 ± 19.5	87.4 ± 15.7	NS
Blood Pressure(on admission), (mean ± SD)	122.6 ± 21.1	124.3 ± 25	NS
Presence of hematemesis, n (%)	68/269 (25.3)	21/147 (14.3)	0.012 (1.18-3.47)
Presence of shock on admission, n (%)	24 (4.9)	10 (2.8)	NS
Hematocrit (on admission), (mean ± SD)	30 ± 7.3	28.5 ± 7.2	0.047
Creatinine (on admission), (mean ± SD)	1.3 ± 0.8	1.5 ± 0.9	0.006
Urea (on admission), (mean ± SD)	85.0 ± 55	105.0 ± 66.0	0.001
Recent NSAIDs use, n (%)	152/269 (56.5)	79/147 (53.7)	NS
Recent aspirin use, n (%)	101/269 (37.5)	58/147 (39.4)	NS
Oral-anticoagulants use, n (%)	34/269 (12.6)	10/147 (6.8)	NS
Antiplatelet drugs use, n (%)	53/269 (19.7)	23/147 (15.6)	NS
Combined use of NSAIDs and antiplatelet drugs, n (%)	28/269 (10.4)	3/147 (2.0)	0.004 (1.7-18.7)
Combined use of NSAIDs and oral anticoagulants, n (%)	10/269 (3.7)	1/147 (0.6)	NS

AUGIB: Acute upper gastrointestinal bleeding; NSAIDs: Non-steroidal anti-inflammatory drugs; NS: No significance.

the causes of death in these patients.

Continuous variables were expressed as mean ± SD and were compared by using Student's *t* test. Categorical variables were expressed as percentages and the differences between the groups were tested for significance by using the chi-squared test. Clinical, biochemical and endoscopic factors that might have contributed to the mortality were evaluated. All these parameters were correlated with in hospital mortality initially by using univariate analysis. Variables found to be significant in the univariate analysis ( $P < 0.05$ ) were included in a multivariate stepwise logistic regression model. All analyses were conducted by using statistical software (SPSS, version 10.0).

## RESULTS

From the total number of 638 patients with acute upper gastrointestinal bleeding hospitalized during the year 2006 in both hospitals, 416 patients (268 males and 148 females) were over 65 years (65.2%). Among these patients 269 (64.7%) were between 65 and 80 years of age (Group A) and 147 (35.3%) were over 80 years (Group B, Table 1). Endoscopy was performed in the majority of patients in both groups. In only 17 patients endoscopy was impossible (15/147 in Group B and 2/269 in Group A,  $P < 0.0001$ , Table 2). Endoscopy was not performed in these patients due to the presence of concomitant severe disease (12/15 in Group B and 1/2 in Group A), their personal or family disagreement (3/15 in Group B and 1/2 in Group A).

### Demographic-clinical characteristics of patients

The percentage of female patients in the octogenarians' group (Group B) was significantly higher than that in Group A (44.2% vs 30.8%,  $P = 0.009$ ). There was no statistically significant difference in the history of previous ulcer between the two groups (Table 1).

The presence of hematemesis before admission was less common in older than in younger patients (14.3% vs 25.3%,  $P = 0.012$ ). Mean hematocrit during admission was less in octogenarians ( $P = 0.047$ ), while mean creatinine and urea levels were higher in comparison with those in Group A ( $P < 0.01$ , Table 1).

The percentage of patients taking NSAIDs and antiplatelet drugs before the bleeding episode wasn't different between the two groups, but the combined use of both drugs was less common in octogenarians (2.0% in Group B vs 10.4% in Group A,  $P = 0.004$ , Table 1).

The percentage of patients with coexisting diseases was higher in octogenarians (Group B) in comparison with Group A, reaching a statistically significant level (94.6% vs 87.7%,  $P = 0.04$ , Table 3). The proportions of patients having hypertension (42.8% vs 32.3%,  $P = 0.04$ ) and cardiovascular diseases (59.2% vs 46.8%,  $P = 0.02$ ) were higher in Group B than in Group A, while that of cirrhotic patients was less frequent (2.0% in Group B vs 7.0% in Group A,  $P = 0.05$ , Table 3).

### Causes of bleeding-endoscopic findings

The main cause of bleeding in both groups was peptic ulcer (51.5% in Group B vs 52.8% in Group A,  $P > 0.05$ , Table 2). Variceal bleeding was statistically less frequent in octogenarians than in younger patients (0.7% vs 7.4%, respectively,  $P = 0.0094$ ) but esophagitis (6.8% vs 1.8%,  $P = 0.025$ ) and vascular diseases such as angiodysplasia (9.0% vs 2.2%,  $P = 0.005$ ) were more common in the octogenarians group. High risk of active stigmata or recent bleeding in peptic ulcer bleeding patients were less common in Group B (17.6%) as compared to younger patients (25.5%), but this difference did not reach statistical significance. Endoscopic hemostasis was administered to 78 patients in the octogenarians' group and was helpful in stopping bleeding and/or preventing rebleeding. There was no complication directly related to diagnostic or therapeutic endoscopy.

**Table 2 Causes of acute upper gastrointestinal bleeding (AUGIB)-endoscopic findings according to age n (%)**

	<b>Group A (65-80 yr)</b>	<b>Group B (&gt; 80 yr)</b>	<b>P (odds ratio)</b>
No endoscopy	2/269 (0.7)	15/147 (10.2)	< 0.0001 (0.015-0.29)
No findings	10/267 (3.7)	6/132 (4.5)	NS
Peptic ulcer	141/267 (52.8)	68/132 (51.5)	NS
Gastric ulcer	68/141 (48.2)	37/68 (54.4)	NS
Duodenal ulcer	73/141 (51.7)	33/68 (48.5)	NS
Varices	20/267 (7.4)	1/132 (0.7)	0.0094 (1.4-79.92)
Erosive gastroduodenitis	61/267 (22.8)	25/132 (18.9)	NS
Angiodysplasia	6/267 (2.2)	12/132 (9.0)	0.005 (0.08-0.62)
Mallory-Weiss	3/267 (1.1)	0/132 (0.0)	NS
Polyps	6/267 (2.2)	1/132 (0.7)	NS
Esophagitis	5/267 (1.8)	9/132 (6.8)	0.025 (0.08-0.79)
Neoplasia	15/267 (5.6)	10/132 (7.5)	NS
Stigmata of bleeding in peptic ulcer bleeding patients			
High risk stigmata	36/141 (25.5)	12/68 (17.6)	NS
Low risk stigmata	105/141 (74.4)	56/68 (82.3)	NS
F1a	7/141 (4.9)	3/68 (4.4)	NS
F1b	13/141 (9.2)	5/68 (7.3)	NS
F2a	16/141 (11.3)	4/68 (5.8)	NS
F2b	32/141 (22.6)	13/68 (19.1)	NS
F2c	8/141 (5.6)	7/68 (10.2)	NS
F3	65/141 (46.0)	37/68 (54.4)	NS
Ulcer diameter < 1 cm	117/141 (82.9)	57/68 (83.8)	NS
Ulcer diameter > 1 cm	24/141 (17.0)	11/68 (16.1)	NS
Endoscopic therapy	78/267 (29.2)	28/132 (21.2)	NS

NSAIDs: Non-steroidal anti-inflammatory drugs; F: Forrest; NS: No significance.

**Table 3 Co-morbidity of patients with acute upper gastrointestinal bleeding n (%)**

	<b>Group A (65-80 yr)</b>	<b>Group B (&gt; 80 yr)</b>	<b>P (95% CI)</b>
No co-morbidity (Stage 0)	33/269 (12.3)	8/147 (5.4)	0.04 (1.09-5.4)
Presence of co-morbidity	236/269 (87.7)	139/147 (94.6)	0.04 (1.09-5.4)
Stage 1	56/269 (20.8)	34/147 (23.1)	NS
Stage 2	146/269 (54.3)	84/147 (57.1)	NS
Stage 3	34/269 (12.6)	21/147 (14.3)	NS
Hypertension	87/269 (32.3)	63/147 (42.8)	0.04 (0.42-0.96)
Cardiovascular diseases	126/269 (46.8)	87/147 (59.2)	0.02 (0.4-0.91)
Malignancy	30/269 (11.1)	14/147 (9.5)	NS
Pulmonary disease	26/269 (9.6)	12/147 (8.1)	NS
Neurologic diseases	21/269 (7.8)	14/147 (9.5)	NS
Cirrhosis	19/269 (7.0)	3/147 (2.0)	0.05 (1.06-12.54)
Diabetes mellitus	65/269 (24.1)	28/147 (19.0)	NS
Renal disease	12/269 (4.4)	11/147 (7.5)	NS

NS: No significance.

### Clinical outcome

Blood transfusion requirements (red blood cell units per patient) were not significantly different between the two groups ( $2.9 \pm 3.2$  in Group B vs  $2.6 \pm 3.2$  in Group A,  $P > 0.05$ ), while octogenarians required more hospitalization days ( $8.1 \pm 5.5$  d vs  $6.9 \pm 4.1$  d,  $P = 0.013$ ).

Rebleeding rates after endoscopic hemostasis in octogenarians were low and not different from those in younger patients (7.5% vs 6.7%). Eleven patients rebled in the octogenarians' group and a second course of endoscopic hemostasis was successful in 5 out of 6 patients. Emergency surgical hemostasis for continuing or recurrent bleeding was rare in bleeding patients and was not different between the two groups (2.7% in Group B vs 2.6% in Group A).

In-hospital complications were more common in octogenarians (38.1% vs 28.3%,  $P = 0.05$ ), whilst there were no significant differences between octogenarians and younger patients in the proportions of the main complications observed; infection (27.9% vs 19.7%), oligemic shock (4.1% vs 1.1%), ischemic episode (6.8% vs 8.9%) and mental status deterioration (3.4% vs 2.2%), for groups B and A, respectively.

More patients died in the group of octogenarians compared with the younger age group (12.2% in Group B vs 5.2% in Group A,  $P = 0.02$ ). There was no significant difference in the reasons of death between the two groups; cardiovascular/cerebrovascular disease (38.9% in Group B vs 42.8% in Group A), malignancy (22.2% vs 28.6%), septic shock (22.2% vs 7.1%), oligemic

shock (16.7% vs 4.3%), whilst one patient in Group A died from hepatorenal syndrome.

In univariate analysis, factors related to mortality in octogenarians were no endoscopic examination ( $P = 0.002$ ), presence of high risk for rebleeding stigmata ( $P = 0.004$ ), urea on admission ( $P = 0.036$ ), rebleeding ( $P = 0.004$ ) and presence of severe coexisting disease ( $P < 0.0001$ ). In multivariate analysis, only the presence of severe co-morbidity was independently related to mortality ( $P = 0.032$ ).

## DISCUSSION

Demographic features and pattern of illness in patients hospitalized with AUGIB have changed during the last decades. A fall in the incidence of AUGIB during the last years has been reported, as well as a striking increase in the proportion of older patients being presented with AUGIB<sup>[11,12]</sup>. About 35%-45% of all patients presented with AUGIB were over 60 years old in previous studies<sup>[13-15]</sup>. In our study this proportion is even higher. Patients over 65 years of age constitute 65.2% of the total population with AUGIB. Additionally, more than a quarter of patients were over 80 years of age. Ten years earlier in our area this percentage was 9.8%<sup>[16]</sup>. It is clear therefore that now we have to deal with an older population with a higher risk of deterioration due to the presence of higher co-morbidities, making their management a clinical challenge.

This shift to older ages is related not only to the increased life expectancy in the western population, but also to changes in the epidemiology of peptic ulcer disease.

Over half of the cases of AUGIB are due to peptic ulcer bleeding irrespective of the patients' age. Increased use of NSAIDs in the elderly, reduced incidence of non-NSAIDs related peptic ulcers as well as better management of the chronic peptic ulcer disease, especially in younger patients, have contributed to this shift. Eradication of *H pylori*, which can be achieved in over 90% of patients with peptic ulcer, reduces ulcer recurrences as well as ulcer bleeding and rebleeding rates in young patients with idiopathic peptic ulcer disease<sup>[17,18]</sup>.

On the other side, indications of aspirin or non-aspirin non-steroidal anti-inflammatory drugs have been increasing during the last years and this has had detrimental consequences, mainly to the elderly. Age is an independent risk factor for NSAID related GI tract toxicity and ulcer formation. In older patients the risk of serious adverse events, such as peptic ulcer bleeding while taking NSAIDs, is 5.5 times that of controls, whereas in younger patients it is only 1.5 times<sup>[19]</sup>. Moreover, concurrent use of NSAIDs and antiplatelet drugs or oral anticoagulants, often used for thromboembolic prophylaxis in the geriatric population, increases the risk of bleeding<sup>[20]</sup>. Two thirds of our patients had taken NSAID and/or oral-anticoagulant or antiplatelet drugs. In a recently published British study, prescriptions for NSAID have been increased by about 13%, aspirin 75 mg by 460% and the prescriptions for

oral anticoagulants by 200% between 1990 and 1999 in the general population<sup>[14]</sup>. Despite the preventive effects of proton pump inhibitors on gastrointestinal toxicity from NSAIDs, it has been found that 70%-80% of users at risk for gastroduodenal complication does not receive gastroprotection<sup>[21,22]</sup>. The increased use of NSAIDs by the elderly explains partly the increased frequency observed in females in these ages in comparison with younger patients<sup>[23]</sup>.

Severity of bleeding does not seem to be higher in the octogenarians group. Prognostic factors of rebleeding, rates or need for emergency surgical hemostasis in our study were not more frequent in the very elderly. Variceal bleeding, which is characterized by high rebleeding rates and high mortality rate, is rare in this group. On the other hand, angiodysplasia and esophagitis, which were more frequent causes of bleeding in the very elderly, rarely produce life threatening bleeding and currently they are easily controlled endoscopically<sup>[24,25]</sup>.

In our study, endoscopy was performed on approximately 90% of patients over 80 years of age, endoscopic hemostasis was successfully applied to our patients without complications and the majority of rebleeding cases were treated with a second endoscopic hemostasis. Except for the endoscopy itself, endoscopic treatment seems also to be an effective and generally safe treatment even when it is performed in the elderly<sup>[26,27]</sup>. In a previous study in elderly patients with peptic ulcer bleeding, no rebleeding or morbidity occurred when endoscopic treatment was performed early but there was a significantly greater risk of further bleeding and treatment related morbidity when treatment was performed after the onset of rebleeding<sup>[28]</sup>. On the other side, from the existing data, there is no evidence that peptic ulcer bleeding is more resistant to endoscopic therapy due to possible atherosclerosis of the underlying artery. In contrast, vessels eroded by NSAIDs-related ulcers may be more amenable to endoscopic hemostasis than those eroded by chronic duodenal ulcers. Chronic duodenal ulcers, in contrast to acute ulcers, produce destruction of the duodenal bulb and erode deeper and larger vessels that bleed more severely and make endoscopic hemostasis difficult or even impossible<sup>[29]</sup>. Moreover, in a recently published study, mild to moderate anticoagulation did not increase the rebleeding rates after endoscopic hemostasis, meaning that endoscopic hemostasis is effective in anticoagulated patients admitted to hospital with AUGIB<sup>[30]</sup>.

Blood loss and emergency surgery is poorly tolerated by elderly patients with increased co-morbidity. Successful endoscopic therapy reduces the rebleeding rates and the need for emergency surgical hemostasis, which is a known risk factor for mortality especially in the elderly, due to high rate of co-morbidity in these patients. Only 4 out of 147 patients over 80 years of age required emergency surgical intervention in our study. Despite this, overall mortality was significantly increased in octogenarians in comparison to younger patients; this is due to the higher co-morbidity in these patients. In a recent multicenter study from France, no difference in

overall mortality was found among patients over and less than 75 years old, but in this study only patients with AUGIB subjected to endoscopy were included<sup>[31]</sup>. The percentage of deaths related directly to bleeding was generally low even in the very elderly, and the majority of elderly patients with AUGIB died of unpreventable causes. Stable elderly patients with AUGIB and no significant co-morbid illnesses and low risk endoscopic findings can even be managed safely as outpatients, as reported in a previous study<sup>[32]</sup>.

In conclusion, currently more than half of the patients hospitalized with AUGIB are over 65 years old and a quarter more than 80 years. Severity of bleeding in octogenarians is not different in comparison with younger patients, rebleeding is uncommon and the need for emergency surgical hemostasis rare. Mortality is higher than in the younger population and the presence of severe co-morbidity is the main adverse factor of clinical outcome.

## COMMENTS

### Background

The increase in life expectancy and increased consumption of many drugs by older people, such as non-steroidal anti-inflammatory drugs, have resulted in a rapidly increasing incidence of acute upper gastrointestinal bleeding (AUGIB) in older patients in recent years in the Western World.

### Research frontiers

Elderly patients constitute a subgroup with special characteristics, such as considerable co-morbidity, higher medication use and greater risk for further complications, making their management a clinical challenge. There has been limited information on the clinical outcome of the very elderly patients with AUGIB.

### Innovations and breakthroughs

This study shows that severity of bleeding in octogenarians is not different in comparison with younger patients, rebleeding is uncommon and the need for emergency surgical hemostasis rare. Mortality is higher than the younger population and the presence of severe co-morbidity is the main adverse factor of clinical outcome.

### Applications

Very old patients suffering from AUGIB should be managed as younger patients, but clinicians should have in mind that their patients with severe co-morbidity are at increased risk of adverse outcome.

### Peer review

This paper describes causes and clinical outcome of upper GI bleeding in elderly patients. More specifically, the age groups 65-80 and 80+ are compared. The numbers are quite large (269 vs 147) and some striking differences between the groups were noted.

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RAPID COMMUNICATION

## Successful outcomes of EMR-L with 3D-EUS for rectal carcinoids compared with historical controls

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treatment for rectal carcinoids. In combination with 3D-EUS, safe and complete resection is further assured.

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**Key words:** Endoscopic mucosal resection with a ligation device; Endoscopic therapy; Endoscopic ultrasonography; Rectal carcinoids

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### Abstract

**AIM:** To assess the results of endoscopic mucosal resection with a ligation device (EMR-L) combined with three dimensional endoscopic ultrasonography (3D-EUS) using an ultrasonic probe for rectal carcinoids. In addition, diagnosis of the depth and size of lesions by EUS was evaluated.

**METHODS:** Between January 2003 and March 2007, 20 patients underwent EMR-L with 3D-EUS using an ultrasonic probe (group A). 3D-EUS was combined with EMR-L at the time of injection of sterile physiological saline into the submucosal layer. For comparison, 14 rectal carcinoids that had been treated by EMR-L without 3D-EUS between April 1998 and December 2002 were evaluated as historical controls (group B). EUS was conducted for all of the patients before treatment to evaluate tumor diameter and depth of invasion. The percentage of complete resection and the vertical resection margin were compared between the two groups.

**RESULTS:** The depth of invasion upon histopathological examination was in complete agreement with the pre-operative findings by EUS. The tumor diameter determined by EUS approximated that found in the tissue samples. There were no significant differences in the gender, tumor sites or tumor diameters between the two groups. The rate of complete resection for groups A and B was 100% and 71%, respectively ( $P < 0.05$ ). The vertical resection margin of group A was longer than that of group B.

**CONCLUSION:** EMR-L is effective as an endoscopic

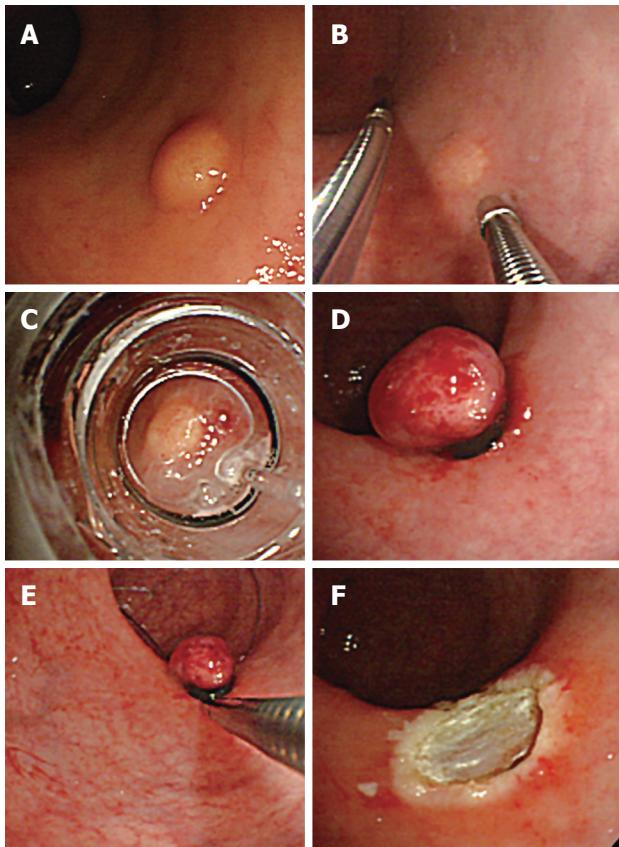
### INTRODUCTION

Recent progress in colonoscopy has facilitated the diagnosis of many rectal carcinoids at an early stage. Rectal carcinoids  $\leq 1$  cm in diameter and a depth of invasion to the submucosal layer are adequately treated by local excision<sup>[1-4]</sup>.

Conventional snare polypectomy or endoscopic mucosal resection (EMR) often result in unsatisfactory complete resection of colonic carcinoids<sup>[1,4-6]</sup>. Although various methods, including EMR with a ligation device (EMR-L), have been developed, problems still remain. We developed a resection technique of EMR-L combined with three-dimensional endoscopic ultrasonography (3D-EUS) using an ultrasonic probe, to confirm accurate injection of saline into the submucosal layer beneath the tumor, for more assured resection. The current study was conducted to evaluate the efficacy of EMR-L in combination with 3D-EUS for endoscopic resection of rectal carcinoids, compared with EMR-L alone.

### MATERIALS AND METHODS

Between April 1998 and March 2007, 36 cases (36 le-

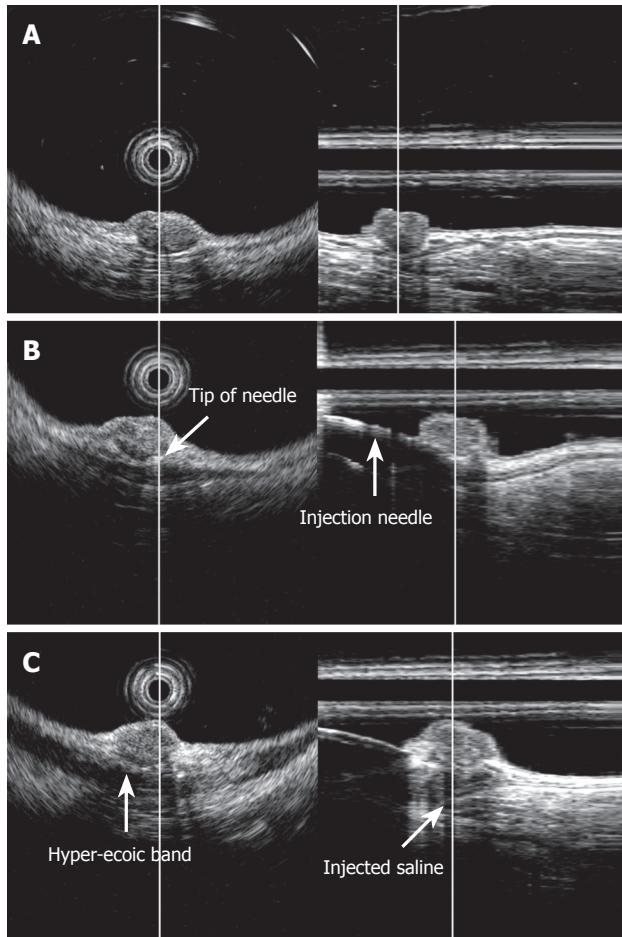


**Figure 1** EMR-L combined with 3D-EUS using an ultrasonic probe. **A:** Endoscopic view of rectal carcinoid; **B:** 3D-EUS was performed at the same time as saline injection into the submucosal layer; **C:** Aspiration of the tumor into the ligation device; **D:** Tumor ligated with the elastic band; **E:** Snaring below the elastic band; **F:** Ulcer after resection.

sions) of rectal carcinoid underwent colonoscopy, and their histopathological appearance was thoroughly examined at our institution. Following observation by conventional colonoscopy, EUS was performed to determine the depth of invasion, tumor size and possible metastasis to the surrounding lymph nodes. To rule out distant metastasis, the procedures were followed by abdominal ultrasonography or CT examination. Those with hepatic metastases, or a complication with severe ulcerative colitis (one case each), were treated surgically. The subjects of the current study were the remaining 34 patients with a tumor diameter  $\leq 10$  mm and a tumor depth that reached the submucosal layer, who underwent EMR-L.

Between January 2003 and March 2007, 20 patients underwent EMR-L combined with 3D-EUS using an ultrasonic probe (group A). For comparison, 14 patients with rectal carcinoids treated by EMR-L alone between April 1998 and December 2002 were included in this study as historical controls (group B).

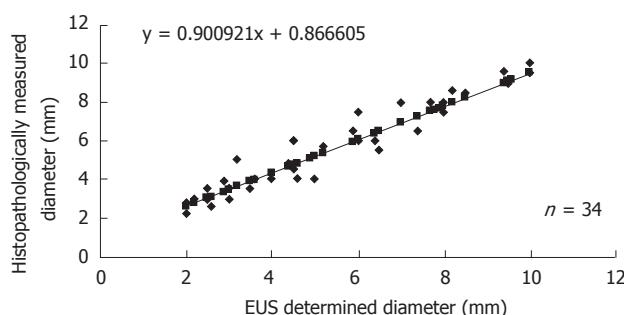
EMR-L, conducted according to Motohashi *et al*<sup>[7]</sup>, was performed as follows: using a small-diameter colonoscope (CF-SV, Olympus, Tokyo Japan) with a ligation device for the treatment of esophageal varices, physiological saline was injected into the submucosal layer. After aspirating as much lesion as possible into the ligation device, the tumor was ligated with an elastic



**Figure 2** EUS image of rectal carcinoid (radial and linear image). **A:** Carcinoid was imaged as a low-echoic region with a depth of invasion to the third layer, i.e. the submucosal layer; **B:** Image upon saline injection into the submucosal layer. The tip of the injection needle was located beneath the tumor in the submucosal layer; **C:** Image after saline injection into the submucosal layer. The injected saline was imaged as a low-echoic area beneath the tumor in the submucosal layer.

band. The section immediately below the elastic band was constricted by snare wire and resected using a high-frequency cutting current.

3D-EUS was combined with EMR-L at the time of injection of sterile physiological saline into the submucosal layer (Figure 1). Specifically, one channel of the two-channel colonoscope (CF-2T230, Olympus) was used for the saline injection, while the 3D ultrasonographic probe (UM-DP20-25R, 20 MHz; Olympus) was inserted through the other channel for scanning. At the time of saline injection, the tip of the injection needle was inserted while observing the ultrasonic radial and linear images, so that it would be located in the middle of the submucosal layer beneath the tumor. By recognizing the rise of the tumor caused by the injection and the adhesion of a hyper-echoic band directly beneath it, the tumor and its dissociation from the muscularis propria were confirmed (Figure 2). We used water-filling methods during EUS examination. For image processing, software for a three-dimensional image display (MAJ 1330, Olympus) was used. For observation of the images, dual plain reconstruction was employed. We com-



**Figure 3** Comparison between EUS-determined and histopathologically measured tumor diameter.

pared tumor diameter and depth, determined by EUS, and the complete resection rate and vertical margin between the two groups.

All resected samples were stained with hematoxylin-eosin for histopathological diagnosis. When the tumor tissue was exposed at the deeper edge of the resected sample, or when evaluation of this section was not possible due to thermal degeneration, a judgment of "positive at the edge" was made. If adhesion of the normal submucosal layer was noted at the lower section of the tumor, it was classified as "negative at the edge". The major axis was measured and recorded as the tumor diameter. The vertical margin was defined as the distance between the tumor and the edge of the specimen. We considered that the vertical margin of positive cases was 0  $\mu$ m.

Following treatment, the patients were followed-up with colonoscopy, EUS and abdominal CT for 6 mo immediately following the treatment, and yearly thereafter.

For statistical analyses, the measured variables were expressed as mean  $\pm$  SD. For group comparisons, the  $\chi^2$  test, Fisher's exact test and Mann-Whitney *U* test were conducted. A corrected  $P < 0.05$  was considered to indicate statistical significance.

## RESULTS

The EUS diagnosis before treatment determined that the tumor had reached deep into the submucosal layer in all 34 subjects. Histopathological examination also found tumor involvement in the submucosal layer in all cases, which demonstrated 100% diagnostic accuracy of EUS. A strong correlation was noted between EUS-determined and histopathologically measured tumor diameters (Figure 3). The two findings were very similar, with the difference between them being  $< 1$  mm in 29 patients (85%) (Table 1).

When two groups were divided according to the method of treatment, there were no significant differences in age, gender or site of involvement and tumor diameter. Group B included four patients with "positive at the edge" and 10 with "negative at the edge," with the rate of complete resection being 71%. All 20 subjects in group A were "negative at the edge" and the complete resection rate was significantly higher when compared with that in group B (Table 2). Mean tumor diameters between

**Table 1 Difference in tumor diameter determined by EUS and histopathology**

Difference in diameter (mm)	Cases ( <i>n</i> = 34)
0-0.5	20
1.0	9
1.5	4
2.0	1

**Table 2 Comparative evaluation and complete resection rate of both groups**

	Group A ( <i>n</i> = 20)	Group B ( <i>n</i> = 14)	<i>P</i> value
Gender (M/F)	11/9	6/8	NS
Age (mean $\pm$ SD)	58.7 $\pm$ 8.7	57.3 $\pm$ 11.3	NS
Location (Rb/Ra)	14/6	11/3	NS
Tumor diameter, mm (mean $\pm$ SD)	6.1 $\pm$ 2.3	5.9 $\pm$ 2.3	NS
Complete resection rate, % (cut edge positive)	100 (0)	71.4 (4)	< 0.05

NS: No statistical significance demonstrated.

positive and negative at the edge in group B were  $5.3 \pm 1.7$  mm and  $6.7 \pm 2.3$  mm. Cases with incomplete resection were found in group B, regardless of tumor diameter. Mean vertical margin of group A was  $1231 \pm 120.87$   $\mu$ m and that of group B was  $634 \pm 147.13$   $\mu$ m.

No procedure-related complications, such as hemorrhage and perforations, were found in either group.

Two patients had severe renal failure and another two did not agree to additional surgery. Therefore, the cases that were "positive at the edge" in group B underwent additional endoscopic treatment by conventional EMR and argon plasma coagulation. None of the additional resected specimens included tumor cells. No local recurrence or lymph node or hepatic metastases were noted in either group during a mean observation period of 48.7 mo.

## DISCUSSION

The word carcinoid was first proposed by Oberndorfer in 1907<sup>[8]</sup>. Currently, carcinoids are characterized as slow-growing malignant neoplasms<sup>[9]</sup>, which are epithelial tumors composed of endocrine cells with a unique histological pattern. In Japan, carcinoids of the digestive tract are frequently seen in the colorectal region, especially in the rectum, within 10 cm from the dentate line<sup>[10]</sup>. With the advancement and mounting popularity of colonoscopy, carcinoid of the digestive tract is being discovered more frequently.

In deciding on treatment for rectal carcinoids, the presence of metastases to lymph nodes or other organs is important. If the tumor is located in the muscularis propria, the rate of lymph node metastasis increases<sup>[11]</sup>. When the tumor diameter is  $\leq 10$  mm, the lesion has most often reached as far as the submucosal layer. For lesions with a diameter  $\leq 10$  mm, the metastatic rate is

significantly lower than for those lesions with diameters  $\geq 11$  mm<sup>[12]</sup>. Therefore, for those lesions with a diameter of  $\leq 10$  mm and a depth that reaches the submucosal layer, local incision, especially endoscopic treatment, is frequently selected.

EUS is useful for measuring the diameter or depth of rectal carcinoids. Carcinoids are imaged by EUS as a low echoic region with a clear surrounding area<sup>[13]</sup>. It has been said that the tumor diameter determined by EUS rarely differs from the actual measurement<sup>[14]</sup>. The capacity of EUS to depict the intramural structure is outstanding, and its usefulness in determining tumor depth has already been widely recognized<sup>[3,13-16]</sup>. The accuracy of the pre-operative depth determination was 100% in the current study.

In endoscopic resection of rectal carcinoids, the complete resection rate for standard snare polypectomy or EMR is often low<sup>[4-6]</sup>. Therefore, various innovations have been made for complete resection. Resection using a two-channel scope<sup>[3,17]</sup>, aspiration lumpectomy<sup>[18-20]</sup>, and EMR-L have been utilized and their efficacy has been reported<sup>[7,21,22]</sup>. Ono *et al* have applied EMR-L to fourteen patients<sup>[22]</sup> and Sakata *et al*<sup>[23]</sup> to eight, and all resulted in complete resection, which testifies to the efficacy of the method.

At our institution, prior to 1998, we resected rectal carcinoids by conventional polypectomy or using a two-channel scope, and EMR-L was adopted in 1998. We had initial success in complete resection in all cases<sup>[24]</sup>, therefore, total complete resection rate has increased. But as the number of cases treated in this manner has increased, we have experienced a few cases with positive margin.

Aspiration lumpectomy can be used for resection from the deep submucosal layer<sup>[25]</sup>. By adding a ligation process with the aid of an elastic band, in theory, EMR-L should be able to reliably and safely resect the lesion and submucosal layer. Motohashi *et al* have stated that a sufficient quantity of saline injected into the submucosal layer results in safe ligation and resection<sup>[7]</sup>. Ono *et al* have reported that the vertical resection margin is greater using the EMR-L technique, however, there are still certain cases that exhibit positive margins regardless of the tumor diameter. It seems that these positive margins are due to technical factors. Moon *et al* have reported a procedure in which a snare device is left under the elastic band, so that the lesion can be ligated to include the deeper submucosal tissue and be resected more completely<sup>[26]</sup>.

We believed that the accurate injection of saline into the submucosal layer beneath the tumor is necessary to elevate it upward, achieve effective ligation of the base of the lesion, and to resect the deep submucosal layer. Therefore, 3D-EUS was utilized to assist with the procedure at the time of the saline injection. While observing radial and linear ultrasonic images in real time, we objectively confirmed the insertion of a puncture needle into the middle section of the submucosal layer beneath the tumor, injection of physiological saline, elevation of the tumor, adhesion of a high-echoic band directly beneath

the tumor, and dissociation from the muscularis propria. The lesion was constricted with a snare and resected. All the patients for whom EMR-L was combined with 3D-EUS exhibited negative margins, and compared with those without 3D-EUS, the rate of complete resection was significantly higher. This procedure tended to provide a deeper vertical resection margin.

There have been reports that confirm the dissociation of tumor from the muscularis propria by two-dimensional EUS (2D-EUS) during endoscopic treatment of submucosal tumors<sup>[27,28]</sup>. The state of rectal carcinoid following a local injection may be confirmed by 2D-EUS<sup>[16]</sup>. When 2D-EUS is employed, the scope or probe must be moved while scanning to observe the entire lesion. Thus, a local saline injection to the submucosal layer cannot be conducted simultaneously with ultrasonic observation of the lesion. By employing 3D-EUS, on the other hand, both radial and linear images may be examined in real time while the injection is being administered. In this manner, the location of the tip of the local injection needle can be instantly confirmed or corrected. Furthermore, the state of the tumor being raised following the injection can be analyzed in three dimensions, which proves the efficacy of 3D-EUS.

Onozato *et al* have reported the use of endoscopic submucosal dissection (ESD) for rectal carcinoids<sup>[29]</sup>. However, the indication criteria for endoscopic treatment for rectal carcinoids included size  $\leq 10$  mm, and there is currently no significant difference between ESD and EMR-L for small rectal carcinoids in terms of complete resection rate. In the present study, the number of patients who underwent EMR-L with and without 3D-EUS was small, and the volume of injection was not compared between the two groups. This is a limitation of our retrospective study. Further studies are required to assess the volume of injection, long-term recurrence and patient survival. Also, the difference between ESD and EMR-L combined with 3D-EUS needs to be further evaluated.

EMR-L is significantly beneficial for endoscopic resection of rectal carcinoids. With the aid of 3D-EUS during the procedure, safe and complete resection is assured.

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## COMMENTS

### Background

Recent progress in colonoscopic examination has facilitated the diagnosis of many rectal carcinoids at an early stage. Conventional snare polypectomy or EMR often yields unsatisfactory results in terms of complete resection of colonic carcinoids.

### Research frontiers

Various endoscopic treatments for colonic carcinoids were demonstrated.

### Innovations and breakthroughs

EMR-L is as effective as endoscopic treatment for rectal carcinoids. In combination with 3D-EUS, safe and complete resection is assured.

## Applications

Further studies are required to assess long-term recurrence and patient survival, and the difference between ESD and EMR-L combined with 3D-EUS needs to be further evaluated in a prospective study.

## Terminology

EMR-L is endoscopic mucosal resection with a ligation device. 3D-EUS is three-dimensional endoscopic ultrasonography.

## Peer review

This very interesting study assessed the results of EMR-L combined with 3D-EUS using an ultrasonic probe for rectal carcinoids. When comparing EMR-L with and without 3D-EUS, the volume of injection should be compared between the groups.

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RAPID COMMUNICATION

# Rebamipide enema therapy for left-sided ischemic colitis patients accompanied by ulcers: Open label study

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## Abstract

**AIM:** To attempt rectal administration of rebamipide in the treatment of ischemic colitis patients with ulcers, and evaluate its effects.

**METHODS:** We compared 9 ischemic colitis patients (2 men, 7 women) with ulcers treated by bowel rest only from 2000 to 2005 (conventional therapy group), with 6 patients (2 men, 4 women) treated by rebamipide enema therapy in 2006 (rebamipide enema therapy group) and analyzed the mean duration of fasting and hospitalization, degree of ulcer healing, and decrease in WBC count for the two groups.

**RESULTS:** The mean duration of fasting and hospitalization were  $2.7 \pm 1.8$  d and  $9.2 \pm 1.5$  d in the rebamipide group and  $7.9 \pm 4.1$  d and  $17.9 \pm 6.8$  d in the control group, respectively, and significantly reduced in the rebamipide group ( $t = -2.915$ ;  $P = 0.0121$  and  $t = -3.054$ ;  $P = 0.0092$ ). As for the degree of ulcer healing at 7 d after admission, the ulcer score was reduced by  $3.5 \pm 0.5$  (points) in the rebamipide group and  $2.8 \pm 0.5$  (points) in the control group ( $t = 1.975$ ;  $P = 0.0797$ ), while the decrease in WBC count was  $120.0 \pm 55.8 (\times 10^3/\mu\text{L})$  in the rebamipide group and  $85.9 \pm 56.8 (\times 10^3/\mu\text{L})$  in the control group ( $t = 1.006$ ;  $P = 0.3360$ ).

**CONCLUSION:** In left-sided ischemic colitis patients with ulcers, rebamipide enema therapy significantly reduced the duration of fasting and hospitalization, recommending its use as a new and effective therapeutic alternative.

## INTRODUCTION

Ischemic colitis was described in 1963 by Boley *et al*<sup>[1]</sup> as reversible occlusion of the blood supply to the colon. In 1966, Marston *et al*<sup>[2]</sup> proposed the term ischemic colitis, establishing it as a definite disease entity, and classified the condition into 3 types namely, transient, stricture, and gangrenous. Today, ischemic colitis is considered to be a common disease of the large intestine, and is thought to be caused by various factors including vascular factors such as ischemia and embolism<sup>[3-5]</sup>, and intestinal factors such as constipation<sup>[6,7]</sup>, irritable bowel syndrome<sup>[8,9]</sup>, and history of intestinal surgery<sup>[10,11]</sup>. Endoscopically, ischemic colitis is characterized by edema, erythema, erosion, and ulceration of the colonic mucosa, and pathologically the condition is characterized by diffuse hemorrhage and edema in the submucosal layer, degeneration, desquamation and necrosis of the mucosal epithelium, congestion of the lamina propria, fibrin thrombi in the capillaries, and slight neutrophilic infiltration. According to Reeders *et al* ischemic colitis involves the left colon in 75% of the cases and the right colon in 8%<sup>[12]</sup>.

While surgery is indicated for treatment of the gangrenous type of ischemic colitis, many patients with the transient or stricture types of the disease improve with bowel rest by fasting and parenteral fluid administration alone. However, healing is frequently delayed in patients with ulcerative lesions. We previously reported that ischemic colitis patients with ulcerative lesions require a significantly longer fasting period and

duration of hospitalization as compared with those without ulcerative lesions<sup>[13]</sup>. A longer fasting period and longer duration of hospitalization pose problems, including stress associated with fasting and a high cost of long-term hospitalization.

Rebamipide is an anti-ulcer drug launched in various Asian countries, that has been reported to have ulcer healing effects<sup>[14,15]</sup>, anti-inflammatory effects<sup>[15]</sup>, suppressant effects against free radical production<sup>[16-19]</sup> and mucin secretion-inducing effects<sup>[20,21]</sup>. Recently, the efficacy of rebamipide enema in the treatment of ulcerative colitis has been reported in Japan<sup>[22-25]</sup>.

In this retrospective cohort study, we evaluated the therapeutic effects of rebamipide solution administered as an enema in ischemic colitis patients with ulcerative lesions, with special attention paid to the fasting period and duration of hospitalization.

## MATERIALS AND METHODS

Fifteen ischemic colitis patients (4 men, 11 women; mean age, 68 years) with ulcerative lesions who were admitted to our hospital during the 7 years between 2000 and 2006 were investigated in a non-randomized study. Nine ischemic colitis patients (2 men, 7 women; mean age, 68 years) with ulcerative lesions were treated by fasting and parenteral fluid administration during the 6 years between 2000 and 2005 (conventional therapy group). Six ischemic colitis patients (2 men, 4 women; mean age, 69 years) with ulcerative lesions were assigned to rebamipide enema therapy in addition to conventional therapy in 2006 (rebamipide enema therapy group). Patients who were admitted for other diseases but developed ischemic colitis during the course of those diseases were excluded. The diagnosis of ischemic colitis was made based on colonoscopic and histopathologic findings in biopsy specimens combined with the following three essential criteria: absence of prior antibiotics, negative cultures of feces or biopsy specimens for bacteria, and absence of history of inflammatory bowel disease (IBD). The age at onset, mode of onset, symptoms, and the location of the lesions were used as supplemental information.

Conventional therapy and rebamipide enema therapy groups were compared with respect to age, gender, location of lesions, hematological and blood chemistry findings, performance status (0-4 based on the WHO performance scale: 0, asymptomatic; 1, symptomatic but completely ambulant; 2, symptomatic, up and about > 50% of waking hours; 3, symptomatic, confined to bed or chair > 50% of waking hours, but not bed- or chair-bound; 4, bed- or chair-bound), prevalence of underlying diseases (hypertension, hyperlipidemia, diabetes mellitus, chronic atrial fibrillation, cerebral infarction, constipation, history of abdominal surgery), oral medication, mean fasting period, mean duration of hospitalization, degree of ulcer healing, and decrease in WBC count. The breakdown of the oral medication was: antihypertensive agent 5 cases, digitalis 1 case in the conventional therapy group, and antihypertensive agent 1 case, diuretic 1 case

**Table 1 Stage classification of gastric ulcer by Sakita-Miwa and its translation into numerical expression<sup>[26]</sup>**

Stages	Manifestation
Active stage	A1 (6p) The surrounding mucosa is edematosly swollen and no regenerating epithelium is seen endoscopically
	A2 (5p) The surrounding edema has decreased, the ulcer margin is clear, and a slight amount of regenerating epithelium is seen in the ulcer margin. A red halo in the marginal zone and a white slough circle in the ulcer margin are frequently seen. Usually, converging mucosal folds can be followed right up to the ulcer margin
Healing stage	H1 (4p) The white coating is becoming thin and the regenerating epithelium is extending into the ulcer base. The gradient between the ulcer margin and the ulcer floor is becoming flat. The ulcer crater is still evident and the margin of the ulcer is sharp. The diameter of the mucosal defect is about one-half to two-thirds that of A1
	H2 (3p) The defect is smaller than in H1 and the regenerating epithelium covers most of the ulcer floor. The area of white coating is about a quarter to one-third that of A1
Scarring stage	S1 (2p) The regenerating epithelium completely covers the floor of ulcer. The white coating has disappeared. Initially, the regenerating region is markedly red. Upon close observation, many capillaries can be seen. This is called "red scar"
	S2 (1p) In several months to a few years, the redness is reduced to the color of the surrounding mucosa. This is called "white scar"

in the rebamipide enema therapy group. For evaluation of the degree of ulcer healing, the ulcerative lesions were classified into stages according to the Sakita-Miwa classification of gastric ulcers<sup>[26]</sup> (Table 1), then the stage was converted into numerical scores as follows: A<sub>1</sub>: 6 points, A<sub>2</sub>: 5 points, H<sub>1</sub>: 4 points, H<sub>2</sub>: 3 points, S<sub>1</sub>: 2 points, S<sub>2</sub>: 1 point. The difference between the score at baseline (time of diagnosis) and the score at the follow-up examination was calculated and divided by the number of days between the baseline and the follow-up examinations for evaluation of the therapeutic effects. The result was corrected to the value at 7 d from the baseline and used for the analysis. To further strengthen the objectivity of the evaluation, the decrease in WBC count was calculated by subtracting the WBC count at the follow-up examination from that at the baseline, and divided by the number of days between the baseline and follow-up examinations. The value obtained was corrected to the value at 7 d from the baseline for use in the analysis.

Colonoscopy was performed in all the patients within 3 d of admission or within 5 d of onset of the symptoms. Colonoscopic follow up was conducted in a total of 11 patients, including all the patients in the rebamipide group. Treatment included bowel rest by fasting and parenteral fluid administration in all the patients, oral antiflatulent agents in 4 patients (23.5%), and administration of antibiotics in 4 patients (23.5%); none of the patients needed intravenous hyperalimentation. Treat-

**Table 2 Clinical features of the ischemic colitis patients: Comparison between the rebamipide and conventional therapy groups**

	Conventional group (n = 9)	Rebamipide group (n = 6)	Statistical significance
Age (mean, range, yr)	68 ± 19 (35-87)	69 ± 16 (41-85)	NS
Percentage of females (%)	77.8	66.7	NS
Lesion location: left colon (%)	88.9	100	NS
Performance status 0-1 (%)	77.8	100	NS
WBC count ( $\times 10^3/\mu\text{L}$ )	12.3 ± 4.7	13.5 ± 5.3	NS
CRP (mg/dL)	2.6 ± 4.1	1.7 ± 1.8	NS
K (mEq/L)	3.8 ± 0.4	4.2 ± 0.5	NS
Total cholesterol (mg/dL)	179.3 ± 30.6	191.2 ± 63.0	NS
TG (mg/dL)	93.3 ± 41.0	131.2 ± 74.2	NS
LDH (IU/L)	190.0 ± 42.8	202.8 ± 18.2	NS
CPK (IU/L)	80.7 ± 38.9	118.0 ± 76.6	NS

Values are the mean ± SD. The percentage of females and the lesion location were evaluated by the Fisher's exact test. Other data were evaluated by Student's *t*-test after skewing the values by log transformation. WBC: White blood cell; CRP: C-reactive protein; K: Potassium; TG: Triglyceride; LDH: Lactate dehydrogenase; CPK: Creatinine phosphokinase.

ment was provided in all patients within 5 d of onset of the symptoms. Oral food intake was allowed when the abdominal pain resolved. If no symptomatic recurrence was noted after the start of oral food intake, the patient was discharged.

For rebamipide enema therapy, 150 mg of rebamipide was suspended in 60 mL of warm water and administered through Nelaton's catheter once daily. The subjects remained in the left lateral decubitus position for 30 min. The period of administration varied depending on individual patient needs. The mean duration of treatment was 7 ± 1.6 d in the rebamipide enema therapy group. This study was allowed by the institutional ethical committee. Prior to the administration of rebamipide enema, all the patients were provided with a thorough explanation of the treatment, and informed consent was obtained from each of them.

### Statistical analysis

Data are expressed as mean ± SD or percentage. The percentage of females and the distribution of the lesion location, performance status, and underlying systemic diseases in the two groups were evaluated by the Fisher's exact test. Other data were evaluated by Student's *t*-test after log-transformation of skewed variables. All data analyses were performed with the StatView 5.0. Statistical significance was set at  $P < 0.05$ .

## RESULTS

Fifteen patients (4 men, 11 women; mean age, 68 years) were eligible for the analyses. The location of the lesions with allowance given for partial overlapping of the lesions, was the sigmoid colon in 66.7%, descending colon in 66.7%, transverse colon in 20.0%, and the rectum or ascending colon in 0% of the patients. In relation to the number of episodes of ischemic colitis experienced by

**Table 3 Underlying disease: Comparison between the rebamipide and conventional therapy groups n (%)**

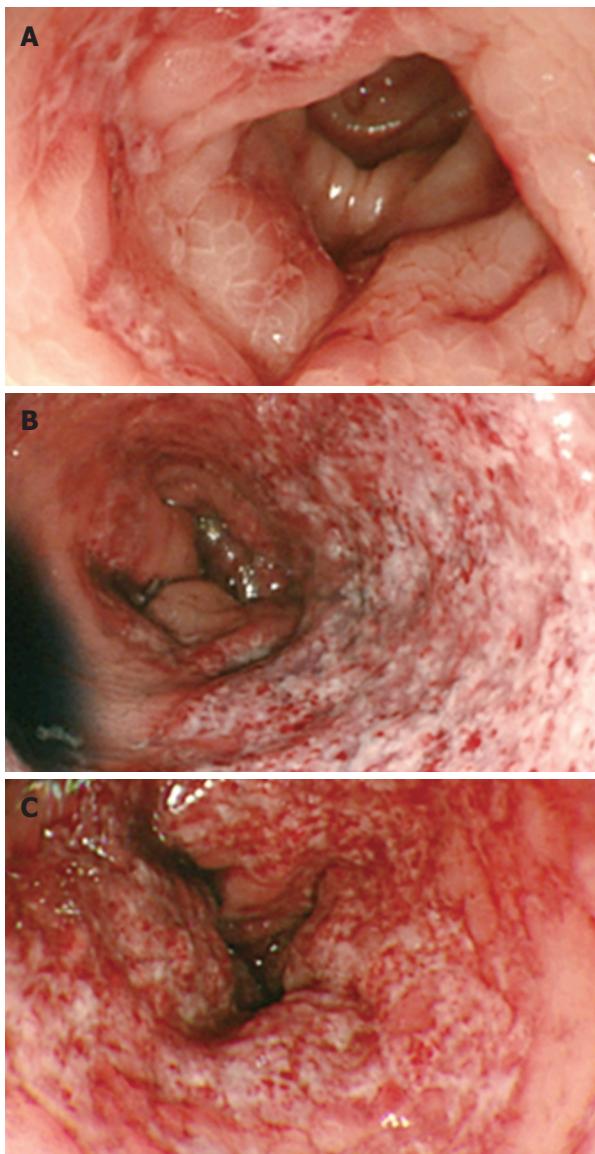
	Conventional group (n = 9)	Rebamipide group (n = 6)	Statistical significance
Hypertension	6 (66.7)	2 (33.3)	NS
Hyperlipidemia	0	3 (50.0)	NS
Diabetes mellitus	1 (11.1)	1 (16.7)	NS
Atrial fibrillation	1 (11.1)	0	NS
Cerebral infarction	1 (11.1)	0	NS
Chronic constipation	5 (55.6)	1 (16.7)	NS
Medications			
Antihypertensive agents	5 (55.6)	1 (16.7)	NS
Others	1 (11.1)	1 (16.7)	NS
History of abdominal surgery	4 (44.4)	3 (50.0)	NS

Values are expressed in percentages. Percentages were evaluated by the Fisher's exact test.

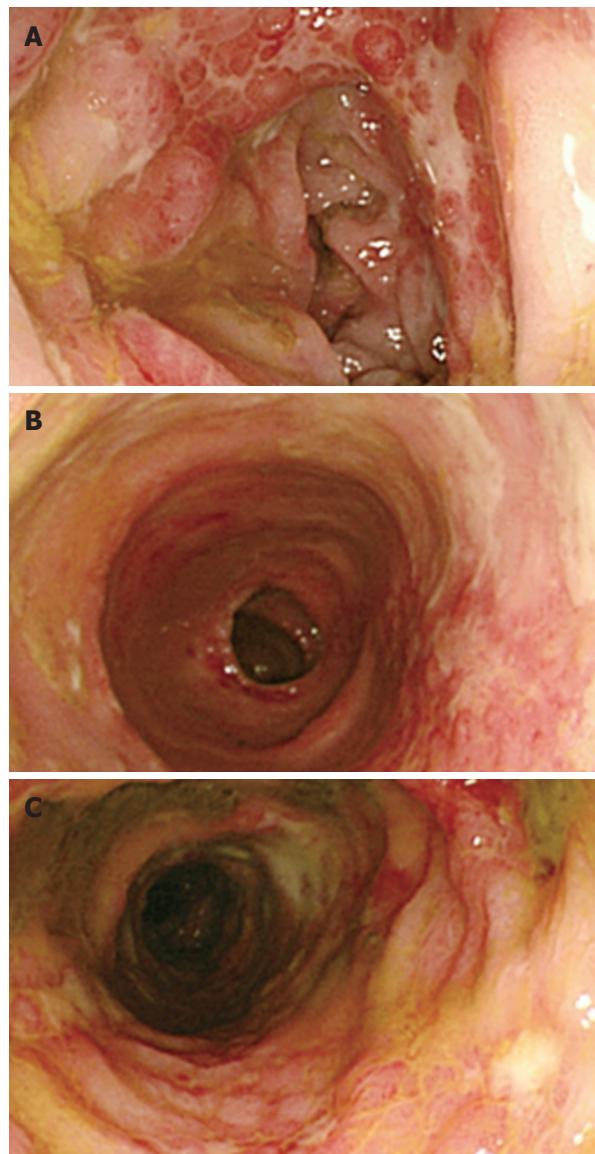
the patients, 93.3% of the patients had suffered from one episode and 6.7% had suffered from two episodes.

The rebamipide enema therapy group consisted of 6 patients (2 men, 4 women), with an average age of 69 years (41-85), and the lesion was located in the left colon in all the patients. The conventional therapy group consisted of 9 patients (2 men, 7 women), with an average age of 68 years (35-87), and the lesion was located in the left colon in 8 patients. There were no statistically significant differences in any of the background factors between the groups, including the prevalence of hypertension, hyperlipidemia, diabetes mellitus, atrial fibrillation, cerebral infarction and chronic constipation, history of abdominal surgery, oral medication, performance status, and hematological and blood chemistry findings (Tables 2 and 3).

The mean fasting period and mean duration of hospitalization were 2.7 ± 1.8 d and 9.2 ± 1.5 d respectively, in the rebamipide enema therapy group, as compared with 7.9 ± 4.1 d and 17.9 ± 6.8 d respectively, in the conventional therapy group. Both the fasting period ( $P = 0.0121$ ) and the duration of hospitalization ( $P = 0.0092$ ) were significantly shorter in the rebamipide enema therapy group (Table 4). As for the degree of ulcer healing, the ulcer score was reduced by 3.5 ± 0.5 (points) in the rebamipide enema therapy group and 2.8 ± 0.5 (points) in the conventional therapy group ( $P = 0.0797$ ), with the interval between the baseline and follow-up examinations being 6.5 ± 0.8 d and 7.7 ± 2.7 d respectively ( $P = 0.3293$ ). The WBC count was decreased by 12.0 ± 5.6 ( $\times 10^3/\mu\text{L}$ ) in the rebamipide enema therapy group and 8.6 ± 5.7 ( $\times 10^3/\mu\text{L}$ ) in the conventional therapy group ( $P = 0.3360$ ), with the interval between the baseline and follow-up examinations of 5.8 ± 1.7 d and 5.4 ± 1.5 d, respectively ( $P = 0.7515$ ). Thus, both the degree of ulcer healing and the decrease in WBC count tended to be favorable in the rebamipide enema therapy group compared to the conventional therapy group, but there was no significant difference in either parameter between the two groups (Table 4). No adverse events were noted in any of the patients.



**Figure 1** Colonoscopic findings at the time of diagnosis of ischemic colitis. **A:** Transverse colon; **B:** Descending colon; **C:** Sigmoid colon.



**Figure 2** Colonoscopic findings at the time of evaluation at 1 wk after the start of rebamipide enema therapy. **A:** Transverse colon; **B:** Descending colon; **C:** Sigmoid colon.

**Table 4 Duration of fasting, duration of hospitalization and reduction in the ulcer score: Comparison between the rebamipide and conventional therapy groups**

	Conventional group (n = 9)	Rebamipide group (n = 6)	Statistical significance
Duration of fasting (d)	7.9 ± 4.1	2.7 ± 1.8	P = 0.0121
Duration of hospitalization (d)	17.9 ± 6.8	9.2 ± 1.5	P = 0.0092
Degree of ulcer healing <sup>1</sup> (points) (n = 5)	2.8 ± 0.5	3.5 ± 0.5	P = 0.0797
WBC decrease <sup>2</sup> (× 10 <sup>3</sup> /μL)	8.6 ± 5.7	12.0 ± 5.6	P = 0.3360

Values are the mean ± SD. <sup>1</sup>The ulcer lesion was scored according to the Sakita-Miwa classification of the stage of gastric ulcer, then the difference between the baseline score (at the time of diagnosis) and at the time of the follow-up colonoscopy was divided by the number of days between the baseline and the follow-up examination, to correct the results to the value on d 7 (A1: 6 points, A2: 5 points, H1: 4 points, H2: 3 points, S1: 2 points, S2: 1 point). <sup>2</sup>Difference in value between that measured at admission and that measured at the time of the follow-up was divided by the number of days between the two examinations to correct the results to the value on d 7.

In a representative case of a 41-year-old man with ischemic colitis with ulcerative lesions located in the transverse colon, descending colon and sigmoid colon, who was treated by rebamipide enema for 7 d, the fasting period was 6 d and the duration of hospitalization was 10 d (Figures 1 and 2).

## DISCUSSION

With the aging of population in Japan and around the world, atherosclerosis and malignancy are two of the major diseases facing our nation. Epidemiological data in Japan reveal that malignant neoplasms rank first as the cause of death, while heart diseases and cerebrovascular diseases together account for about 30% of all deaths, which represents the percentage of deaths caused by malignant neoplasms. Since the prevalence of atherosclerotic diseases may be expected to increase further with the accelerated aging of populations, an increase in the incidence of ischemic colitis is also

inevitable. In fact, we have documented an annual increase in the prevalence of ischemic colitis in our care-providing area.

We previously reported that the fasting period and the duration of hospitalization were significantly prolonged in ischemic colitis patients with ulcerative lesions<sup>[13]</sup>, and sought suitable treatment methods to shorten these parameters. In this study, we evaluated the effects of rebamipide enema therapy in these patients. Rebamipide is an anti-ulcer drug used to treat peptic ulcer, that has been demonstrated to enhance the production of endogenous prostaglandin E2 via COX-2 and thereby act as a gastric mucosal protectant<sup>[27-29]</sup>, to promote mucin production<sup>[20,21]</sup>, and to suppress the production of free radicals, such as hydroxyl radicals and superoxide ions<sup>[16-19]</sup>. Farhadi *et al* reported that rebamipide suppressed the production of reactive oxygen species by neutrophils in the presence of plasma and rectal perfusate in patients with ulcerative colitis<sup>[30]</sup>. It has been reported that in ulcerative colitis, excessive oxygen and free radicals produced by activated neutrophils and macrophages intensify the microcirculatory disorder and lipid peroxidation to aggravate the intestinal mucosal disorder; thus they play important roles in the mechanism of development of inflammation in this disease. Makiyama *et al* reported that the use of rebamipide enema therapy in addition to conventional therapy in patients with ulcerative colitis yielded favorable responses, and classified this therapy as a promising new strategy for the treatment of moderate or less than severe distal colitis or as a supplemental therapy during steroid withdrawal<sup>[22,23]</sup>. Furuta *et al* and Miyata *et al* likewise reported that rebamipide enema therapy was effective in patients with ulcerative colitis<sup>[24,25]</sup>. Oral administration of rebamipide at a dose of 300 mg daily has also been reported to result in remarkable improvement in patients with multiple small intestinal erosions<sup>[31]</sup>.

Limited studies have discussed the mechanism of development of ischemic colitis. Ichihara *et al* produced an experimental model of ischemic colitis in rats, and reported that the development of lesions was clearly suppressed when the superoxide scavenger, liposomal-encapsulated superoxide dismutase (L-SOD), was administered. Based on this they suggested that the production of superoxide anions may be closely involved in the pathogenesis of ischemic colitis<sup>[32]</sup>. In hydrogen peroxide-induced hemorrhagic lesions of the gastric mucosa in the rat, rebamipide suppressed inactivation of SOD activity in a dose-dependent manner<sup>[33]</sup>, suggesting that rebamipide might contribute to healing in ischemic colitis by suppressing the production of free radicals.

This study is an open label study by the non-randomization, and the small study population may be a weak point. In this study, the degree of ulcer healing and decrease in WBC count were used as objective indicators in the evaluation of healing. No significant difference in either parameter was observed between the treatment and previously untreated groups. However, the results did suggest that the healing of the lesions was accelerated to a greater extent in the rebamipide enema therapy

group compared to the conventional therapy group.

The mechanism underlying the efficacy of rebamipide in the treatment of ischemic colitis remains unclear; however, it is assumed that the effect of the drug in suppressing free radical production and accelerating ulcer healing account for its efficacy in patients of ischemic colitis with ulcerative lesions.

Figures 1 and 2 show colonoscopic photographs of the large intestine in a patient who responded to rebamipide enema therapy. In this patient, skip ulcer lesions were present extending from the transverse to the sigmoid colon. Comparison of colonoscopic images of the large intestine obtained at the time of diagnosis (Figure 1) with those obtained at the time of follow-up 7 d after the initiation of rebamipide enema therapy (Figure 2) clearly revealed a more pronounced healing tendency toward the anal end. In regard to the effect of 5-ASA enema therapy and the site of the colon reached by the drug given as an enema in patients with ulcerative colitis, Bodegraven *et al*<sup>[34]</sup> and Otten *et al*<sup>[35]</sup> reported that following administration of 60 mL and 50 mL of 5-ASA, respectively, the drug reached between the sigmoid colon and the descending colon. The present results of rebamipide enema therapy are considered to be related to its effect of accelerating ulcer healing within the range of the colonic segments reached by the drug. As for the dosage of rebamipide, the dose was set at 150 mg in this study, although a dose finding study may be necessary in the future.

In conclusion, rebamipide enema therapy significantly shortened the fasting period and the duration of hospitalization in left-sided ischemic colitis patients with ulcerative lesions. Based on these findings, rebamipide enema therapy appears to be a promising new therapy in ischemic colitis patients with ulcerative lesions.

## COMMENTS

### Background

Authors have recently reported that the duration of fasting and hospitalization were significantly prolonged in ischemic colitis patients with ulcers.

### Research frontiers

In this study, the authors attempted rectal administration of rebamipide for the treatment of ischemic colitis patients with ulcers, and evaluated its effects, including on the duration of fasting and hospitalization.

### Applications

Rebamipide enema therapy shortened the fasting and hospitalization period in left-sided ischemic colitis patients with ulcerative lesions. We recommend rebamipide enema therapy in left-sided ischemic colitis patients with ulcerative lesions.

### Peer review

This is a retrospective study involving a small number of patients with left-sided ischemic colitis with ulcer, collected over a period of 5-6 years, compared with rebamipide therapy. The study shows promising results. It's an interesting paper.

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RAPID COMMUNICATION

# Ultrasonically activated scalpel *versus* monopolar electrocautery shovel in laparoscopic total mesorectal excision for rectal cancer

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drainage volume and VAS in postoperative d 1 ( $P = 0.431$ ,  $P = 0.426$ ) and d 3 ( $P = 0.844$ ,  $P = 0.617$ ) between ES group and US group. The occurrence of surgery-related complications such as anastomotic leakage and wound infection was the same in the two groups.

**CONCLUSION:** ES is a safe and feasible tool as same as US used in laparoscopic TME with anal sphincter preservation for rectal cancer on the basis of the skillful laparoscopic technique and the complete understanding of laparoscopic pelvic anatomy. Application of ES can not only reduce the operation costs but also benefit the popularization of laparoscopic operation for rectal cancer patients.

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**Key words:** Laparoscopy; Ultrasonically activated scalpel; Monopolar electrocautery; Rectal cancer; Total mesorectal excision

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Zhou BJ, Song WQ, Yan QH, Cai JH, Wang FA, Liu J, Zhang GJ, Duan GQ, Zhang ZX. Ultrasonically activated scalpel *versus* monopolar electrocautery shovel in laparoscopic total mesorectal excision for rectal cancer. *World J Gastroenterol* 2008; 14(25): 4065-4069 Available from: URL: <http://www.wjgnet.com/1007-9327/14/4065.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4065>

## Abstract

**AIM:** To investigate the feasibility and safety of monopolar electrocautery shovel (ES) in laparoscopic total mesorectal excision (TME) with anal sphincter preservation for rectal cancer in order to reduce the cost of the laparoscopic operation, and to compare ES with the ultrasonically activated scalpel (US).

**METHODS:** Forty patients with rectal cancer, who underwent laparoscopic TME with anal sphincter preservation from June 2005 to June 2007, were randomly divided into ultrasonic scalpel group and monopolar ES group, prospectively. White blood cells (WBC) were measured before and after operation, operative time, blood loss, pelvic volume of drainage, time of anal exhaust, visual analogue scales (VAS) and surgery-related complications were recorded.

**RESULTS:** All the operations were successful; no one was converted to open procedure. No significant differences were observed in terms of preoperative and postoperative d 1 and d 3 WBC counts ( $P = 0.493$ ,  $P = 0.375$ ,  $P = 0.559$ ), operation time ( $P = 0.235$ ), blood loss ( $P = 0.296$ ), anal exhaust time ( $P = 0.431$ ), pelvic

## INTRODUCTION

Since the successful introduction of laparoscopic colectomy by Jacobs *et al*<sup>[1]</sup>, laparoscopic surgery, especially laparoscopic rectal surgery has been developed considerably<sup>[2-6]</sup>. Laparoscopic total mesorectal excision (TME) for rectal cancer whether hand-assisted, laparoscopy-assisted or robotic-assisted laparoscopic technique can offer advantages over open TME,

such as greater comfort and an earlier return to daily activities while preserving the oncologic radicality of the procedure<sup>[7-10]</sup>. Moreover, the laparoscopy allows good exposure of the pelvic cavity because of magnification and good illumination. The laparoscope seems to facilitate pelvic dissection including identification and preservation of critical structures such as the autonomic nervous system<sup>[11-12]</sup>. Laparoscopic TME for rectal cancer is feasible and safe; the short-, mid- and long-term outcomes of the operation are favorable compared with those of conventional open surgery<sup>[13-17]</sup>. However, the laparoscopic TME for rectal cancer is complicated and has some technical difficulties during operation; surgeons had to invent some methods to resolve the problem<sup>[18-22]</sup>. As ultrasonically activated scalpel (US) is able to grasp and divide tissues while sealing small vessels, the laparoscopic operation has become simpler, but the expensive medical instrument and high costs of the disposable materials can greatly increase the cost of laparoscopic TME for rectal cancer<sup>[23-25]</sup>. In order to reduce the cost of the operation, we tried to use monopolar electrocautery shovel (ES) in laparoscopic TME with anal sphincter preservation for rectal cancer, and compared it with US.

## MATERIALS AND METHODS

### **Patients**

Forty-two rectal cancer patients who were treated between June 2005 and June 2007 were chosen prospectively. The inclusion criteria are: patients with confirmed diagnosis of rectal cancer, non-emergency surgery, the tumor margin from the anal margin being more than 5 cm, no preoperative examination of liver, and other distant organ metastasis, and suitable for sphincter-saving surgery. Removal criteria are: patients whose tumor involved the bladder, uterus or pelvic metastasis, and not suitable for radical surgery. One patient in each group was excluded according to the removal criteria. The patients were randomly divided into laparoscopic US group and laparoscopic ES group.

### **Surgical techniques**

The two groups of patients used the same method for preoperative preparation. All the procedures were performed by the same operation team. Each of them conformed to the radical treatment principles including en bloc resection, no-touch isolation technique, proximal lymph-vascular ligation, complete lymphadenectomy, wound protection, and adequate resected margin of the rectum and TME for rectal cancer.

### **Study parameters**

The following parameters were measured prospectively in the two groups: white blood cells (WBC) in the peripheral blood before and after operation, the operative time, blood loss, postoperative pelvic drainage volume and the time of anal exhaust. The pain degree of patients after operation was assessed by visual analogue scales (VAS), "0" represents painless, and the "10" represents the most intense pain<sup>[26]</sup>.

**Table 1 Demographic data of ES and US groups (*n* = 20, %)**

Parameters	ES group	US group	$\chi^2$ or <i>t</i>	P
Mean age (yr)	59.2 ± 12.6	58.8 ± 14.9	0.080	0.937
Gender				
Male	14 (70)	11 (55)		
Female	6 (30)	9 (45)	0.960	0.327
Distance from tumor to anal margin				
5-10 cm	13 (65)	12 (60)		
> 10 cm	7 (35)	8 (40)	0.107	0.744
Dukes' stage				
A, B	7 (35)	9 (45)		
C	13 (65)	11 (55)	0.417	0.519

### **Statistical analysis**

All the statistical analyses were performed using SPSS 11.5 software package. The data were expressed as mean ± SD. Student's *t* test was used to analyze quantitative variables and  $\chi^2$  test was used to analyze qualitative variables. *P* < 0.05 was considered statistically significant.

## RESULTS

### **Comparison of demographic data**

The demographic data of the two groups are shown in Table 1. There was no significant difference in age, gender, tumor location of rectum and Duke's staging.

### **Comparison of surgical safety and postoperative recovery between ES group and US group**

Comparison of the WBC counts before operation between ES group and US group showed no significant differences. The WBC counts of the two groups in postoperative d 1 and d 3 were higher than that of preoperation, but without significant difference. There was no significant difference between ES group and US group in operation time, blood loss, the anal exhaust time, the pelvic drainage volume, postoperative d 1 and d 3 VAS and postoperative complications such as anastomotic fistula and the occurrence of wound infection (Table 2).

## DISCUSSION

The electric power can be converted to mechanical energy by ultrasound frequency generator in US, which can generate 55.5 kHz mechanical oscillation. By the oscillation, the tissues can be cut and coagulated and vascular closure can be made. US can precisely cut and stop bleeding and produce less heat, thus not damaging the surrounding tissues because of the small thermal spread. When US produces less smog and less eschar, the operative field become more clearly. But US also has obvious drawbacks: US instrument is expensive, needs disposable material; and significantly increases costs of the laparoscopic operation<sup>[23,27,28]</sup>. In clinical practice, we found that the laparoscopic rectal cancer operation can almost be conducted at the same anatomical space of rectum; monopolar electrocautery can also be applied in

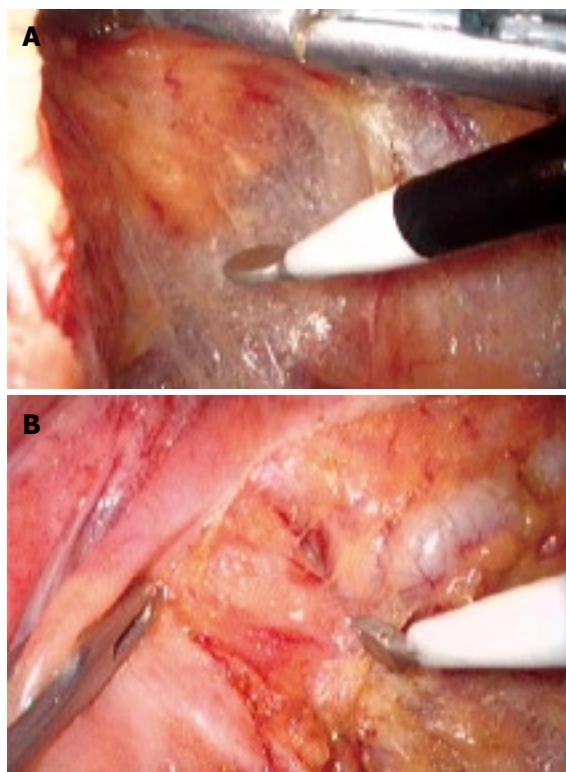
**Table 2 Comparison of surgical safety and postoperative recovery (*n*= 20, mean  $\pm$  SD)**

Parameters	ES group	US group	$\chi^2$ or <i>t</i>	<i>P</i>
WBC counts ( $\times 10^9/L$ )				
Preoperative	5.37 $\pm$ 0.84	5.55 $\pm$ 0.76	0.693	0.493
Postoperative d1	12.77 $\pm$ 2.32	12.17 $\pm$ 1.89	0.898	0.375
Postoperative d3	7.93 $\pm$ 2.15	7.57 $\pm$ 1.66	0.590	0.559
Operation time (min)	184.5 $\pm$ 28.3	173.7 $\pm$ 28.5	1.206	0.235
Blood loss (mL)	60.8 $\pm$ 41.8	48.9 $\pm$ 28.3	1.069	0.296
Anal exhaust time (h)	33.7 $\pm$ 5.9	31.8 $\pm$ 6.8	0.963	0.431
Pelvic drainage volume (mL)				
Postoperative d1	90.5 $\pm$ 27.1	81.9 $\pm$ 39.7	0.796	0.431
Postoperative d3	5.4 $\pm$ 4.6	5.7 $\pm$ 4.9	0.198	0.844
VAS				
Postoperative d1	5.54 $\pm$ 1.37	5.21 $\pm$ 1.17	0.805	0.426
Postoperative d3	2.44 $\pm$ 1.04	2.27 $\pm$ 1.10	0.504	0.617
Anastomotic leakage ( <i>n</i> )	1	1	0.00	1.00
Wound infection ( <i>n</i> )	1	1	0.00	1.00

laparoscopic TME with anal sphincter preservation for rectal cancer because there is no large blood vessel in the space around the rectum.

The electric current through the tissue can produce high temperature from 100°C to 200°C because the resistance of tissues, the tissue degeneration, necrosis, drying, evaporation, carbonation, eschar, monopolar electrocautery can cut or stop bleeding<sup>[29,30]</sup>. The laparoscopic surgery, according to different operations, can choose different shaped monopolar electrocautery components such as monopolar electrocautery knife, monopolar electrocautery hook, monopolar electrocautery scissors and monopolar ES. ES is particularly suitable for laparoscopic TME with anal sphincter preservation for rectal cancer. First of all, the metal tip of ES only exposes a small area, it has a complete laparoscopic vision so as to avoid injuring the tissues outside the vision during operation in the narrow pelvic space, therefore, the laparoscopic operation has become more secure. Secondly, ES with blunt tip and a flat disk shape, can be used for blunt dissection without electricity supply and sharp dissection with electricity supply, avoiding frequent exchange of surgical instruments through trocars, so it is very suitable for dissection in the space around the rectum. Finally, the relatively sharp edge of ES can be used for sharp dissection, the disk of ES can oppress the bleeding point and achieve electrocoagulation when the tissue was bleeding, it has a good hemostatic effect. During the laparoscopic operation, ES should always use electrocoagulation in order to reduce the smog and extravasate from space wound. This study showed no significant difference in the blood loss, the pelvic drainage volume, the time of anal exhaust flow with defecation, the VAS and WBC counts between the ES group and the US group, indicating that the local and systemic impact of the body due to ES had no significant difference compared with US in laparoscopic TME with anal sphincter preservation for rectal cancer.

ES can produce a higher surface temperature when managing the tissues; the theoretical distance of thermal conduction is longer than that of US, which makes it



**Figure 1** Intraoperative photograph. **A:** Showing monopolar ES dissect the space around the rectum; **B:** Showing monopolar ES dissect the seminal vesicle and the rectum.

easy to damage the surrounding tissues<sup>[31]</sup>. Therefore, it is particularly important for surgeons to understand how to use ES in laparoscopic TME with anal sphincter preservation for rectal cancer. The correct method is to maintain a certain tension on both sides of the separation space; let the ES slightly contact the tissue with its sharp edge, and then gently slide along the surface of tissue in order to form a large space (Figure 1A). The tissue was cut immediately into slide aside to expose deep layer tissue and the important organs in the deep layer tissue can easily be uncovered and avoid injury, thus reducing the possibility of the surrounding tissue necrosis due to “heat chemotactic effect”. At the important anatomical position, the operative speed must be slow, use “mm-class” dissection and sharp dissection in combination with blunt dissection in order to reduce bleeding and avoid injury of major blood vessels and organs (Figure 1B). There was no significant difference in postoperative complications such as anastomotic leakage and wound infection between ES group and US group in our study, indicating that ES is as safe as US in laparoscopic TME with anal sphincter preservation for rectal cancer. The operative time was almost the same between the two groups, although the cutting speed of ES was faster than that of US. On the other hand, the intraperitoneal CO<sub>2</sub> must be exchanged regularly due to heavy smog generated by ES in order to maintain the clarity of operative vision, although it might slow the operative speed of ES in a certain extent. During operation, it must rely on the movement of metacarpophalangeal joint opening and closing repeatedly in order to control US. But in ES

operation, surgeons might feel more comfortable as they can grasp ES, and rely on the push-pull movement of the arm, and the hand movement intensity might significantly decrease. Compared with US, ES has prominent advantages, including sturdiness, durability, low cost, and being suitable for laparoscopic TME with anal sphincter preservation for rectal cancer in less developed settings.

In conclusion, ES is a safe and feasible tool similar to US used in laparoscopic TME with anal sphincter preservation for rectal cancer on the basis of skillful laparoscopic technique and complete understanding of laparoscopic pelvic anatomy. Application of ES can not only reduce the operation costs but also benefit the popularization of laparoscopic operation for rectal cancer patients.

## COMMENTS

### Background

Since the successful introduction of laparoscopic colectomy by Jacobs et al, laparoscopic surgery, especially laparoscopic rectal surgery has been developed considerably. Compared with open operation, the laparoscopic operation has many advantages such as less pain, little blood loss, small incision, good exposure of the pelvic cavity, an earlier return to daily activities, etc. while preserving the oncologic radicality of the procedure. However, the laparoscopic operation is more difficult than the open operation. Ultrasonically activated scalpel (US) is able to grasp and divide tissues while sealing small vessels, making the laparoscopic operation simpler, whereas the expensive medical instrument and high costs of the disposable materials can greatly increase the cost of laparoscopic rectal cancer operation.

### Research frontiers

It has been shown that the laparoscopic rectal cancer operation had the same short-term and long-term outcomes compared with open surgery, so how to overcome the operation difficulty and reduce the cost of laparoscopic rectal operation has become hotspots in laparoscopic surgery.

### Innovations and breakthroughs

In this study, authors tried to use cheap and durable monopolar electrocautery shovel (ES) in laparoscopic operation for rectal cancer in order to reduce the cost of the laparoscopic operation, they also summarized the technique and skill of using monopolar ES, and compared with US.

### Applications

ES is a safe and feasible tool similar to US used in laparoscopic TME for rectal cancer on the basis of skillful laparoscopic technique and complete understanding of laparoscopic pelvic anatomy. Application of ES can not only reduce the operation costs but also facilitate the popularization of laparoscopic operation for rectal cancer.

### Terminology

US is an expensive medical instrument which can incise tissue and seal small vessels, it is often used in complicated laparoscopic operations, but it costs high for the disposable materials. So in laparoscopic operation, US can greatly increase the cost of operation. ES is often used in simple laparoscopic operations because it is not as good as US for hemostasis, but it is cheap and durable.

### Peer review

This is an interesting manuscript looking into two different methods of dissecting the rectum laparoscopically. It is a good paper.

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RAPID COMMUNICATION

## Microsatellite alterations in phenotypically normal esophageal squamous epithelium and metaplasia-dysplasia-adenocarcinoma sequence

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**Author contributions:** Cai JC designed the research; Cai JC, Liu D, Liu KH, and Zhong S performed the research; Cai JC, Zhang HP and Xia NS analyzed the data; Cai JC and Liu D wrote the paper.

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reactions (PCR) were performed, and microsatellite analysis was carried out.

**RESULTS:** No statistically significant difference was found in microsatellite instability (MSI) and loss of heterozygosity (LOH) of un-diluted DNA between SCC and ADC. The levels of MSI and LOH were high in the metaplasia-dysplasia-adenocarcinoma sequence of diluted DNA. The more the diluted DNA was, the higher the rates of MSI and LOH were at the above 7 loci, especially at D3S1616, D5S346, D2S123, D3S1300 and D18S58 loci.

**CONCLUSION:** The sequence of metaplasia-dysplasia-adenocarcinoma is associated with microsatellite alterations, including MSI and LOH. The MSI and LOH may be the early genetic events during esophageal carcinogenesis, and genetic alterations at the D3S1616, D5S346 and D3S123 loci may play a role in the progress of microsatellite alterations.

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**Key words:** Microsatellite alteration; Dilution PCR; Metaplasia-dysplasia-adenocarcinoma sequence; Esophageal squamous epithelium; Squamous cell carcinoma

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## INTRODUCTION

Barrett esophagus, thought to be primarily due to uncontrolled gastric acid and biliopancreatic reflux into the esophagus, is a precancerous condition<sup>[1,2]</sup>. It is estimated that the risk of neoplasia in Barrett esophagus, through the intermediate step of dysplastic

transformation of the columnar epithelium, is 125-fold higher than that in general population<sup>[1]</sup>. There is evidence that adenocarcinoma (ADC) of the esophagus arises in similar metaplastic epithelium, which has been termed “short segment Barrett esophagus” by some investigators<sup>[3,4]</sup>. However, this etiology alone is insufficient to explain the rising incidence of this highly lethal form of cancer<sup>[5,6]</sup>. Although several studies focusing on the squamous cell carcinoma (SCC) and the advanced stages of progression from dysplasia to ADC have investigated the role of genetic alterations in esophageal cancer (EC)<sup>[7-10]</sup>, scarce data are available on genetic alterations occurring in metaplasia-dysplasia-adenocarcinoma sequence of Barrett esophagus. To better understand the pathogenesis of esophageal ADC, we investigated the microsatellite alterations in these lesions. We dissected Barrett esophageal columnar epithelium (metaplasia) adjacent to dysplastic and neoplastic changes, dysplasia, and ADC, from the formalin-fixed, paraffin-embedded tissue blocks. Microsatellite alterations were evaluated at D2S123, D3S1616, D3S1300, D5S346, D17S787, D18S58 and BATRII loci, using the technique of dilution polymerase chain reaction (PCR). Our aim was to assess the early microsatellite alterations characterizing Barrett esophagus.

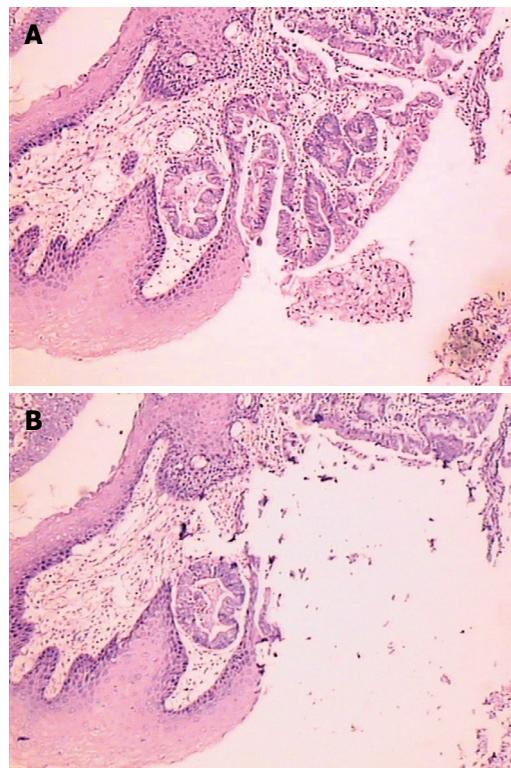
## MATERIALS AND METHODS

### Tissue specimens

Forty-one specimens were obtained from EC patients identified from Xiamen First Hospital affiliated to Fujian Medical University, Fujian Province, China, during 2002-2005. All the patients did not receive chemotherapy or radiation before surgery. Formalin-fixed, paraffin-embedded archival esophageal tissue specimens from 41 EC patients were accrued according to the guidelines established by the Ethics Committee of Xiamen First Hospital Affiliated to Fujian Medical University. Histopathological assessment identified 23 SCC and 18 ADC, including only 8 ADC with metaplasia and dysplasia adjacent to ADC. Strict criteria were used to diagnose ADC of primary esophageal origin as previously described<sup>[11]</sup>. Briefly, these included the presence of Barrett epithelium and dysplasia adjacent to ADC, at least 75% of tumor mass located in the distal one third of esophagus, invasion of periesophageal tissue, minimal gastric involvement, and clinical symptoms of dysphagia due to esophageal obstruction. Tissue histopathology was reviewed independently by 2 gastrointestinal pathologists.

### Tissue cellularity, DNA isolation and DNA dilution

Paraffin-embedded normal squamous epithelium, Barrett esophageal columnar epithelium (metaplasia), dysplasia and esophageal tumor tissues were dissected from the surrounding tissues under microscopic guidance in order to enrich the appropriate cell population for subsequent DNA extraction (Figure 1). All the hematoxylin-eosin



**Figure 1** Photomicrograph of epithelial dysplasia (HE,  $\times 200$ ) in Barrett esophagus (A) and after dissected under microscopic guidance (B).

(HE) stained sections of the above tissues from each EC patient were reviewed and cellularity was determined by estimating the relative density of neoplastic cells to normal tissue components (infiltrating stroma, muscle cells, white blood cells) per microscopic field. For the majority of cases, carcinoma specimens with at least 75% neoplastic cellularity were selected for microsatellite analyses. A series of unstained 10  $\mu\text{m}$ -thick sections of the above tissues were prepared from the corresponding paraffin-embedded tissue block with the microtome appropriately cleaned between cases in order to prevent tissue contamination. Previously selected areas on HE stained sections were then properly oriented and traced carefully on the corresponding unstained sections using a mark pen. Paraffin shaving from the unstained 10  $\mu\text{m}$ -thick sections was collected and pooled together in 0.5 mL Eppendorf tubes and DNA was extracted using proteinase K digestion buffer (10 mmol/L Tris-HCl, pH 8.0, 100 mmol/L KCl, 2.5 mmol/L MgCl<sub>2</sub>, 0.45% Tween20 and 2.5 mg/mL proteinase K) as previously described<sup>[12]</sup>. Briefly, samples were deparaffinized at 95°C for 10 min, cooled 1 h to room temperature and incubated at 65°C for 1 h with the proteinase K digestion buffer (100-200  $\mu\text{L}$ ). Samples were then denatured at 95°C for 10 min to remove excess proteinase K. The mixture was centrifuged at 5000 r/min for 5 min and the supernatant was carefully transferred by aspiration to a clean microcentrifuge tube. The quality of DNA was 1.07-1.24 ( $A_{260}/A_{280}$ ), the quantity of DNA was 255.4-407.0 ng/ $\mu\text{L}$ . To increase its sensitivity, DNA samples were diluted at 1:100, 1:1000, 1:5000, 1:10 000 and 1:50 000, respectively.

**Table 1** Microsatellite alterations of un-diluted and diluted DNA in different esophageal tissues

Tissue DNA	Number studied	MSI			LOH		
		Negative	At ≥ 1 locus	At ≥ 2 loci	Negative	At ≥ 1 locus	At ≥ 2 loci
<b>Classification</b>							
SCC	23	12	5	6	17	3	3
ADC	18	11	4	3 <sup>1</sup>	15	2	1 <sup>2</sup>
<b>Normal</b>							
Undilution	8	8	0	0	8	0	0
Dilution	8	3	2	3 <sup>3</sup>	4	2	2 <sup>4</sup>
<b>Metaplasia</b>							
Undilution	8	7	1	0	7	1	0
Dilution	8	1	4	3 <sup>5</sup>	2	3	3 <sup>6</sup>
<b>Dysplasia</b>							
Undilution	8	7	1	0	7	1	0
Dilution	8	1	3	4 <sup>7</sup>	2	2	4 <sup>8</sup>
<b>Carcinoma</b>							
Undilution	8	6	2	0	6	2	0
Dilution	8	0	3	5 <sup>9</sup>	0	4	4 <sup>10</sup>

Normal: Normal esophageal squamous epithelium DNA; Metaplasia: Barrett esophageal columnar epithelium DNA; Dysplasia: Dysplastic esophageal epithelium DNA; Carcinoma: Esophageal adenocarcinoma DNA; <sup>1</sup>P = 0.758, <sup>2</sup>P = 0.696, <sup>3</sup>P = 0.026, <sup>4</sup>P = 0.069, <sup>5</sup>P = 0.009, <sup>6</sup>P = 0.034, <sup>7</sup>P = 0.009, <sup>8</sup>P = 0.029, <sup>9</sup>P = 0.004, <sup>10</sup>P = 0.004 vs other groups.

### Dilution PCR and microsatellite analysis

Seven microsatellite markers (D2S123, D3S1616, D3S1300, D5S346, D17S787, D18S58 and BATRII loci) were used in this study. The oligonucleotide primers used for PCR amplifications were obtained from Research Genetics (Huntsville, AL). Each PCR reaction contained 1 μL of DNA samples each diluted at 1:100, 1:1000, 1:5000, 1:10000 or 1:50000, respectively, 0.4 μCi [r-<sup>32</sup>p] ATP-radiolabeled microsatellite primer, 0.2 mmol/L dNTP, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L MgCl<sub>2</sub>, 50 mmol/L KCl, and 0.4 units of Taq polymerase in a total reaction volume of 10 μL (that was called dilution PCR). The hot start PCR was used to eliminate nonspecific amplification. The samples underwent 35 cycles of PCR amplification (95°C for 30 s, 58°C for 40 s, and 72°C for 60 s) followed by an extension at 72°C for 2 min. Several PCR amplifications were performed for each DNA dilution. PCR products were diluted at 1:1 in a formalin loading buffer containing 95% formalin, 0.05% bromophenol blue, 0.05% xylene cyanol and 20 mmol/L EDTA, and 5 μL aliquots of PCR products was electrophoresed on 7% denaturing polyacrylamide gels (19:1 acrylamide:bisacrylamide) containing 5.6 mol/L urea, 32% formamide and 1 × TBE [0.089 mol/L Tris (pH 8.3)], 0.089 mol/L borate and 0.002 mol/L EDTA] at 75 W for 2-4 h. Gels were vacuum-dried at 80°C and exposed to a Kodak MR film (Kodak, Xiamen, China) for 6-24 h. Microsatellite analysis was carried out. A sample was scored positive for microsatellite instability (MSI) if additional new alleles were observed in Barrett esophageal epithelium or dysplasia or tumor DNA compared to control DNA from the same individual. Loss of heterozygosity (LOH) was defined as a visible reduction (at least 2 folds or more) in the ratio of signal intensities of the 2 alleles in metaplasia or dysplasia or tumor DNA relative to that observed in the corresponding normal DNA of the same individual. In all positive results analyzed, since

losses were readily visible or obvious, quantification was unnecessary. To ensure that the allele in intensities was identified in microsatellite analysis, multiple exposures of each autoradiograph were carried out. The autoradiographs were independently scored by JCC and HPZ.

### Statistical analysis

Fisher's exact test was performed to assess any statistically significant correlation of the frequency of the 2 histological subtypes (SCC vs ADC) or 2 subgroups (un-dilution vs dilution) by SPSS 10.0. P < 0.05 was considered statistically significant.

## RESULTS

### Microsatellite alterations in undiluted DNA of SCC and ADC

A varying level of MSI, ranging from 0% (0/23) to 28.6% (6/23) at the 7 loci tested, was detected in 23 SCC. The rate of MSI was 25% (3/18) at the D2S123 or D3S1616 or D5S346 locus in 18 ADC. The rate of MSI was 0 at the D3S1300, D17S787, D18S58 and BAT-R II loci in 18 ADC. Only informative cases (heterozygous for the microsatellite markers used) were considered for LOH analysis. The rate of LOH was 23.5% (4/17 informative cases) at the D3S1300 locus, and very low for the other loci in 23 SCC. The rate of LOH was 10.0% (1/10 informative cases) at the D2S123 or D3S1300 or D3S1616 locus, the others were 0 in 18 ADC. The results of the microsatellite analysis are summarized in Tables 1 and 2. No statistically significant difference was found in the rate of MSI and LOH at one or more positive loci, or at two or more positive loci between SCC and ADC.

### Microsatellite alterations in diluted DNA of phenotypically normal esophageal squamous epithelium and metaplasia-dysplasia-adenocarcinoma sequence

Dilution PCR showed that the more sensitive strategy

**Table 2** Microsatellite alterations of undiluted and diluted DNA at 7 loci in different esophageal tissues

Tissue DNA	Number studied	D2S123		D3S1616		D3S1300		BATR II		D5S346		D17S787		D18S61	
		MSI	LOH	MSI	LOH	MSI	LOH	MSI	LOH	MSI	LOH	MSI	LOH	MSI	LOH
Classification															
SCC	23	3	1	6	2	3	4	0	0	2	1	2	1	2	0
ADC	18	4	1	3	1	2	1	0	0	2	0	1	0	0	0
Normal															
Undilution	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dilution	8	2	1	1	2	2	1	0	0	1	0	1	1	1	1
Metaplasia															
Undilution	8	0	0	1	1	0	0	0	0	0	0	0	0	0	0
Dilution	8	2	2	2	3	1	2	1	0	1	1	2	1	1	0
Dysplasia															
Undilution	8	0	0	1	1	0	0	0	0	0	0	0	0	0	0
Dilution	8	4	3	4	3	4	3	1	0	1	1	4	2	1	1
Carcinoma															
Undilution	8	1	1	1	1	0	0	0	0	0	0	0	0	0	0
Dilution	8	6	4	6	7	6	4	2	0	4	0	6	3	3	2

Normal: Normal esophageal squamous epithelium DNA; Metaplasia: Barrett esophageal columnar epithelium DNA; Dysplasia: Dysplastic esophageal epithelium DNA; Carcinoma: Esophageal adenocarcinoma DNA.

for analyzing microsatellite changes was developed, reasoning that if a rare cell in a population harbored microsatellite alterations, the new microsatellite alleles would not be detected amid the large background of normal alleles<sup>[13,14]</sup>. High levels of MSI and LOH were very commonly found in phenotypically normal esophageal squamous epithelium and metaplasia-dysplasia-adenocarcinoma sequence of diluted DNA. The more the dilution of DNA (up to 1:5000) was, the higher the rates of MSI and LOH were at the above 7 loci, especially at D3S1616, D5S346, D3S123, D2S1300 and D18S58 loci. However, the rates of MSI and LOH were low when DNA was diluted at 1:10 000 and up, because most PCR products were not available. The data described here could document a profound microsatellite alteration in phenotypically normal esophageal squamous epithelium and metaplasia-dysplasia-adenocarcinoma sequence, although un-diluted DNA was not discovered. The results of microsatellite analysis are summarized in Tables 1, 2 and Figure 2.

## DISCUSSION

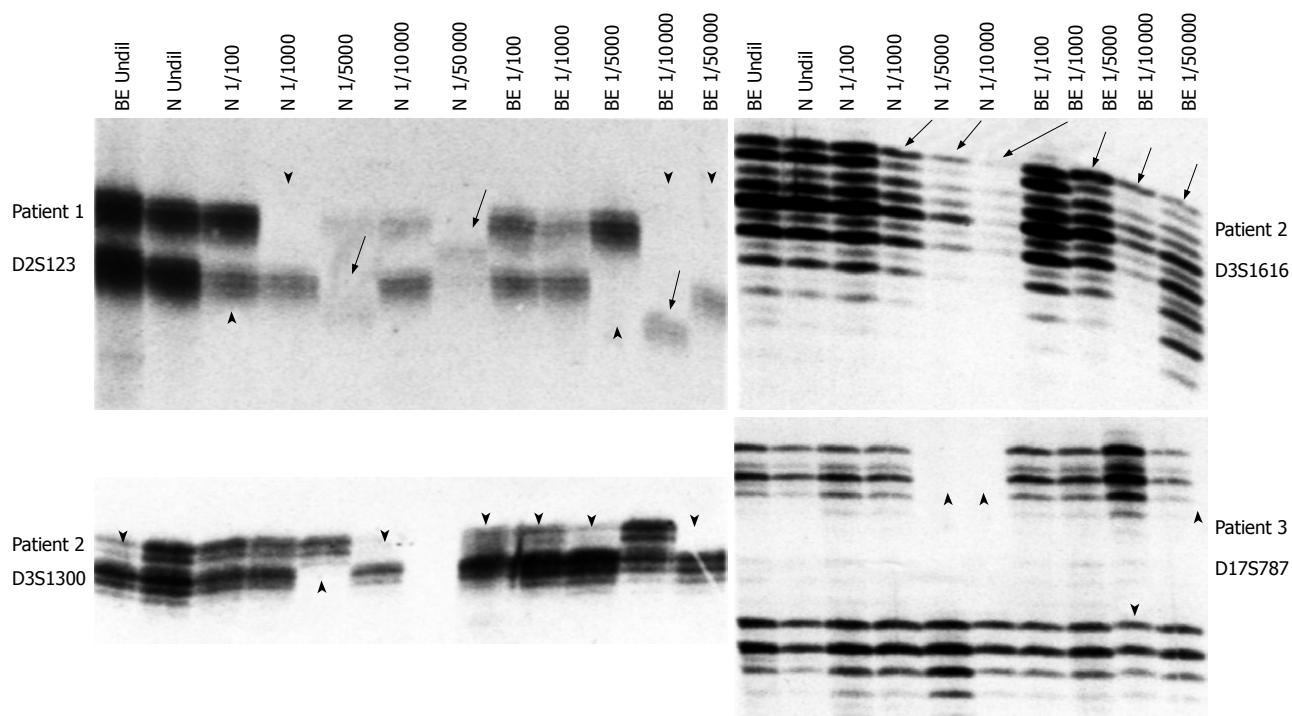
Tumor of Barrett esophagus occurs via a multi-step pathway, and is histopathologically recognized as a metaplasia-dysplasia-adenocarcinoma sequence<sup>[15]</sup>. DNA and certain molecular abnormalities under the form of proto-oncogene alterations, LOH and mutations of tumor suppressor genes are thought to represent the genetic background of metaplastic and dysplastic changes<sup>[16-19]</sup>. In recent years, frequent or infrequent MSI at multiple microsatellite loci corresponding to diverse tumor suppressor genes has been demonstrated in ADC of colon and rectum, small intestine, stomach, gallbladder and esophagus<sup>[20-24]</sup>. However, few data are available on the correlation between microsatellite alterations and metaplasia-dysplasia-adenocarcinoma sequence in Barrett esophagus, especially in normal squamous epithelium and metaplastic tissue adjacent to dysplasia and neoplasm.

Our study was to assess the microsatellite alterations in metaplasia-dysplasia-adenocarcinoma sequence of Barrett esophagus, as well as in normal squamous epithelium adjacent to metaplastic tissue. Tissue was dissected under microscopic guidance with a new technique which ensures enrichment and purity of the tissues to be studied and the specificity of the molecular event encountered.

In this study, the frequency of MSI was investigated in 23 SCC and 18 ADC, including 8 ADC with Barrett esophageal columnar epithelium and dysplasia. MSI of at least one microsatellite locus was detected in 10 out of 23 SCC (47.6%) and 5 out of 18 ADC (41.7%). MSI of two or more microsatellite loci was found in 5 out of 23 SCC (23.8%) and 3 out of 18 ADC (25.0%). MSI was found in 1 out of 8 Barrett esophageal columnar epithelium tissue samples, 2 out of 8 dysplasia tissue samples adjacent to ADC only at one microsatellite locus. Although no significant difference was found in the frequency of MSI between SCC and ADC in this study, the results are in agreement with the findings of some previous studies showing frequent MSI in EC<sup>[25-28]</sup>, but not consistent with some recent reports showing infrequent MSI in EC<sup>[24,29]</sup>.

It was reported that tumors develop *via* a process of clonal expansion resulting from the selection of increasingly abnormal sub-populations<sup>[10]</sup>. According to this theory, microscopic tumor usually represents the clonal expansion of one or a few cells, indicating that cells of a given neoplasm should share some common genetic abnormalities<sup>[10]</sup>. In order to find whether microsatellite alterations are an early event in esophageal ADC, we diluted DNA of metaplasia, dysplasia, ADC and normal esophageal squamous epithelium from the same 8 patients, and performed dilution PCR which showed surprisingly high levels of MSI and LOH expression at the early stage of metaplasia-dysplasia-adenocarcinoma sequence.

However, some studies also suggested that PCR



**Figure 2** Microsatellite alterations in diluted DNA. Arrow and arrowhead indicate MSI and LOH, respectively. N: Normal esophageal squamous epithelium DNA; BE: Barrett esophageal columnar epithelium (metaplasia) DNA; Undil: Un-diluted; 1/100-1/50 000: Dilution fold.

artifacts are found when paraffin-embedded tissues are used, especially when the number of cells analyzed is small<sup>[30,31]</sup>. With a larger number of target cells, enough non-damaged templates dominate the amplification process. With a smaller amount of cells, only fragmented DNA presents and requires a few PCR cycles to achieve an *in vitro* repaired template that would yield an exponential amplification<sup>[30,31]</sup>. Thus, to minimize the potential for artificial microsatellite alterations resulting from the diluted DNA, DNA was diluted at 1:100 to 1:50 000 for the dilution PCR. The concentration was estimated to be 25.5-40.7 pg/µL DNA when diluted at 1:10 000. PCR amplification was performed for each diluted DNA. For example, in patient 1, a concentration of 34.3-40.7 pg/µL DNA diluted at 1:10 000 was used.

Interestingly, microsatellite alterations were detected in all the eight patients having the “sequence” of metaplasia-dysplasia-adenocarcinoma at each representative DNA dilution. Similarly, microsatellite alterations were detected both in metaplasia and in dysplasia adjacent to ADC at the 7 microsatellite loci. The more the dilution of DNA was, the higher the rate of microsatellite alterations was at the D3S1616, D5S346, D2S123, D3S1300 and D18S58 loci depending on development of the metaplasia-dysplasia-adenocarcinoma sequence, clearly revealing that there are extensive microsatellite alterations at the D3S1616, D5S346, D2S123, D3S1300 and D18S58 loci in the metaplasia-dysplasia-adenocarcinoma sequence of Barrett esophagus, even in the histopathologically normal esophageal squamous epithelium. The observations of shared molecular abnormalities in metaplasia, dysplasia and ADC suggested a process of

colonial expansion in the proposed histological pathway of tumor development, which may be partly interpreted by the fact that only partial cells undergo molecular alterations under a similar circumstance.

In conclusion, high levels of MSI and LOH are frequently found in metaplasia, dysplasia and adenocarcinoma. Development of esophageal ADC is associated with microsatellite alterations, including MSI and LOH. These alterations occurring in recognized precursor lesions provide evidence for support of the proposed metaplasia-dysplasia-adenocarcinoma sequence in Barrett esophagus. MSI and LOH may be the early genetic events during esophageal carcinogenesis. Additional genetic alterations at the D3S1616, D5S346 and D3S123 loci probably play a role in the progression of these diseases. D3S1616, D5S346, D2S123, D3S1300 and D18S58 loci may be used as predictive markers for the early detection of esophageal ADC.

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## COMMENTS

### Background

It is estimated that the risk of neoplasia in Barrett esophagus is 125-fold higher than that in the general population, through the intermediate step of dysplastic transformation of the columnar epithelium. There is evidence that adenocarcinoma (ADC) of the esophagus arises in metaplastic epithelium, termed “short segment Barrett esophagus”. However, this etiology alone is insufficient to explain the rising incidence of this highly lethal form of cancer.

Although several studies focusing on the squamous cell carcinoma (SCC) and the advanced stages of progression from dysplasia to ADC, have investigated the role of genetic alterations in esophageal cancer (EC), scarce data are available on genetic alterations occurring in metaplasia-dysplasiaadenocarcinoma sequence of Barrett esophagus.

### Research frontiers

Tumor occurring in Barrett esophagus via a multi-step pathway is histopathologically recognized as a metaplasia-dysplasia-adenocarcinoma sequence. Certain molecular abnormalities in the form of proto-oncogene alterations, loss of heterozygosity (LOH) and mutations of tumor suppressor genes are thought to represent the genetic background of metaplastic and dysplastic changes. In recent years, frequent or infrequent microsatellite instability (MSI) at multiple microsatellite loci corresponding to the diverse tumor suppressor genes has been found in ADC of colon and rectum, small intestine, stomach and esophagus. However, few data are available on the correlation between microsatellite alterations and metaplasia-dysplasia-adenocarcinoma sequence in Barrett esophagus, especially in normal squamous epithelium and metaplastic tissue adjacent to dysplasia and neoplasm.

### Innovations and breakthroughs

Dilution polymerase chain reaction (PCR), the more sensitive strategy for analyzing microsatellite changes, was developed, reasoning that if a rare cell in a population harbored microsatellite alterations, the new microsatellite alleles would not be detectable amid the large number of normal alleles. High levels of MSI and LOH are frequently found in phenotypically normal esophageal squamous epithelium and metaplasia-dysplasia-adenocarcinoma sequence of diluted DNA in this study.

### Applications

MSI and LOH might be the early genetic events during esophageal carcinogenesis. Additional genetic alterations at the D3S1616, D5S346 and D3S123 loci may play a role in progression of ADC. The microsatellite markers (D3S1616, D5S346, D2S123, D3S1300 and D18S58 loci) may be used as predictive markers in the early detection of esophageal ADC.

### Terminology

Dilution PCR refers to diluted DNA of metaplasia, dysplasia, ADC and normal esophageal squamous epithelium in the same patient before PCR is performed.

### Peer review

The study was well designed and provided a great finding at metaplasia-dysplasia-adenocarcinoma sequence in Barrett esophagus. It is interesting.

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RAPID COMMUNICATION

# Sphincter of Oddi hypomotility and its relationship with duodenal-biliary reflux, plasma motilin and serum gastrin

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group ( $t = 5.254, 3.438$  and  $3.527, P < 0.001$ ). SOD of the reflux group was shorter than the control group ( $t = 2.049, P < 0.05$ ). The level of serum gastrin and plasma motilin of the reflux group was much lower than the control group ( $t = -2.230$  and  $-2.235, P < 0.05$ ). There was positive correlation between the level of plasma motilin and SOBP and between the level of serum gastrin and SOBP and CBDP.

**CONCLUSION:** About 35.9% of the patients with a T tube after cholecystectomy and choledochotomy have duodenal-biliary reflux. Most of them have sphincter of Oddi hypomotility and the decreased level of plasma motilin and serum gastrin. The disorder of gastrointestinal hormone secretion may result in sphincter of Oddi dysfunction. There is a close relationship between sphincter of Oddi hypomotility and duodenal-biliary reflux.

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**Key words:** Sphincter of Oddi; Tc99m-labeled diethylene triamine penta-acetic acid; Pressure; Motilin; Gastrin

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## Abstract

**AIM:** To detect whether patients with a T tube after cholecystectomy and choledochotomy have duodenal-biliary reflux by measuring the radioactivity of Tc99m-labeled diethylene triamine penta-acetic acid (DTPA) in the bile and whether the patients with duodenal-biliary reflux have sphincter of Oddi hypomotility, by measuring the level of plasma and serum gastrin of the patients. Finally to if there is close relationship among sphincter of Oddi hypomotility, duodenal-biliary reflux and gastrointestinal peptides.

**METHODS:** Forty-five patients with a T tube after cholecystectomy and choledochotomy were divided into reflux group and control group. The level of plasma and serum gastrin of the patients and of 12 healthy volunteers were measured by radioimmunoassay. Thirty-four were selected randomly to undergo choledochoscopy manometry. Sphincter of Oddi basal pressure (SOBP), amplitude (SOCA), frequency of contractions (SOF), duration of contractions (SOD), duodenal pressure (DP) and common bile duct pressure (CBDP) were scored and analyzed.

**RESULTS:** Sixteen (35.6%) patients were detected to have duodenal-biliary reflux. SOBP, SOCA and CBDP in the reflux group were much lower than the control

## INTRODUCTION

Endoscopic sphincterotomy (EST) is an important surgery to treat choledocholithiasis. About 50% of the patients after EST had biliary gas indicating reflux of duodenal contents<sup>[1]</sup>. The duodenal-biliary reflux rate was as high as 100% in the patients after EST<sup>[2]</sup>. The stone recurrence rate was 9.8% in the patients with EST<sup>[3]</sup>, but in the patients after cholecystectomy for gallbladder stones, the choledocholithiasis recurrence rate was only about 2.5%<sup>[4]</sup>. Thus, EST destroyed the sphincter of Oddi, resulting in duodenal-biliary reflux which may have close relationship with the recurrence of choledocholithiasis.

The stone recurrence rate was about 10.3% in the patients with T tubes after cholecystectomy and choledochotomy without apparent difference from that of patients after EST<sup>[3]</sup>. To understand whether sphincter of Oddi hypomotility and duodenal-biliary reflux existed in these patients and if these patients had abnormal secretion of gastrointestinal hormones it needs further studies. To detect if the duodenal-biliary reflux existed the radioactivity of Tc99m-labeled diethylene triamine pentaacetic acid (DTPA) in the bile from the T tubes. Besides, we investigated whether the patients with duodenal-biliary flux had sphincter of Oddi hypomotility and we measured the level of plasma motilin and serum gastrin of the patients. Finally, we discuss the relationship of duodenal-biliary flux with sphincter of Oddi hypomotility, serum gastrin and plasma motilin.

## MATERIALS AND METHODS

### **Patients**

Forty-five patients (16 men, 29 women, and mean age 58.9 years), who underwent cholecystectomy and choledochotomy at least 1.5 mo (mean 2.5 mo) after T tube drainage, were selected at the Second Affiliated Hospital of China Medical University between April 2004 and August 2005. They were divided into reflux group and control group. The amount of radioactivity of DTPA in the bile was measured and their blood was taken at fasting state in the morning for detecting serum gastrin and plasma motilin. The blood of 12 healthy volunteers (9 men, 3 women and mean age 51.6 years) was taken as control. Thirty-four patients (12 men, 22 women, and mean age 58.3 years) were selected for sphincter of Oddi manometry randomly and double-blindedly.

### **Observation of duodenal-biliary reflux**

The patients were fasted overnight and took 1 mL water orally containing 185 MBq (5mCi) <sup>99m</sup>Tc-DTPA, then drunk 240 mL water and took a prostrate position. The bile of 2 h was collected and 20 mL of the bile were taken for detection. The intensity of radioactivity was counted in the RM905 radioactivity-detecting meter. Patient was thought to have duodenal-biliary reflux when radioactivity was detected in the bile. <sup>99m</sup>Tc-DTPA was prepared just before administration and radiochemical purity was detected by radiochromatography. The radiochemical purity of <sup>99m</sup>Tc-DTPA was more than 99% while the free <sup>99m</sup>Tc was less than 1%.

### **Detection of plasma motilin and serum gastrin**

Four mL vein blood was obtained from the patients and healthy volunteers in the early morning and was put into two test tubes undividedly. One of them was filled with 30 μL 10% ethylene diamine tetraacetic acid disodium and 30 μL aprotinin. Then they were centrifuged at 1500 r/min for 15 min, serum and plasma isolated were put into Eppendorf tubes and stored in freezing refrigerator at -70°C.

Plasma motilin and serum gastrin were measured by radioimmunoassay. The sample of serum, plasma misce bene was collected before measurement, then

centrifuged at 1500 r/min for 15 min, The supernatant was taken and tested. The motilin radioimmunoassay kit was supplied by Peking Huaying Biotechnology Research Institute. The gastrin radioimmunoassay kit was provided by Isotope Research Center of the China Atomic Energy Academy.

### **Method and apparatus of sphincter of Oddi manometry**

A triple lumen polyethylene manometry catheter 200 cm in length with an outer diameter of 1.7 mm was used for manometry. The three side holes in the distal end were located 2 mm apart. Sterile water was infused through the catheter at a flow rate of 0.5 mL/min by a hydraulic capillary infusion system. PC polygraph HR (Swedish CTD-Synectics Medical Company) and relevant program were used to record and analyze the tracings. Manometry was performed after all the stones were removed from the common bile duct. The catheter was introduced via the side-pore of choledochoscope into duodenum directly, when the pressure was stable, duodenal pressure-curve was recorded. It was then withdrawn in a stepwise fashion; the position of catheter in the sphincter could be confirmed by direct observation under choledochoscope or by the characteristic pressure changes on the screen. The Oddi's sphincter and common bile duct motility tracings were recorded respectively.

Sphincter of Oddi basal pressure (SOBP), amplitude of phasic contractions (SOCA), frequency of phasic contractions (SOF), duration of phasic contractions (SOD), duodenal pressure (DP) and common bile duct pressure (CBDP) were recorded and analyzed with a special computer program.

Statistical analysis was carried out using Student's *t* test. Data were analyzed with software SPSS11.5. The results were expressed as mean ± SD. Levene's test was used to test the homoscedasticity of the data; *t* test would be used when data had no homoscedasticity. A two-tailed *P* value of < 0.05 was considered significant statistically. Pearson correlation test was used to test correlation of the data.

## RESULTS

### **Duodenobiliary reflux**

There were 16 (35.6%) patients with duodenal-biliary reflux among 45 patients who underwent cholecystectomy, choledochotomy and T tube drainage. The mean technetium count of the bile was 132.73 ± 246.07 KBq (counting performed at the second hour after the isotope ingested). The rest 29 bile samples detected had no radioactivity.

### **Plasma motilin and serum gastrin**

Plasma motilin (377.54 ± 130.44) and serum gastrin (68.33 ± 28.56) of the reflux group is apparently lower than that of the control group and the non-reflux group (Table 1).

### **Manometry of SO**

Thirty-four patients were selected (12 men, 22 women,

**Table 1** Plasma motilin and serum gastrin of patients with a T tube after cholecystectomy and choledochotomy (pg/mL)

	Cases	Level of plasma motilin	Level of serum gastrin
Reflux group	16	377.54 ± 130.44 <sup>a,d</sup>	68.33 ± 28.56 <sup>a,d</sup>
Non-reflux group	29	525.37 ± 184.47	97.64 ± 26.93
Control group	12	499.02 ± 157.77	92.95 ± 25.67

<sup>a</sup>P < 0.05 vs the control group; <sup>d</sup>P < 0.01 vs the nonreflux group.

and mean age 58.3 years) for sphincter of Oddi manometry randomly and double-blindedly. Among them 13 were classified into the reflux group and 21 into the control group (Table 2).

The sphincter of Oddi's basal pressure, amplitude, common bile duct' pressure in the reflux group were significantly lower than that of the control group ( $P < 0.001$ ), the duration of contractions of the reflux group was shorter than that of control group while there was no significant difference in the frequency of contractions and duodenal pressure between the two groups. The change of SOCA was most remarkable; and the mean value of the reflux group was lower than that half of the control group. In the reflux group, there were 10 (76.9%) patients with DP higher than SOBP and 8 (61.5%) patients with DP higher than CBDP while no patient with DP higher than SOBP and only 2 (9.5%) patients with DP higher than CBDP in the control group (Table 2).

#### Pearson correlation test

There was no evident correlation between plasma gastrin and serum motilin (the Coefficient of Correlation was 0.146) while plasma motilin showed obviously positive correlation with sphincter of Oddi basal pressure (the Coefficient of Correlation was 0.366) ( $P < 0.05$ ). Moreover serum gastrin showed apparent positive correlation with sphincter of Oddi basal pressure (the Coefficient of Correlation is 0.429) ( $P < 0.05$ ) and common bile duct pressure (the Coefficient of Correlation is 0.359) ( $P < 0.05$ ).

## DISCUSSION

Sphincter of Oddi was at the lower end of the bile duct and pancreatic duct. It plays a major role in controlling bile and pancreatic juice flowing into the duodenum and equally importantly to prevent reflux of duodenal contents into the biliary and pancreatic duct. One-hundred years ago, a variety of names were given to malfunction of sphincter of Oddi, including odditis, biliary dyskinisia, postcholecystectomy and sphincter of Oddi stenosis. Now based on manometry, sphincter of Oddi dysfunction was subdivided into two groups; one of a stenotic pattern and the other a dyskinetic pattern<sup>[5]</sup>. Reports about sphincter of Oddi hypomotility and sphincter of Oddi dilation were rare. Does sphincter of Oddi hypomotility exist? Does it have close relationship with duodenal-biliary reflux? Does it have close relationship with recurrent cholangitis and recurrent gallstone? Our

study would discuss these questions profoundly.

Digestive juice reflux is an abnormal phenomenon of the digestive tract. Duodenal-gastric reflux and gastric-esophagus reflux were most common among them which would result in reflux gastritis and esophagitis<sup>[6,7]</sup>. Under some conditions, such as after intestinal anastomosis and EST, duodenobiliary reflux may occur, resulting in ascending cholangitis. But whether duodenobiliary reflux existed in patients with gallstones and without EST and in healthy people, has been rarely studied. Feng *et al*<sup>[8]</sup> found only two of 30 patients with gallbladder stones had duodenobiliary reflux. Wang *et al*<sup>[9]</sup> studied 53 patients who suffered from the recurrent inflammation of biliary duct after operation and found that the incidence rate of duodenobiliary reflux was as high as 66.67%. The change of biliary tract anatomy could result in pneumatosis of the duct of Wirsung, suggesting duodenopancreas reflux<sup>[10]</sup>. Li *et al*<sup>[11]</sup> found that there was apparent duodenobiliary reflux during the process of T tube drainage and the main bacterium infecting bile was G<sup>-</sup> bacterium (especially the *E. coli*). We employ <sup>99m</sup>Tc-DTPA for the first time to determine duodenobiliary reflux in patients undergoing choledocholithotomy plus T-tube drainage. The only machine used was RM905 radioactivity meter. For its high molecular weight, <sup>99m</sup>Tc-DTPA had no other pathway to enter into the bile and it was hard for it to penetrate the intestinal mucosa<sup>[12]</sup>. If there was radioactivity detected in patients' bile, we could infer they had duodenobiliary reflux. The method was simple, less expensive and safe. It was easy to repeat within a short period for the short half-life of the technetium. The results could be easily understood. It had a high specificity (100%), and the sensibility was still unknown since the occurrence of duodenobiliary reflux also could be influenced by many factors, such as the state of the sphincter of Oddi. This means the real duodenobiliary reflux incidence among patients undergoing choledocholithotomy may be higher than our results. We found that the morbidity of duodenobiliary reflux among these people was 35.9% (28/78). It was so high, resulting in bacterial infection and recurrence of stone.

As a valve to prevent duodenal juice reflux, does state of Oddi sphincter have intimate relationship with duodenobiliary reflux? There were numerous and thorough researches on sphincter of Oddi disorder<sup>[13-17]</sup>, but reports about sphincter of Oddi hypomotility and sphincter of Oddi dilation were relatively rare. Early in the 1890, Courvoisier found that 14% of the patients with common bile duct stones had dilation of the duodenal ampulla. Deng *et al*<sup>[18]</sup> found that the mean contraction period was shortened ( $P < 0.02$ ), and that the frequency ( $P < 0.005$ ) and amplitude ( $P < 0.001$ ) of sphincter phasic waves during phase III were decreased in the patients with proximal duodenal transection performed during gastrectomy; this suggested that duodenal transection produced significant changes in interdigestive sphincter of Oddi motility, possibly contributing to augmented duodenobiliary reflux and then lithogenesis. There were many case reports about

**Table 2 Results of sphincter of Oddi manometry of patients with a T tube after cholecystectomy and choledochotomy (mmHg)**

Groups	n	M/F	Age (yr)	SOBP	SOCA	SOD	SOF	CBDP
Reflux group	13	7/6	62.4 ± 8.4	-3.48 ± 4.77	33.81 ± 29.51	4.34 ± 1.26	7.46 ± 2.80	-1.64 ± 4.91
Control group	21	6/15	55.7 ± 14.2	12.26 ± 10.08 <sup>b</sup>	91.40 ± 66.97 <sup>b</sup>	5.27 ± 1.31 <sup>b</sup>	7.08 ± 2.78	6.70 ± 7.58 <sup>b</sup>

<sup>b</sup>P < 0.01 vs control group.

patients who had sphincter of Oddi hypomotility which expressed pneumobilia, lower SOBP and SOCA (patients with chronic intestinal pseudo-obstruction<sup>[19]</sup>, common bile duct stones<sup>[20]</sup> and progressive systemic sclerosis<sup>[21]</sup>). Factors correlated to sphincter of Oddi hypomotility including dragging of SO by postoperative adhesion, intra-abdominal abscess, pancreatitis, incompetence of SO resulting from tumors, retrograde peristalsis of duodenum, functional disorder of nerves of the sphincter, passing of stones and actions of drugs.

We found that 16 patients had duodenal-biliary reflux among 45 patients who underwent cholecystectomy and T tube drainage (35.6%), a large number of whom had sphincter of Oddi hypomotility. There were ten (76.9%) patients with DP higher than SOBP and 8 (61.5%) patients with DP higher than CBDP (61.5%) while no patient with DP higher than SOBP and only two (9.5%) patients with DP higher than CBDP (9.5%) in the control group. Sphincter of Oddi structure of human is resembled to that of cats. The bile flow stopped at the contraction state while expelling to the duodenum in the intervals of contraction, but the sphincter of opossum can expel bile during the contraction stage. Sphincter of cats and opossums could expel bile into the duodenum when the duodenum pressure was as high as 45 cmH<sub>2</sub>O and no duodenobiliary reflux occurred<sup>[22]</sup>. Sphincter of Oddi function played an important role in preventing the occurrence of duodenobiliary reflux. It would lose its function of non-return valve in the hypomotility state and could not prevent the duodenal fluid refluxing into the biliary duct and pancreatic duct.

It has been verified that when duodenal juice was added to lithogenic bile, gallstones formed<sup>[23]</sup>. Bacterial pathogens gained access into the biliary system by descending *via* the portal venous circulation or through the sphincter of Oddi. Bacteria thrived as glycocalyxenclosed microcolonies, coalescing to form an adherent biofilm. The establishment of biofilm was a key event in the formation of biliary sludge and pigment gallstones<sup>[24]</sup>. There were about 35.9% of the patients with a T tube after cholecystectomy and choledochotomy who had duodenal-biliary reflux. Most of them had sphincter of Oddi hypomotility. We thought that sphincter of Oddi hypomotility may be one of the most important factors which would result in duodenal-biliary reflux, and reflux of duodenum juice into biliary duct is a main cause of stone recurrence.

Motilin is a kind of multiple peptide hormones which contains 22 amino acids and is secreted by the endocrine cells of gut (mainly produced by cells of sinus ventriculi, duodenum and upper part of small intestine).

It had the effect of strongly stimulating the contraction of the upper digestive tract and physiological myoelectric activity of its smooth muscle cells. Motilin can invoke contractions of smooth muscle of gallbladder and stimulate secretions of bile. Motilin had prokinetic effect on the sphincter of Oddi and its function had intimate relationship with the dosage used<sup>[25-27]</sup>. When large dose of motilin used, the sphincter of Oddi of animals contracted, even spasmed. Gastrin is a kind of gastrointestinal hormone, which has been studied earlier and intensively. It is mainly secreted by the G cells of the sinus ventriculi mucosa and small intestines. The researches about effect of gastrin on sphincter of Oddi are rare<sup>[28-30]</sup>. A few reports showed gastrin could increase SOBP and SOCA. Chen suggested that the patients with post-cholecystectomy pain had sphincter of Oddi dysfunction with characteristics of high tension and it was related to elevation of serum gastrin<sup>[31]</sup>. Our study found that many patients with a T tube after cholecystectomy and choledochotomy had duodenal-biliary reflux. Most of them had sphincter of Oddi hypomotility, and decreased plasma motilin and serum gastrin. Plasma motilin showed obviously positive correlation with sphincter of Oddi basal pressure while serum gastrin showed apparent positive correlation with sphincter of Oddi basal pressure and common bile duct pressure. Disorder of gastrointestinal hormone secretion might result in sphincter of Oddi dysfunction and the sphincter of Oddi hypomotility caused by it had intimate relationship with duodenal-biliary reflux and recurrence of gallstones.

## COMMENTS

### Background

Sphincter of Oddi is at the lower end of the bile duct and pancreatic duct. It plays a major role in controlling the flowing of bile and pancreatic juice into the duodenum and equally importantly to prevent the reflux of duodenal contents into the biliary and pancreatic duct. Based on manometry, sphincter of Oddi dysfunction was subdivided into two groups; one characterized by a stenotic pattern and the other a dyskinetic pattern. The reports about sphincter of Oddi hypomotility and sphincter of Oddi dilation are fairly rare.

### Research frontiers

Whether sphincter of Oddi hypomotility and duodenal-biliary reflux existed in patients with a T tube after cholecystectomy and choledochotomy and if they had abnormal secretion of gastrointestinal hormones needed further researches. Does sphincter of Oddi hypomotility exist? Does it have close relationship with duodenal-biliary reflux? Does it have close relationship with recurrent cholangitis and recurrent gallstone? This study discussed these questions profoundly.

### Innovations and breakthroughs

This study found many patients with a T tube after cholecystectomy and choledochotomy had duodenal-biliary reflux. Most of them had sphincter of Oddi hypomotility, plasma motilin and serum gastrin of them decreased. The disorder

of gastrointestinal hormone secretion might result in sphincter of Oddi dysfunction and sphincter of Oddi hypomotility caused by it had intimate relationship with duodenal-biliary reflux and recurrence of gallstones.

### Applications

The reports about sphincter of Oddi hypomotility and sphincter of Oddi dilation are relatively rare. Authors found the disorder of gastrointestinal hormone secretion might result in sphincter of Oddi dysfunction and sphincter of Oddi hypomotility caused by it had intimate relationship with duodenal-biliary reflux and recurrence of gallstones. So regulating the level of hormones might be helpful in the treatment of sphincter of Oddi dysfunction.

### Peer review

It is a very interesting study, and the presentation of manuscript is good. Title accurately reflects the major topic and contents of the study. Although it is not a multi-center study the results are very interesting because they demonstrated more about the function of the Sphincter of Oddi. Discussion is well organized, and references are appropriate and relevant.

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RAPID COMMUNICATION

## Changes of serum p53 antibodies and clinical significance of radiotherapy for esophageal squamous cell carcinoma

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### Abstract

**AIM:** To explore the relationship between serum p53 antibodies (p53-Abs) and clinicopathological characteristics and therapeutic effect in patients with esophageal carcinoma (EC), and to investigate sequential changing regularity of serum p53-Abs after radiotherapy.

**METHODS:** The serum p53-Ab levels were detected in 46 EC patients and 30 healthy adults by enzyme linked immunosorbent assay (ELISA). The blood samples were collected on the day before radiotherapy and on the administration of an irradiation dose of 20 Gy/10 f/12 d, 40 Gy/20 f/24 d and 60 Gy/30 f/36 d after radiotherapy.

**RESULTS:** The level and positive rate of serum p53-Abs in EC patients were significantly higher than those in normal individuals ( $P < 0.05$ ). Serum anti-p53 antibodies were positive in 18 of 46 EC patients (39.1%). The positive rate of p53-Abs in EC was related to histological grade, disease stage and lymph node metastasis ( $P < 0.05$ ), but it was not significantly related to sex, age and to the size and site of tumor. The level and positive rate of p53-Abs had significant differences between before radiotherapy and after administration of an irradiation dose of 40 Gy/20 f/24 d and 60 Gy/30 f/36 d ( $P < 0.05$  or  $P < 0.01$ ). The positive rate of p53-Abs in EC patients with effect was significantly lower than that in those without effect after radiotherapy ( $P < 0.0001$ ).

**CONCLUSION:** Detection of serum p53-Abs is helpful to the diagnosis of esophageal carcinoma. Monitoring for sequential change of serum p53-Abs before and after radiotherapy in patients with esophageal carcinoma is also useful to evaluate the response to the treatment and prognosis of the patients.

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**Key words:** Esophageal carcinoma; Radiotherapy; Serum p53 antibodies; Enzyme linked immunosorbent assay

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### INTRODUCTION

Esophageal carcinoma (EC) is one of the most common malignant diseases in China. The prognosis of this disease is unfavorable in spite of advances in therapies. Mutations of the p53 gene are common gene alterations in most malignant tumors, including EC. Mutant p53 protein is accumulated in cells because of its longer half-life compared with wild-type protein. Therefore, p53 overexpression can be detected by immunohistochemical staining for p53. The anti-p53 antibody (p53-Ab) assay is based on the initial results of Crawford *et al*<sup>[1]</sup> who detected p53-Abs in the sera of patients with breast carcinomas. Because mutation in the p53 gene and the consequent overexpression of p53 are associated with tumor tissues, both wild type and mutant p53 may act as targets of tumor specific humoral and cellular immune responses<sup>[2,3]</sup>. The presence of p53-Abs in the sera of patients has been noted in various types of carcinomas. Non-carcinoma patients do not exhibit such antibodies<sup>[4-7]</sup>. Close correlations were observed between the presence of such antibodies and other factors related to a poor prognosis, such as high histological grade and

the absence of hormone receptors<sup>[3,8]</sup>. The presence of serum p53-Abs was reported to be an independent prognostic factor for breast carcinoma<sup>[9]</sup>. Studies have revealed that overexpression of p53 protein closely related to induction of drug resistance in cancer patients and it can be used as a predictor for chemosensitivity of tumor. Specific p53-Ab was found in the sera of cancer patients with p53 protein overexpression. Furthermore, the presence of serum p53-Ab is closely correlated with p53 protein overexpression<sup>[10,11]</sup>. Thus, serum p53-Ab could theoretically be useful in predicting radiosensitivity in cancer patients. However, the clinical relevance of p53-Ab and radiotherapy for EC has not been fully studied. In the present study, the serum p53-Ab level was detected in 46 EC patients and 30 healthy adults as control by enzyme linked immunosorbent assay (ELISA). We explored the relationship between serum p53-Abs and clinicopathological characteristics and therapeutic effect in EC patients, and investigated the sequential changing regularity of serum p53-Abs after radiotherapy.

## MATERIALS AND METHODS

### Clinical data

A total of 46 consecutive patients with primary EC were enrolled in this study. They consisted of 38 males (82.6%) and 8 females (17.4%), with a mean age of 56.4 years (range, 46-70 years), who were treated at Gansu Provincial Tumor Hospital between April 2005 and June 2007. The patients' eligibility for this study was as follows: (1) Primary lesion was squamous carcinoma in histology. There was no previous malignant disease or clinical evidence of a second primary tumor elsewhere; (2) Karnofsky performance status  $\geq 70$ , white blood cell and hemoglobin levels within the normal range; and (3) Having not received radiotherapy, chemotherapy and operation. Tumor, nodes and metastasis (TNM) classification was established on the basis of pathologic examinations of biopsied specimens. Lymph node status in the patients was determined by computed tomography (CT). According to the UICC (2002) classification, 10 patients were in stage I, 17 in stage II, 14 in stage III and 5 in stage IV. Twenty-eight patients had no signs of lymph node metastasis, and the other 18 patients had metastasis of mediastinal and/or supraclavicular lymph nodes. Thirty-two had primary lesions  $< 6$  cm and 14 were  $\geq 6$  cm in size. Five tumors were located in the upper thorax, 21 in the middle thorax and 20 in the lower thorax. To compare the reliability of seronegative testing, a total of 30 healthy volunteers, 19 males and 11 females, with a mean age of 36 years (range, 21-62 years) from our hospital staff and medical students served as controls. Informed consent was obtained from all patients. The blood samples were collected on the day before radiotherapy and on the administration of an irradiation dose of 20 Gy/10 f/12 d, 40 Gy/20 f/24 d and 60 Gy/30 f/36 after radiotherapy, and stored at -80°C until Assay.

### Radiotherapy

Radiation source was a 6MV linear accelerator. The

design of the radiation fields was based on the diagnosis by CT and barium examination. For all patients, a three-field approach was administered: one anterior and two posterior oblique portals. The width of the fields was adjusted to cover gross tumors with 3-4 cm extended margins so as to include subclinical lesions. The length of the field covered clinical tumors with a 3-5 cm extended margin at both ends of the lesion. All patients received conventional fractions, 2.0 Gy per fraction, five fractions per week. The total dose given to the tumor was 60-70 Gy/6-7 W.

### Assay of serum anti-p53 antibodies

Anti-p53 antibodies in sera of patients was detected using a commercially available sandwich enzyme-linked immunosorbent assay kit (Immunotech, France). The assay was done according to the manufacturer's instructions, with the following specifications: patient serum was added for 60 min at 37°C to microtiter wells coated with recombinant wild type human p53 protein to detect specific anti-p53 antibodies or with a control protein to detect nonspecific interactions. After washing, goat antihuman immunoglobulin G (IgG) antibody conjugated with peroxidase was added and stayed for 60 min at 37°C. Next, the substrate 3,3,5,5-tetramethyl benzidine (TMB) was added for 10 min. The enzymatic process was halted by adding 2N hydrogen chloride. Light absorption was measured at 450 nm using a photospectrometer. Because human serum may give rise to variable background signals, an internal control was used to measure the nonspecific background of each sample. This background reflects nonspecific interactions of serum components either with the plastic or with the components used in the ELISA. In the assay, the nonspecific background corresponds to the absorbance measured on wells coated with control protein. The presence of anti-p53 antibodies in a sample is determined by different parameters, i.e., for the specific signal of the sample, index = specific signal of the positive control (low); for p53 net absorbance of the sample, ratio of net absorbance = control protein net absorbance of the sample. An index of 1.1 and a ratio of net absorbance of 1.6 were determined to be positive for the presence of anti-p53 antibodies.

### Clinical evaluation of radiation response

At the end of RT, all patients received esophageal barium examination and the clinical radiation response was evaluated according to standard X-ray diagnosis of EC after radiotherapy<sup>[12]</sup>. A complete response (CR) was defined as the disappearance of the mass shadow, no narrowing observed in the esophageal lumen, and none or slight rigidity of the esophageal wall without residual ulceration. Partial response (PR) was > 50% reduction in tumor bulk, but < 100% resolution of the disease and a residual shallow ulcer with a diameter  $< 1.5$  cm, despite the disappearance of the mass shadow. No response (NR) was defined as no improvement in the X-ray findings, with a deep and large residual ulcer or complete

**Table 1 Sequential change of positive rate of serum p53-Abs in EC patients before and after radiotherapy**

Groups	n	-	+	%	$\chi^2$	P
Before RT	46	28	18	39.1	0.000	1.000 <sup>1</sup>
20 Gy	46	28	18	39.1	0.187	0.666 <sup>2</sup>
40 Gy	46	30	16	34.8	4.696	0.030 <sup>3</sup>
60 Gy	46	39	7	15.2	6.646	0.010 <sup>4</sup>

<sup>1</sup>Before RT vs 20 Gy; <sup>2</sup>Before RT vs 40 Gy; <sup>3</sup>40 Gy vs 60 Gy; <sup>4</sup>Before RT vs 60 Gy.

**Table 2 Relationship between positive rate of serum p53-Abs and clinical pathophysiological characteristics in EC patients**

	n	-	+	Positive (%)	$\chi^2$	P
Age (yr)					0.27	0.870
< 50	16	10	6	37.5		
≥ 50	30	18	12	40.0		
Sex					0.11	0.918
Male	38	23	15	39.5		
Female	8	5	3	37.5		
TNM stage					6.09	0.014
I + II	27	21	6	22.2		
III + IV	19	8	11	57.9		
Histological grade					10.43	0.002
I	15	13	2	13.3		
II	17	11	6	35.3		
III	14	4	10	71.4		
Lymph node metastasis					6.00	0.015
Positive	18	7	11	61.1		
Negative	28	21	7	25.0		
Size of tumor					2.74	0.102
< 6 cm	32	22	10	31.3		
≥ 6 cm	14	6	8	57.1		
Site of the tumor					0.02	0.953
Upper thorax	5	3	2	40.0		
Middle thorax	21	13	8	38.1		
Lower thorax	20	12	8	40.0		

TNM: Tumor, nodes and metastasis.

obstruction of the esophageal lumen, regardless of the residual state of the mass shadow.

### Statistical analysis

The Statistical Package for Social Sciences, version 10.0 was used for statistical analysis. Serum p53-Abs levels were expressed as means ± SD. Student's *t* test, Pearson Chi-square and logistic analysis were used to determine the significance of difference between the two groups. A *P* value < 0.05 was considered significant.

## RESULTS

### Comparison of index and ratio of serum p53-Abs

The index and ratio of serum p53-Abs for EC patients were  $1.5847 \pm 0.5133$  and  $3.0293 \pm 0.7013$ , and those for healthy controls were  $0.2418 \pm 0.1438$  and  $1.0361 \pm 0.2175$ , the former was higher than the latter (*P* < 0.0001).

### Comparison of positive rate of serum p53-Abs

The positive rate of serum p53-Ab was 39.1% (18 of 46)

**Table 3 Logistic analysis on the relationship between positive rate of serum p53-Abs and clinical pathophysiological characteristics in EC patients**

Variables	B	OR	95% CI	P
Age	0.0241	1.0237	0.9583-1.0816	0.4659
Sex	-0.6230	0.5363	0.0863-3.3401	0.5165
TNM stage	1.8389	6.2899	2.4658-3.8954	0.0248
Histological grade	-4.1430	0.0159	0.0039-0.5867	0.0156
Lymph node metastasis	-0.4891	0.5973	0.3327-1.1406	0.1314
Size of tumor	0.6954	1.8924	0.7859-4.5968	0.1602
Site of tumor	-0.0695	0.9530	0.3240-2.6575	0.7986

for EC patients and 0% (0 of 30) for healthy controls, with a significant difference (*P* < 0.0001). The sensitivity, specificity, accurate rate, positive predictive value and negative predictive value of p53-Ab detection in EC were 39.1%, 100%, 63.2%, 100% and 52%, respectively.

### Comparison of before and after radiotherapy sequential change

The level and positive rate of serum p53-Abs had significant differences between before radiotherapy, after administration of an irradiation dose of 40 Gy/20 f/24 d and after administration of an irradiation dose of 60 Gy/30 f/36 d (Table 1).

### Relationship between positive rate of serum p53-Abs and clinical pathophysiological characteristics

The positive rate of serum p53-Abs in EC was related to histological grade, disease stage and lymph node metastasis, but it was not significantly related to sex, age and to the size and site of tumor (Table 2).

### Logistic analysis

Logistic analysis showed that the positive rate of serum p53-Abs in EC had positive correlation with disease stage, negative correlation with histological grade and independence with sex, age and with the size and site of tumor (Table 3).

### Clinical response to RT and serum p53-Abs

At the end of RT, CR was achieved in 21 patients, RR in 18 and NR in 7. In patients with CR plus PR, 61.1% (11/39) were positive for serum p53-Abs, but 100% (7/7) of patients with NR were positive for serum p53-Abs. The positive rate of serum p53-Abs in EC patients with effect was significantly lower than those without effect after radiotherapy (*P* < 0.0001).

## DISCUSSION

p53 protein plays a crucial role in the regulation of the cell cycle and has been implicated in cell differentiation, apoptosis, DNA synthesis, and repair<sup>[13,14]</sup>. This nuclear protein, because of its critical function in triggering cell death via apoptosis in cells affected by irreparable genomic damage, has been designated as the "guardian of the genome" by Lane<sup>[15]</sup>.

Mutations in the p53 tumor suppressor gene are

among the most common genetic alterations in human malignancies. In ovarian carcinoma, alterations in this tumor suppressor gene occur in approximately 50% of cases<sup>[16-18]</sup>. The commonly termed overexpression of p53 corresponds to a cellular accumulation of a biologically inactive protein stabilized either by a decreased rate of degradation of mutated gene product or by complex formation with certain proteins, such as viral oncoproteins or heat-shock protein 70<sup>[14,19,20]</sup>. In normal cells, p53 protein, through its short half-life, is present at such low levels that it is undetectable by conventional immunohistochemical methods. However, the results obtained in a number of studies on the prognostic impact of overexpression of p53 protein in ovarian carcinoma tissue were controversial. Whereas some investigators reported impaired clinical outcome in patients with p53 overexpressing tumors<sup>[21-23]</sup>, others failed to demonstrate a relation between the course of the disease and p53 expression<sup>[16,24]</sup>.

Recently, circulating p53-Ab has been detected in the serum or plasma of patients with various carcinomas. Detection is made by a simple and rapid ELISA procedure. The positive rate for p53-Ab was reported to be 24% for lung cancer, 19% for pancreatic cancer and 25% for colorectal cancer. The frequency of positive p53-Ab in patients with EC ranges from 25% to 53% in the literature. Our study detected p53-Ab in 18 (39.1%) of the 46 patients with EC, while it was not detected in any of the 30 healthy subjects. This positive rate for p53-Ab in esophageal cancer patients is thus similar to that in the published literatures<sup>[25,26]</sup>. The index and ratio of serum p53-Abs for patients with EC before radiotherapy were obviously higher than those for healthy controls ( $P < 0.05$ ). This suggests that the detection of serum p53-Ab in patients with EC is helpful to its diagnosis. We also found that the positive rate of serum p53-Abs in EC was related to the histological grade, disease stage and lymph node metastasis ( $P < 0.05$ ), but it was not significantly related to sex, age and the size and site of tumor ( $P > 0.05$ ). The lower the histological grade and clinical stage, the higher the level and positive rate of serum p53-Abs in EC, indicating that the positive serum p53-Abs is a poor characteristic and identifying marker of unfavorable prognosis for patients with EC.

At the end of RT, CR was achieved in 21 patients, RR in 18 and NR in 7. In patients with CR plus PR, 61.1% (11/39) were positive for serum p53-Abs, but 100% (7/7) of patients with NR were positive for serum p53-Abs. The positive rate of serum p53-Abs in EC patients with effect was significantly lower than those without effect after radiotherapy. This suggests that serum p53-Ab overexpression can be one of the reference indicators for predicting radiosensitivity in patients EC. Future studies will be required to reveal and confirm the correlation between radiosensitivity and serum p53-Abs.

We also found that the serum p53-Abs level was useful for the monitoring of treatment. In the present study, the level and positive rate of serum p53-Abs had significant differences between before radiotherapy,

after administration of an irradiation dose of 40 Gy/20 f/24 d and after administration of an irradiation dose of 60 Gy/30 f/36 d. It was reported that the presence of p53-Ab correlates closely with p53 overexpression and/or mutation<sup>[2,27]</sup>. This suggests that the p53 immune response in the patients can be attributed to accumulation of mutant p53 in the nucleus of tumor cells. The p53 protein was decreased and the tumor cell was destroyed through effective radiotherapy, the source of mutation of the p53 gene was eliminated, and then the immune response was weakened. Thus, monitoring of sequential change of serum p53-Abs before and after radiotherapy in patients with EC is helpful to evaluating the response to treatment and prognosis. A larger series of studies will be required to reveal and confirm the correlation between radiosensitivity and serum p53-Abs.

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## COMMENTS

### Background

Mutations of the p53 gene are common gene alterations in most malignant tumors, including esophageal carcinoma. Mutant p53 protein is accumulated in the cell because of its longer half-life compared with wild-type protein. p53 protein plays a crucial role in the regulation of the cell cycle and has been implicated in cell differentiation, apoptosis, DNA synthesis and repairment. In addition, it has been shown that patients with various types of neoplasias have p53 antibodies in their sera. ELISA was used to detect anti-p53 antibodies in the sera. The authors explored the relationship between serum p53-Abs and clinicopathological characteristics and therapeutic effect in patients with esophageal carcinoma, and investigated the sequential changing regularity of serum p53-Abs after radiotherapy.

### Research frontiers

Studies have revealed that overexpression of p53 protein is closely related to induction of drug resistance in cancer patients and it can be used as a predictor for chemosensitivity to tumor treatment. Specific p53-Ab was found in the sera of cancer patients with p53 protein overexpression. Furthermore, the presence of serum p53-Ab is closely correlated with p53 protein overexpression. Thus, serum p53-Ab could theoretically be useful in predicting radiosensitivity in cancer patients. However, the clinical relevance of p53-Ab and radiotherapy for esophageal carcinoma has not been fully studied.

### Innovations and breakthroughs

From the results of this study, the following conclusions can be drawn that the positive rate of serum p53-Abs in esophageal carcinoma is related to histological grade, disease stage and lymph node metastasis, but it is not significantly related to sex, age and the size and site of tumor. The level and positive rate of serum p53-Abs had significant differences between before radiotherapy, after administration of an irradiation dose of 40 Gy/20 f/24 d and after administration of an irradiation dose of 60 Gy/30 f/36 d. The positive rate of serum p53-Abs in esophageal carcinoma patients with effect is significantly lower than those without effect after radiotherapy.

### Applications

Detection of serum p53-Abs is helpful to diagnosis of esophageal carcinoma. Monitoring of sequential change of serum p53-Abs before and after radiotherapy in patients with esophageal carcinoma is also useful in evaluating the response to the treatment and prognosis of the patients.

### Peer review

The authors detected the serum p53 antibody level in patients with esophageal

squamous cell carcinoma and its sequential changes before and after radiotherapy. The results showed that changes of serum p53-Abs are helpful to the diagnosis of esophageal carcinoma. Monitoring of sequential change of serum p53-Abs before and after radiotherapy in patients with esophageal carcinoma is helpful to evaluating the response to the treatment and prognosis of the patients. Therefore, this study has values in clinical management of the patients.

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CASE REPORT

## Takayasu's arteritis following Crohn's disease in a young woman: Any evidence for a common pathogenesis?

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## INTRODUCTION

Takayasu's arteritis is a granulomatous panarteritis affecting large vessels, namely the aorta and its main branches, pulmonary and coronary arteries. It is characterised by stenotic and, occasionally, aneurysmal lesions. Manifestations of the disease range from no symptoms, to bruits, hypertension, cerebrovascular events, congestive heart failure and blindness<sup>[1]</sup>. Takayasu's arteritis classically progresses through three stages, namely a systemic non-vascular phase, a vascular inflammatory phase and a quiescent "burnt out" phase<sup>[1]</sup>. First described by Yamamoto in 1830<sup>[1]</sup>, the disease has a worldwide distribution, although it is most commonly diagnosed in young women of Asian decent. The incidence in North America has been estimated at 2.6 cases per million<sup>[2]</sup>.

Crohn's disease is a chronic, relapsing, transmural inflammatory disease, affecting primarily the gastrointestinal mucosa. The incidence of Crohn's disease is approximately 2 in 100 000, and its prevalence is 50 in 100 000<sup>[3]</sup>, with geographical and ethnic variation, most commonly seen in Caucasian populations. There is a slight female preponderance, with a peak onset of disease around 20 years of age, with another peak occurring between 50 and 70 years of age<sup>[4]</sup>.

Both Takayasu's arteritis and Crohn's disease are chronic granulomatous inflammatory processes involving different target organs, namely large elastic arteries and the gastrointestinal tract respectively. They may coexist and in 29 reported cases, Crohn's disease

## Abstract

Takayasu's arteritis and Crohn's disease are chronic inflammatory diseases of uncertain aetiology. They rarely occur together, with only twenty nine cases of co-existent Takayasu's arteritis and Crohn's disease reported in the literature. In 88% of these cases, Takayasu's arteritis was diagnosed simultaneously or following a diagnosis of Crohn's disease. We present a case of a young Caucasian medical student, incidentally found to have bilateral carotid bruits on auscultation by a colleague. Magnetic resonance angiography revealed stenoses of the common carotid arteries with established collaterals, and a diagnosis of Type 1 Takayasu's arteritis was made. An <sup>18</sup>F-fluorodeoxyglucose positron emission tomography scan revealed no active disease. Nine months later, she presented with a short history of abdominal pain, vomiting and abdominal distension. Barium follow-through and computer tomography revealed a terminal ileal stricture and proximal small bowel dilation. An extended right hemicolectomy was performed and histopathology supported a diagnosis of Crohn's disease. This case report is presented with a particular focus on the temporal relationship between these two disease processes and explores whether their concurrence is more than just co-incidence.

preceded Takayasu's arteritis in 88% cases, raising the possibility that it could be regarded as an extraintestinal manifestation of Crohn's disease. In contrast, herein we present a rare case of Crohn's disease in a patient with clinically inactive Takayasu's arteritis.

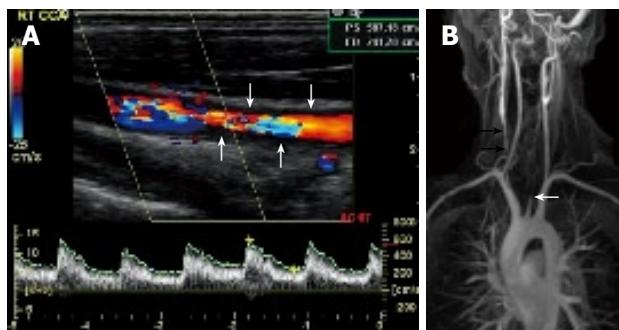
## CASE REPORT

A 24-year-old Caucasian medical student was found incidentally to have bilateral carotid bruits whilst practising for final clinical examinations. Her past medical history was unremarkable. Physical examination revealed normal peripheral pulses, equal normal blood pressure in both arms and no cardiac murmurs. A C-reactive protein of 13 mg/L, haemoglobin of 11.8 g/dL and a plasma viscosity of 1.73 mmHg were recorded.

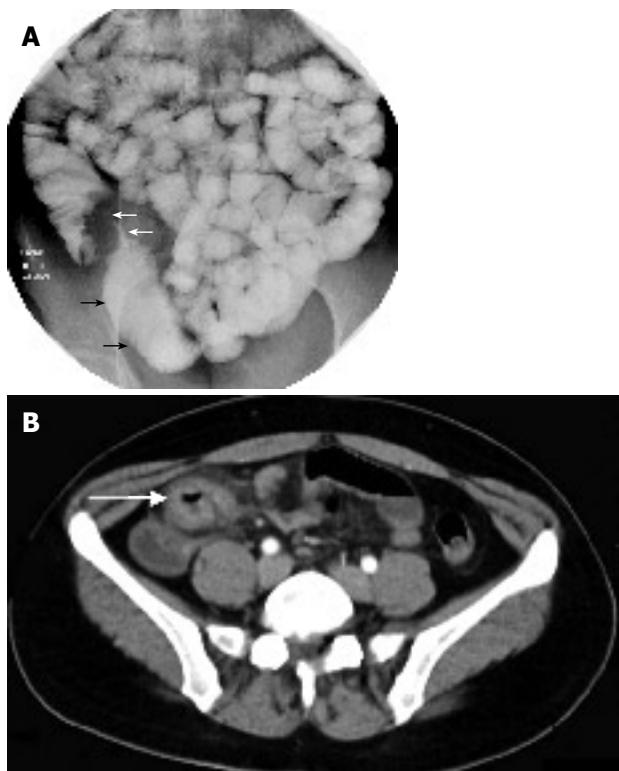
A duplex scan showed thickening of the proximal common carotid with severe stenoses in the mid common carotid arteries bilaterally (Figure 1A). Reduced internal carotid flow was compensated by retrograde flow in both external carotid arteries. Gadolinium-enhanced magnetic resonance angiography confirmed these findings along with a normal aortic arch, prominent bilateral vertebral arteries, mild irregularity in the right subclavian artery and collaterals feeding both external carotid arteries (Figure 1B). These findings led to the diagnosis of Type I Takayasu's arteritis<sup>[1]</sup>. Subsequent echocardiography confirmed the absence of aortic regurgitation and computer tomography-angiography of the subdiaphragmatic aorta was unremarkable. A normal full body  $^{18}\text{F}$ -fluorodeoxyglucose positron emission tomography ( $^{18}\text{F}$ -FDG-PET) scan revealed no active disease.

Nine months later, she presented with a short history of colicky abdominal pain, vomiting and abdominal distension. Barium studies and a computer tomography scan revealed a terminal ileal stricture and proximal small bowel dilation (Figure 2). At operation, the obstructing lesion was found to have extended to the ileocaecal valve with tethering to the proximal transverse colon. A laparoscopic extended right hemicolectomy was performed with a primary anastomosis, from which a full recovery was made and the patient remains symptom free.

The resected specimen showed a three-centimetre long stricture at the distal ileum extending to the ileocaecal valve (Figure 3A), and the strictured bowel had a thickened wall with fat wrapping. Along the stricture, the small bowel mucosa was replaced by intensely inflamed granulation tissue and inflamed transmural fibrous tissue focally replaced the muscularis propria (Figure 3A). Within this tissue, there were scattered medium-sized blood vessels showing intramural chronic inflammation with focal obliteration. In addition, one blood vessel showed a cluster of multinucleated giant cells along the outer edge of the media (Figure 3D). However, the majority of similarly-sized vessels at and around the stricture and the ileocolic artery were normal. The ileum just proximal to the stricture showed focal ulceration, transmural chronic inflammation and ulcer-associated cell lineage (also known as gastric pyloric gland metaplasia) (Figure 3C). Well-formed granulomas were present in the

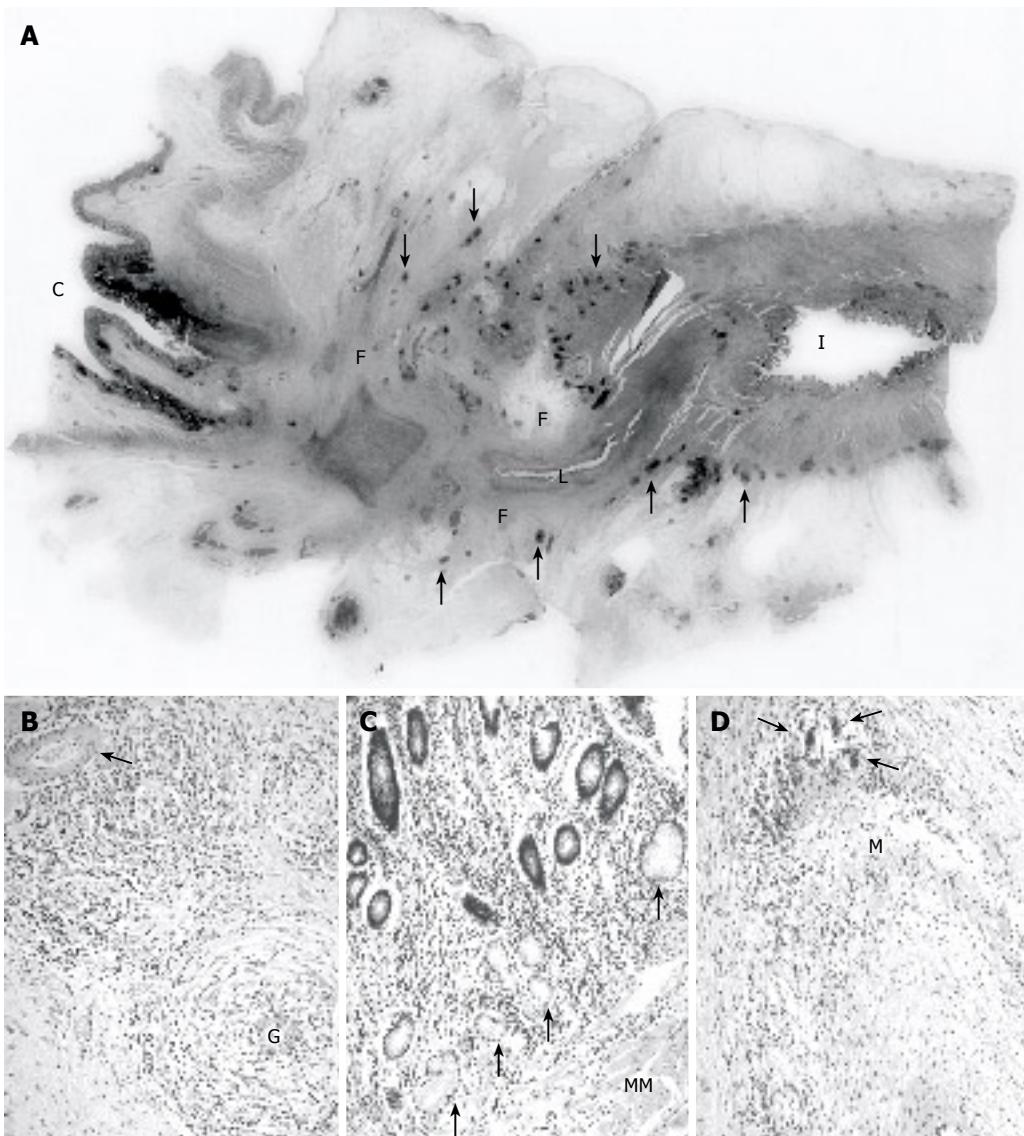


**Figure 1** Ultrasound image of the right common carotid artery showing wall thickening (arrows) and blood flow with a significantly enhanced peak systolic velocity (PS  $> 587 \text{ cm/s}$ ), indicating a severe stenosis (A) and magnetic resonance angiography showing occlusion of the left common carotid (white arrows) and stenosis of the right common carotid (black arrows) arteries (B).



**Figure 2** Barium follow through showing a stricture at the terminal ileum (white arrows) typical of Crohn's disease with dilated ileum just proximal to it (black arrows) (A) and axial CT image showing the thickened terminal ileum (arrow) and associated inflammatory change (B).

caecal submucosa just distal to the stricture and in the subserosal tissues surrounding the stricture (Figure 3B). The presence of a fistula connecting the stricture to the transverse colon was confirmed histologically. Seventeen lymph nodes harvested showed reactive changes only, with no evidence of granulomas. Several features of this specimen strongly support a diagnosis of Crohn's disease. The stricture occurred at a site classically affected by Crohn's disease, and showed several characteristic features of this disease, namely fat wrapping, fistula formation, transmural fibrosis and inflammation, multiple granulomas (which could not be attributed to crypt rupture or extravasated intestinal contents),



**Figure 3** **A:** Whole histological mount of the terminal ileal stricture showing part of its stenosed lumen (L), with the caecum (C) and ileum (I) at either end. The stricture showed transmural chronic inflammation (examples of the numerous lymphoid follicles are arrowed) with extensive fibrosis (F); **B:** Well-formed granulomas (G) with surrounding chronic inflammation in the subserosal tissues around the stricture with no vasculitis in an arrowed small muscular artery; **C:** Ileal mucosa proximal to the stricture showing extensive ulcer-associated cell lineage (arrowed) and chronic inflammation extending into the hypertrophied muscularis mucosae (MM); **D:** A medium-sized muscular artery within the fibrous tissue of the stricture showing chronic inflammation and multinucleated giant cells (arrowed) concentrated around the outer edge of the media (M) and the lumen of the artery in the bottom right corner.

and widespread adjacent ulcer-associated cell lineage. By contrast, there was no evidence that Takayasu's arteritis directly caused the ileal stricture and inflammation. Takayasu's arteritis is a vasculitis of large vessels. If the arteritis involved a large vessel supplying the abnormal terminal ileum, namely the superior mesenteric artery or its proximal branches, the most likely intestinal manifestation would have been infarction, and a much longer segment of bowel. The largest blood vessel included in the resection specimen (i.e. the ileocolic artery) did not show vasculitis. In fact, the only abnormal vessels seen were occasionally inflamed and focally occluded medium-sized arteries around the stricture, and even these were more likely to represent endarteritis obliterans secondary to the inflammation. Finally, 'granulomatous vasculitis', as was seen in one medium-sized artery beside the stricture, is a recognised feature of Crohn's disease.

## DISCUSSION

Co-existence of Takayasu's arteritis and Crohn's disease has previously been reported in 29 cases. The expected prevalence of Crohn's disease in patients with Takayasu's

arteritis, if present by chance alone, is approximately 0.05%-0.2%<sup>[3]</sup>. Thus, it has been suggested that this unexpected association is more than just a coincidence. The epidemiology, aetiology and pathogenesis of these two diseases point to a role of both environmental and hereditary factors, interacting to trigger the inflammatory process.

Takayasu's arteritis and Crohn's disease present more commonly in young females, suggesting a potential hormonal influence. To this end, the oral contraceptive pill has been reported, somewhat controversially, to increase the risk of Crohn's disease<sup>[4]</sup>. Geographically, Takayasu's arteritis is common in Japan, Korea and India, whereas Crohn's disease has a much higher prevalence in Northern Europe and North America<sup>[1,4]</sup>. Such ethnic predilection has led to the investigation of human leucocyte antigens (HLA) in the pathophysiology. However, to date no common HLA genotype links these two disease processes. Likewise infectious agents have been implicated. In particular, mycobacterium tuberculosis (MTB) or autoimmunity evoked by this micro-organism. Although increased reactivity to, and circulating antibodies against mycobacterium heat shock protein, 65 have been identi-

fied in Takayasu's arteritis and Crohn's disease patients, there is no convincing evidence for a role of MTB in the pathogenesis<sup>[5,6]</sup>.

Cell-mediated immunological mechanisms play an important role in both diseases. In Takayasu's arteritis, autoantibodies against aortic endothelial cells may contribute to vascular dysfunction by increasing local cytokine synthesis, cellular adhesion molecule expression and endothelial cell apoptosis<sup>[7]</sup>. Pro-inflammatory cytokines such as tumour necrosis factor (TNF)  $\alpha$ , interleukin (IL)-6, IL-8, IL-12 and IL-18, are common to both, amplifying the inflammatory process<sup>[7,8]</sup>. Moreover, the anti-TNF $\alpha$  monoclonal antibody infliximab is an effective therapeutic agent for both Takayasu's arteritis and Crohn's disease, suggesting the presence of common inflammatory pathways<sup>[3,7]</sup>. Wakefield *et al*<sup>[9]</sup> have found evidence of granulomatous vasculitis, also present in our case, in 15 out of 24 patients when examining resected intestine from patients with Crohn's disease. Granulomatous inflammation is also seen in Takayasu's arteritis<sup>[10]</sup>. Despite these similarities, the question of why the arch of the aorta is the predominant site of tissue injury in Takayasu's arteritis, and the bowel wall in Crohn's disease remains to be answered.

In our patient, Takayasu's arteritis was asymptomatic and the presence of established collaterals, a negative  $^{18}\text{F}$ -FDG-PET scan and no MRA evidence for vascular wall inflammation, suggested that the peak activity was present some years ago. Thus, Crohn's disease appears to have presented apparently independent of Takayasu's arteritis. It was reported that only 3 of 24 previous cases with known details have Takayasu's arteritis preceded Crohn's disease<sup>[3,11-17]</sup>. An association between the conditions may simply reflect two diseases typically presenting in the same age group. However, a shared common cause resulting in a granulomatous vasculitis and end organ damage remains a possible explanation.

In summary, the temporal relationship between Takayasu's arteritis and Crohn's disease remains of interest, with the majority of cases presenting initially with Crohn's disease. Possible explanations for their association have been explored. However, no data can confirm an aetiological link between the two diseases. We present an interesting case of Crohn's disease arising in a patient apparently some years after Takayasu's arteritis, as indicated by the established carotid arterial collaterals. As the active phase of Takayasu's arteritis may be relatively short, and the disease can 'burn out', many cases may go undetected, as would this case had it not been for the astute medical student.

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CASE REPORT

# Gastric arterio-venous malformation emerging from splenic artery

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patient admission to the general surgery departments. There are various pathologies which lead to bleeding; however, bleeding due to gastric arterio-venous malformation is not commonly seen. In this article we present a patient with sustained hematemesis, whose medical workup revealed the bleeding source to be the stomach. The bleeding could not be stopped by upper endoscopy. Celiac trunk angiography demonstrated arterio-venous malformation emerging from the splenic artery and embolization of the vessel was performed. A search of the English medical literature for congenital anomalies such as arterio-venous malformations supplied by the splenic artery did not yield any similar descriptions.

## CASE REPORT

A 24-year-old previously healthy male was transferred to our general surgery department due to unsuccessful treatment of acute upper gastrointestinal bleeding. Ten days ago, he had been admitted to hospital due to hematemesis and melena. He underwent an upper endoscopy, which demonstrated a small gastric ulcer covered by a blood clot, without a visible vessel. The ulcer was endoscopically coagulated using Argon beam. Four days later the patient had sustained hematemesis again. He underwent a second endoscopy, which showed a large clot covering a gastric ulcer. During hospitalization he was treated with ten units of packed red blood cells. Due to the relapse of symptoms and massive transfusion of blood products an angiography was performed. An aberrant gastric arterio-venous malformation supplied by the splenic artery was demonstrated. Attempts to embolize the vessel failed and the patient was referred to our institute. Upon arrival, his pulse was 102 bpm, and blood pressure 140/80 mmHg. Blood tests showed hemoglobin level of 10.2 mg/dL. Naso-gastric tube was inserted and drained fresh blood. Celiac trunk angiography was performed and revealed an arterio-venous malformation emerging from the splenic artery (Figure 1). Arterial embolization was achieved by selective injection of embospheres (diameter of 0.5-0.7mm) and placement of several detachable coils until flow arrest was obtained (Figure 2). The procedure was performed without any immediate complications. Two days following the procedure the patient developed a fever of 38°C, and suffered from left

## Abstract

In this case report, we present a patient who suffered from gastrointestinal bleeding. The bleeding source was a gastric arterio-venous malformation emerging from the splenic artery. Attempts to stop the bleeding failed and therapeutic angiography succeeded in occluding the vessel. A search at the literature has not yielded any other case report describing this anatomical anomaly.

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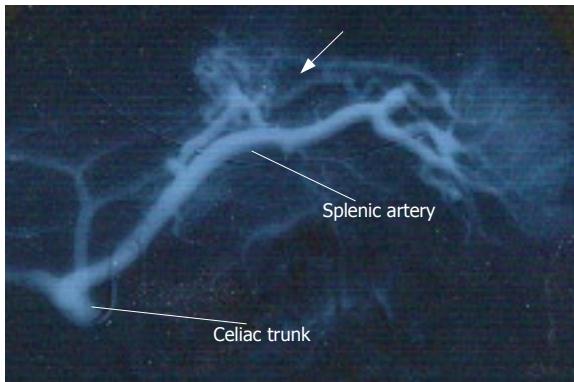
**Key words:** Arterio-venous; Malformation; Gastrointestinal; Bleeding; Angiography

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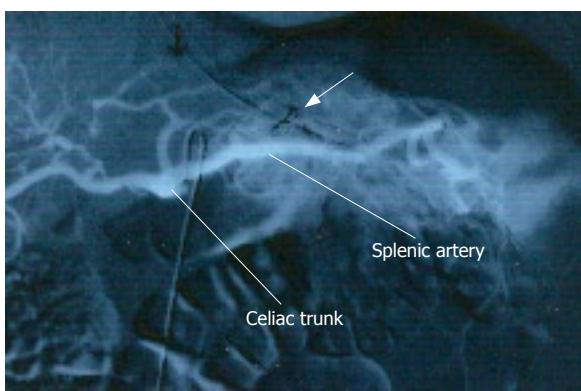
Elazary R, Verstandig A, Rivkind AI, Almogy G. Gastric arterio-venous malformation emerging from splenic artery. *World J Gastroenterol* 2008; 14(25): 4091-4092 Available from: URL: <http://www.wjgnet.com/1007-9327/14/4091.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4091>

## INTRODUCTION

Upper gastrointestinal bleeding is a common cause for



**Figure 1** Arteriography of the celiac trunk demonstrating the arterio-venous malformation emerging from the splenic artery (arrow).



**Figure 2** Embolization of the arterio-venous malformation was achieved using intra-arterial coils (arrow).

upper quadrant abdominal pain, both were attributed to segmental infarction of the spleen. Blood tests showed a constant level of hemoglobin. Eight days after the procedure he underwent elective upper endoscopy which showed mild fundal gastritis without an ulcer. On a six-month follow up, the patient presented no symptoms.

## DISCUSSION

Upper gastrointestinal bleeding is a common cause for admission of patients to emergency departments<sup>[1]</sup>. The most common etiologies are esophageal or gastric varices due to portal hypertension, erosive gastritis, peptic ulcers and gastric benign or malignant tumors. Other rare pathologies are aorto-enteric fistula which can cause massive upper gastrointestinal bleeding, gastric arterio-venous malformation<sup>[2]</sup> which is very common in patients who suffer from end stage renal failure, or Dieulafoy's

syndrome<sup>[3]</sup> which is a congenital dysplastic large caliber artery often lying at the submucosa of the gastric fundus, where erosion of the overlying mucosa is believed to cause significant bleeding. The pathogenesis of arterio-venous malformations of the splenic artery<sup>[4]</sup> is described in the medical literature as a cause of left sided portal hypertension (arterio-venous malformation is caused by blunt or penetrating trauma to the splenic artery which produces a fistula between the artery and the vein). A high flow of blood directly from the artery through the portal venous system elevates the portal blood pressure. Other pathology of the splenic artery which can cause massive upper gastrointestinal or intra-abdominal bleeding, is aneurysm of the artery. Aneurysms are the third most common intra-abdominal aneurysms, they are more common in multi-gravid women or patients with pancreatitis or abdominal trauma, and rarely cause any symptoms. Erosion into the gastric wall initiates gastric bleeding which may be massive.

The arterial supply of the vascular malformation described in this case emerged from the splenic artery, the patient did not sustain any abdominal trauma. It seems that an embryological developmental defect caused this congenital anomaly. Searching the English medical literature for articles describing this rare pathology has not found any similar descriptions. We assume that growing use of angiographic modalities for diagnosis and treatment of pathologies will show in the future more congenital anomalies which have not been characterized yet. The arterio-venous malformation has also supplied part of the spleen. According to this, we recommend not to discharge the patients too early after the procedure because of possible occurrence of complications related to partial infarction of the spleen. Usually such an infarction necessitates only observation and analgesia.

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CASE REPORT

# Non-Hodgkin lymphoma as a cause of obstructive jaundice with simultaneous extrahepatic portal vein obstruction: A case report

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no evidence of the disease for 7 mo after operation and will receive additional courses of chemotherapy.

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**Key words:** Non-Hodgkin lymphoma; Obstructive jaundice; Portal vein obstruction

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## Abstract

Non-Hodgkin lymphoma is a rare cause of biliary obstruction. To the best of our knowledge, non-Hodgkin lymphoma in the peripancreatic region causing obstructive jaundice with simultaneous portal vein (PV) invasion has not yet been reported. We present a 50-year-old patient with obstructive jaundice whose extrahepatic portal vein was obstructed by the invasion of a peripancreatic non-Hodgkin lymphoma. The patient denied any other symptoms such as recurrent fever, night sweat and loss of body weight. Computed tomography (CT) revealed a 10 cm mass in the retroperitoneal space behind the head of the pancreas causing obstruction of the distal bile duct and the PV. A pylorus-preserving pancreaticoduodenectomy combined with a PV resection was performed. The PV was reconstructed using an autologous right internal jugular vein graft. The resected specimen showed endoluminal invasion of both the bile duct and the PV. Histological examination showed the mass consisting of diffuse sheets of large malignant lymphoid cells. These cells were positive for CD20 and CD79a, partially positive for CD10, and negative for CD3, CD4, CD5, CD8 and CD30. The pathologic diagnosis was diffuse large B-cell type non-Hodgkin lymphoma and the patient was transferred to the Department of Hematology and Oncology for chemotherapy. He received four cycles of combined chemotherapy including cyclophosphamide, doxorubicin, vincristine and prednisone plus rituximab, and three cycles of intrathecal chemoprophylaxis including methotrexate, cytosine arabinoside and prednisone. The patient is alive with

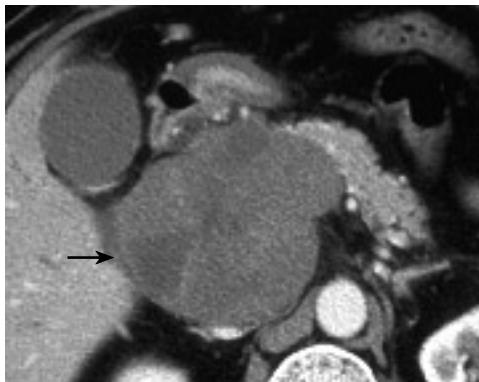
## INTRODUCTION

Non-Hodgkin lymphoma is a rare cause of biliary obstruction<sup>[1,2]</sup>. In a retrospective study, biliary obstruction secondary to non-Hodgkin lymphoma was found in 3 (0.3%) of 1123 patients with malignant biliary obstruction, and obstructive jaundice was found in one of them<sup>[1]</sup>. On the other hand, vascular invasion due to non-Hodgkin lymphoma has been described only as case reports including a case of primary non-Hodgkin lymphoma of the liver with bile duct invasion and portal venous tumor thrombus<sup>[3,4]</sup>.

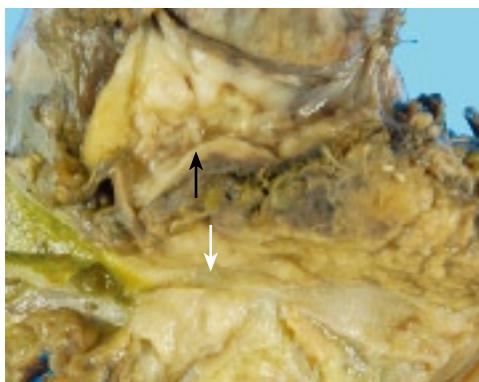
To the best of our knowledge, non-Hodgkin lymphoma in the peripancreatic region causing obstructive jaundice with simultaneous portal vein (PV) invasion has not yet been reported. Here we present a patient with obstructive jaundice whose extrahepatic PV was obstructed by a peripancreatic non-Hodgkin lymphoma.

## CASE REPORT

A 50-year-old man was reported to have an elevated serum lactate dehydrogenase (LDH) level (269 IU/L)



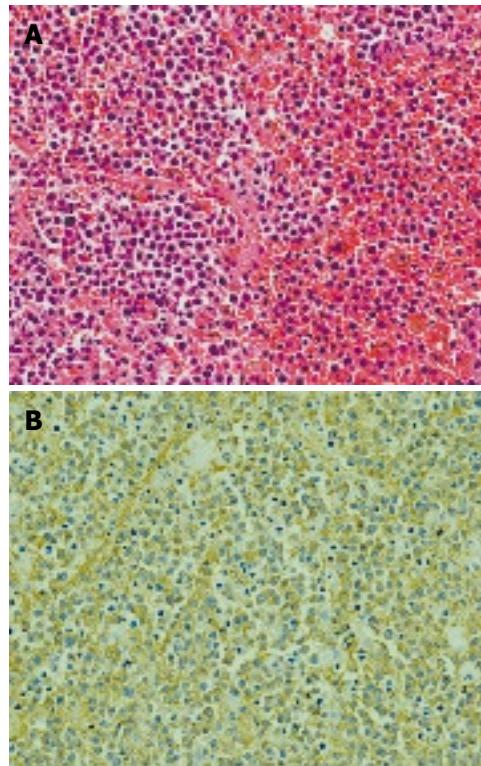
**Figure 1** Preoperative enhanced computed tomography. A low density mass (arrow), 10 cm in diameter, can be seen in the retroperitoneal space behind the pancreas head.



**Figure 2** A photograph of the resected specimen. Endoluminal invasion of both the bile duct (white arrow) and the portal vein (black arrow) can be observed.

by medical checkup in July 2007. He consulted with a physician in the following month. Serum LDH level continued to increase (313 IU/L). Abdominal ultrasonography was scheduled but he did not show up. On October 9, 2007, he presented with jaundice and was referred to our hospital on the next day. The patient denied any other symptoms such as recurrent fever, night sweat, and loss of body weight. His past history was unremarkable excluding a history of nephritis in his childhood. He had smoked 30 cigarettes per day, and was not alcoholic. His height was 173 cm and body weight 73 kg, blood pressure 124/79 mmHg and pulse rate 68 beats/min. No superficial lymphadenopathy was palpable. Computed tomography (CT) revealed a low-density mass, 10 cm in diameter, in the retroperitoneal space behind the pancreas head (Figure 1), due to which the distal bile duct and the PV were obstructed.

On October 22, 2008, a pylorus-preserving pancreaticoduodenectomy combined with a PV resection was performed. The PV was reconstructed using an autologous right internal jugular vein graft. The resected specimen showed endoluminal invasion of both the bile duct and the PV (Figure 2). Histological examination showed the mass consisting of diffuse sheets of large malignant lymphoid cells (Figure 3A). Immunohistochemical studies revealed that these cells were positive for CD20 (B-cell associated antigen, Figure 3B) and CD79a (B-cell associated



**Figure 3** Photomicrographs of the tumor. A: The tumor consists of diffuse sheets of large malignant lymphoid cells (HE, x 20); B: Immunostaining of the tumor. The tumor cells are positive for CD20 (immunostaining, x 20).

antigen), and partially positive for CD10 (B-cell associated antigen), and negative for CD3 (T-cell associated antigen), CD4 (T-cell associated antigen) CD5 (T-cell associated antigen), CD8 (T-cell associated antigen) and CD30 (a universal feature of anaplastic large cell lymphoma). The pathologic diagnosis was diffuse large B-cell type non-Hodgkin lymphoma. The patient was transferred to the Department of Hematology and Oncology.

On January 9, 2008, combined chemotherapy including cyclophosphamide, doxorubicin, vincristine, and prednisone plus rituximab (CHOP-R) was started. During the next 4 mo, he received 4 cycles of CHOP-R, and 3 cycles of combination of intrathecal chemoprophylaxis including methotrexate, cytosine arabinoside, and prednisone. The patient is alive with no evidence of the disease for 7 mo after the operation, and will receive additional courses of chemotherapy.

## DISCUSSION

Because of its rarity, non-Hodgkin lymphoma is seldom considered as a differential diagnosis in patients presenting with jaundice<sup>[2]</sup>. In one retrospective study by Ravindra *et al*, 6 of 9 patients with non-Hodgkin lymphoma presenting obstructive jaundice underwent laparotomy before the diagnosis was made as in the present case<sup>[2]</sup>, whereas, the other 3 patients were diagnosed by biopsy and could avoid laparotomy due to the following clinical features. The first patient with obstructive jaundice also had cervical lymphadenopathy, and cervical lymph node biopsy was performed, the second patient was considered too old to withstand the operation, and un-

derwent an ultrasonographically guided percutaneous biopsy, and the third patient had a history of lymphoma and jaundice 1 year after complete remission, which suggested recurrence<sup>[2]</sup>. In our case, the patient had none of such clinical findings as described above. Furthermore, our case presented with not only obstructive jaundice but also simultaneous portal venous obstruction. Such clinical features were not observed in the Ravindra's cases<sup>[2]</sup>. The common etiology of malignant biliary obstruction occurring simultaneously with extrahepatic PV invasion is generally pancreatic carcinoma or cholangiocarcinoma<sup>[5]</sup>, and we had no idea of lymphoma for a differential diagnosis in this case before operation.

Chemotherapy is the mainstay of treatment for non-Hodgkin lymphoma<sup>[6,7]</sup>. Surgical intervention delayed the initiation of chemotherapy in the present case, which could miss an opportunity to cure the disease. Although it remains unclear whether chemotherapy should precede the biliary drainage procedures in patients with non-Hodgkin lymphoma presenting with jaundice, chemotherapy alone usually alleviate the obstructive jaundice without biliary drainage<sup>[7]</sup>. Dudgeon *et al* described five patients with non-Hodgkin lymphoma causing obstructive jaundice, who were treated with combined chemotherapy without prior surgical or endoscopic biliary decompression, or radiation therapy. Jaundice was relieved rapidly (within 2-59 d) in all the patients with acceptable toxic effects, and all the five patients achieved remissions<sup>[8]</sup>.

Fine needle aspiration (FNA) with percutaneous or endoscopic ultrasound (EUS) guidance should precede the operation in the present case. FNA with percutaneous or EUS guidance permits both morphologic and cytologic analysis of lesions at various locations such as within or adjacent to the gastrointestinal tract, and intra-abdominal and retroperitoneal masses<sup>[9-11]</sup>. Erickson and colleagues studied 18 patients with retroperitoneal lesions who underwent EUS and EUS-guided FNA<sup>[9]</sup>. EUS-guided FNA was done in 15 (83%) of the 18 patients, among whom four were diagnosed as having lymphomas and avoided surgical intervention<sup>[9]</sup>, whereas, one patient ultimately had to have exploratory surgery for biopsy because neither EUS-guided FNA nor CT-guided percutaneous biopsy could provide enough tissues for definitive classification of a follicular centroblastic centrocytic lymphoma<sup>[9]</sup>. Similarly, in one retrospective study, 9 patients with non-Hodgkin lymphoma presented with obstructive jaundice<sup>[2]</sup>. Among them, two underwent percutaneous biopsy, and both avoided laparotomy.

One disadvantage of FNA with percutaneous or EUS guidance is a risk of complications. The possible complications associated with FNA included infection, intracystic hemorrhage, retroperitoneal hematoma, and pancreatitis<sup>[10,11]</sup>. Eloubeidi *et al* studied 547 patients who underwent EUS-guided FNA in the preoperative evaluation of suspected pancreatic cancer<sup>[10]</sup>. In their report, 11 (2%) patients developed a major complication, and one patient required surgical debridement for necrosis<sup>[10]</sup>. Guo *et al* reported that one (1.5%) of 68 patients had a minor complication (hematoma) from the radiologically guided percutaneous FNA biopsy of pelvic and retro-

peritoneal masses<sup>[11]</sup>.

Another disadvantage of FNA with percutaneous or EUS guidance is a risk of tumor seeding along a needle tract<sup>[12,13]</sup>. Although the EUS-guided FNA is considered to have a lower risk of peritoneal carcinomatosis compared with percutaneous one<sup>[12]</sup>, a case of tumor seeding of a pancreatic adenocarcinoma because of EUS-guided FNA has been reported<sup>[13]</sup>. Therefore, FNA with percutaneous or EUS guidance should be indicated only when additional information accessible by the procedure significantly affects the subsequent management of patients.

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## Meetings

### Events Calendar 2008-2009

FALK SYMPOSIA 2008  
January 24-25, Frankfurt, Germany  
Falk Workshop: Perspectives in Liver Transplantation

International Gastroenterological Congresses 2008  
February 14-16, Paris, France  
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies  
[www.easl.ch/hepatitis-conference](http://www.easl.ch/hepatitis-conference)

February 14-17, Berlin, Germany  
8<sup>th</sup> International Conference on New Trends in Immunosuppression and Immunotherapy  
[www.kenes.com/immuno](http://www.kenes.com/immuno)

February 28, Lyon, France  
3<sup>rd</sup> Congress of ECCO - the European Crohn's and Colitis Organisation Inflammatory Bowel Diseases 2008  
[www.ecco-ibd.eu](http://www.ecco-ibd.eu)

February 29, Québec, Canada  
Canadian Association of Gastroenterology  
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK  
British Society of Gastroenterology Annual Meeting  
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China  
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea  
Asian Pacific Association for the Study of the Liver  
18<sup>th</sup> Conference of APASL: New Horizons in Hepatology  
[www.apaslseoul2008.org](http://www.apaslseoul2008.org)

March 29-April 1, Shanghai, China  
Shanghai-Hong Kong International Liver Congress  
[www.livercongress.org](http://www.livercongress.org)

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco  
OESO 9<sup>th</sup> World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas  
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA  
SAGES 2008 Annual Meeting - part of Surgical Spring Week  
[www.sages.org/08program/html/](http://www.sages.org/08program/html/)

April 18-22, Buenos Aires, Argentina  
9<sup>th</sup> World Congress of the International Hepato-Pancreato Biliary Association  
Association for the Study of the Liver  
[www.ca-ihpba.com.ar](http://www.ca-ihpba.com.ar)

April 23-27, Milan, Italy  
43<sup>rd</sup> Annual Meeting of the European Association for the Study of the Liver  
[www.easl.ch](http://www.easl.ch)

May 2-3, Budapest, Hungary

Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA  
Digestive Disease Week 2008

May 21-22, California, USA  
ASGE Annual Postgraduate Course  
Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion  
E-mail: education@sgo.org

June 4-7, Helsinki, Finland  
The 39<sup>th</sup> Nordic Meeting of Gastroenterology  
[www.congrex.com/ngc2008](http://www.congrex.com/ngc2008)

June 5-8, Sitges (Barcelona), Spain  
Semana de las Enfermedades Digestivas  
E-mail: sepd@sepde.es

June 6-8, Prague, Czech Republic  
3<sup>rd</sup> Annual European Meeting: Perspectives in Inflammatory Bowel Diseases  
E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey  
ESGAR 2008 19<sup>th</sup> Annual Meeting and Postgraduate Course  
E-mail: fca@netvisao.pt

June 11-13, Stockholm, Sweden  
16<sup>th</sup> International Congress of the European Association for Endoscopic Surgery  
E-mail: info@aes-eur.org

June 13-14, Amsterdam, Netherlands  
Falk Symposium 165: XX International Bile Acid Meeting. Bile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic  
Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management  
E-mail: idca2008@guarant.cz

June 25-28, Barcelona, Spain  
10<sup>th</sup> World Congress on Gastrointestinal Cancer  
Imedex and ESMO  
E-mail: meetings@imedex.com

June 25-28, Lodz, Poland  
Joint Meeting of the European Pancreatic Club (EPC) and the International Association of Pancreatology (IAP)  
E-mail: office@epc-iap2008.org  
[www.e-p-c.org](http://www.e-p-c.org)  
[www.pancreatology.org](http://www.pancreatology.org)

June 26-28, Bratislava, Slovakia  
5<sup>th</sup> Central European Gastroenterology Meeting  
[www.ceurgem2008.cz](http://www.ceurgem2008.cz)

July 9-12, Paris, France  
ILTS 14<sup>th</sup> Annual International Congress  
[www.ilts.org](http://www.ilts.org)

September 10-13, Budapest, Hungary  
11<sup>th</sup> World Congress of the International Society for Diseases of the Esophagus  
E-mail: isde@isde.net

September 13-16, New Delhi, India  
Asia Pacific Digestive Week  
E-mail: apdw@apdw2008.net

III FALK GASTRO-CONFERENCE  
September 17, Mainz, Germany

Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany  
Falk Symposium 166: GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic  
Prague Hepatology Meeting 2008  
[www.czech-hepatology.cz/phm2008](http://www.czech-hepatology.cz/phm2008)

September 20-21, Mainz, Germany  
Falk Symposium 167: Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France  
Third Annual Meeting  
European Society of Coloproctology  
[www.escp.eu.com](http://www.escp.eu.com)



October 8-11, Istanbul, Turkey  
18<sup>th</sup> World Congress of the International Association of Surgeons,  
Gastroenterologists and Oncologists  
E-mail: orkun.sahin@serenash.com.tr

October 18-22, Vienna, Austria  
16<sup>th</sup> United European Gastroenterology Week  
[www.negf.org](http://www.negf.org)  
[www.acv.at](http://www.acv.at)

October 22-25, Minnesota, USA  
Anstralian Gastroenterology Week 2008  
E-mail: gesa@gesa.org.au

October 22-25, Brisbane, Australia  
71<sup>st</sup> Annual Colon and Rectal Surgery Conference  
E-mail: info@colonrectalcourse.org

October 31-November 4, Moscone West Convention Center, San Francisco, CA  
59<sup>th</sup> AASLD Annual Meeting and Postgraduate Course  
The Liver Meeting  
Information: [www.aasld.org](http://www.aasld.org)

November 6-9, Lucerne, Switzerland  
Neurogastroenterology & Motility Joint International Meeting 2008  
E-mail: ngm2008@mci-group.com  
[www.ngm2008.com](http://www.ngm2008.com)

November 12, Santiago de Chile, Chile  
Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

November 28-29, Cairo, Egypt  
1<sup>st</sup> Hepatology and Gastroenterology Post Graduate Course  
[www.egyptgastrohep.com](http://www.egyptgastrohep.com)

December 7-9, Seoul, Korea  
6<sup>th</sup> International Meeting Hepatocellular Carcinoma: Eastern and Western Experiences  
E-mail: sglee@amc.seoul.kr

INFORMATION FOR ALL  
FALK FOUNDATION e.V.  
E-mail: [symposia@falkfoundation.de](mailto:symposia@falkfoundation.de)  
[www.falkfoundation.de](http://www.falkfoundation.de)

Advanced Courses - European

Institute of Telesurgery EITS - 2008  
Strasbourg, France  
January 18-19, March 28-29, June 6-7, October 3-4

N.O.T.E.S  
April 3-5, November 27-29  
Laparoscopic Digestive Surgery

June 27-28, November 7-8  
Laparoscopic Colorectal Surgery

July 3-5  
Interventional GI Endoscopy Techniques  
Contact address for all courses:  
E-mail: info@eits.fr

International Gastroenterological Congresses 2009  
March 23-26, Glasgow, Scotland  
Meeting of the British Society of Gastroenterology (BSG)  
E-mail: bsg@mailbox.ulcc.ac.uk

May 17-20, Denver, Colorado, USA  
Digestive Disease Week 2009

November 21-25, London, UK  
Gastro 2009 UEGW/World Congress of Gastroenterology  
[www.gastro2009.org](http://www.gastro2009.org)



### Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London, The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.



## Instructions to authors

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*World Journal of Gastroenterology* (*World J Gastroenterol* ISSN 1007-9327 CN 14-1219/R) is a weekly open access peer-reviewed journal supported by an editorial board consisting of 1208 experts in gastroenterology and hepatology from 60 countries. The aim of the journal is to deliver the most clinically relevant original and commentary articles to readers, and to make the full text publicly available to all clinicians, scientists, patients and biomedical students on an unrestricted platform, so that they can access and learn about the most recent key advances in the field.

In addition to the open access nature, another key characteristic of *WJG* is its reading guidance for each article which includes background, research frontier, related reports, breakthroughs, applications, terminology, and comments of peer reviewers for the general readers.

*WJG* publishes articles on esophageal, gastrointestinal, hepatobiliary and pancreatic tumors, and other esophageal, gastrointestinal, hepatic-biliary and pancreatic diseases in relation to epidemiology, immunology, microbiology, motility & nerve-gut interaction, endocrinology, nutrition & obesity, endoscopy, imaging and advanced hi-technology.

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#### Indexed and abstracted in

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#### Published by

The WJG Press

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Full manuscript title, running title, all author(s) name(s), affiliations, institution(s) and/or department(s) where the work was carried out; author contributions; disclosure of any financial support for the research; and the name, full address, telephone and fax numbers and email address of the corresponding author should be included. Titles should be concise and informative (remove all unnecessary words), emphasize what is new, and avoid abbreviations. A short running title of less than 40 letters should be provided. List the author(s)' name(s) as follows: initial and/or first name, middle name or initial(s), and full family name.

**Author contributions:** The format of this section should be like this: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed research; Wang CL, Zou CC, Hong F and Wu XM performed research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed data; and Wang CL, Liang L and Fu JF wrote the paper.

**Peer reviewers:** All articles received are subject to peer review. Normally, three experts are invited for each article. Decision for acceptance is made only when at least two experts recommend an article for publication. Reviewers for accepted manuscripts are acknowledged in each manuscript, and reviewers of articles which were not accepted will be acknowledged at the end of each issue. To ensure the quality of the articles published in *WJG*, reviewers of accepted manuscripts will be announced by publishing the name, title/position and institution of the reviewer in the footnote accompanying the printed article. For example, reviewers: Professor Jing-Yuan Fang, Shanghai Institute of Digestive Disease, Shanghai, Affiliated Renji Hospital, Medical Faculty, Shanghai Jiaotong University, Shanghai, China; Professor Xin-Wei Han, Department of Radiology, The First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan Province, China; and Professor Anren Kuang, Department of Nuclear Medicine, Huaxi Hospital, Sichuan University, Chengdu, Sichuan Province, China.

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An informative, structured abstract of no more than 350 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections: AIM: Only the purpose should be included. METHODS: The materials, techniques, instruments and equipment, and the experimental procedures should be included. RESULTS: The observed and experimental results, including data, effects, outcome, etc. should be included. Authors should present *P* value where necessary, and also include any significant data. CONCLUSION: Accurate view and the value of the results should be included.

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#### Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

#### Text

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and case reports, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the body text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, should be found at: <http://www.wjgnet.com/wjg/help/instructions.jsp>.

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Figures should be numbered as 1, 2, 3, etc., and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: <http://www.wjgnet.com/1007-9327/13/4520.pdf>; <http://www.wjgnet.com/1007-9327/13/4554.pdf>; <http://www.wjgnet.com/1007-9327/13/4891.pdf>; <http://www.wjgnet.com/1007-9327/13/4986.pdf>; <http://www.wjgnet.com/1007-9327/13/4498.pdf>. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: ...etc. It is our principle to publish high resolution-figures for the printed and E-versions.

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### Notes in tables and illustrations

Data that are not statistically significant should not be noted. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 should be noted (*P* > 0.05 should not be noted). If there are other series of *P* values, <sup>c</sup>*P* < 0.05 and <sup>d</sup>*P* < 0.01 are used. A third series of *P* values can be expressed as <sup>e</sup>*P* < 0.05 and <sup>f</sup>*P* < 0.01. Other notes in tables or under illustrations should be expressed as <sup>1</sup>F, <sup>2</sup>F, <sup>3</sup>F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, □, ▲, △, etc., in a certain sequence.

### Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscripts and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

## REFERENCES

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The author should number the references in Arabic numerals according to the citation order in the text. Put reference numbers in square brackets in superscript at the end of citation content or after the cited author's name. For citation content which is part of the narration, the coding number and square brackets should be typeset normally. For example, "Crohn's disease (CD) is associated with increased intestinal permeability<sup>[1,2]</sup>". If references are cited directly in the text, they should be put together within the text, for example, "From references<sup>[19,22-24]</sup>, we know that..."

When the authors write the references, please ensure that the order in text is the same as in the references section, and also ensure the spelling accuracy of the first author's name. Do not list the same citation twice.

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### DOI requirement

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### Style for journal references

Authors: the name of the first author should be typed in bold-faced letters. The family name of all authors should be typed with the initial letter capitalized, followed by their abbreviated first and middle initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR). The title of the cited article and italicized journal title (journal title should be in its abbreviated form as shown in PubMed), publication date, volume number (in black), start page, and end page [PMID: 11819634 DOI: 10.3748/wjg.13.5396].

### Style for book references

Authors: the name of the first author should be typed in bold-faced letters. The surname of all authors should be typed with the initial letter capitalized, followed by their abbreviated middle and first initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR) Book title. Publication number. Publication place: Publication press, Year: start page and end page.

### Format

#### Journals

English journal article (list all authors and include the PMID where applicable)

- 1 Jung EM, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

- 2 Lin GZ, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixudiarrhoea. *Shijie Huaren Xiaohua Zaishi* 1999; **7**: 285-287

In press

- 3 Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

- 5 Vallancien G, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

Volume with supplement

- 7 Geraud G, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment

of migraine and in comparison with sumatriptan. *Headache* 2002; 42 Suppl 2: S93-99 [PMID: 12028325]

*Issue with no volume*

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (401): 230-238 [PMID: 12151900]

*No volume or issue*

- 9 Outreach: Bringing HIV-positive individuals into care. *HRS&A Careaction* 2002; 1-6 [PMID: 12154804]

## Books

*Personal author(s)*

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

*Chapter in a book (list all authors)*

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

*Author(s) and editor(s)*

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

*Conference proceedings*

- 13 **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

*Conference paper*

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

*Electronic journal (list all authors)*

- 15 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/eid.htm>

*Patent (list all authors)*

- 16 **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

## Inappropriate references

Authors should always cite references that are relevant to their article, and avoid any inappropriate references. Inappropriate references include those linked with a hyphen when the difference between the two numbers is greater than five. For example, [1-6], [2-14] and [1, 3, 4-10, 22] are all considered inappropriate references. Authors should not cite their own unrelated published articles.

## Statistical data

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Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as  $\chi^2$  (in Greek), related coefficient as *r* (in italics), degree of freedom as *v* (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

## Units

Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h, blood glucose concentration, *c* (glucose)  $6.4 \pm 2.1$  mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 24.5  $\mu$ g/L;  $\text{CO}_2$  volume fraction, 50 mL/L  $\text{CO}_2$ , not 5%  $\text{CO}_2$ ; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantums can be found at: <http://www.wjgnet.com/wjg/help/15.doc>.

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Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of

Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

## Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg 1*, *c myc*, *c fos*, etc.

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Biology: *H pylori*, *E coli*, etc.

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## Mechanisms of heme iron absorption: Current questions and controversies

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### Abstract

Iron is a critical micronutrient, and iron derived from heme contributes a large proportion of the total iron absorbed in a typical Western diet. Heme iron is absorbed by different mechanisms than non-heme iron, but despite considerable study over many years these mechanisms remain poorly understood. This review provides an overview of the importance of heme iron in the diet and discusses the two prevailing hypotheses of heme absorption; namely receptor mediated endocytosis of heme, and direct transport into the intestinal enterocyte by recently discovered heme transporters. A specific emphasis is placed on the questions surrounding the site of heme catabolism and the identity of the enzyme that performs this task. Additionally, we present the hypothesis that a non-heme iron transport protein may be required for heme iron absorption and discuss the experiences of our laboratory in examining this hypothesis.

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**Key words:** Iron; Heme absorption; Receptor mediated endocytosis; Heme transporter; Heme oxygenase

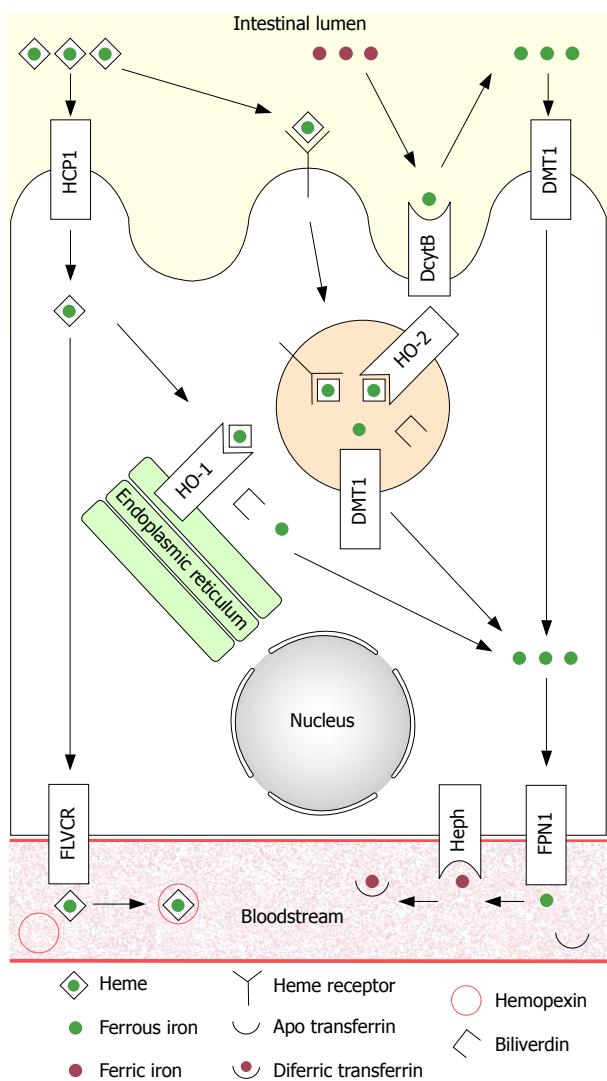
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### THE IMPORTANCE OF DIETARY IRON AND HEME

Iron is a vitally important element in biological terms (for review see<sup>[1]</sup>). Iron is a transition metal with the ability to readily accept and donate electrons, allowing it to function as an oxidant or reductant in a large number of biochemical reactions. In mammals, iron is notably required for oxygen transport as a component of hemoglobin, DNA synthesis as a component of ribonucleotide reductase, and as an electron acceptor/donor in the cytochromes that are essential for energy transduction. Currently, iron deficiency is the most common diet related health problem in the world<sup>[2]</sup>, and the effects on human health are wide ranging. Iron deficiency manifests as anaemia in up to 2 billion people, impairs physical and mental development in children, and can exacerbate many other health problems.

Heme is a biologically important iron containing compound and a key source of dietary iron. Historically, it was doubted that heme iron could be absorbed by the enterocyte and it was not until 1955 that the absorption of heme-derived iron was demonstrated for the first time<sup>[3]</sup>. Currently, the importance of heme iron in the diet cannot be underestimated. Studies estimate that in Western societies, iron derived from heme sources such as myoglobin and hemoglobin make up two-thirds of the average person's total iron stores despite only constituting one-third of the iron that is actually ingested<sup>[4-6]</sup>. This likely explains why vegetarians are more prone to iron deficiency than those who regularly consume red meat<sup>[7]</sup>.

The relative importance of dietary heme is attributable to its high bioavailability compared with



**Figure 1** Summary diagram of established and putative iron absorption pathways in the intestinal enterocyte. Non-heme iron: All non-heme iron is ultimately taken up from the lumen by DMT1 situated on the microvillus membrane, before joining the labile iron pool in the cytoplasm. Ferric iron must first be reduced to the ferrous form by DcytB before uptake. Ferrous iron in the labile iron pool is then transferred to the circulation by FPN1, which requires hephaestin for oxidation to the ferric form in order to bind to circulating apotransferrin. Heme iron: Heme iron is hypothesized to be taken up by receptor mediated endocytosis. Internalised heme is degraded by HO-2 inside the vesicles, releasing non-heme iron and generating biliverdin. The non-heme iron is then transported to the cytoplasm by DMT1. Heme iron may also be taken up by PCFT/HCP1 directly into the cytoplasm. Intact heme may be transported across the basolateral membrane by FLVCR where it binds circulating hemopexin. Alternatively, heme may be catabolized to non-heme iron and biliverdin by HO-1 located on the endoplasmic reticulum. Any iron released from heme inside the enterocyte, regardless of the mode of uptake, ultimately joins the labile iron pool and is transferred to the bloodstream by FPN1 in the same fashion as non-heme iron.

non-heme iron in the predominantly alkaline conditions found in the lumen of the small intestine. In aqueous solutions at or above pH 7.0, non-heme iron is present as Fe(II) and Fe(III). Fe(II) readily oxidizes to Fe(III) which precipitates from solution as ferric hydroxide or forms soluble hydroxyl-iron dimers which are not directly available for absorption<sup>[8]</sup>. Further, many dietary components (particularly humic substances such as tannins and phytate) can chelate iron making it non-

bioavailable<sup>[9-11]</sup>, while only select reductants in the diet (such as ascorbate) can act as solubilizing agents<sup>[12]</sup>.

In contrast polymerization of heme, which reduces its absorption, is minimised in alkaline conditions<sup>[13]</sup> while humic substances<sup>[14,15]</sup> and chelators such as desferrioxamine<sup>[16,17]</sup> do not reduce heme bioavailability. Heme solubility is also increased significantly by the presence of protein<sup>[13,18-20]</sup> which is important considering heme-rich foods typically contain high quantities of protein. Paradoxically, the absorption of heme iron cannot upregulate to the same extent as non-heme iron during iron deficiency<sup>[14,15,21-25]</sup>. This is possibly due to rate limitations at the step of heme catabolism (see 'Heme Catabolism' below) although the extent to which enterocyte adaptation (particularly transfer to the circulation by ferroportin<sup>[26-29]</sup> and humoral regulation by hepcidin<sup>[30-33]</sup>) affects heme iron absorption is very poorly characterised compared to non-heme iron.

Despite the clear importance of dietary heme as a source of body iron, the mechanism by which the enterocyte takes up heme and catabolizes it to utilise the iron is still poorly understood. Heme is taken up as an intact metalloporphyrin<sup>[34,35]</sup> and comparative studies show that a molecule with a similar size, structure, and ionic charge to heme (namely Vitamin B12) was not absorbed by enterocytes because of the absence of specific carriers on the apical membrane, whereas in identical conditions up to 19% of an equivalent dose of heme was absorbed<sup>[36]</sup>. This strongly suggests that heme uptake is a facilitated process, as opposed to simple diffusion.

Currently, there are two prevailing hypotheses explaining the mechanisms of this process; firstly, a long-standing hypothesis that heme is taken up by receptor mediated endocytosis; secondly, the recent discovery of a heme transporter that may have the capability of transferring heme from the small intestinal lumen directly into the cytoplasm. These pathways are summarised in Figure 1 and discussed in detail below.

## HEME RECEPTOR MEDIATED ENDOCYTOSIS

The hypothesis of heme uptake by receptor mediated endocytosis originated in 1979 from the discovery of a heme binding protein on the microvillus membrane of the upper small intestine of both pigs and humans<sup>[37]</sup>. Characterisation of this binding protein demonstrated that it was not albumin, hemopexin, glutathione-S-transferase, or aggregated heme<sup>[38]</sup>. The dissociation constant for the binder/heme complex was found to be  $10^{-6}$  to  $10^{-7}$  mol/L using radioactive ligands, and  $10^{-9}$  mol/L in spectral studies<sup>[39]</sup>. This high affinity binding, along with loss of binding capacity with trypsin digestion, indicated the presence of a protein receptor for heme on the microvillus membrane. Heme uptake by rat duodenal enterocytes increases with iron deficiency and correlates with a modest but statistically significant increase in heme binding capacity<sup>[36]</sup>, suggesting that the quantity of

receptor present on the microvillus membrane contributes to regulation of heme iron absorption.

A heme binding protein has also been characterised on the membrane of erythroleukemia cells with very similar properties to that observed in the duodenum<sup>[40]</sup>. These cell types are capable of internalising heme intact, as evidenced by the binding and uptake of heme-embedded latex beads<sup>[41,42]</sup>. Heme uptake is also temperature<sup>[43]</sup> and ATP<sup>[44]</sup> dependent, and these combined data provide strong evidence for the ability of cells to actively take up heme by endocytosis.

Morphological evidence corroborates this assessment. In two similar studies, heme or hemoglobin were administered into closed duodenal loops of rats or dogs, and duodenal tissue samples collected over a time period of up to 3 h thereafter<sup>[45,46]</sup>. Heme in the duodenal mucosa was reacted with DAB to produce an electron dense precipitate that was then observed by electron microscopy. Both studies reported the appearance of heme initially on the microvillus membrane, then within tubulovesicular structures in the apical cytoplasm, before collecting in vesicles identified as secondary lysosomes<sup>[46]</sup>. Heme disappeared from within these vesicles approximately 2–3 h after the initial dose. There was no heme observed in the basal cytoplasm or the extracellular space, consistent with heme uptake at the microvillus membrane by an endocytotic pathway and its catabolism within the apical cytoplasm of the cell.

One important criticism of the receptor mediated endocytosis hypothesis is that it assumes iron released from heme is transported out of the internalised vesicles in order to join the labile iron pool. Currently, no such transport process has been identified. However, it is possible that divalent metal transporter 1 (DMT1) may fulfil this role in a manner analogous to its role in the transferrin receptor cycle<sup>[47–50]</sup> (see ‘A Possible Role for DMT1?’ below).

## HEME TRANSPORTERS

In recent years, two mammalian heme transporters have been discovered, namely PCFT/HCP1<sup>[51,52]</sup> and FLVCR<sup>[53]</sup>. These appear to function independently of the putative heme receptor and receptor mediated endocytosis in that they act as a direct transfer process across plasma membranes. At this early stage the physiological relevance of these transporters to intestinal heme iron absorption is unclear, but the information that is available will be considered below.

The PCFT/HCP1 cDNA was initially isolated by the subtractive suppressive hybridisation of ileal cDNA from duodenal cDNA in hypotransferrinaemic mice<sup>[51]</sup>. Heme transport capability by PCFT/HCP1 has been demonstrated *in vitro*, with expression in Xenopus oocytes and the HeLa cell line resulting in a 2-fold to 3-fold increase in heme uptake. Heme uptake by HeLa cells expressing PCFT/HCP1 was greatly reduced at 4°C compared to 37°C which was interpreted as energy dependence, although without the specific use of metabolic inhibitors to distinguish between temperature

dependent kinetic properties of the transporter and a requirement for metabolic fuels, this interpretation is ambiguous.

Uptake of radio-labelled heme by transfected CHO cells was competitive with the uptake of unlabelled heme, zinc protoporphyrin, and protoporphyrin suggesting that transport is selective for the porphyrin ring, although no non-porphyrin competitors appear to have been assessed. However, the addition of PCFT/HCP1 siRNA to CHO cells did not reduce the substantial basal heme uptake of control cells<sup>[51]</sup>. Clearly, CHO cells have significant pre-existing heme acquisition pathways that are not related to PCFT/HCP1. *In vivo*, heme uptake from closed duodenal loops in normal and hypoxic mice was modestly reduced (30%–40%) by the addition of a PCFT/HCP1 antibody but no decrease was seen with pre-immune serum. PCFT/HCP1 gene expression was significantly increased by hypoxia but was not significantly altered by iron deficiency which appears contrary to the upregulation of heme binding and uptake shown in other studies<sup>[36]</sup>, although regulation of heme transport may instead be regulated by the sub-cellular location of PCFT/HCP1.

PCFT/HCP1 has been independently characterised as a folate/proton symporter and appears to play a key role in intestinal folate absorption<sup>[52,54]</sup>. This is evidenced by a 55%–80% reduction in pH dependent folate uptake in the enterocyte-like CaCo-2 cell line following RNA interference for PCFT/HCP1. Additionally, human patients diagnosed with hereditary folate malabsorption carry a point mutation to PCFT/HCP1 that results in the formation of a non-functional splice variant. Interestingly, the folate transport capabilities of PCFT/HCP1 are at least an order of magnitude higher than that observed for heme, suggesting that folate may be the more physiologically relevant target of this transport protein. It is clear that the generation of a knockout model for PCFT/HCP1 is required to assess the importance of this heme/folate transporter *in vivo*.

FLVCR was initially characterised as the cell surface protein receptor for feline leukaemia virus subgroup C which causes severe anaemia in infected cats<sup>[55,56]</sup>. It has since been demonstrated that loss of FLVCR function in erythropoietic cells is associated with impairment of erythroid maturation and increased apoptosis, and that heme content of erythropoietic primary cultures is dependent upon FLVCR expression<sup>[53]</sup>. Since cells expressing FLVCR are capable of actively exporting heme, it was concluded that FLVCR acts as an overflow valve for excess manufactured heme that would otherwise result in cellular toxicity by producing oxidative stress before it can bind to globins for hemoglobin production.

With regard to intestinal heme iron absorption, no studies have yet directly examined FLVCR function *in vivo*, though the polarized intestinal cell line CaCo-2 does express the protein<sup>[53]</sup>. It has also been demonstrated that CaCo-2 cells are capable of heme transport in both directions, equivalent to both absorption and secretion *in vivo*, and that the secretory pathway is significantly

more active under control conditions<sup>[57]</sup>. Presumably, FLVCR is acting on the basolateral membrane to regulate heme content when heme synthesis rates are at their peak just prior to differentiation, and the potential for cell damage from oxidative stress is greatest if HO-1 activity is impaired<sup>[58-60]</sup> (see 'Heme Oxygenase' below). In this case it is highly unlikely that FLVCR is involved with heme uptake at the apical membrane. Studies of FLVCR *in vivo* are required to confirm this assessment.

## HEME CATABOLISM IN THE ENTEROCYTE

It was initially hypothesized that following uptake, heme passed directly into the portal circulation where it bound hemopexin and was most likely sequestered by hepatocytes using the hemopexin receptor and degraded, based on early observations in guinea pigs<sup>[23]</sup>. However, this theory is questionable for other species, with strong evidence that heme is catabolized within the enterocyte in most omnivorous and carnivorous mammals. This is best demonstrated by experiments in which dogs were administered an intragastric dose of radio-labelled hemoglobin, and 90% of the recoverable radioactivity in samples of portal blood over a period of 3 h was present as non-heme iron<sup>[35]</sup>. Similar observations have been made in human<sup>[14,34]</sup> and rat<sup>[17]</sup> experiments.

The presence of a heme splitting substance in the mucosa was first demonstrated in 1968<sup>[61]</sup>. The high molecular weight of this substance (MW about 64 kDa) and the kinetic properties of the reaction indicated that the heme splitting substance was an enzyme. Initial studies suggested that xanthine oxidase could play a role by generating hydrogen peroxide to chemically degrade heme, resulting in iron release and a non-specific mixture of four bilirubin isomers<sup>[62-64]</sup>. However, this hypothesis was problematic since *in vivo* heme degradation typically results in a single dominant isomer, namely bilirubin IX- $\alpha$ <sup>[65,66]</sup>.

Further research generated a strong case that the heme splitting substance in the mucosa was microsomal heme oxygenase<sup>[24]</sup>. This is based on the fact that heme oxygenase almost exclusively generates the expected bilirubin IX- $\alpha$  isomer and that heme oxygenase activity is highest in the location where heme iron absorption is highest, the duodenum<sup>[17,23,37]</sup>. In addition, iron deficiency results in an increase in both heme iron absorption and mucosal heme oxygenase activity, whereas xanthine oxidase activity decreases dramatically.

Based on morphological studies, it appears that heme is degraded inside internalised vesicles within 2-3 h of heme uptake by receptor mediated endocytosis<sup>[45,46]</sup>. Acid ferrocyanide staining, which exclusively detects non-heme iron, indicates that iron is released from heme inside the vesicle, before transport to the labile iron pool by unknown mechanisms (see 'A Possible Role for DMT1?' below). The iron is then thought to undergo identical transport through the enterocyte and into the circulation as for internalised non-heme iron.

A study tracking the absorption of <sup>59</sup>Fe-hemoglobin in closed duodenal loops has suggested that heme degradation is the rate limiting step in heme iron absorption, as opposed to hemoglobin degradation, heme uptake or iron transfer to the circulation<sup>[67]</sup>. This is based on increasing doses of hemoglobin resulting in the accumulation of <sup>59</sup>Fe-heme, but not <sup>59</sup>Fe, within the mucosa. However, since this study utilized whole-mucosal homogenates to assess relative heme and non-heme iron content there may not be sufficient sensitivity to detect the possible accumulation of non-heme iron inside endocytotic vesicles which would result in decreased heme oxygenase activity by end-product inhibition<sup>[61]</sup>. Nonetheless, the hypothesis that heme oxygenase is limiting for heme iron absorption is consistent with the decrease in absorption that is observed with inhibitors of heme oxygenase activity<sup>[68]</sup>.

## HEME OXYGENASE

Heme oxygenase is a microsomal enzyme (corresponding to the endoplasmic reticulum *in vivo*) that catalyses the mixed function oxidation of heme using cytochrome P-450, NADPH and molecular oxygen producing CO, iron and biliverdin IX- $\alpha$  which is rapidly reduced to bilirubin IX- $\alpha$ <sup>[69-71]</sup>. There are two well characterised isoforms of heme oxygenase, referred to as HO-1 and HO-2<sup>[72,73]</sup>, and these isoforms are products of different genes<sup>[74,75]</sup>. A third isoform has been described as HO-3<sup>[76]</sup>, but this appears to be a brain-specific pseudogene derived from HO-2<sup>[77]</sup>.

HO-1 expression is induced by numerous factors including oxidative stress, inflammation, cytokines, nitric oxide, prostaglandins, an elevated level of substrate, iron deficiency, metals including Cd, Co, Cr, Cu, Fe, Hg, Ni, Pd, Pt, Sn and Zn, hyperoxia, and exposure to UV light (for review see<sup>[78]</sup>). HO-1 is also induced by hyperthermia, leading to the use of the alternate name heat shock protein 32<sup>[79]</sup>. Considering these combined factors, induction of HO-1 expression appears to be related to preventing cell damage under many circumstances by reducing levels of the pro-oxidant heme and generating the antioxidant bilirubin<sup>[80]</sup>. This assessment is confirmed by genetic knockout of HO-1 in mice<sup>[81]</sup>, and humans with impaired HO-1 expression<sup>[82-84]</sup>, which present with reduced defence against external stresses.

In contrast, HO-2 expression is not inducible<sup>[79,85]</sup>. HO-2 is primarily found in the brain<sup>[86]</sup> and testis<sup>[85]</sup> and appears to function as a sensor for O<sub>2</sub>, CO, and NO<sup>[87-90]</sup>. In the intestine these functions are relevant in the interstitial cells of Cajal, where HO-2 regulates levels of CO, which in turn affects potassium currents and resting membrane potential of intestinal smooth muscle, and thus intestinal motility (for review see<sup>[78]</sup>).

In relation to the catabolism of dietary heme, most work regarding heme oxygenase was performed before the different isoforms were known. As such, the specific isoform involved with heme iron absorption has not been established with certainty. However, it has long

been assumed that HO-1 is the key player<sup>[91]</sup> based on corroboration of physiological evidence; specifically, HO-1 and heme iron absorption both upregulate in iron deficiency<sup>[92]</sup>. Further, HO-1 activity<sup>[24]</sup> and heme iron absorption<sup>[17,23,37]</sup> are both highest in the duodenum, which is also the site of highest expression of the putative heme receptor and PCFT/HCP1<sup>[37,51,52]</sup>.

However, HO-1 is generally considered to be a membrane bound protein associated with microsomes<sup>[69]</sup> with a cytoplasmic catalytic site<sup>[93]</sup>. Thus under normal circumstances HO-1 would have no topological association with the vesicles that are thought to contain internalised heme, namely endosomes and/or lysosomes. As such, the possibility that HO-2 plays a role in heme iron absorption should be strongly considered, since both HO-1 and HO-2 generate the appropriate isoform of bilirubin<sup>[72]</sup>. Further, the participation of a non-inducible enzyme could explain why the ability to upregulate heme iron absorption is limited compared to non-heme iron<sup>[14,15,21-25]</sup>, and heme splitting is speculated to be rate limiting<sup>[67]</sup>.

In a recent study our laboratory examined the sub-cellular location of HO-1 and HO-2 in enterocytes in relation to endocytotic markers during the course of heme iron absorption<sup>[94]</sup>. We observed that HO-1 was distributed evenly throughout the cytoplasm of enterocytes and did not co-localise with endocytotic markers. In contrast, HO-2 presented as a dense band in the apical cytoplasm that co-localised extensively with endosomes. This strongly suggests that HO-2 could be exposed to all heme taken up by the enterocytes, either by way of receptor mediated endocytosis or by transport directly into the cytoplasm. Clearly, the role of HO-2 in heme iron absorption needs to be investigated further using more direct methods, particularly using knockout models for HO-1 and HO-2.

## A POSSIBLE ROLE FOR DMT1?

The uptake of non-heme iron by the enterocytes occurs through the function of DMT1<sup>[95,96]</sup>. DMT1 is also capable of transporting other divalent cations<sup>[96-98]</sup> and is responsible for cellular iron acquisition during the transferrin receptor cycle<sup>[47-50]</sup>. DMT1 functions as a Fe(II)/proton symporter<sup>[96]</sup> and is highly expressed in the duodenum during iron deficiency<sup>[99]</sup>. This is consistent with the duodenum being the principal site of iron absorption<sup>[100]</sup> and the least alkaline section of the small intestine due to the close proximity to acidic gastric secretions. Ultimately, Fe(III) is also transported by DMT1 after reduction to Fe(II) by DcytB<sup>[101]</sup>, although the physiological significance of this pathway is the subject of continued debate<sup>[102,103]</sup>.

The physiological relevance of DMT1 in iron metabolism, including iron absorption, is confirmed in the Belgrade (*b*) rat and *mk* mouse which both exhibit a microcytic, hypochromic anaemia due to a G185R mutation to DMT1, resulting in a dramatic decrease in DMT1 function<sup>[47,104-106]</sup>. Considering *b/b* rats, the primary symptoms are mostly attributable to

decreased iron uptake by reticulocytes<sup>[107,108]</sup> and earlier erythroid precursors<sup>[109]</sup>. Further research has shown that endosomal iron transport during the transferrin receptor cycle is significantly reduced in *b/b* rats<sup>[108,110-112]</sup>, and these findings are entirely consistent with the functional role<sup>[47,105]</sup> and sub-cellular location<sup>[48,50]</sup> of DMT1 in relation to the transferrin receptor cycle.

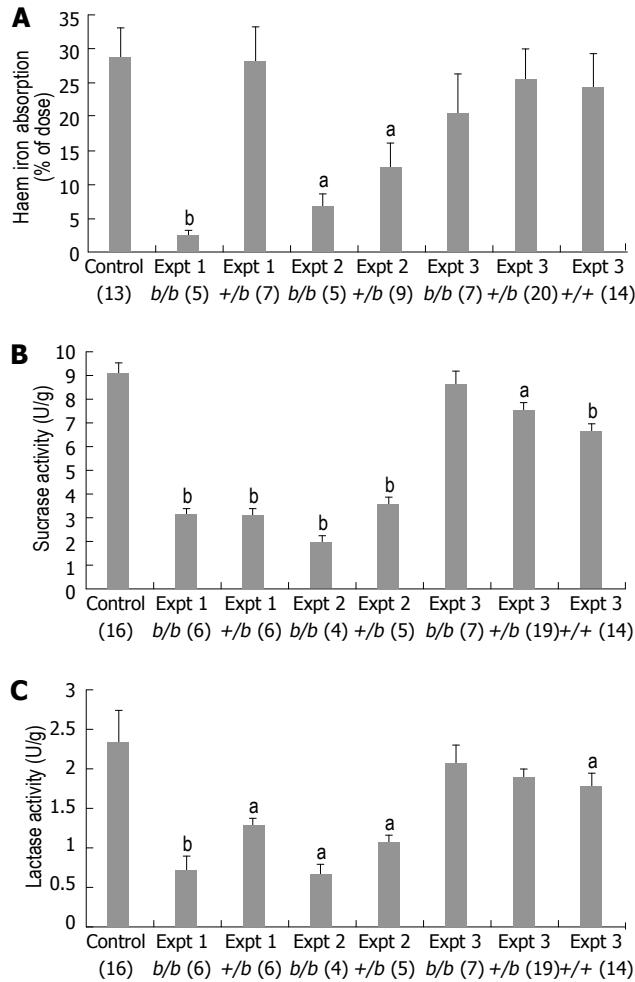
In addition to the striking effects on reticulocyte development, *b/b* rats also exhibit a significant decrease in the quantity of megakaryocytes in their bone marrow<sup>[113]</sup>, and their overall hematological status is similar to that observed in a rare preleukaemic syndrome<sup>[114]</sup>. The subsequent high clearance rates of prematurely senescent erythrocytes in turn causes splenomegaly. Aside from hematological factors, *b/b* rats exhibit a universal reduction in iron uptake by body tissues<sup>[115]</sup>, including the brain<sup>[116]</sup>. The extent to which this affects overall health and development, independent of hematological parameters, is not currently known.

The final important aspect of defective iron metabolism by Belgrade rats is their decreased dietary non-heme iron absorption at the stage of uptake into the enterocytes<sup>[115,117]</sup>. This is consistent with the location<sup>[99]</sup> and function<sup>[95]</sup> of DMT1 on the microvillus membrane of enterocytes. Decreased iron absorption can be viewed as the 'second hit' for iron deficiency anaemia in *b/b* rats. However, the residual activity of mutated DMT1 means that appropriate dietary iron supplementation and iron dextran injections can improve general health and survival rates without returning hematological status to normal<sup>[118]</sup>.

Over the past few years our laboratory has been using the Belgrade rat to examine whether DMT1 is the transporter required to utilise the iron released from heme during receptor mediated endocytosis. This hypothesis suggests that the function of DMT1 is directly comparable to its role in the transferrin receptor cycle<sup>[47-50]</sup> and that *b/b* rats would exhibit decreased heme iron absorption compared with *+/b* and control rats due to a reduced ability to transport iron out of internalised vesicles.

## THE BELGRADE RAT AND HEME IRON ABSORPTION

In preliminary experiments, *b/b* rats exhibited a statistically significant decrease in hemin chloride, hemoglobin, and ferrous iron absorption when compared with *+/b* and Wistar (*W*) rats, as assessed by whole body retention of an oral gavage of radio-labelled iron. This strongly suggested that there was a requirement for DMT1 in heme iron absorption (see Expt 1 in Figure 2A). However, it was unclear whether this effect was due to a reduction of DMT1 function across the membranes of endocytotic vesicles, or was secondary to pathological effects of the iron deficiency and anaemia experienced by *b/b* rats. A general defect in the mucosal biology of *b/b* rats may have consequences for other proteins and biosynthetic processes including



**Figure 2** Results from Belgrade rats for heme iron absorption (A), sucrase activity (B) and lactase activity (C) over a series of experiments. Figures in brackets indicate n values, and data is mean  $\pm$  SE. Groups marked with 'a' or 'b' are significantly different from the control group (1-way ANOVA  $^aP < 0.05$  and  $^bP < 0.005$ , respectively). In early experiments 1 and 2, b/b and +/b rats had significantly lower heme iron absorption than Wi controls, initially suggesting a possible role for DMT1. However, sucrase and lactase activity was also lower in b/b and +/b rats indicating a more general defect in the mucosa of the Belgrade strain. In experiment 3, +/+ rats were used as an improved control to account for strain differences between Wi and Belgrade strains, but in this (and subsequent) experiments there was an apparent recovery in both heme iron absorption as well as sucrase and lactase activities. We could find no explanation for this dramatic phenotypic change, making it difficult to correlate DMT1 function with heme iron absorption.

reduced expression of the heme receptor and/or heme transporters, reduced capacity to internalise a heme/receptor complex and reduced heme oxygenase activity -all of which would affect heme iron absorption.

It was observed that in addition to reduced heme iron absorption both b/b and +/b rats also exhibited significantly lower activity of sucrase and lactase<sup>[119]</sup> when compared with Wistar rats (see Expt 1 in Figure 2B, C). Previous studies examining the Belgrade strain have only observed phenotypic differences in b/b rats, whereas +/b rats appear identical to control laboratory rat strains such as Wistar and Sprague-Dawley for all measured parameters<sup>[115,117,120,121]</sup>. This raised the strong possibility that there are as yet uncharacterized strain differences between Belgrade and other laboratory

rat strains that are unrelated to reticulocyte iron uptake and non-heme iron absorption.

Experiments assessing heme iron absorption and the expression of sucrase and lactase were repeated shortly after with similar results; b/b rats again exhibited significantly decreased heme iron absorption compared to Wi, but so did +/b rats contrasting with the previous experiment. Further, both b/b and +/b rats had significantly decreased sucrase and lactase activities compared to Wi controls, making it difficult to correlate DMT1 function with heme iron absorption (see Expt 2 in Figure 2A-C). At this time it was decided to use a more appropriate control than Wi rats in order to account for any strain differences, thus we established a breeding program that would generate b/b, +/b and +/+ Belgrade rats. We concurrently began genotyping all experimental rats by sequencing reverse transcribed DMT1 mRNA; in all cases the measured genotype matched the observed phenotype.

The experiment including +/+ rats yielded unexpected and conflicting results. b/b rats demonstrated a dramatic recovery of both heme iron absorption and sucrase and lactase activity, such that they were directly equivalent to +/b and +/+ rats, and approximately comparable to Wi controls from previous experiments (see Expt 3 in Figure 2A-C). Subsequent experiments were able to replicate this recovery (data not shown). The dramatic phenotypic change in the Belgrade rat strain could not be attributable to any gross developmental characteristics including body mass, spleen mass and mucosa mass, nor any change in the measures of iron deficiency and anaemia. Additionally, we were unable to find evidence for the contraction of a chronic infection, significant changes in other external factors or any variation in our well-controlled measurement of heme iron absorption and enzyme activity.

Thus the colony of Belgrade rats available to us has been unable to provide any conclusive evidence for a role of DMT1 in heme iron absorption. Similarly, we were not able to observe any relocation of DMT1 to endosomes or lysosomes during the course of heme iron absorption using confocal microscopy<sup>[94]</sup>. However, bearing in mind that there are still large deficiencies in the understanding of heme iron absorption, we feel that a potential role for DMT1 should still be seriously considered in the future. This will require more reliable animal models than the Belgrade rat, possibly including the mk mouse or the *Slc11a2<sup>int/int</sup>* mouse which exhibits selective knockout of intestinal DMT1<sup>[95]</sup>. Further, it would be highly desirable to use more sensitive techniques than confocal microscopy (such as immunoelectron microscopy) to determine whether DMT1 is internalised to endosomes or lysosomes during heme iron absorption.

## CONCLUSION

Since the discovery of DMT1 in 1997 iron metabolism has experienced a renaissance, and the subsequent discovery of ferroportin and the humoral regulator

hepcidin have provided a very solid and complete theory for the mechanisms of non-heme iron absorption. The absorption of heme iron has yet to undergo a similar revolution, and considerably more work needs to be done in the future for a complete understanding of the absorption of this critical micronutrient. Primarily, the physiological relevance of recently discovered heme transporters will need to be confirmed *in vivo*, along with continued searching for additional heme transporters and receptors. Concurrent efforts to explicitly identify the enzyme responsible for heme catabolism, as well as the sub-cellular location of this enzyme's catalytic site, will help ascertain whether heme is taken up by receptor mediated endocytosis or is transported directly into the cytoplasm of enterocytes. Finally, the potential role of iron transporters such as DMT1 in heme iron absorption will need to be investigated further; particularly if receptor mediated endocytosis proves to be an important heme uptake pathway.

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## Liver in systemic disease

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viral hepatitis, alcohol intake, nonalcoholic fatty liver disease, autoimmune liver diseases and hereditary diseases such as hemochromatosis,  $\alpha_1$ -antitrypsin deficiency and Wilson's disease. Many patients with liver injury are likely to be treated with several drugs, increasing the possibility that their liver injuries are drug-induced. Some patients with liver injury, however, have underlying systemic diseases, which may also affect their livers. Knowledge of liver involvement in systemic diseases is important for the accurate diagnosis of liver injury and to avoid unnecessary examination and treatment. This review will describe liver injury caused by various systemic diseases.

### Abstract

Potential causes of abnormal liver function tests include viral hepatitis, alcohol intake, nonalcoholic fatty liver disease, autoimmune liver diseases, hereditary diseases, hepatobiliary malignancies or infection, gallstones and drug-induced liver injury. Moreover, the liver may be involved in systemic diseases that mainly affect other organs. Therefore, in patients without etiology of liver injury by screening serology and diagnostic imaging, but who have systemic diseases, the abnormal liver function test results might be caused by the systemic disease. In most of these patients, the systemic disease should be treated primarily. However, some patients with systemic disease and severe liver injury or fulminant hepatic failure require intensive treatments of the liver.

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**Key words:** Abnormal liver function tests; Jaundice; Systemic disease

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### INTRODUCTION

Potential causes of abnormal liver function tests include

### CARDIOVASCULAR DISEASES

#### *Ischemic hepatitis*

The pathophysiology of ischemic hepatitis, also known as "shock liver", is poorly understood<sup>[1]</sup>. Patients usually show rapid (within 24-48 h) and dramatic transient increases in serum aminotransferase, lactate dehydrogenase (LDH) levels and bilirubin following periods of hemodynamic instability or hypoxia. Three features distinguish ischemic hepatitis from acute viral hepatitis: LDH elevation is more marked in ischemic than in viral hepatitis; serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations rapidly return to normal in ischemic hepatitis, usually within 7-10 d; and ischemic hepatitis is more often complicated by renal damage<sup>[2]</sup>. Ischemic hepatitis has several etiologies (Table 1)<sup>[2]</sup>.

#### *Liver congestion*

Liver congestion is caused by acute or chronic right-sided heart failure and is manifested by hepatomegaly (95%-99%), ascites (7%-49%), splenomegaly (12%-25%) and/or jaundice (< 20%)<sup>[3,4]</sup>. In addition, patients often show signs indicative of heart failure, including peripheral edema and pleural effusion. Liver function tests show that serum bilirubin is elevated, usually to 1-5 mg/dL and mostly in the unconjugated form, in 24%-81% of patients, depending on the severity of heart failure<sup>[3-5]</sup>. Bilirubin concentrations rapidly return to normal 3-7 d after improvement of right-sided heart failure. In addition, 3%-50% of patients with heart failure show elevated levels of serum aminotransferases, with the elevation of AST more marked than that of ALT<sup>[3-5]</sup>. Elevated serum alkaline phosphatase (ALP) is observed in 10%-20% of patients with right-sided heart failure, but these levels return to normal within 1 wk after improvement of heart failure<sup>[3-5]</sup>.

**Table 1 Etiologies of ischemic hepatitis<sup>[2]</sup>**

<b>Etiologies of ischemic hepatitis</b>
Left ventricular failure
Acute myocardial infarction
Cardiac arrhythmia
Prosthetic valvular dysfunction
Cardiomyopathy
Pericardial tamponade
Other causes of shock
Trauma
Burns
Dehydration
Hemorrhage

### Cardiac cirrhosis

Cardiac cirrhosis may occur after longstanding hepatic congestion, but its incidence is relatively low. Although liver cirrhosis has no characteristic biochemical markers, a multivariate analysis found that elevated concentrations of AST and bilirubin are prognostic of poor outcome<sup>[6]</sup>.

### HEATSTROKE

The liver is extremely sensitive to thermal injury and is a frequent site of tissue injury in patients with heatstroke, with almost all of these patients experiencing liver injury<sup>[7]</sup>. Elevated serum ALT concentration is the most common feature in patients with heatstroke, and may lead to acute hepatic failure<sup>[8]</sup>. Liver function tests usually return to normal after 2 wk, but may remain elevated after 1 mo<sup>[9]</sup>.

### CONNECTIVE TISSUE DISEASES

Connective tissue diseases often involve the liver, and various forms of hepatic involvement have been reported. Laboratory markers and liver diseases associated with connective tissue diseases are summarized in Table 2<sup>[10,11]</sup>. For example, liver disease has been shown to be a common complication of systemic lupus erythematosus (SLE). Elevated serum ALT has been observed in 21% of patients with SLE<sup>[12]</sup>, and 4.4% of patients with SLE may have serious chronic liver diseases, including chronic active hepatitis and liver cirrhosis<sup>[13-16]</sup>. The most common liver histologic manifestation of SLE is steatosis, which may not be associated with corticosteroid therapy<sup>[17]</sup>. It is important to distinguish lupus-related chronic hepatitis from autoimmune hepatitis (AIH), because patients with the latter often rapidly progress to liver cirrhosis unless they are treated with appropriate and sufficient doses of corticosteroids. Inasmuch as corticosteroid treatment can improve liver biochemical abnormalities in either disease, a response to the drug does not contribute to the differential diagnosis. The presence of anti-smooth muscle antibody, which is found in patients with AIH but not in those with lupus-related liver disease, may be helpful for distinguishing these two diseases<sup>[18]</sup>. There have been reports of patients with overlapping SLE and autoimmune hepatitis<sup>[13-16]</sup>, thus confusing the diagnosis

**Table 2 Connective tissue disease-associated abnormal liver function tests and liver diseases<sup>[10,11]</sup>**

<b>Connective tissue disease</b>	<b>Abnormal liver function tests</b>	<b>Liver disease or histology</b>
Juvenile rheumatoid arthritis	Elevated aminotransferases	Drug hepatotoxicity Massive hepatomegaly Infiltration of portal tracts by chronic inflammatory cells Acute necro-inflammatory disease
Felty syndrome	Elevated ALP and aminotransferases	Chronic hepatitis Drug hepatotoxicity Nodular regenerative hyperplasia Portal fibrosis Sinusoidal lymphocytosis Amyloidosis Macronodular cirrhosis Hepatomegaly
Rheumatoid arthritis	Elevated ALP, correlate with severity of arthritic activity	Drug hepatotoxicity Autoimmune chronic hepatitis (type 1) Nodular regenerative hyperplasia Primary biliary cirrhosis Amyloidosis Spontaneous hepatic rupture/ Necrotizing arteritis Steatosis
Polymyalgia rheumatica	Elevated ALP and aminotransferases	Drug hepatotoxicity Steatosis Lymphocytic infiltration of portal tracts Granulomas Hyperplasia of perisinusoidal stellate cells Primary biliary cirrhosis
Sjögren's syndrome	Elevated ALP and aminotransferases	Primary; 7% have liver dysfunction, mostly PBC Secondary; 40%-70% have PBC
Scleroderma	Hepatic involvement is low; Elevated ALP, mild elevation of aminotransferases and bilirubin	Drug hepatotoxicity CREST syndrome (PBC) Spotty calcification Idiopathic portal hypertension Cirrhosis Nodular regenerative hyperplasia Hepatomegaly
Systemic lupus erythematosus	Elevated aminotransferases in up to 50% of patients	Drug hepatotoxicity Autoimmune chronic hepatitis (type 1) Venous congestion Nodular regenerative hyperplasia Hepatic infarction Steatosis Venous thrombosis Granulomatous hepatitis Centrilobular necrosis Cirrhosis
Adult Still's disease	Elevated ALP and aminotransferases	Moderate portal mononuclear cell infiltration with occasional focal hepatocyte necrosis

of liver disease in SLE patients. Criteria are needed for the differential diagnosis of AIH and lupus-related liver disease in patients with SLE.

## HEMATOLOGICAL DISEASES

### Hodgkin disease

Liver infiltration of malignant cells has been reported in 14% of patients with Hodgkin disease, and hepatomegaly in 9% of patients with stage I-II and in 45% of patients with stage III-IV disease<sup>[17]</sup>. In addition, mild elevations of aminotransferases and moderate elevation of ALP can occur, due to tumor infiltration or extrahepatic bile duct obstruction<sup>[17]</sup>. However, cholestasis in zone 3, which was not associated with extrahepatic obstruction or tumor infiltration, has been described; this cholestasis may be due to vanishing bile duct syndrome<sup>[18]</sup>.

### Non-Hodgkin lymphoma

Lymphoma cell infiltration of the liver is more common in non-Hodgkin than in Hodgkin disease, with 16%-43% of non-Hodgkin patients showing hepatic involvement<sup>[17]</sup>. Extrahepatic obstruction is also more common in non-Hodgkin than in Hodgkin disease. Moreover, hepatic infiltration is more common in low-grade B-cell lymphomas (small cell) than in high-grade (diffuse large B-cell, T-cell histiocytic) lymphomas<sup>[19]</sup>. Liver function tests show mild to moderate elevations in serum ALP, and hepatomegaly may occur<sup>[17]</sup>. Although liver involvement in both Hodgkin and non-Hodgkin lymphomas may present as acute hepatic failure<sup>[20-25]</sup>, liver transplantation should be avoided<sup>[26]</sup>. Jaundice due to non-Hodgkin lymphoma can be distinguished from that due to viral hepatitis or drug hepatotoxicity by the presence of liver enlargement and lactic acidosis in lymphoma<sup>[27]</sup>.

### Chronic lymphoid leukemia (CLL)

Patients with CLL often show mild to moderate liver enlargement and extensive lymphocytic infiltration in the portal tracts, with functional impairment of the liver in late stages<sup>[28,29]</sup>.

### Hairy cell leukemia

Leukemia cells often infiltrate the liver, in both the portal tracts and sinusoids, and liver enlargement has been observed in up to 40% of patients with this disease<sup>[30]</sup>.

### Acute leukemia

Although hepatic involvement in acute leukemia is usually mild and silent at the time of diagnosis<sup>[27]</sup>, a post mortem study showed liver infiltration in > 95% of ALL and up to 75% of AML patients<sup>[31]</sup>. In ALL, infiltration was confined to the portal tracts, whereas, in AML, infiltration was observed in both portal tracts and sinusoids. Massive leukemic cell infiltration of the liver may present as fulminant hepatic failure<sup>[32]</sup>. In patients with acute leukemia, drug-induced liver injury and bacterial or fungal infections may also affect the liver.

### Multiple myeloma

Hepatomegaly has been observed in 15%-40% of patients with multiple myeloma and may sometimes be accompanied by splenomegaly<sup>[33,34]</sup>.

### Primary myelofibrosis

Liver involvement is common in patients with primary myelofibrosis, and liver enlargement is observed in almost all patients. The mechanisms of liver involvement have been associated with extramedullary hematopoiesis, increased hepatic blood flow and hemosiderosis caused by multiple blood transfusions<sup>[27]</sup>. Ascites and esophageal varices secondary due to portal hypertension has been found in 7% of these patients<sup>[35,36]</sup> and nodular regenerative hyperplasia of the liver following obstruction of intrahepatic portal vein branches may augment the portal hypertension<sup>[37]</sup>. The most common liver function abnormality in patients with primary myelofibrosis is elevated ALP, which has a frequency of 40%-60% and which may be associated with the severity of sinusoidal dilatation<sup>[38]</sup>.

### Polycythemia vera

Although direct liver involvement is uncommon, some patients may present with acute or chronic Budd-Chiari syndrome<sup>[39]</sup>.

### Chronic myeloid leukemia (CML)

About 50% of patients with CML show mild to moderate hepatomegaly at presentation, with no liver function abnormalities<sup>[40]</sup>. At the time of blastic crisis, however, liver sinusoidal infiltration by immature cells may lead to liver enlargement and elevated serum ALP levels<sup>[41]</sup>.

### Myelodysplasias

In patients with sideroblastic or refractory anemia, iron deposition in the liver may occur due to repeated transfusion or decreased iron utilization by bone marrow<sup>[27]</sup>.

### Sickle-cell disease

The liver is commonly involved in sickle-cell disease. This may be due to iron overload caused by multiple blood transfusions, gallstones, or cardiac dysfunction due to secondary hemochromatosis<sup>[42]</sup>.

### Thalassemia

The major cause of liver injury in patients with thalassemia is hemochromatosis due to ineffective erythropoiesis, with massive iron deposits found in the liver<sup>[27]</sup>.

## LUNG DISEASES

### Pneumonia

Lobar pneumonia caused by *Legionella pneumophila*, *Mycoplasma pneumoniae* or *Pneumococcus* may be associated with elevated concentrations of serum aminotransferase and bilirubin<sup>[43]</sup>.

Jaundice has been observed in 3%-25% of patients with *Pneumococcus* pneumonia<sup>[44]</sup>, often developing between days 3 and 6 of illness.

In *Legionnaire's* disease, liver function tests are likely to show abnormalities, with elevated concentration

of serum ALP and aminotransferase in up to 50% of patients<sup>[45]</sup>.

*Mycoplasma pneumoniae* is a frequent cause of community-acquired pneumonia. Liver involvement is not common, but some patients may have elevated levels of serum aminotransferases<sup>[43]</sup>. Cholestatic hepatitis and mild hepatitis without pneumonia have been described<sup>[43]</sup>.

Cytomegalovirus pneumonia can also result in jaundice and elevated levels of ALP and aminotransferases<sup>[46]</sup>.

### Chronic pulmonary disease

Serum bilirubin, ALT,  $\gamma$ -glutamyl transpeptidase (GGT) and ALP may be elevated in patients with chronic pulmonary disease or status asthmatics<sup>[47-49]</sup>, and these liver abnormalities may be associated with secondary heart failure or hypoxia.

## RENAL DISEASES

Even in the absence of liver metastasis, renal cancer causes hepatomegaly and abnormal liver function test results. Following tumor resection, however, these liver abnormalities return to normal, suggesting that the previously observed abnormalities were caused by a hepatotoxic hormone secreted from the tumor<sup>[50]</sup>.

## SYSTEMIC INFECTION-BACTEREMIA AND SEPSIS

Cholestasis is a common complication in patients with extrahepatic bacterial infection and sepsis, regardless of whether the infectious agent is gram-negative (*E.coli* and *Klebsiella*) or gram-positive (*S aureus*)<sup>[51,52]</sup>. Proinflammatory cytokines, including tumor necrosis factor-alpha (TNF- $\alpha$ ) and interleukin-6 (IL-6), as well as nitric oxide, are thought to induce cholestasis by inhibiting the canalicular excretion of conjugated bilirubin<sup>[53]</sup>. Liver biopsy may show mild portal inflammation, but bile ducts often appear normal and there is usually no cholangitis<sup>[54]</sup>.

Laboratory findings in sepsis include mild elevation of ALP (mostly 1 to 3 times the ULN) and modest elevation of ALT. Peak serum bilirubin concentrations typically range from 5 to 10 mg/dL, but levels as high as 30 to 50 mg/dL have been reported<sup>[51]</sup>. Importantly, the serum concentrations of ALP and bilirubin may be discordant, with deeply jaundiced patients often having normal ALP levels, while anicteric patients may show marked elevation of ALP or GGT<sup>[55-57]</sup>. Compared with infected patients without bacteremia, those with bacteremia had significantly higher serum levels of GGT and ALP and significantly lower serum concentrations of albumin, cholesterol and cholinesterase. These alterations were observed within several days after the onset of bacteremia, but concentrations returned to normal following adequate treatment of the infection<sup>[52]</sup>. Although the major pathogens were *S aureus* and *E.coli*, *P aeruginosa* infection may cause cholestasis more frequently than other organisms, with 26% to 52%

of patients severely infected with *P aeruginosa* having jaundice<sup>[58]</sup>. At autopsy, these patients showed periportal cholestasis with minimal liver cell damage.

## LIVER DAMAGE IN INFECTION BY SPECIFIC PATHOGENS

### *Clostridium perfringens* infection

*C perfringens* may directly affect the liver by forming an abscess or causing necrotizing massive gas gangrene of the liver, leading to fulminant hepatic failure<sup>[59]</sup>.

### *Salmonella typhi* infection

While sepsis can cause liver dysfunction, it can also occur following *Salmonella typhi* infection, a condition known as *Salmonella* hepatitis<sup>[60]</sup>. Although severe elevation of liver function tests is rare, jaundice has been observed in 33% of these patients. Although the clinical features of *Salmonella* hepatitis may be identical to those of acute viral hepatitis, these diseases may be distinguished by the ALT/LDH ratio, which is < 4.0 in *Salmonella* hepatitis, significantly lower than the ratio in acute viral hepatitis, > 5.0<sup>[61]</sup>.

### Lyme disease

Hepatic involvement is common in Lyme disease caused by *Borrelia burgdorferi*, and mild elevations of GGT and aminotransferase are commonly observed especially in patients with early stage disease<sup>[62,63]</sup>.

### Q fever

Nearly 50% of patients with Q fever had accompanying hepatitis, but the liver function abnormalities were nonspecific. Although these patients usually show anicteric hepatitis, one third may have jaundice if the disease is prolonged<sup>[64]</sup>.

### Syphilis

Acute cholestatic syphilitic hepatitis, sometimes accompanied by jaundice, has been reported in patients with secondary syphilis<sup>[65]</sup>. Patients with tertiary syphilis may present with gummas formation in the liver, which resemble metastatic tumors<sup>[66]</sup>.

### *Campylobacter* infection

Mild to severe liver biochemical abnormalities have been observed following infection with *Campylobacter* organisms<sup>[67]</sup>.

### *Chlamydia* or *Neisseria* infection

Perihepatitis has been observed in patients infected with *Chlamydia trachomatis*<sup>[68]</sup> and *Neisseria gonorrhoeae*<sup>[69]</sup>, with the formation of liver granulomas in the former<sup>[65]</sup>.

### HIV infection

Liver injury in patients with HIV infection can be caused by HIV itself, by coinfection with hepatitis viruses such as HBV and HCV, or by hepatic involvement of

**Table 3 Liver involvement in deep fungal infections<sup>[73]</sup>**

Liver involvement in deep fungal infections
Opportunistic mycoses
Candidiasis-includes hepatosplenic candidiasis
Cryptococcosis
Less common: aspergillosis, mucormycosis, trichosporonosis
Pathogenic mycoses
Histoplasmosis-disseminated histoplasmosis
Paracoccidioidomycoses
Less common: coccidioidomycosis, african histoplasmosis, blastomycosis, penicilliosis

systemic infections, including *M tuberculosis*, *M avium complex*, *Toxoplasma gondii*, or *Cryptosporidium*. In addition, liver injury in HIV-infected patients may be due to the toxicities of drugs prescribed for the treatment of HIV or coinfecting microbes. In addition, biliary tract injuries caused by tuberculosis, *M avium* and *Cryptosporidium* have been reported<sup>[70]</sup>.

#### ***Mycobacteria infection***

Liver involvement is frequent in patients with mycobacterial infections, not only with *Mycobacterium tuberculosis* infection, but also with *M avium intracellulare* or *M genavense* infection<sup>[65]</sup>. The clinical spectrum of liver disease due to *Mycobacterium spp.* ranges from the absence of symptoms to liver failure, with multiple granulomas in the parenchyma of the liver being the most common lesion<sup>[71]</sup>. These patients show elevated serum ALP concentrations and hepatomegaly. Although the number of intrahepatic granulomas is greater in patients with than without disseminated military tuberculosis, hepatic tuberculosis can occur, even in the absence of apparent tuberculosis elsewhere<sup>[72]</sup>.

#### ***Fungal infection***

The liver is often involved in deep fungal infections, possibly due to enrichment of the blood flow through the liver or the invasion of fungi, including *C albicans* and *C tropicanus*, into the liver from the gut by penetrating through degenerated barriers of gastrointestinal mucosa<sup>[73]</sup>. Patients with liver involvement of fungal infection may show elevated serum concentrations of ALP and GGT, due to the formation of multiple small abscesses or granulomas in the liver. Liver involvement following deep fungal infections is summarized in Table 3<sup>[73]</sup>.

### **ACUTE HEPATIC FAILURE CAUSED BY VIRUSES OTHER THAN HEPATITIS A TO E**

The major causes of acute hepatic failure include drugs, hepatitis A, hepatitis B, and hepatitis E. Although Epstein-Barr virus and cytomegalovirus can also cause severe hepatitis during primary infection, other microbes, which mainly affect other organs, can cause acute hepatic failure. Among these are *Salmonella paratyphi A*<sup>[74]</sup>, herpes simplex virus<sup>[75,76]</sup>, parvovirus B19<sup>[77]</sup>, coxsackie virus B2<sup>[78]</sup>, human herpesvirus-6<sup>[79]</sup>, Varicella-Zoster

virus<sup>[80]</sup>, and Dengue virus<sup>[81]</sup>. Parvovirus B19 and human herpesvirus-6 are often found in patients with non-A to non-E acute hepatic failure.

### **TOTAL PARENTERAL NUTRITION (TPN)**

TPN may cause steatosis or cholestasis, and liver disease is more severe in infants than in adults. Elevated serum aminotransferase concentrations are common during the first 1-3 wk of TPN, and bilirubin increases in some adults after 10 wk or more of TPN<sup>[82-84]</sup>. Chronic cholestasis has been observed in 55% of patients receiving TPN for at least 2 years<sup>[85]</sup>, and cholestasis can lead to acalculous and calculous cholecystitis or TPN-induced cholelithiasis<sup>[86]</sup>.

### **ENDOCRINE DISEASES**

#### ***Thyroid disease***

Patients with hyperthyroidism frequently experience liver injury, which may be caused by increased hepatocyte oxygen demand without an associated increase in hepatic blood flow. Liver injury can be either cholestatic or hepatocellular. Up to 64% of these patients show elevated serum ALP, and up to 35% show elevated ALT. Interestingly, only 17% of these patients show elevated GGT<sup>[17]</sup>, and most of the increased ALP is bone-derived<sup>[87]</sup>.

In contrast to hyperthyroidism, liver biochemistry abnormalities are less prominent in patients with hypothyroidism. However, modest elevations in serum AST and ALT have been reported in 84% and 60%, respectively, of patients with hypothyroidism<sup>[88]</sup>. Some patients may show low serum ALP<sup>[87]</sup>, but their most characteristic symptom is ascites, which is caused by unknown mechanisms<sup>[87]</sup>. In addition, cholestatic jaundice has been described in case reports of patients with severe hypothyroidism<sup>[17]</sup>.

#### ***Cushing syndrome***

Hypercortisolism causes fatty infiltration of the liver in half of the patients, which may progress to NASH. The prevalence of NASH in these patients has been estimated to be 20% to 50%<sup>[89]</sup>.

#### ***Adrenal insufficiency***

Elevated serum aminotransferase concentrations have been reported in patients with adrenal insufficiency; these abnormalities usually resolve with appropriate hormone replacement<sup>[90,91]</sup>.

#### ***Diabetes mellitus***

Elevated liver chemistries have been observed in 10%-20% of patients with DM<sup>[92,93]</sup>, more frequently in patients with type 2 than type 1 DM<sup>[94]</sup>. One study reported that 16.5%, 9%, 11% and 6% of these patients had elevations in serum GGT, ALP, ALT and AST, respectively. Non-alcoholic fatty liver disease is a complication in 32% to 78% of patients with type 2

DM, and 50% of these patients may have non-alcoholic steatohepatitis (NASH)<sup>[95,96]</sup>. NASH in DM patients may lead to liver cirrhosis and eventually to hepatocellular carcinoma<sup>[94,97]</sup>.

## POSTOPERATIVE JAUNDICE

Jaundice often occurs after surgery, especially after cardiac surgery; of the latter, approximately 26.5% show conjugated hyperbilirubinemia<sup>[98]</sup>. Factors thought to contribute to the development of jaundice after surgery include: (1) Liver congestion due to preexisting right-sided heart failure; (2) Degree of perioperative hypotension and hypoxia; (3) Destruction of transfused erythrocytes; (4) Hemolysis secondary to mechanical prostheses; (5) Type of operation-The incidence of postoperative jaundice is dependent on the type of operation. For example, patients who underwent mitral valve replacement or multiple valve surgery had a higher rate of jaundice than those who underwent coronary bypass graft surgery; (6) Perioperative infection; (7) Resorption of hematoma; (8) Worsening of jaundice in Gilbert's syndrome-Gilbert's syndrome is the most common form of inherited hyperbilirubinemia, with 5% to 10% of Caucasians and Japanese estimated to have this syndrome. Although serum bilirubin levels are usually below 3 mg/dL, with the unconjugated form being dominant, jaundice may be worsened by a stress caused by surgery or infection; (9) Total parenteral nutrition; (10) Drug-induced liver injury, and (11) Benign postoperative intrahepatic cholestasis.

Postoperative jaundice usually occurs within 1-2 wk after major surgery. Serum concentrations of mainly conjugated bilirubin may increase to 40 mg/dL, but these resolve within a few days to weeks without specific treatment<sup>[99]</sup>.

## GASTROINTESTINAL DISEASES

Abnormal liver function test results are observed in over 50% of patients with inflammatory bowel diseases requiring surgery. The hepatobiliary diseases accompanying ulcerative colitis and Crohn's disease are shown in Table 4<sup>[100]</sup>.

Patients who undergo jejunoileal resection for the treatment of severe obesity may experience liver damage<sup>[101]</sup>. The liver often shows NASH, leading to liver cirrhosis and liver failure. Similar liver injuries have been observed in patients undergoing gastrectomy via Billroth-II reconstruction. Bacterial overgrowth in the blind-loop of the intestine can induce endotoxin and intrinsic ethanol<sup>[102,103]</sup>, leading to Kupffer cell activation and hepatocyte damage.

## GRANULOMA FORMATION IN THE LIVER

Several systemic diseases and drugs have been shown to induce granulomas in the liver, causing liver enlargement. The most consistently abnormal liver biochemistry result is elevated serum ALP. The diagnosis and causes of hepatic granulomas may be determined by

**Table 4 Hepatobiliary disorders associated with inflammatory bowel disease<sup>[100]</sup>**

Hepatobiliary disorders	Ulcerative colitis	Crohn's disease
Primary sclerosing cholangitis (PSC)	+	+
Large duct PSC	+	+
Small duct PSC (pericholangitis)	+	+
Cirrhosis	+	+
Hepatocellular carcinoma	+	+
Cholangiocarcinoma	+	+
Miscellaneous disorders		
Fatty liver	+	+
Granulomas		+
Amyloidosis		+
Hepatic abscess		+
Gallstones		+
Autoimmune hepatitis	+	

a histological examination of the liver. Five etiological categories have been identified<sup>[104]</sup>: (1) Immunological: Sarcoidosis, primary biliary cirrhosis, giant cell hepatitis, Wegener's granulomatosis, chronic granulomatous disease and allergic granulomatosis; (2) Infectious: Hepatitis C virus, cytomegalovirus, Epstein-Barr virus, tuberculosis, *Mycobacterium avium-intracellulare* in patients with HIV infection, leprosy, brucellosis, typhoid fever, Whipple's disease, tularaemia, yersiniosis, cat-scratch disease, histoplasmosis, blastomycosis, coccidiomycosis, candidiasis, Q fever, leishmaniasis, toxoplasmosis, syphilis, and schistosomiasis; (3) Medications: Penicillins, diphenylhydantoin and allopurinol; (4) Neoplastic: Hodgkin's disease and hypernephroma, and (5) Foreign body: Beryllium, suture material used in operation and thorotrast.

## AMYLOIDOSIS

Hepatic involvement has been demonstrated in about one-fifth of patients with AA amyloidosis and about half of those with the AL type, and liver function tests can remain normal even in patients with substantial amyloid deposits and hepatomegaly. Elevated serum ALP and GGT occur first in patients with massive amyloid deposits, followed by modest elevations of serum AST and ALT<sup>[105]</sup>.

## CONCLUSION

Abnormal liver function often occurs in patients without hepatitis virus infection and without excessive alcohol intake. Although US, CT or MR imaging should be used to assess the occurrence of fatty liver disease, hepatobiliary malignancies or infection and gallstones, diagnosis of autoimmune liver disease, especially with atypical presentation, is sometimes difficult. Moreover, intake of drugs including herbal medicines or supplemental nutrients may cause liver injury. However, abnormal liver function tests do not necessarily indicate serious liver disease. Asymptomatic patients with isolated, mild elevation of unconjugated bilirubin (e.g. Gilbert's syndrome) or GGT generally do not have liver disease and do not require further examination<sup>[106]</sup>. In

contrast, the liver may be involved in systemic diseases that mainly affect other organs. Therefore, in patients without etiology of liver injury by screening serology and diagnostic imaging, but who have systemic diseases, the abnormal liver function test results might be caused by the systemic disease. In most of these patients, the systemic disease should be treated primarily. However, some patients with systemic disease and severe liver injury or fulminant hepatic failure require intensive treatments of the liver.

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EDITORIAL

## Hepatic steatosis: A benign disease or a silent killer

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### INTRODUCTION

Both hepatitis C infection and non-alcoholic fatty liver disease (NAFLD) are major causes of liver related morbidity and mortality. Hepatitis C virus (HCV) is a major cause of chronic liver disease with about 170 million people infected worldwide. The severity of disease varies widely from asymptomatic chronic infection to cirrhosis and hepatocellular carcinoma (HCC)<sup>[1]</sup>.

NAFLD represents a spectrum of liver diseases characterized mainly by macrovesicular steatosis that occurs in the absence of alcoholic consumption. The hepatic histology varies from isolated hepatic steatosis alone “first hit” to fatty liver accompanied by hepatocellular damage plus inflammation known as steatohepatitis “second hit” which is followed by the development of fibrosis.

Adipose tissue is now recognized as not simply a storage depot for excess energy, but rather an active endocrine organ that secretes a number of molecules termed, adipocytokines. A number of these adipocytokines have been linked to alterations in insulin sensitivity, including adiponectin, leptin, resistin, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )<sup>[2,3]</sup>.

Insulin resistance (IR) is a major pathogenic feature leading to hepatic fat accumulation. In the meantime, hepatitis C infection promotes IR. Two types of IR are found in chronic hepatitis C patients: “viral” and “metabolic” IR. IR in chronic hepatitis C is relevant because it promotes steatosis and fibrosis<sup>[4]</sup>. Metabolic IR is thought to be triggered by hepatic FFA accumulation that may exacerbate overall IR<sup>[5]</sup>.

### METABOLIC SYNDROME

Metabolic Syndrome (X-syndrome) is a cluster of disorders including central obesity, IR with or without type 2 DM, dyslipidemia and hypertension. Recent findings linking the components of the metabolic syndrome with NAFLD and the progression to non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis will be reviewed. Metabolic syndrome was first described in 1988 by Reaven GM<sup>[6]</sup>. Whether hepatic IR causes cellular injury and inflammation in the liver or is

### Abstract

Steatosis is a common feature of many liver diseases, namely non-alcoholic steatohepatitis (NASH) and hepatitis C virus (HCV) infection, but the pathogenic mechanisms differ. Insulin resistance (IR), a key feature of metabolic syndrome, is crucial for NASH development, associated with many underlying genetically determined or acquired mitochondrial and metabolic defects and culminates to inflammation and progression to fibrosis. This may have potential implications for new drug therapy. In HCV-related disease, steatosis impacts both fibrosis progression and response to treatment. Steatosis in HCV-related disease relates to both viral factors (HCV genotype 3), and host factors (alcohol consumption, overweight, hyperlipidemia, diabetes). Among others, IR is a recognized factor. Hepatic steatosis is reported to be associated with disturbance in the signaling cascade of interferon and downregulation of its receptors. Thus, hepatic steatosis should not be considered a benign feature, but rather a silent killer.

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**Key words:** Metabolic steatosis; Hepatitis C virus steatosis; Insulin resistance; Fibrosis progression

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the result of both inflammation and steatosis is still unrevealed<sup>[7]</sup>. Hepatic steatosis is caused by imbalance between the delivery of fat in the liver and its subsequent secretion or metabolism. In other words, fat accumulates when the delivery of fatty acids to the liver, either from the circulation or by de novo synthesis within the liver, exceeds that capacity of the liver to metabolize the fat by  $\beta$ -oxidation or secrete it as very low-density lipoproteins (VLDL). Derangements in any of these pathways alone or in combination causes fat to accumulate in the liver.

#### **Delivery of fatty acids from peripheral stores to the liver**

Triglycerides (TAG) are stored in adipose tissue and released as FFAs into the circulation through the actions of lipoprotein lipase. FFAs released from peripheral stores are hydrophobic and are strongly bound to circulating albumin. FFAs are transported by albumin to the liver where they can then be used as a substitute for  $\beta$ -oxidation, stored as TAG, or exported as VLDL.

Excess glucose is converted to the liver, the backbone of most amino acids, can be converted to pyruvate and then to acetyl-coenzyme A (acetyl-CoA), which feeds directly into cytosolic fatty acid synthesis.

Processes that can lead to excessive FFAs delivery or impaired  $\beta$ -oxidation or secretion can lead to hepatic steatosis, increased mitochondrial reactive oxygen species (ROS) and lipid peroxidation products<sup>[8,9]</sup>.

#### **Fate of fatty acids in the liver**

In the fasting state, adipocyte TAG is hydrolyzed to release FFAs, which are transported to the liver where they can serve as substrates for mitochondrial  $\beta$ -oxidation.  $\beta$ -oxidation of fatty acids is a major source of energy needed to maintain liver viability during fasting. It is also the source of the ketone bodies, acetoacetate and acetone. These are released into the blood and are essential fuel sources for peripheral tissues, when glucose is in short supply. Defects in hepatic  $\beta$ -oxidation cause microvesicular steatosis of the liver, increase in oxidative stress due to extramitochondrial oxidative stress. ROS and peroxidation products lead to cytotoxic events, release of proinflammatory cytokines and activation of hepatic stellate cells and fibrosis<sup>[8,9]</sup>.

#### **Formation and secretion of VLDL**

In the fed state,  $\beta$ -oxidation of fatty acids is not required as an energy source, and fatty acids delivered to the liver are mainly converted to TAG. Insulin regulates the metabolic path that fatty acids take in the liver. Without insulin, creatinine palmitoyl transferase-I (CPTI) commits fatty acids to mitochondrial  $\beta$ -oxidation; when insulin levels are high, glycerol-3-phosphate acetyltransferase commits fatty acids to the formation of TAG. This bulk of acetyl CoA entering the citric acid cycle, results in delivery of electrons to the respiratory chain, where they generate ROS.

## **DEVELOPMENT OF STEATOHEPATITIS**

Steatosis *per se* does not always lead to hepatocellular injury

suggesting that a two-step model involving additional secondary insults is required for the inflammatory component of steatohepatitis “second hit”<sup>[10]</sup>.

Factors that lead to progressive liver injury are multifactorial and may include increased lipid peroxidation, FA toxicity, mitochondrial impairment, cytokine mediated hepatotoxicity, and oxidative injury. Hyperinsulinemia in the insulin-resistant state leads to increased FA oxidation promoting the development of ROS and oxidative injury<sup>[11-13]</sup>. Although there are no reliable human data supporting a causal role of oxidative injury in human NASH, animal data have suggested a potential role for oxidative injury<sup>[14-16]</sup>.

Excess fat in the liver predisposes to hepatocellular injury. This may be caused by direct cellular cytotoxicity of excess FFAs, oxidative stress, lipid peroxidation or other mechanisms. In the meantime, IR may contribute to hepatic fat accumulation and plays a key role in the development of steatohepatitis and disease progression<sup>[4]</sup>. Furthermore, TNF- $\alpha$  secreted by the macrophages of the adipose tissue which directly impairs insulin signaling and is crucial for the passage of steatosis to steatohepatitis<sup>[17]</sup> through induction of several proinflammatory cytokines. In addition, FFAs may lead to hepatocyte apoptosis which is one mechanism of cell injury in NAFLD<sup>[5]</sup>.

Hepatocellular injury may cause inflammatory response with subsequent cytokine induction, mitochondrial dysfunction and progressive fibrosis in a subset of patients<sup>[10]</sup>.

In animal models, development of steatohepatitis depends on additional factors, such as endotoxin exposure, acute liver injury (for example, ischemia-reperfusion), alcohol, excess dietary polyunsaturated fatty acids, or aging. A unifying hypothesis envisages that all causes capable of changing the redox equilibrium of the hepatocyte may result in liver inflammation and fibrogenesis activation. ROS may promote hepatic stellate cell activation and collagen fiber deposition<sup>[18]</sup>. Lipid peroxidation products may elicit activation of nuclear factors that lead to procollagen type I overexpression<sup>[19]</sup>.

Some investigators have used a taxonomic distinction of secondary NASH, or that attributable to readily identifiable drugs, toxins, or genetic abnormalities, and primary NASH, which is probably related to IR<sup>[20]</sup>.

## **FIBROSIS PROGRESSION IN NASH**

Progression of fibrosis in NASH has been histologically demonstrated in 32%-37% of the patients<sup>[21,22]</sup>. Estimated rates of cirrhosis development over 10 years of 5%-20% have been reported by 3 independent studies<sup>[23-25]</sup>. NASH patients with advanced fibrosis are at risk of developing liver complications<sup>[25]</sup>. Obesity, diabetes, IR and the initial severity of the fibrosis are the factors most conspicuously associated with fibrotic progression<sup>[23-25]</sup>.

The mechanisms by which IR promotes fibrosis progression include: steatosis, hyperleptinemia, increased

TNF production, impaired expression of PPAR- $\gamma$  receptors<sup>[4]</sup>. Hepatic injury in NASH induces oxidative stress, ROS and peroxidation products which lead to cytotoxic events, release of proinflammatory cytokines that activate hepatic stellate cells and deposition of collagen<sup>[8,9]</sup>.

HCC has been detected in several NASH patients, most often at the time of diagnosis, and rarely, during follow up<sup>[23,26,27]</sup>. In the larger Olmsted County Community Study<sup>[26]</sup>, 2 of 420 NAFLD patients developed HCC during a 7-year follow-up period. The estimated rate of liver-related deaths over 10 years was 12% for NASH patients<sup>[24,25]</sup>.

## ROLE OF HYPERTENSION

Hypertension is one of the main components of metabolic syndrome. The renin-angiotensin system (RAS) plays a role in progression of chronic liver disease to fibrosis, and HCC and this action is mediated *via* several mechanisms such as direct effect on activated HSCs and neovascularization<sup>[28]</sup>.

RAS is frequently activated in patients with chronic liver disease. In animal models, evidence has shown that angiotensin 2 receptor antagonist and angiotensin-converting enzyme (ACE) inhibitors display antifibrotic characteristics *via* the hepatic stellate cell proliferation<sup>[29]</sup>. In a pilot study examining the therapeutic efficacy of angiotensin 2 receptor antagonist, losartan was studied in patients with NASH and hypertension. Seven patients were treated with losartan (50 mg/d) for 48 wk. After 48 wk, patients not only showed a significant decrease in blood markers of hepatic fibrosis, but also an improvement in serum aminotransferase levels<sup>[30]</sup>. However, recent evidence that angiotensin 2 receptor antagonists and ACE inhibitors are antifibrotic in animal models of hepatic fibrosis suggests that these agents are worth examining in clinical trials<sup>[29]</sup>. Hypertension should be sought and treated appropriately in patients with NAFLD, particularly those with type 2 DM in whom tight blood pressure control (< 140/80 mmHg) with an ACE inhibitor or a  $\beta$ -blocker significantly reduces the risk of cardiovascular morbidity, sudden death, stroke and peripheral vascular disease<sup>[31,32]</sup>.

## BIOLOGICAL ROLE OF INSULIN

Insulin, after binding its receptor, induces the phosphorylation of receptor substrates in the liver and muscles, and triggers several steps toward the transactivation of glucose transporter-4 (GLUT-4). This increases glucose uptake by cells and its storage as glycogen, and inhibits the net production of glucose by the liver, thus blocking glycogenolysis and neoglycogenesis. Moreover, insulin promotes lipid storage by inhibiting lipolysis. When insulin is unable to induce glucose uptake, pancreatic  $\beta$ -cells increase insulin production and the hyperinsulinemic state prevents hyperglycemia. Thus, IR depends on insulin secretion and insulin sensitivity.

## IR

IR is defined as a condition in which higher-than-normal insulin concentrations are needed to achieve normal metabolic responses or, alternatively, normal insulin concentrations are unable to achieve normal metabolic responses<sup>[33]</sup>.

Hyperinsulinemia appears as a consequence of the inability of insulin to induce its effect on glucose metabolism, and hence, an abnormally large amount of insulin is secreted to achieve a biological response with consequent several abnormalities in target organs such as the liver, endothelium, and kidneys, and this represents the main feature in the metabolic syndrome<sup>[4]</sup>.

IR is measured by many ways. The most accurate is euglycemic-hyperinsulinemic clamp method whereas the less accurate, but widely applied, is Homeostasis Model Assessment (HOMA). Mean HOMA index increases with the stage of fibrosis and could help to differentiate stages of fibrosis<sup>[34]</sup>.

### **Pathogenesis of IR**

Pathogenesis of IR is not fully understood. IR is thought to be the key pathogenic feature leading to hepatic fat accumulation. It causes an increase in FFA influx into the liver that drives hepatic triglyceride production. Increased serum insulin and glucose levels also promote *de novo* lipogenesis by upregulating lipogenic transcription factors. NAFLD may in turn result in hepatic IR, which is thought to be triggered by hepatic FFA accumulation and their metabolites that may exacerbate overall IR<sup>[5]</sup>.

TNF- $\alpha$  is liberated by macrophage of adipose tissues of obese persons and worsens IR. Typically patients with NASH have also low serum adiponectin which is considered a potent insulin enhancer<sup>[17]</sup>.

### **IR in hepatitis C**

HCV directly associates with IR independent of the visceral fat area in non-obese and non-diabetic patients<sup>[35]</sup>. The mechanisms by which hepatitis C induces increased IR and the risk for development of diabetes has not been completely understood. Liver fibrosis progression has been considered, for a long time, responsible for the appearance of IR and type 2 diabetes in patients with chronic liver diseases<sup>[4]</sup>.

Recent data support a connection between HCV replication and IR, and HOMA decreased when the virus was eradicated. Besides, the incidence of diabetes type 2 is different in cured patients than in non-responders, supporting a better control of IR after HCV clearance<sup>[36]</sup>. Therefore, hepatitis C promotes IR and IR induces interferon resistance, steatosis and fibrosis progression in a genotype-dependent manner<sup>[4]</sup>.

Extensive evidence supports a central role of TNF- $\alpha$  and other proinflammatory cytokines in the development of obesity-associated IR and fatty liver<sup>[37]</sup>.

HCV infection promotes IR, mainly by increased TNF- $\alpha$  production together with enhancement of suppressor of cytokine (SOC)-3; both events block PI3K and Akt phosphorylation. Two types of IR can

be found in chronic hepatitis C patients: "viral" and "metabolic" IR<sup>[4]</sup>.

Both HCV and TNF- $\alpha$  downregulate IRS-1, 2 phosphorylation *via* upregulation of SOCS-3, which degrades the insulin receptor. Furthermore, HCV core protein *per se*, or its inflammatory cytokine (TNF- $\alpha$ ), interfere with normal insulin signaling which impairs translocation of GLUT-4 transporters to plasma membrane limiting glucose uptake and increases blood glucose/insulin level leading to IR. In the mean time, TNF- $\alpha$  impairs expression of PPAR- $\gamma$  receptor which in turn, decreases insulin sensitivity<sup>[4]</sup>.

## STEATOSIS IN CHRONIC HEPATITIS C

In chronic hepatitis C patients, the prevalence of steatosis ranges from 40% to 86% (mean, 55%)<sup>[38,39]</sup>. The majority of patients with steatosis (78%) have mild steatosis affecting less than 30% of hepatocytes. Thus, steatosis occurs more frequently in patients with chronic hepatitis C (55%) than in the general population (20%-30%) of adults in the Western world<sup>[40]</sup>. Macrovesicular steatosis is found in the periportal region of the liver-different from the centrilobular distribution characteristic of NASH patients. Mild steatosis had been reported in nearly 40% of patients with HCV genotype 4<sup>[41]</sup>.

Moderate or severe steatosis is significantly less frequent in genotype 4 than 3 chronic hepatitis C patients and similar between genotype 4 and 1. In non-diabetic, overweight patients, moderate or severe steatosis is present in only 10%-15% of genotype 4 or 1 compared with 40% of genotype 3 patients. Thus, hepatic steatosis in genotype 4 is mostly associated with metabolic factors, similar to those in genotype 1<sup>[41,42]</sup>.

It has been shown that HCV genotype 3 is associated with higher quasispecies heterogeneity than genotype 1<sup>[43]</sup>. Serum levels of apolipoprotein B and cholesterol are reduced in patients in whom steatosis responds to antiviral therapy<sup>[44]</sup>. Hypocholesterolemia in patients with chronic hepatitis C (especially genotype 3) has been reported by others<sup>[45,46]</sup>. Instead, after antiviral treatment, virus-related steatosis disappears whereas host associated steatosis remains unaffected<sup>[11]</sup>. Thus the disappearance of steatosis correlates with normalization of apolipoprotein B and cholesterol levels.

### Pathogenesis of steatosis in chronic hepatitis C

IR emerges as a very important host factor, mainly because it has been related to steatosis development, fibrosis progression and non-response to peg-interferon plus ribavirin. HCV directly associates with IR independent of the visceral fat area in non-obese and non-diabetic patients. HCV is directly associated with IR in a dose-dependent manner, independent of the visceral adipose tissue area<sup>[35]</sup>.

Factors associated with steatosis in chronic hepatitis C are: (1) viral factor (HCV genotype 3); (2) host factors (alcohol consumption, overweight, hyperlipidemia, diabetes, insulin resistance), and (3) drug therapy

(corticosteroids, amiodarone, methotrexate, *etc*)<sup>[11]</sup>. The mechanisms underlying the development of parenchymal steatosis in HCV infection are not exactly known<sup>[47]</sup>.

The first mechanism supposes that HCV core protein may block assembly of Apo-A<sub>1</sub>-A<sub>2</sub> with TAG. This will result in decreased export of TAG bound to apolipoprotein- $\beta$  (Apo- $\beta$ ) as VLDL out of hepatocyte, which is corrected by antiviral therapy<sup>[11]</sup>. Others propose that the core protein induces oxidative stress within the mitochondria that contributes to lipid accumulation<sup>[48]</sup>. Though the exact mechanism remains elusive, it seems that HCV itself can directly induce steatosis in genotype 3<sup>[49]</sup> by the cytopathic effect of high titer of intracytoplasmic negative strand HCV RNA<sup>[39]</sup>.

The second proposed mechanism recognizes IR as the major mechanism in the pathogenesis of hepatic steatosis<sup>[49-53]</sup>. It has been reported that IR plays a central role in NAFLD. However, the mechanisms of development of IR in patients with chronic HCV infection are not well understood<sup>[54,55]</sup>. IR causes impaired metabolic clearance of glucose, compensatory hyperinsulinemia, and increased lipolysis. The latter leads to increased plasma FFAs; increased hepatic uptake of FFAs by the liver which results in steatosis<sup>[9]</sup>. Furthermore, it has been suggested that IR may result from excess FFAs, TNF- $\alpha$  and suppressor of cytokine signaling (SOCS) which could downregulate insulin receptor substrate (IRS)-1 signaling<sup>[20]</sup>. This will result in the impaired translocation of GLUT-4 transporters to the plasma membrane which limits glucose uptake; increases blood glucose and cause a compensatory increase in insulin<sup>[56-58]</sup>. Recently, Pazienza *et al* reported that both genotype 3a and 1b downregulates IRS-1 through genotype-specific mechanisms<sup>[59]</sup>. Furthermore, Fartoux L *et al* reported that IR depends mainly on the age of the patient<sup>[34]</sup>. It has been suggested that age associated decline in mitochondrial function could contribute to IR<sup>[34,60]</sup>.

The main deleterious effect of IR in chronic hepatitis C is the ability to promote fibrosis progression. High serum glucose levels have been found associated with an increased rate of fibrosis progression, even greater than overweight<sup>[61]</sup>.

### Steatosis and fibrosis progression in HCV

High levels of TNF- $\alpha$  have also been observed in human chronic hepatitis C patients<sup>[40]</sup>. TNF- $\alpha$  has been shown to induce IR in experimental animals and cultured cells<sup>[62,63]</sup>. Inhibition of tyrosine phosphorylation of IRS 1 and 2 may be one of the mechanisms by which a high level of TNF- $\alpha$  causes IR<sup>[63-65]</sup>. Administration of an anti-TNF- $\alpha$  antibody restores insulin sensitivity<sup>[66]</sup>. These results provide direct experimental evidence for the contribution of HCV in the development of IR. There are experimental arguments for a direct role of insulin in fibrosis progression in HCV infection<sup>[11]</sup>.

Epidemiological studies indicating that the state of IR now associated with NASH is also associated with an increased risk of HCC. It is worth mentioning that diabetes increases the risk of chronic liver disease and HCC<sup>[67]</sup>.

## RESPONSE TO INTERFERON THERAPY IN HCV INFECTION

The current treatment for patients with chronic hepatitis C is the addition of ribavirin to interferon-based therapies for 24 to 48 wk. Unfortunately, a sustained virological response (SVR) is achieved in only 42%-52% of treatment-naïve patients, and the rest either show no response or experience a relapse when therapy is stopped<sup>[68]</sup>.

The mechanisms underlying the failure of interferon therapy are not well understood, but evidence indicates that in addition to viral factors, several host factors are also involved<sup>[69]</sup>. Among host factors, IR has been found to impair virological response to combined therapy in chronic hepatitis C patients<sup>[70]</sup>.

Hyperinsulinemia interferes with IFN signaling cascade<sup>[71]</sup> through upregulation of SOCS and activation of phosphatidylinositol-3-kinase (PI3K) that inhibit phosphorylation of STAT1.

In addition to IR, HCV core protein and TNF- $\alpha$  cytokine, derived primarily from macrophages of adipose tissue, upregulate SOCS-3 which binds to Janus Kinase inhibiting phosphorylation of STAT1, eventually interfering with interferon signaling<sup>[4]</sup>. SOCS-3 also causes degradation of IRS-1, in turn leading further to interference with insulin signaling and hyperinsulinemia that interferes eventually with interferon signaling<sup>[71]</sup>.

Recent data support a connection between HCV replication and IR and HOMA decreased when the virus was eradicated<sup>[4]</sup>. HCV directly associates with IR independent of the visceral fat area in non-obese and non-diabetic patients<sup>[35]</sup>.

In addition to IR, obesity and hepatic steatosis have been recognized as independent risk factors for a poor response to IFN- $\alpha$  therapy<sup>[72,73]</sup>. In obese individuals, subcutaneous fat could cause a reduction in the initial absorption and bioavailability of interferon given subcutaneously<sup>[72]</sup>. In the meantime, obesity and hepatic steatosis impair immune responses to HCV and increase fibrosis progression in obese patients<sup>[73]</sup>.

Furthermore, hyperleptinemia in obese is an independent risk factor for non-response to antiviral therapy<sup>[74]</sup>. Hyperferritinemia downregulates the response to interferon therapy<sup>[75,76]</sup>.

From this review, we have noted that steatosis, either metabolic or cytopathic, contributes to the development of NASH and progression to fibrosis, cirrhosis and HCC. Accordingly, we can conclude with confidence that hepatic steatosis is not a benign disease, but rather a silent killer.

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## Use of the Crohn's disease activity index in clinical trials of biological agents

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### Abstract

The Crohn's disease activity index (CDAI) has been commonly used to assess the effects of treatment with different agents in Crohn's disease (CD). However, these studies may be compromised, if the results compared to a placebo or standard therapy group (in the absence of a placebo) substantially differ from the expected response. In addition, significant concerns have been raised regarding the reliability and validity of the CDAI. Reproducibility of the CDAI may be limited as significant inter-observer error has been recorded, even if measurements are done by experienced clinicians with expertise in the diagnosis and treatment of CD. Finally, many CDAI endpoints are open to subjective interpretation and have the potential for manipulation. This is worrisome as there is the potential for significant financial gain, if the results of a clinical trial appear to provide a positive result. Physicians caring for patients should be concerned about the positive results in clinical trials that are sponsored by industry, even if the trials involve respected centers and the results appear in highly ranked medical journals.

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**Key words:** Crohn's disease; Crohn's disease activity index; Clinical trials; Infliximab; Adalimumab; Corticosteroids; Azathioprine

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### INTRODUCTION

The Crohn's disease activity index (CDAI) is a numerical calculation derived from the sum of products from a list of 8 items (Table 1), and multiplied by weighting factors for each item to define the severity of "disease activity" in patients with Crohn's disease (CD)<sup>[1]</sup>. Essentially, the CDAI represents a numerical estimation of a physician's interpretation of patient symptoms. Initially, the CDAI was correlated with a physician's overall global assessment for a group of 112 patients with CD after a total of 186 patient visits done in 13 different hospitals in the United States<sup>[1]</sup>. This instrument was first applied in the National Cooperative Crohn's Disease Study (NCCDS), published in 1979, and used to quantitatively compare the results in a placebo group to groups treated with the following drugs: sulphasalazine, prednisone and azathioprine.

Index values of 150 and below were associated with quiescent or non-active disease (i.e. "remission"). Values over 150 were indicative of active disease, and over 450, extremely severe disease. Of particular note, in patients randomized to placebo in the NCCDS, 32% achieved a spontaneous remission at the end of 17 wk, and 53% of these were still in remission at the end of 24 mo<sup>[2]</sup>. The results of drug treatment using the CDAI in active disease showed that the response to prednisone or sulfasalazine was significantly better than placebo, while the response to azathioprine was better than placebo, but did not reach statistical significance<sup>[3]</sup>. Patients with colonic involvement were especially responsive to sulfasalazine, and those with small bowel involvement were especially responsive to prednisone<sup>[3]</sup>. Since these studies were reported, other publications<sup>[4-7]</sup> have appeared employing the CDAI as well as other indices<sup>[8-12]</sup> to assess disease activity in the evaluation of

**Table 1 CDAI items and weighting factors**

<b>Item (daily sum per week)</b>	<b>Weighting factor</b>
Number of liquid or very soft stools	2
Abdominal pain score in one week (rating, 0-3)	5
General well-being (rating, 1-4)	7
Sum of physical findings per week:	20
Arthritis/arthralgia	
Mucocutaneous lesions (e.g. erythema nodosum, aphthous ulcers)	
Iritis/uveitis	
Anal disease (fissure, fistula, etc)	
External fistula (enterocutaneous, vesicle, vaginal, etc)	
Fever over 37.8°C	
Antidiarrheal use (e.g. diphenoxylate)	30
Abdominal mass (no = 0, equivocal = 2, yes = 5)	10
47 minus hematocrit (males) or 42 minus hematocrit (females)	6
1-x (1-body weight divided by a standard weight)	1

therapeutic effectiveness of different pharmacological and biological agents<sup>[13]</sup>.

## PLACEBO RESPONSE

Knowledge of the expected response to a placebo may be useful, but may not always be available. The NCCDS compared each of the treatment groups to a placebo group. Therapy with a new agent may now require comparison to the standard treatment, instead of a placebo. In part, at least, this relates to the ethical need to ensure that all participants in a clinical trial have access to proven therapy. Placebo alone may be difficult to justify, especially if patients are significantly symptomatic. And, if a new treatment shows a statistically positive result, it could reflect a limited effect of standard treatment (or the placebo). As in day-to-day clinical practice, the standard treatment may not always perform as well (or as badly) in each clinical trial. To best appreciate the results of a clinical trial involving a new agent, the expected response to placebo and treatment groups, as in the NCCDS, may be both important and informative.

## OBSERVER DIFFERENCES

Although the CDAI appeared to correlate with the global clinical assessment in a limited number of CD patients, it was appreciated that the same observer or different observers might derive different results at different times. Thus, efforts were made by the CDAI investigators and later groups to evaluate the reliability and validity of the CDAI. In the initial report of its development<sup>[14]</sup>, the consistency of the CDAI was examined in 2 successive visits for 32 patients. A positive association with the physician global assessment (with some overlap) was noted with a total of 50 index points being the apparent difference between slight clinical improvement (or worsening) and no actual change in symptoms for 2 successive visits. Subsequent studies also suggested that the reliability of the CDAI is within a defined moderate to good range, but not in a defined

very good to excellent range<sup>[15,16]</sup>. Later efforts to recalculate the CDAI by these investigators showed no significant difference from the original estimation, so no further changes in the CDAI were recommended<sup>[16]</sup>.

The CDAI appeared to be relatively reliable if used by these observers well experienced in its application. However, significant inter-observer differences were later noted in a series of separate studies, published as a single paper<sup>[17]</sup>. In one study, the CDAI was calculated from 10 "paper cases" evaluated by 5 consultants in surgery or gastroenterology, and 2 research assistants. Major discrepancies in calculated CDAI values were noted for each of the 7 different cases ranging from 166 to 430 points! In a second study, 15 members of the IOIBD prospectively evaluated a single case. The range of estimated values was 320 to 391, or 71 points. Improvement in observer differences, however, could be achieved with terminology discussion prior to calculation. A final study assessed the ability of 6 experienced gastroenterologists to independently elicit patient data, rather than being provided by the information from the "paper case" format. Wide variations in the estimated CDAI were seen ranging (in 1 case) up to 500 points! "Good agreement" was believed to have been achieved even in the best 2 patients, the difference was over 50 points. In spite of these critical concerns, the CDAI is used today in most clinical trials to evaluate different agent effects on symptom activity in CD.

## OTHER CDAI ISSUES

There are other recognized difficulties with the CDAI *per se*. First, as noted elsewhere<sup>[18]</sup>, a significant component of the total CDAI score is derived from highly subjective items, such as "general well being" and "intensity of abdominal pain". Many symptoms overlap with those that might be ascribed to some other functional causes. To overcome this issue, it has been logically recommended that these patients should be randomly distributed to each of the treatment groups<sup>[18]</sup>. But, in practice, this may be difficult to accomplish and may not be done. Second, the CDAI is computed using a diary card that must be developed by the patient for 7 d prior to submission. In practice, this may be difficult for some patients to prospectively do well without close monitoring which may not be feasible. Some clinical study coordinators apparently assist patients in retrospective completion of 7-d diaries on the study visit<sup>[18]</sup> and this could clearly impact results. As noted<sup>[18]</sup>, there are no data on the prevalence of this practice in clinical trial centers and it is not generally mentioned in the Methods section of the published reports. Third, some symptoms may be difficult to interpret or quantitate easily. For example, "liquid" stools may be difficult to precisely define<sup>[17]</sup>. Finally, even though enterocutaneous fistulae may be very troublesome for the patient, their impact on the CDAI may be limited. "General well being" may be severely impacted by perianal disease, but the patient may be fully functional. As a result, other indices have been developed to directly evaluate this component<sup>[19]</sup>. Even here, however, the endpoint measured may be criticized. For example, an

open fistula may be differentiated from a closed fistula based on expression of purulent material from the tract following application of “gentle pressure”. Data on intra-observer and inter-observer agreement for such endpoints in clinical trials are not available. Moreover, full disappearance of fistula tracts is rarely documented.

## IMPACT OF INDUSTRY

Another significant issue is the impact of the pharmaceutical industry in the conduct of clinical trials. This issue has been addressed in detail elsewhere<sup>[20]</sup>. In 1979, the NCCDS was reported to be largely supported through peer-review national research grant agencies for all patient study costs while the pharmaceutical industry was simply acknowledged for their donation of the study medications. Of course, it is not known if there were other financial benefits provided by industry then since requirements for reporting of industry support were minimal. Now, virtually all clinical trials are conducted almost entirely through industry support, either from private or shareholder-owned public companies. Many clinical trials are largely authored by a select group of “experts”, often, but not always, affiliated with university centers. Some authors are fully employed by the sponsoring industry and have the responsibility of collecting and monitoring all data collected, evaluating results and even drafting the manuscript. Some, but not all, journals require that all investigators declare income received from industry in the form of honoraria and other forms of financial support, although the precise amounts are never disclosed. Others may actually be owners or shareholders of the company concerned. This declaration is meant to alert the reader that the investigator may have a conflict of interest, not increase investigator credibility. If the journal did not require the declaration, it would not be provided. The problem, however, is even deeper. While hospitals involved in provision of research facilities may not be directly involved in conduct of the clinical trial, a “fee” is usually attached to the clinical trial costs for use of facilities, and so, indirectly, the institution (and its reputation possibly earned over many decades) is also being “purchased” by industry. Many teaching institutions, limited in resources, often consider industry funding as a positive asset for faculty members in the promotion process. In everyday clinical practice, it may be difficult to wade through all of these issues. The CDAI provides a simple numerical means to statistically evaluate treatment response, but this measurement has the potential to be manipulated as a positive spin will surely impact the share price in a positive direction.

## TOP-DOWN AND BOTTOM-UP CDAI TRIALS

A recent clinical trial compared infliximab to “conventional therapy” for CD<sup>[21]</sup>. An industry marketing label for these respective approaches has already appeared in the literature: “top-down” and “bottom-up”. In this study, both investigators and patients were aware of the drugs

being used for treatment. The study reported a positive result in favor of infliximab and azathioprine. The potential conflict of interest for a positive study result was clearly evident for all concerned in this open label study. Patients can actively influence the results of open label clinical trials, especially if an ordinarily expensive treatment is offered at no cost. Many of the authors noted financial support from a number of companies, including those with a vested commercial interest in the direct global marketing of infliximab. Even the editorialist<sup>[22]</sup> listed support from the same companies, certainly not an independent view of the data.

This open label study illustrated more about the modern conduct of clinical trials, their potential for conflict of interest and the credibility of the investigators associated with the results of this trial. Clinicians will need to be more suspicious of the results of clinical trials for CD, especially if based partly, or solely, on the CDAI, an index whose reliability and validity may be quite limited. Moreover, as the CDAI was used originally to assess the effectiveness of therapeutic agents having been used by physicians for decades, the present use of the CDAI seems to have evolved from its original intent. Now, potent biologicals (rather than pharmaceuticals) are being explored in clinical trials to determine if CDAI numbers, reflecting clinical symptoms, can be altered sufficiently to produce a statistically relevant result. Interestingly, even in an early report with infliximab in CD, a statistically positive result was observed with a specific dose of the agent, but in the same study, a dose-response could not be defined with higher doses<sup>[13]</sup>. This lack of dose-response with this biological agent was not explained. However, in retrospect, the study design clearly provided an advantage to infliximab treatment. There were three different infliximab groups that could be compared to only one placebo group. A statistically significant result for any one of these infliximab groups compared to the placebo would have been considered positive in favor of the new agent. Also, it was reported that 33% of the infliximab-treated patients had a CDAI-defined remission compared to only 4% for placebo. While statistically different, the remission rate for the infliximab-treated group was remarkably similar to the earlier reported placebo rate in the NCCDS. It is not known if these results reflected, to some degree, the inherent limitations of the CDAI to estimate patient symptoms, even in a blinded clinical trial, and permit comparisons with earlier studies.

Where will this approach related to the “chemotherapy of the CDAI” take us? No one really knows. However, the impact of an investigator bearing therapeutic “gifts” for the patient in a clinical trial, while, at the same time, personally receiving “gifts” from industry should raise concerns among physicians that are responsible for patient care.

## NEED FOR LONG-TERM STUDIES ON NATURAL HISTORY OF CD

A final paragraph might be added to raise a separate issue.

It is probably not sufficient to simply raise criticism and concern regarding the CDAI. Others have explored the use of other indices, some perhaps simpler to apply in a clinical setting. For example, the Harvey-Bradshaw Index<sup>[23]</sup> evaluates symptoms and signs during the preceding 24 h, rather than the previous 7 d, reduces the original 8 items in the CDAI to 5 (i.e. excluding antidiarrheal use, hematocrit and body weight), and eliminates the weighting factor. Its use appears to correlate well with the CDAI<sup>[23,24]</sup>. Another, the Cape Town Index (or South African Index) was developed as another alternative to avoid the “single parameter” bias inherent in the Harvey-Bradshaw Index<sup>[25]</sup>. Another index, the Van Hees Index<sup>[26]</sup> was developed in an attempt to avoid subjective clinical criteria by employing a physician global assessment variable. Most important, each of these different indices really attempts to measure only a very limited temporal window in the clinical course of CD. A longer term view of CD is required<sup>[27,28]</sup> so that objective measurement of the effects of different therapeutic efforts can be seen to positively or negatively alter the natural history of this chronic inflammatory process.

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## Diagnostic approaches for cholangiocarcinoma

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### Abstract

Cholangiocarcinomas arise from the epithelial cells of the bile ducts and are associated with poor prognosis. Despite new diagnostic approaches, the definite diagnosis of this malignancy continues to be challenging. Cholangiocarcinomas often grow longitudinally along the bile duct rather than in a radial direction. Thus, large tumor masses are frequently absent and imaging techniques, including ultrasound, CT, and MRI have only limited sensitivity. Tissue collection during endoscopic (ERCP) and/or percutaneous transhepatic (PTC) procedures are usually used to confirm a definitive diagnosis of cholangiocarcinoma. However, forceps biopsy and brush cytology provide positive results for malignancy in about only 50% of patients. Percutaneous and peroral cholangioscopy using fiber-optic techniques were therefore developed for direct visualization of the biliary tree, yielding additional information about endoscopic appearance and tumor extension, as well as a guided biopsy acquisition. Finally, endoscopic ultrasonography (EUS) complements endoscopic and percutaneous approaches and may provide a tissue diagnosis of tumors in the biliary region through fine-needle aspiration. In the future, new techniques allowing for early detection, including molecular markers, should be developed to improve the diagnostic sensitivity in this increasing tumor entity.

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**Key words:** Diagnosis; Brush cytology; Forceps biopsy;  
Cholangiocarcinoma

**Peer reviewers:** James M Millis, Professor, University

### INTRODUCTION

Cholangiocarcinomas are topographically categorized as intrahepatic or extrahepatic carcinomas. Extrahepatic cholangiocarcinomas are further subdivided into hilar, middle and distal carcinomas. The most common type of hilar cholangiocarcinoma is classified into 4 stages according to the bismuth classification<sup>[1]</sup>. Surgery is the only curative treatment in patients with cholangiocarcinoma. The results are more favourable for patients with early-stage disease. Therefore, a reliable diagnostic procedure is of great importance for these patients. However, confirmation of cholangiocarcinoma can be very difficult because of a wide spectrum of alternative diagnoses, including other carcinomas, metastasis and benign biliary strictures. Therefore, multidisciplinary investigative approaches are needed to overcome this problem. Cholangiocarcinomas often grow longitudinally along the bile duct rather than in a radial direction away from the bile duct. Consequently, imaging techniques including ultrasound, CT, and MRI are of limited sensitivity for the detection of cholangiocarcinoma<sup>[2]</sup>. Biliary tissue collection during endoscopic procedures is widely used for distinction between benign and malignant strictures and provides the only definitive diagnosis that can be used for establishing therapeutic strategies. To obtain tissue samples, brush cytology and/or forceps biopsy were routinely performed in patients with suspected malignant biliary strictures.

### BIOCHEMICAL INVESTIGATIONS

Obstructive jaundice is typically associated with an increase of serum bilirubin, alkaline phosphatase and gamma-

glutamyl transpeptidase. These laboratory parameters are unspecific and do not allow a distinction between benign and malignant bile duct strictures. The most widely studied tumor markers are carbohydrate antigen (CA) 19-9 and carcinoembryonic antigen (CEA). Both tumor markers may be elevated in cholangiocarcinoma<sup>[3-5]</sup>. However, CA19-9 and CEA are not specific for cholangiocarcinoma. CA19-9 is also raised in pancreatic cancer, colorectal cancer, gastric cancer, and gynaecological malignancies<sup>[6]</sup>. Additionally, CA19-9 may be elevated in patients with acute cholangitis<sup>[7]</sup>. In a series of patients without primary sclerosing cholangitis, the sensitivity of a serum CA19-9 level of more than 100 U/mL in diagnosing cholangiocarcinoma was 53%<sup>[3]</sup>. Furthermore, the authors reported in patients with unresectable cholangiocarcinoma a significantly greater mean CA19-9 concentration compared to patients with resectable cholangiocarcinoma. Recently, John *et al*<sup>[8]</sup> reported that sensitivity and specificity were 67.5% and 86.8%, respectively, when using a cut-off value of 100 U/mL. In another report that included 37 patients with primary sclerosing cholangitis, a serum CA19-9 concentration above 100 U/mL sensitivity was 89% and specificity was 86% for the diagnosis of cholangiocarcinoma<sup>[4]</sup>. CEA also has unsatisfactory diagnostic sensitivity and specificity for cholangiocarcinoma<sup>[9]</sup>. In conclusion, the diagnostic value of tumor markers in cholangiocarcinoma is limited. However, CA19-9 is useful in following the effect of treatment and to detect disease recurrence.

## IMAGING

### Ultrasonography

Patients suffering from jaundice usually undergo transabdominal ultrasonography to evaluate the bile duct diameter and hepatic parenchyma. Furthermore, gallstones can be excluded. In most patients cholangiocarcinomas are not directly detectable, but indirect signs are visible in the majority of patients. Distal lesions cause dilation of both intrahepatic and extrahepatic bile ducts, whereas proximal lesions only cause dilation of intrahepatic bile ducts. The localization of the bile duct lesion can be suggested if there is an abrupt change in ductal diameter. The diagnostic accuracy of ultrasonography was investigated in 429 patients with obstructive jaundice. In this series ultrasonography demonstrated ductal obstruction in 89%, and the sensitivity for localizing the site of obstruction was 94%<sup>[10]</sup>. The sensitivity and specificity of ultrasonography depends on tumor localization, the quality of the equipment and the experience of the investigator<sup>[11]</sup>. Ultrasound findings are limited in patients with liver cirrhosis and primary sclerosing cholangitis due to a lack of visible dilated bile ducts. Doppler ultrasonography provides information on hepatic and portal vessel patency. Recent studies reported that contrast enhanced ultrasonography provides sensitive and specific criteria for the differentiation between malignant and benign liver lesions<sup>[12-15]</sup>. Preliminary

data for cholangiocarcinoma suggest a behavior that is not dissimilar to metastatic lesions<sup>[14,16]</sup>. However, the limited number of cases in the reported series does not allow conclusive considerations for cholangiocarcinoma. Therefore, further studies with appropriate numbers of patients are needed.

### Computed tomography

Computed tomography (CT) is a commonly used approach for the detection and staging of cholangiocarcinoma. The radiological findings depend on localization and morphology of the tumor. CT scan permits identification of bile duct dilatation as well as assessment of lymph node, liver parenchyma, vascular encasement and metastasis<sup>[17]</sup>. Additionally, computed tomography is useful for detecting the presence of liver atrophy. Dilatation of bile ducts combined with atrophy suggests the obstruction of the portal vein<sup>[18]</sup>. However, conventional computed tomography is limited in the ability to estimate the extent of cholangiocarcinoma and resectability. Tillich *et al*<sup>[17]</sup> reported a series of 29 patients with hilar cholangiocarcinoma who underwent multiphasic helical CT, including arterial and portal venous phase. In these patients resectability was correctly predicted in only 60%. In another series, Yamashita *et al*<sup>[19]</sup> reported only 59% sensitivity in identifying a primary lesion by using contrast-enhanced computed tomography. Recently, the accuracy of preoperative high-resolution computed tomography to determine resectability in patients with hilar cholangiocarcinoma was evaluated<sup>[20]</sup>. In this series negative and positive predictive values of high-resolution computed tomography to determine resectability were 92% and 85%, respectively. Thus, only new CT scanning techniques should be taken into account since radiological procedures have had a considerable improvement in the last years.

### Magnetic resonance imaging and magnetic resonance cholangiopancreatography

In recent years, magnetic resonance imaging (MRI), especially in combination with magnetic resonance cholangiopancreatography (MRCP) has improved diagnosing cholangiocarcinoma and determining resectability<sup>[21-23]</sup>. Magnetic resonance imaging can assess the local tumor extension, lymph nodes, metastasis and liver parenchyma. It is important to use sequences with thin-slice thickness (3-4 mm) that provide sufficient signal to obtain good quality images and are sufficiently thin to detect subtle abnormalities. At present, good quality MRI in the hands of experienced centers, can be an excellent imaging approach for the diagnosis and staging of cholangiocarcinoma<sup>[24]</sup>. Moreover, magnetic resonance angiography (MRA) provides good assessment for infiltration of blood vessels. Magnetic resonance cholangiography can provide a three-dimensional reconstruction of the biliary tree without injection of intravenous and biliary contrast fluid. Therefore, the risk for cholangitis is reduced<sup>[21]</sup>, and additionally there is no

risk for contrast induced nephropathy. MRCP allows the assessment of bile ducts above and below a total obstruction. Therefore, MRCP should be considered for planning the treatment of patients suffering from cholangiocarcinoma. Zidi *et al*<sup>[25]</sup> reported a correct malignant hilar tumor stage using MRCP in 78% of the investigated patients. Furthermore, in this series an underestimated tumor extension was reported in 22%<sup>[25]</sup>. Biliary stent placement and percutaneous drainage results in mild inflammation of bile duct walls, which appears as an increased gadolinium enhancement with an appearance indistinguishable from the superficial spread of cholangiocarcinoma. To avoid this problem MRI and MRCP should be performed before endoscopic stenting and percutaneous transhepatic drainage<sup>[23]</sup>.

### **Positron emission tomography (PET)**

Several studies reported intensive accumulation of nucleotide tracer 18-fluorodeoxyglucose (FDG) in cholangiocarcinoma<sup>[26-28]</sup>. PET scanning with focal FDG accumulation permits visualization of cholangiocarcinomas. PET scan can detect cholangiocarcinomas as small as 1 cm<sup>[29,30]</sup>. FDG-PET is of value for staging of bile duct cancers, especially for discovering distant metastasis and malignant lymph nodes. In one series, PET led to a change of therapeutic management in 30% of patients suffering from cholangiocarcinoma because of detection of primary unsuspected metastases<sup>[26]</sup>. The limitation of FDG-PET is false positive results in patients with biliary tract infections, primary sclerosing cholangitis, and biliary stenting via endoscopic retrograde cholangiography (ERC) and PTBD<sup>[26,31]</sup>. The diagnostic sensitivity can be increased by using 18-fluorodeoxyglucose (FDG) in combination with CT scanning (FDG-PET/CT). Reinhardt *et al*<sup>[28]</sup> evaluated the effectiveness of this new dual-modality technique for noninvasive differentiation of extrahepatic bile duct strictures. This series included 14 patients with histological proven cholangiocarcinoma and 8 patients with benign bile duct strictures. In this series, all patients with cholangiocarcinoma presented with focal increased tracer uptake compared to patients with benign bile duct stricture. Overall, our experience is that <sup>18</sup>F-FDG PET/CT does not provide high accuracy for noninvasive detection of perihilar cholangiocarcinoma in extrahepatic bile duct strictures, which may be mainly due to the small size of the tumors.

## **ENDOSCOPIC APPROACHES**

### **Endoscopic retrograde cholangiography**

Retrograde injection of contrast fluid into the biliary tract allows the assessment of localization and morphology of bile duct strictures. Malignancy is suggested when there are findings of asymmetric, irregular strictures. Moreover, resectability can be evaluated. However, the differentiation in benign and malignant bile duct stricture may be difficult. Park *et al*<sup>[32]</sup> identified 20 out of 27 malignant bile duct strictures using ERC alone. In this series diagnostic

sensitivity and specificity for endoscopic retrograde cholangiography was 74% and 70%, respectively. Other authors have reported similar results for detecting malignant bile duct strictures by direct cholangiography<sup>[33]</sup>. Compared to non-invasive imaging techniques, endoscopic retrograde cholangiography allows tissue collection for cytological and histological investigation. Additionally, ERC allows biliary stent implantation for palliative treatment in irresectable tumors.

### **Percutaneous transhepatic cholangiography (PTC)**

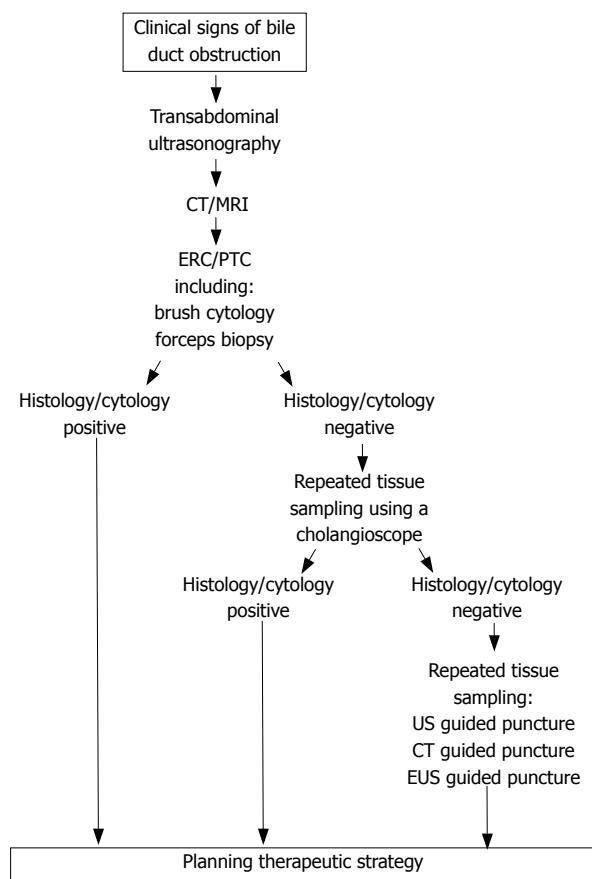
In patients with difficult bile duct access percutaneous transhepatic approaches offer a valuable alternative for bile duct access. The effectiveness of this procedure in diagnostic and therapy of complex biliary obstruction has been well documented<sup>[34,35]</sup>. Because percutaneous transhepatic bile duct access is an invasive technique, potential complications including bleeding, cholangitis, biliary leakage, duodenal perforation and death can occur. In previous series, procedure related death ranging from 0.6% to 5.6% was reported<sup>[36-39]</sup>. Therefore, endoscopic retrograde cholangiography is usually favoured above percutaneous transhepatic cholangiography. Percutaneous transhepatic approaches also allow tissue collection and biliary drainage.

### **Cholangioscopy**

Cholangioscopy using fiber-optic techniques provide direct visualization of the biliary tree. Differentiation between benign and malignant bile duct stricture using a cholangioscope has not been well defined. However, typical signs for malignancy including mucosal ulcerations, irregular mucosa and asymmetric stricture may be visible. Moreover, cholangioscopic guided forceps biopsy and brush cytology may enhance the diagnostic accuracy of tissue diagnosis. The most common approach is percutaneous transhepatic cholangioscopy. Another possibility is to perform peroral transpapillary cholangioscopy using a mother baby endoscope. Fukuda *et al*<sup>[40]</sup> evaluated the utility of peroral cholangioscopy for distinguishing malignant from benign biliary disease. The authors identified 22 out of 38 malignant bile duct strictures using ERC in combination with tissue sampling. The addition of peroral cholangioscopy correctly identified all 38 malignant strictures in this series.

### **Intraductal ultrasonography**

Intraductal ultrasonography (IDUS) is a promising imaging modality for the evaluation of a variety of biliary disorders<sup>[41,42]</sup>. Intraductal ultrasonography does not provide definite diagnoses. However, the characterization of biliary structures provided by IDUS can be used in combination with other diagnostic approaches to develop appropriate therapeutic strategies. Intraductal ultrasonography can provide the local staging to select patients with cholangiocarcinoma who benefit from surgical resection<sup>[43-46]</sup>. Recently, Stavropoulos *et al*<sup>[47]</sup> reported that intraductal ultrasonography increased the



**Figure 1** The diagnostic algorithm in patients with suspected extrahepatic bile duct obstruction.

accuracy of ERCP in distinguishing between benign and malignant strictures from 58% to 90%. This high rate of diagnostic accuracy using intraductal ultrasonography has been confirmed by others<sup>[48,49]</sup>.

#### EUS guided fine-needle aspiration

Endoscopic ultrasonography (EUS) complements the role of endoscopic and percutaneous transhepatic approaches and may provide a tissue diagnosis through fine-needle aspiration (FNA). The yield of EUS-FNA in patients with suspected cholangiocarcinoma was evaluated by Eloubeidi *et al*<sup>[50]</sup>. The authors reported a diagnostic sensitivity of 86%. However, another group reported lower rates of diagnostic sensitivity (45%) for detection of bile duct lesions by using ultrasound guided fine needle aspiration<sup>[51]</sup>. EUS-FNA may represent an alternative approach in the diagnosis of cholangiocarcinoma, especially in patients with negative brush cytology and forceps biopsy findings. One of the major limitations of endoscopic brush cytology from bile duct strictures is the poor quality of cytologic samples. Therefore, negative cytological results do not permit reliable exclusion of malignancy.

#### Brush cytology and forceps biopsy

Tissue collection during endoscopic and/or percutaneous transhepatic procedures are the most common techniques for providing a definitive diagnosis of

cholangiocarcinoma<sup>[52]</sup>. Brush cytology, first described in 1975, is the most common tissue sampling technique in patients with suspected bile duct strictures<sup>[53]</sup>. It is generally safe, requires little time, and is technically easier compared to forceps biopsy. The sensitivity of brush cytology for diagnosis of malignant biliary strictures ranges from 30% to 60% in most published series<sup>[54-56]</sup>. Tissue samples for histological investigation can be obtained from biliary strictures by using forceps. This technique is more time consuming than brushing and is less widely used, but it provides a sample of subepithelial stroma. In patients with malignant biliary stricture the overall cancer detection rate of forceps biopsy is often higher than for brush cytology, ranging from 43% to 81%<sup>[57-59]</sup>. In these published series, the sensitivity of brush cytology and forceps biopsy was evaluated in a heterogeneous patient group with several malignant bile duct strictures. Recently, the diagnostic sensitivity of transpapillary brush cytology and forceps biopsy was evaluated in patients with hilar cholangiocarcinomas<sup>[60]</sup>. In this series, the sensitivity of transpapillary brush cytology was 41.4% and the sensitivity of forceps biopsy was 53.4%. In combined approaches the diagnostic sensitivity increased to only 60.3%.

#### Fluorescence in situ hybridization (FISH)

Recently, investigators have attempted to improve diagnostic assessment with an advanced cytological technique for the detection of malignant pancreaticobiliary strictures<sup>[61]</sup>. Fluorescence *in situ* hybridization (FISH) has been shown to increase the sensitivity for the diagnosis of malignant pancreaticobiliary strictures compared to conventional cytology. Kipp *et al*<sup>[62]</sup> used a multitarget FISH probe set which has previously shown high impact in monitoring recurrent urothelial carcinoma<sup>[63]</sup>. This advanced technique identifies malignant cells by detecting aneusomy and deletion of the locus 9p21. By applying this technique for brush cytology and bile aspirate specimens in 131 patients with bile duct strictures (including 71 with primary sclerosing cholangitis, FISH analysis showed sensitivity of 35% and specificity of 91%. When patients with primary sclerosing cholangitis were excluded, sensitivity for malignancy detection by FISH was 16%<sup>[64]</sup>. This indicates that probe sets specific for biliary neoplasms will be required for higher sensitivity. However, not all malignant tumors present aneusomy or aneuploidy. In the biliary tract, the percentage of cancers displaying aneuploidy has been estimated to be approximately 80%<sup>[65]</sup>.

#### CONCLUSION

Figure 1 demonstrates the diagnostic algorithm used in our hospital for patients with suspected extrahepatic bile duct obstruction. Cholangiocarcinomas are still difficult to diagnose. In the future we need better early detection methods including molecular markers and improved histological techniques. Furthermore, new imaging and endoscopic techniques should be

developed to improve the diagnostic accuracy and tumor extension.

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TOPIC HIGHLIGHT

Miguel A Muñoz-Navas, Profesor, Series Editor

## Expanding role of capsule endoscopy in inflammatory bowel disease

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### Abstract

Capsule endoscopy has been shown to detect small bowel inflammatory changes better than any other imaging modality. Selection criteria have been optimized to increase the yield of capsule endoscopy in patients suspected to have Crohn's disease. Capsule endoscopy allows for earlier diagnosis of Crohn's disease of the small bowel and improved diagnosis of colitis in patients where it is unclear if they suffer from Crohn's or ulcerative colitis. A test capsule is available to assess for small bowel strictures and thus avoid capsule retention. A common language has been developed and a new scoring index will be added to capsule software. It is envisioned that the manner in which we treat Crohn's disease in the future will change, based on earlier diagnosis and treatment aimed at mucosal healing rather than symptom improvement.

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**Key words:** Capsule endoscopy; Crohn's disease; Capsule scoring index

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### INTRODUCTION

Capsule endoscopy (CE) was initially marketed in 2001 and in short time, this procedure gained a reputation for providing state-of-the-art imaging of the small intestine. It is recommended as the third test used in the investigation of obscure gastrointestinal bleeding after colonoscopy and upper endoscopy<sup>[1]</sup>. Capsule endoscopy has also been shown to be of use in patients with suspected Crohn's disease. Many papers have been published on the utility of capsule endoscopy for the diagnosis of Crohn's disease in cases of both suspected and known illness<sup>[2-5]</sup>. In a pooled data analysis, CE had a miss rate for ulcers of 0.5%<sup>[6]</sup>. A meta-analysis of eleven studies including 223 patients, comparing CE to other imaging modalities of the small bowel for inflammatory bowel disease established that CE has an incremental diagnostic yield of 25%-40% over other modalities such as barium studies and CT scanning (Table 1)<sup>[7]</sup>. This ability to visualize ulcers and other subtle inflammatory lesions led to the use of this technology to study the adverse effects of non-steroidal anti-inflammatory drugs (NSAID) on the small intestine<sup>[8]</sup>. The International Conference on Capsule Endoscopy (ICCE) consensus statement concluded that capsule endoscopy identifies small-bowel mucosal lesions not seen with other imaging modalities and may therefore play an important diagnostic role in the evaluation and monitoring of patients with known or suspected Crohn's disease<sup>[9]</sup>. Secondly, they concluded that capsule endoscopy may have a unique role in assessing mucosal healing after medical therapy, for assessing early postoperative recurrence and in guiding therapy, and finally the consensus statement concluded that capsule endoscopy may identify sub-clinical markers in asymptomatic family members and contribute to the understanding of the natural history inflammatory bowel disease (IBD).

### SUSPECTED CROHN'S DISEASE

With capsule endoscopy able to identify mucosal changes before other technologies, it is often used in patients with suspected Crohn's disease. This is a group that previously had not been formally defined. Suspicion of Crohn's disease was previously left to the discretion of the treating physician and usually was considered when a

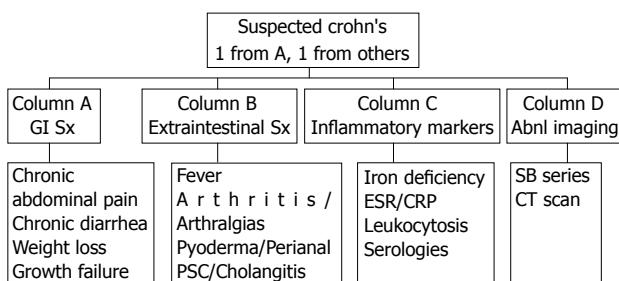
**Table 1** The incremental yield of capsule endoscopy over other testing (Treister *et al*<sup>[7]</sup>)

	Total yield CE (%)	Total yield other modality (%)	% IY for CE (95% CI)
Small bowel series	66	24	42 (0.30-0.54)
Ileoscopy	61	46	15 (0.02-0.27)
CT enterography	75	37	38 (0.23-0.54)
Push enteroscopy	51	7	44 (0.31-0.57)
Small bowel MRI	60	40	20 (0.41-0.81)

patient had either abdominal pain or persistent diarrhea. Yields of capsule endoscopy are low when performed in patients with abdominal pain alone<sup>[10]</sup> and in patients with abdominal pain and diarrhea alone<sup>[11]</sup>. When other criteria are added this yield increases. The addition of a sign or symptom of inflammation increases the yield of capsule endoscopy. In the CEDAP-Plus study of 50 patients with suspected Crohn's disease, signs of inflammation included elevated erythrocyte sedimentation rate, elevated C-reactive protein, thrombocytosis and leukocytosis and one of these markers increased the yield of capsule endoscopy with an odds ratio of 3.2<sup>[12]</sup>. The landmark paper by Fireman enrolled patients with abdominal pain, diarrhea, anemia, and weight loss. These patients had had symptoms for an average of 6.3 years and all had normal colonoscopies, upper endoscopies and small bowel series. Crohn's disease was diagnosed in 12 of the 17 by capsule endoscopy. It is clear that selective criteria were needed. The first consensus statement of the ICCE addressed the proper selection of patients and the group of suspected Crohn's disease was defined<sup>[9]</sup>. More recently, the ICCE convened in part to expand their definition of patients who should be considered as being suspect for Crohn's disease<sup>[13]</sup>. An algorithm was formulated (Figure 1). Patients should be considered for capsule endoscopy to diagnose or exclude the diagnosis of Crohn's disease if they had symptoms plus either extraintestinal manifestations, inflammatory markers or abnormal imaging studies.

## INDETERMINATE COLITIS

Another emerging use of capsule endoscopy in the field of inflammatory bowel disease is in patients with indeterminate colitis. Colonoscopic and pathologic criteria cannot differentiate Crohn's from ulcerative colitis in 10%-15% of colitic cases<sup>[14]</sup>. Proper identification of the disease state is important especially when choosing a surgical intervention. A few studies have examined the potential role of CE to rule out small bowel lesions suggestive of Crohn's disease in the setting of indeterminate colitis. Mow reported finding small bowel lesions in 40% of indeterminate cases<sup>[4]</sup>. Manoury reported 30 patients with indeterminate colitis and negative serologies in whom CE identified 5 cases with Crohn's<sup>[15]</sup>. This problem is especially difficult in children where the diagnosis cannot be assured in up to 30% of cases<sup>[16]</sup>. Use of capsule endoscopy in children has increased. Studies show that swallowing is possible in almost all cases, and in small children, the capsule is placed endoscopically<sup>[17]</sup>.

**Figure 1** Criteria for suspected Crohn's disease (Mergener *et al*<sup>[13]</sup>).

## RETENTION

Despite the ability to identify ulcers where no other technology could, there have been limitations to the application of this new technology. Fear of retention is one. Having a capsule retained in the small bowel remains a major concern for physicians performing capsule endoscopy since it could possibly lead to surgery in a patient who may otherwise have been treated medically for the same illness. This has been felt to be true especially for patients with Crohn's disease or NSAID enteropathy. The ICCE consensus statement on capsule retention reported a 1.5% risk of retention when capsule endoscopy is performed in the setting of suspected Crohn's disease<sup>[18]</sup>. Cheifetz reported retention in 13% of exams performed in the setting of previously known Crohn's disease<sup>[19]</sup>.

The ICCE consensus statement defined capsule retention as having a capsule endoscope remain in the digestive tract for a minimum of two weeks<sup>[18]</sup>. Retention was also defined as the capsule permanently remaining in the bowel lumen unless extracted by endoscopic or surgical methods or if passed as a result of medical therapy. There is no data on the success of medical therapies for retention such as initiating a course of steroids or infliximab, stopping NSAIDs, or using prokinetics or cathartics to aid in passage of the capsule. There is no time limit to institute management for capsule removal and capsules have stayed in patients asymptotically for over 3 years.

It is up to the physician and patient together to decide the best management for capsule retention. The choice of surgical, endoscopic, or medical management once capsule retention has been diagnosed depends on the cause of the retention, the indication for the exam in the first place, and the extent of previous treatment. If retention occurs behind a tumor or mass, surgical intervention is typically pursued quickly. If retention occurs behind a Crohn's stricture and the patient has had pronounced bleeding, again surgical intervention may prove the most efficacious method of not only removing the capsule but also dealing with the cause of hemorrhage. This is equally true for retention in a patient with known Crohn's disease and recurrent symptoms but without documented disease by any other method and failure to respond to medical therapy prior to the capsule exam. Those with known Crohn's disease who have already maximized their treatment with biologics and steroids for ongoing

**Table 2 Parameters and weightings for the capsule endoscopy scoring index (Gralnek *et al*<sup>[26]</sup>)**

	<b>Parameters</b>	<b>Number</b>	<b>Longitudinal extent</b>	<b>Descriptors</b>
First tertile	Villous appearance	Normal: 0	Short segment: 8	Single: 1
		Edematous: 1	Long segment: 12	Patchy: 14
	Ulcer	None: 0	Whole tertile: 20	Diffuse: 17
		Single: 3	Short segment: 5	< 1/4: 9
		Few: 5	Long segment: 10	1/4-1/2: 12
		Multiple: 10	Whole tertile: 15	> 1/2: 18
Second tertile	Villous appearance	Normal: 0	Short segment: 8	Single: 1
		Edematous: 1	Long segment: 12	Patchy: 14
	Ulcer	None: 0	Whole tertile: 20	Diffuse: 17
		Single: 3	Short segment: 5	< 1/4: 9
		Few: 5	Long segment: 10	1/4-1/2: 12
		Multiple: 10	Whole tertile: 15	> 1/2: 18
Third tertile	Villous appearance	Normal: 0	Short segment: 8	Single: 1
		Edematous: 1	Long segment: 12	Patchy: 14
	Ulcer	None: 0	Whole tertile: 20	Diffuse: 17
		Single: 3	Short segment: 5	< 1/4: 9
		Few: 5	Long segment: 10	1/4-1/2: 12
		Multiple: 10	Whole tertile: 15	> 1/2: 18
Stenosis-Rated for Whole Study	Stenosis	None: 0	Ulcerated: 24	Traversed: 7
		Single: 14	Non-Ulcerated: 2	Not traversed: 10
		Multiple: 20		

symptoms are not likely to improve without surgical intervention. Finally, for the patient in whom bleeding is not pronounced or in whom prior disease was only suspected but not treated, capsule retention behind a NSAID or Crohn's stricture can be managed with double balloon enteroscopy<sup>[20]</sup>. This technique allows for capsule retrieval and then the patient can be treated medically for their underlying illness.

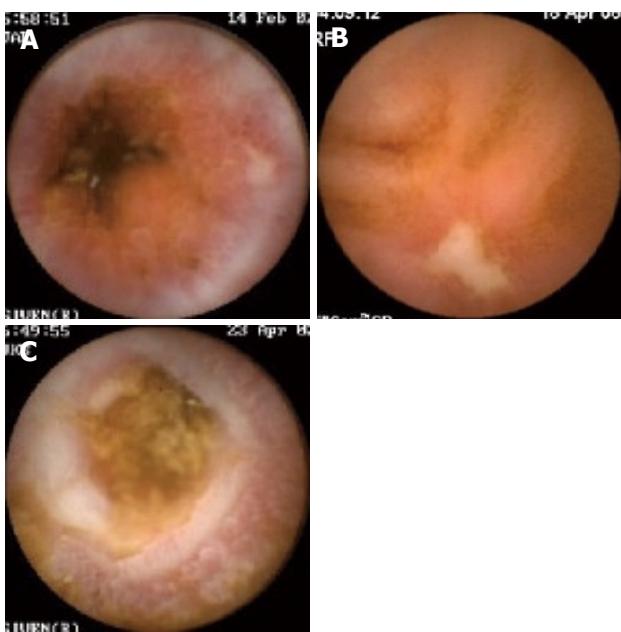
In an effort to avoid such situations, a dissolving test capsule called a patency capsule has been developed<sup>[21]</sup>. This capsule is the same size as a video capsule. It is constructed of cellophane with wax plugs at either end and it contains lactose mixed with 10% barium to make it radio-opaque. The wax plugs have holes that allow sucus entericus to dissolve the lactose and thus collapse the capsule into its various parts. A 2 mm × 10 mm radiotag inside the capsule allows the patient to be scanned externally to see if it is present in the body. Typically the patency capsule is swallowed by the patient and he or she is scanned 30 h after ingestion. At 30 h the capsule begins to dissolve. Thirty-eight percent of patency capsules are dissolved by 35 h and all are dissolved by 36-72 h.

## CAPSULE SCORING INDEX

The other major limitation to the adoption of this technology has been the lack of standardization when describing small bowel inflammatory lesions, in terms of their extent and severity. Specifically, no one language for findings has been developed, and no severity scale of mucosal disease activity or even a threshold for disease diagnosis has been agreed upon. There are many clinical scoring indices for Crohn's disease including the Crohn's

disease activity index (CDAI), the Harvey-Bradshaw and van Hees indices among others<sup>[22]</sup>. Since prior to CE, there has been no good direct measure of mucosal disease activity in the small intestine, these indices are based on clinical symptoms and some laboratory parameters. There is high interobserver variability in these scores due to their subjective nature<sup>[23]</sup>. Capsule endoscopy has provided the ability to detect mucosal inflammatory change of the small intestine often missed by other techniques. Interpretation and comparison of previous reports on the yield of CE have been limited due to the lack of a standardized and validated scoring index. The landmark papers by Fireman and Eliakim describing the yield of CE in suspected Crohn's disease did not outline the findings necessary to make the diagnosis<sup>[2,24]</sup>. Goldstein, who compared the effect of naproxen *versus* celecoxib on the small intestine, counted the number of mucosal breaks to measure adverse drug effects<sup>[8]</sup>. Mow used the cut off of three ulcers of any size to establish a diagnosis of Crohn's disease<sup>[4]</sup>. Fidder defined a positive capsule study for Crohn's disease as four or more ulcers, erosions, or a region with clear exudate and mucosal hyperemia and edema<sup>[25]</sup>. The meta-analysis of CE in the setting of Crohn's disease recognized this lack of a uniform method of categorizing findings at capsule endoscopy<sup>[7]</sup>.

A scoring index has been developed to assess mucosal inflammatory disease in the small bowel detected by CE and this will be included in the next version of capsule endoscopy software (Table 2)<sup>[26]</sup>. This scoring index is based on three capsule endoscopic variables: villous appearance, ulceration and stenosis (Figure 2). In addition, each variable is assessed by other parameters including size and extent of the change. The changes in villous appearance and



**Figure 2** Examples of endoscopic findings of Crohn's disease at capsule endoscopy. **A:** Edema; **B:** Ulceration; **C:** Strictures.

ulceration are assessed by tertiles, dividing the small bowel transit time into three equal time allotments. The stenosis evaluation is done for one entire study. The endoscopic variables have been specifically defined. Villous appearance is defined as edema where villous width is equal or greater than villous height. Villous appearance is based on mucosa distinct and separated from an ulcer rather than contiguous to a mucosal break. Ulcerations are defined as mucosal breaks with white or yellow bases surrounded by red or pink collars. Ulcer size is based on the entire lesion including its surrounding collar and is measured according to the percentage of the capsule image occupied by the ulcerated lesion. Ulcer size is based on the largest ulcer seen in each tertile. The number of lesions was defined as single, few (2-7 lesions) or multiple (8 or more lesions). The index was created in four separate steps. First, as outlined above, the characteristics of inflammatory change in the small bowel were identified. The terminology used accepted structured language developed for capsule endoscopy<sup>[27]</sup>. Second, blinded readers graded the presence or absence of each parameter on de-identified videos prospectively and also graded a perceived global assessment of overall severity of the findings. Third, the individual parameters and their descriptors were ranked in order of severity. In the fourth step, values for each parameter were created using the descent gradient method, a mathematic method to optimize numbers assigned to a rank order of variables. The premise was to assure that a final numerical score reflected the global assessment and that the global assessment agreed with the ranking of finding severity.

The score provides a common language to quantify mucosal changes associated with any inflammatory process. The index does not diagnose or measure a disease; it measures mucosal change. In addition, this scoring index does not have the discriminatory ability to differentiate these illnesses. At the same time however,

the index could be used for a number of different purposes including differentiating normal small bowel from disease states. This scoring index may be able to help establish the diagnosis of small bowel Crohn's disease when combined with other clinical signs/symptoms including patient history, clinical presentation and laboratory values. The index could also be potentially used to measure and document mucosal healing in response to therapy. The CE scoring index can provide one more point of evaluation along with other patient-level data to assist in determining appropriate patient management. Finally, the score could be a standardized method of communication both for treating physicians and for research purposes when assessing therapies and outcomes of patient's with small bowel Crohn's disease.

## THE FUTURE OF CAPSULE ENDOSCOPY IN IBD

Capsule endoscopy has the opportunity to propel a coming paradigm shift in the treatment of Crohn's disease. It is clear that capsule endoscopy identifies the earliest inflammatory changes in the small bowel. At the same time, the average time from the onset of a patient's symptoms until diagnosis historically lags an average of 35 mo<sup>[28]</sup>. Thus capsule endoscopy has the opportunity to diagnose Crohn's disease earlier than ever before. What remains unclear is if early diagnosis provides patient benefit. Does earlier diagnosis and thus earlier intervention change the natural history of the disease? This is not known, though studies in children with fistulous disease had greater response to therapy the earlier they were diagnosed<sup>[29]</sup>. Thus it is theorized that early diagnosis will bring earlier treatment and thus improved outcomes. Another paradigm shift in the making is the method of assessing disease activity. Previously, physicians have used patient symptoms to guide treatment. Unfortunately placebo response rates by symptoms average 18% (0%-50%)<sup>[30]</sup>. Remission has been defined as symptom improvement typically using the CDAI. But remission does not correlate with mucosal healing. In Rutgeerts' trial of 75 patients treated with infliximab, 67% of healed patients were in symptom remission, while 56% of remission patients did not heal<sup>[31]</sup>. Prior to capsule endoscopy there was no reliable method to determine the extent or severity of the disease in the small bowel though colonic evaluation has been available. It has been proposed that in the future, patient management decisions may be based on measures of mucosal healing rather than symptom response<sup>[32]</sup>. A variety of tools are available to assess overall disease activity including fecal and serum biomarkers, endoscopy, radiology, and capsule endoscopy.

In summary, capsule endoscopy has been shown to detect small bowel inflammatory changes better than any other imaging modality. A common language has been developed. It is envisioned that the manner in which we treat Crohn's disease in the future will change, based on earlier diagnosis and treatment aimed at mucosal healing rather than symptom improvement.

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## TOPIC HIGHLIGHT

Miguel A Muñoz-Navas, Profesor, Series Editor

# The future of wireless capsule endoscopy

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## Abstract

We outline probable and possible developments with wireless capsule endoscopy. It seems likely that capsule endoscopy will become increasingly effective in diagnostic gastrointestinal endoscopy. This will be attractive to patients especially for cancer or varices detection because capsule endoscopy is painless and is likely to have a higher take up rate compared to conventional colonoscopy and gastroscopy. Double imager capsules with increased frame rates have been used to image the esophagus for Barrett's and esophageal varices. The image quality is not bad but needs to be improved if it is to become a realistic substitute for flexible upper and lower gastrointestinal endoscopy. An increase in the frame rate, angle of view, depth of field, image numbers, duration of the procedure and improvements in illumination seem likely. Colonic, esophageal and gastric capsules will improve in quality, eroding the supremacy of flexible endoscopy, and become embedded into screening programs. Therapeutic capsules will emerge with brushing, cytology, fluid aspiration, biopsy and drug delivery capabilities. Electrocautery may also become possible. Diagnostic capsules will integrate physiological measurements with imaging and optical biopsy, and immunologic cancer recognition. Remote control movement will improve with the use of magnets and/or electrostimulation and perhaps electromechanical methods. External wireless commands will influence capsule diagnosis and therapy and will increasingly entail the use of real-time imaging. However, it should be noted that speculations about the future of technology in any detail are almost always wrong.

## INTRODUCTION

Wireless capsule is an incompletely developed technology, which may change endoscopy forever and has the capacity to replace a good deal of conventional endoscopy. Will capsule endoscopy replace much of conventional upper gastrointestinal endoscopy and colonoscopy? The answer is probably yes but the time frame is unclear. Will capsule endoscopy be able to deliver therapy? Again, the answer is probably yes.

There are major challenges to the expansion of capsule technology into a position where it can compete with conventional diagnostic gastroscopy and colonoscopy<sup>[1-5]</sup>. The first challenge is power management. At present the Pillcam capsule contains two small 3 volt hearing aid batteries which allow about 8 h of continuous imaging at 2 frames per second. The use of complementary oxide silicone (CMOS) technology for the video has the advantage of requiring extremely low power levels when compared with a charge coupled device (CCD). Slowing the video frame rate to two frames per second also increases the life span of the capsule. The esophageal capsule takes 14 frames a second and has a life span of about 20 min. Recent quite radical design changes have included the development of an esophageal capsule with 18 frames per second and small bowel and colon capsules, both with 4 frames per second, yet with an increase in capsule life to about 11 h. These changes have been accomplished

mainly by more efficient power management. More batteries or increased battery volume may be of help but would increase the size and weight of the capsule. The two small silver oxide batteries in current use are not the most efficient in terms of power to weight ratio, but were chosen because of their safety record. Lithium batteries could run the capsule for longer periods. There have been important "break-throughs" in battery design with the advent of carbon nanotubes and Buckytubes which have the intrinsic characteristics desired in the material used as electrodes in batteries and capacitors, two technologies of rapidly increasing importance. Buckytubes have a tremendously high surface area (approximate 1000 m<sup>2</sup>/g), good electrical conductivity, and their linear geometry makes the surfaces highly accessible to the electrolyte. It may be that better battery design and better power management will give the capsule the power required for additional performance and functions.

The development of external power transmission methods using electrical field induction, radio-frequency, microwaves or ultrasound technology could free the capsule from the requirement for batteries or at least prolong the functional lifespan. This would substantially lighten the capsule, allow space and power for other functions such as biopsy or drug delivery but above all allow the capsule to be powered for indefinite periods which would permit increase in the present rather low frame rate, and in particular make the problem of prolonged or implanted capsule endoscopy and remote controlled therapeutic capsule endosurgery much easier to solve.

The development of the capsule endoscopy was made possible by miniaturization of digital chip camera technology, especially CMOS chip technology. The continued reduction in size, increases in pixel numbers and improvements in imaging with the two rival technologies-CCD and CMOS is likely to change the nature of endoscopy. The current differences are becoming blurred and hybrids are emerging.

The main pressure is to reduce the component size, which will release space that could be used for other capsule functions such as biopsy, coagulation or therapy. New engineering methods for constructing tiny moving parts, miniature actuators and even motors into capsule endoscopes are being developed.

Although semi-conductor lasers that are small enough to swallow are available, the nature of lasers which have typical inefficiencies of 100-1000 percent makes the idea of a remote laser in a capsule capable of stopping bleeding or cutting out a tumour seems to be something of a pipe dream at present, because of power requirements. The construction of an electrosurgical generator small enough to swallow and powered by small batteries is conceivable but currently difficult because of the limitations imposed by the internal resistance of the batteries. It may be possible to store power in small capacitors for endosurgical use, and the size to capacity ratio of some capacitors has

recently been reduced by the use of tantalum. Small motors are currently available to move components such as biopsy devices but need radio-controlled activators. One limitation to therapeutic capsule endoscopy is the low mass of the capsule endoscope (3.7 g). A force exerted on tissue for example by biopsy forceps may push the capsule away from the tissue. Opening small biopsy forceps to grasp tissue and pull it free will require different solutions to those used at flexible endoscopy-the push force exerted during conventional biopsy is typically about 100 g and the force to pull tissue free is about 400 g.

Future diagnostic developments are likely to include capsule gastroscopy, attachment to the gut wall, ultrasound imaging, biopsy and cytology, propulsion methods and therapy including tissue coagulation. Narrow band imaging and immunologically or chemically targeted optical recognition of malignancy are currently being explored by two different groups supported by the European Union as FP6 projects: -the VECTOR and NEMO projects. These acronyms stand for: VECTOR = Versatile Endoscopic Capsule for gastrointestinal TumOr Recognition and therapy<sup>[6]</sup> and NEMO = Nano-based capsule-Endoscopy with Molecular Imaging and Optical biopsy.

Both projects also include the development of remote manipulation methods.

## CHALLENGE TO USING CAPSULE ENDOSCOPY FOR COLONOSCOPY

There are challenges to using wireless capsule endoscopy for colonoscopy but most of these have been solved. Currently the capsule acquires images for 8 h and usually reaches the right side of the colon before the battery expires. The capsule may have to run for 24-48 h in order to perform a complete examination of the colon unless we can speed up the total gut transit. The power problem can be addressed in several ways including the use of more batteries, batteries with a delay mode which are switched on when the capsule reaches the distal small intestine, external power transmission and methods to move the capsule faster in the colon. A very effective timed colon cleaning will be necessary. Deletion of identical frames would make it easier to examine the images since the capsule in the colon may remain stationary for prolonged periods. Currently, wireless capsule colonoscopy has generated images from all areas of the colon and has imaged pathology especially in the right side of the colon, but also in the rectum. Initial clinical studies have provided promising results.

Autonomous wireless capsule laparoscopy is technically feasible and has been tried *in vivo* but needs to develop further and offer advantages over conventional laparoscopy. The obvious advantages might be that port numbers can be reduced for selected laparoscopic operations. It can be used to improve visualization during NOTES, and may allow multiple camera angles

during surgery, and views of structures that are difficult to see with conventional rigid laparoscopes such as the retroesophageal space or the pouch of Douglas. Wireless imaging of cardiac or vascular structures is conceivable, but would require substantial development and control strategies.

Wireless laparoscopy is a desirable extension of wireless capsule endoscopy. This may allow deconstruction of laparoscopes or NOTES platforms, separating the imager from the surgical effectors, thus reducing the outer diameter of trocars, and making surgery less invasive.

The manufacture of an autonomous video capsule the size of a red blood cell as described in Isaac Asimov's "The Fantastic Voyage" and made into a movie in 1966 by Richard Fleischer with Stephen Boyd, Raquel Welsh and Donald Pleasance is some way in the future. Reduction in size by up to an order of magnitude is currently conceivable with available components. It would be relatively easy to reduce by one-half the current dimensions of the capsule.

## **ATTACHMENT OF CAPSULE ENDOSCOPES TO THE GI TRACT**

It may become possible to stitch or clip the capsule to the wall of the stomach so that prolonged examination of bleeding ulcers or varices becomes possible. An on/off radio-controlled command may be helpful to conserve power. Long-term endoscopy with wireless endoscopes attached to the wall of the gut seems an obvious way to improve the management of gastrointestinal bleeding and other disorders.

## **TISSUE INTERACTIVE DIAGNOSTIC METHODS SUCH AS BIOPSY**

At present, the capsule cannot obtain biopsies, aspirate fluid or brush lesions for cytology. These common endoscopic manoeuvres may become possible during capsule endoscopy. These techniques require real-time viewing, as well as radio-controlled triggering and remote controlled capsule manipulation, if they are to be used with precision. Biopsy using a spring loaded Crosby capsule-like device with an evacuated chamber is feasible with existing capsule technology. In preliminary studies, patients were almost always able to retrieve the capsules from the stools using a net and a magnet. Experimental brush, biopsy and radio-controlled release mechanisms which can be used in autonomous capsules have been tested in "*in vivo*" studies. It may become possible to combine capsule technology with "optical biopsy" such as narrow band imaging or pathology-targeted enhanced tissue markers.

## **CAPSULE COAGULATION**

A prototype coagulation capsule has been built and tested which employs an exothermic chemical reaction to

generate heat using the interaction of calcium oxide and water. It seems possible that other thermal therapeutic applications will be added in the future.

## **ELECTRO-STIMULATION FOR PROPELLING CAPSULE ENDOSCOPE**

One way to manipulate a wireless capsule endoscope autonomously in the gastrointestinal tract is to use electro-stimulation to propel the device, for example with a pair of bipolar electrodes at either end of the capsule. This technology has been tested in humans. Electrodes attached to a PILLCAM capsule have been used to propel the device in the porcine esophagus and small intestine, as well as in the human small intestine. A dumb-bell shaped capsule allows the imaging capsule to view the traction capsule. A radio-controlled electro-stimulation capsule has been developed. Radio-commands can be sent from a transmitter to the receiving traction capsule causing it to propel the video capsule forwards or backwards in the human gastrointestinal tract.

Water-jet propulsion has also been used to propel very light weight (3.7 g) capsules in the gastrointestinal tract. The light weight of the capsule compared with the 1.5 kg of a colonoscope makes it possible to think in terms of developing new types of very light weight colonoscopes, which can be passed on a wire through the anus, and would require much less force on the wall of the colon to reach the cecum.

Olympus in a news release (November 30, 2004) announced the following developments in capsule endoscopy: wireless power supply system, capsule guidance system, drug delivery system, body fluid sampling technology, self-propelled capsule and ultrasound capsule. Competition is likely to stimulate new technical developments which may make capsule endoscopy cheaper. Olympus is using CCD technology in their small bowel capsule that was released recently in Europe and USA, with FDA approval and a CE mark. A Chinese wireless small intestinal capsule (OMOM) has gone through 3 technical design changes, with improving image quality, and currently costs less than the other systems. A Korean small intestinal capsule (MIRO) includes some novel technological features including two external electrodes and a single skin electrode to facilitate transmission of image data using the body as an electrical conductor, thus eliminating the need for radiofrequency (wireless) data transmission, and as a result saving power. Recent technical improvements in the Given Imaging capsules (SB2, ESO2, COLON1) include adaptive light control i.e. if a frame is too dark the light emitting diodes will emit more light in subsequent frames. The frame rates of commercially available Given capsules have increased from 2 to 4 frames per second, for prolonged image transmission of up to 11 h and from 14-18 frames per second for rapid esophageal double imager viewing. Additional lenses have been added to the

redesigned optics to increase the depth of focus and widen the angle of view from 140 to 156 degrees which can more than double the surface area of the mucosa examined. The software continues to improve in order to manage the technical advances. Rapid 5, is the most recent version, indicating that the software has been revised 5 times. Real-time viewing has improved and will be increasingly important for new diagnostic and therapeutic options. At the time of writing (May 2008) more than 750 000 wireless capsule endoscopes have been sold since they were first launched in 2001.

## CONCLUSION

Capsule video-enteroscopy has opened up a new world of diagnostic and other possibilities for the gastroenterologist. It is remarkable to see images of small intestinal abnormalities such as an ulcerated Meckel's diverticulum or active bleeding from a tumour in the middle of the small intestine, which was not possible until recently. The development of wireless capsule endoscopy has changed video endoscopy of the small intestine into a much less invasive and more complete examination. The increasing use of these resources and the comfort and ease with which some of these examinations can be performed makes it likely that wireless capsule video imaging will have a substantial impact on the management of small intestinal disease as well as other parts of the body.

It is usually a mistake to try to predict the future

because the chances of error are very high. "It is impossible to predict the future, and all attempts to do so in any detail look ludicrous within a very few years." These are the opening words of "The Profiles of the Future" by Arthur C. Clarke, who wrote an amusing account of eminent scientists predicting that electric light, human flight and space travel were impossible at the same time as Edison perfected the light bulb, the Wright brothers made their first motor powered flight and Yuri Gagarin circled the earth in a sputnik. Einstein who probably knew more than most about the potential impact of physics on the future of mankind was goaded during an interview in 1929 on board the Belgoland into saying "I never think about the future; it comes soon enough".

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## TOPIC HIGHLIGHT

Miguel Angel Muñoz-Navas, Professor, Series Editor

# Capsule endoscopy in celiac disease

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## Abstract

Video capsule endoscopy is an attractive and patient-friendly tool that provides high quality images of the small bowel. Obscure gastrointestinal bleeding is the primary and most evaluated indication to capsule endoscopy; however, indications are expanding and a small number of preliminary reports have been presented concerning the role of video capsule endoscopy in the diagnosis of celiac disease. The purpose of this review is to update the current knowledge and to hypothesize on future perspectives of the use of video capsule endoscopy in patients with celiac disease.

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**Key words:** Capsule endoscopy; Celiac disease; Diagnosis of celiac disease; Celiac disease complications

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## INTRODUCTION

Celiac disease is a gluten-dependent enteropathy, characterized by chronic small bowel inflammation

and mucosal atrophy. Up to a few years ago, celiac disease was considered a rare pathology. Today, its prevalence has been estimated ranging between 0.2% and 1% in the United States and European population<sup>[1]</sup>. Although celiac disease was believed to be a pediatric syndrome, it is now recognized mainly as an adult disease that involves multiple organs. Abdominal pain, diarrhea, growth failure and malabsorption are its typical clinical presentation. However, the increased interest for this pathology over the last 2 decades allowed diagnosing celiac disease also in those with the silent or "atypical" form. These patients may present vague and subclinical manifestations such as dyspeptic symptoms or esophageal reflux, irritable bowel syndrome, polyneuropathy or iron deficiency anemia<sup>[2]</sup>. The disease can also be totally asymptomatic and the diagnosis can be accidental. The diagnosis of celiac disease is made by demonstrating the characteristic histopathological changes on intestinal biopsy obtained by esophagogastroduodenoscopy. Villous atrophy on a duodenal biopsy represents the internationally accepted gold standard diagnostic test for celiac disease. Serological tests include antigliadin, antiendomysium and anti-tissue transglutaminase antibodies. These tests are highly reliable for the diagnosis of celiac disease and represent a cheap and non-invasive method to identify patients who will be referred to endoscopy, specifically with the purpose of obtaining duodenal biopsy. The positive and negative predictive value of combining the measurement of antibodies from tissue transglutaminase and antiendomysium has been reported to be greater than 96%<sup>[3]</sup>.

Capsule endoscopy is a well tolerated, minimally invasive method for the visualization of the entire small bowel and is currently used to evaluate patients with obscure bleeding, inflammatory bowel diseases, suspected small bowel tumours, polyposis syndromes, nonsteroidal anti-inflammatory drug injury and complicated celiac disease<sup>[4]</sup>. Video capsule endoscopy is provided with an 8-fold magnification capacity lens optical system that allows a magnification similar to that of dissection microscopy<sup>[5]</sup> and is therefore able to assess the small bowel villous structure. For this reason, video capsule endoscopy could offer an alternative to duodenal biopsy in patients who are unable or unwilling to undergo esophagogastroduodenoscopy. This article reviews the current knowledge and future prospects of the use of video capsule endoscopy in patients affected by celiac disease.

## CRITERIA FOR DIAGNOSIS OF CELIAC DISEASE

The first step in pursuing a diagnosis for celiac disease is a serological test. The immunoglobulin A (IgA) anti-human tissue transglutaminase (t-TG) and IgA endomysial antibody immunofluorescence (EMA) are the best tests available. Although these tests are highly sensitive and specific, small bowel biopsy remains the standard for the diagnosis of celiac disease. Demonstration of hyperplastic villous atrophy of the small bowel and clinical remission when a gluten-free diet is followed represent the diagnostic tests for celiac disease<sup>[6]</sup>.

## ENDOSCOPIC SIGNS IN CELIAC DISEASE

Four endoscopic markers suggestive of villous atrophy have been described in celiac disease: loss or reduction in duodenal Kerkring's folds, mosaic mucosal pattern, scalloped configuration of duodenal folds and micronodular pattern of the mucosa<sup>[7,8]</sup>. These markers should serve as a tool to assist endoscopists in deciding when small bowel biopsies may be indicated. Sensitivity of these markers for the diagnosis of celiac disease has been reported to be between 47% and 100%<sup>[8-10]</sup>. Endoscopic markers overall have a wide range in sensitivity mainly because of their absence when lesser degrees of villous atrophy are present. Therefore, endoscopic evaluation without biopsies is not considered sensitive enough for a diagnosis and is considered inadequate to confirm or to exclude celiac disease<sup>[11]</sup>. However, when endoscopic signs are present, they have a high specificity, ranging between 92% and 100%<sup>[2,10,12]</sup>. For this reason, investigators should be aware of the importance of such markers detected during endoscopy. Recognition of endoscopic signs of celiac disease could help to select patients for biopsy and avoid delays in the diagnosis of the disease, preventing long term complications. Recently, endoscopic approaches for the evaluation of duodenal villous abnormalities have been proposed allowing a direct visualization of the duodenal villous structure during routine upper endoscopy. Endoscopic visualization of intestinal villous pattern with the "immersion technique" provides a sensitivity, specificity, positive predictive value and negative predictive value of 85%, 99%, 99% and 90%, respectively<sup>[11,13]</sup>. Moreover, it has been demonstrated that the new high-magnification and high-resolution endoscopes are able to better evaluate the presence or absence of villous pattern. Sensitivity, specificity, positive predictive value and negative predictive value of endoscopic magnification ( $\times 2$ ) for detection of total villous atrophy were all 100%. Similar results were obtained combining the "immersion technique" with magnification<sup>[14]</sup>. Therefore, theoretically, in patients with suspected celiac disease (EMA+ and/or t-TG antibodies+) using these new endoscopic approaches, the biopsy sampling of the small bowel could be avoided when a flat duodenal mucosa is observed and should be reserved only for those patients in whom villi are observed at endoscopy<sup>[11]</sup>.

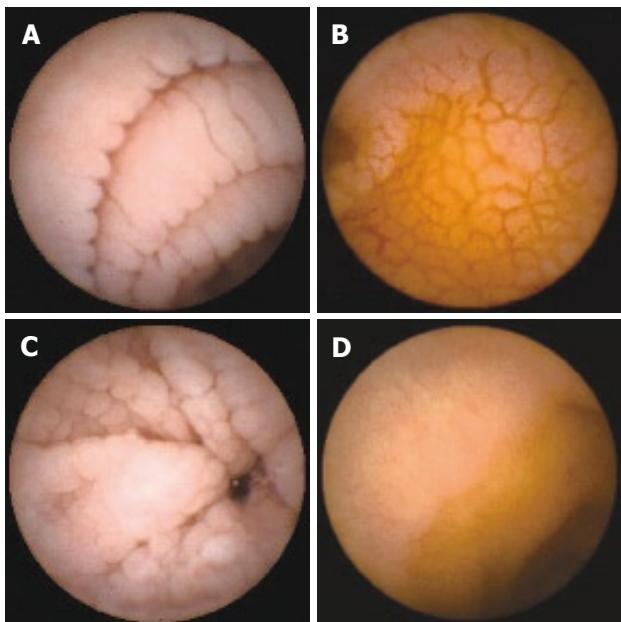


**Figure 1** Normal endoscopic pattern at video capsule endoscopy. Villous pattern can be easily observed.

## CAPSULE ENDOSCOPY

Capsule endoscopy has become an important tool in the investigation of patients with small bowel diseases. Preliminary reports suggest that capsule endoscopy could represent an attractive and non-invasive diagnostic tool in patients with suspected celiac disease<sup>[15-19]</sup>. It can also be used in patients with known celiac disease for monitoring small bowel healing; in patients with alarming symptoms despite a closely followed gluten-free diet and in long-term surveillance to detect malignancies<sup>[20]</sup>. The diagnosis of celiac disease is still based on the recognition of villous atrophy on duodenal biopsy; however duodenal biopsy cannot be considered an optimal gold standard. The major limits are represented by the need to perform an upper gastrointestinal (GI) endoscopy which represents an invasive procedure; the difficulty to obtain proper oriented tissue samples; the occurrence of patchy mucosal lesions that can be missed by the biopsy; and the limited portion of gut examined with the risk of loosing the diagnosis of complications, such as enteropathy-associated T-cell lymphoma and ulcerative jejunileitis. Capsule endoscopy is provided with certain features that may overcome some of the limits of traditional upper GI endoscopy. Capsule endoscopy is a non-invasive, painless and well accepted procedure. The capsule has the magnification power of a dissecting microscope: the optical system has an 8-folds magnification power, therefore the villi can be easily observed (Figure 1). The test is performed without air inflation, with the optical dome of capsule endoscopy close to the mucosa, improving the assessment of the villous architecture. Finally, it allows the visualization of the entire small bowel, providing an estimation of the extent of small bowel involvement and facilitating the diagnosis of complications.

In a preliminary study, Petroniene *et al*<sup>[16]</sup> compared 10 celiac patients with histologically proven villous atrophy (Marsh III) with 10 control patients with normal histology, showing that capsule endoscopy has an excellent accuracy in identifying villous atrophy. When compared to upper GI endoscopy, capsule endoscopy reported an identical specificity (100%); a tendency towards a better sensitivity (70% vs 60%); and a positive



**Figure 2** Endoscopic markers of celiac disease at capsule endoscopy: scalloping (A), mosaic pattern (B), micronodularity (C), and reduction of folds (D).

predictive value and negative predictive value of 100% and 77%, respectively. These data were confirmed by recent studies<sup>[17,19]</sup>. Hopper *et al*<sup>[17]</sup> showed that 17 out of 20 patients with celiac disease had villous atrophy also detected by capsule endoscopy. In this paper, the sensitivity, specificity, positive predictive value and negative predictive value for capsule endoscopy in recognising villous atrophy were 85%, 100%, 100% and 88.9%, respectively. Upper GI endoscopy detected endoscopic markers consistent with celiac disease in 16 out of 20 celiac patients with a sensitivity, specificity, positive predictive value and negative predictive value of 80%, 100%, 100% and 85.7%, respectively. Capsule was more sensitive than conventional endoscopy in identifying endoscopic markers, but the difference observed did not achieve statistical significance. In the largest presented series, Rondonotti *et al*<sup>[19]</sup> reported on 43 consecutive patients with signs and/or symptoms suggestive of celiac disease and positive serological markers who underwent upper GI endoscopy and video capsule endoscopy. 87.5% of patients who had characteristic histological changes were diagnosed with celiac disease by capsule endoscopy. Overall, capsule endoscopy was reported to have a sensitivity of 87.5%, a specificity of 90.9%, a positive predictive value of 96.5%, a negative predictive value of 71.4% and positive and negative likelihood ratios of 9.6 and 0.14, respectively. Capsule endoscopy appeared highly performing in patients with Marsh III lesions, as it is able to correctly diagnose 89% of patients with this type of histological change.

Such promising data are not confirmed in the series presented by Biagi *et al*<sup>[5]</sup>. In this series, the authors classified the mucosal appearance as seen at capsule endoscopy as normal, hypotrophic and atrophic and

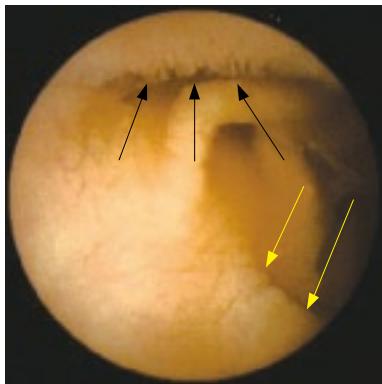
**Table 1** Sensitivity, specificity, PPVs and NPVs of capsule endoscopy in celiac disease from referenced articles (%)

Ref.	Sensitivity	Specificity	PPV	NPV
Petroniene <i>et al</i> <sup>[16]</sup>	70	100	100	77
Biagi <i>et al</i> <sup>[5]</sup>	90.5-95.2	63.6	100	77.8-87.5
Hopper <i>et al</i> <sup>[17]</sup>	85	100	100	88.9
Rondonotti <i>et al</i> <sup>[19]</sup>	87.5	90.9	96.5	71.4

evaluated whether there was a correlation between the degree of villous atrophy at the histology and capsule endoscopy results. Video capsule findings regarding the degree of small bowel mucosal atrophy showed only a moderate agreement with the histologic pattern, with a high sensitivity (range, 90.5%-95.2%), but a low specificity (63.6%). Positive predictive value was 100% and negative predictive value ranged between 77.8% and 87.5%.

Overall, capsule endoscopy seems to be able to recognize the endoscopic markers of celiac disease described in the literature<sup>[15,16,21]</sup>. Scalloping, mosaicism, micronodularity and reduction of folds (Figure 2) can all be seen by capsule endoscopy<sup>[16,19,22]</sup>. In addition to general mucosal pattern, capsule endoscopy can easily recognize finger-like villi (Figure 1)<sup>[16,17]</sup>. Table 1 shows the literature data on sensitivity, specificity, positive predictive value and negative predictive value of capsule endoscopy in celiac disease. Capsule endoscopy has a high sensitivity (range, 70%-95.2%), even better than that of upper GI endoscopy, an overall high specificity (range, 63.6%-100%) and high positive predictive value and negative predictive value, respectively between 96.5%-100% and 71.4%-88.9%. This means that when an atrophic pattern is observed by capsule endoscopy, patients have a very high probability to have celiac disease. However, the relatively low negative predictive value suggests that a normal capsule endoscopy pattern can not exclude definitively villous atrophy.

The accuracy obtained by capsule endoscopy is similar to that of magnification endoscopy or of endoscopy with the "immersion technique"<sup>[11,13]</sup>, however capsule endoscopy has the great advantage of being a non-invasive technique and to visualize the entire small bowel<sup>[15]</sup>. Overall, interobserver agreement was moderate to high in the detection of villous atrophy and ranged between 0.41 and 0.87<sup>[16,19]</sup>. The interobserver agreement is an important factor in assessing the accuracy of the tests employing subjective visual evaluation. Agreement between investigators for the diagnosis of specific endoscopic markers is different: while it is low for erosions ( $\kappa$ , 0.27-0.72) and reduction in the number or loss of duodenal folds ( $\kappa$  = 0.41 and 0.59, respectively), it is good for mosaic pattern ( $\kappa$  = 0.76) and scalloped folds ( $\kappa$  between 0.65 and 0.85)<sup>[5,16,19]</sup>. The lack of an overall high degree of agreement between investigators could mean that the correct visualization of villous atrophy is difficult even for physicians with long-term experience in capsule endoscopy. It has been demonstrated that the interobserver agreement varies significantly between investigators ranging from poor to perfect ( $\kappa$ , 0.26-1.0). The agreement is perfect between experts who have



**Figure 3** "Patchy" atrophy detected by capsule endoscopy. Capsule endoscopy shows a normal villous pattern in the upper part of the image (black arrows) and villous atrophy in the lower part (yellow arrows).

extensive experience with capsule endoscopy<sup>[16]</sup>. Familiarity with capsule endoscopy may be an important factor affecting the accuracy of the procedure and training sessions are needed for those endoscopists who are interested in capsule endoscopy. In fact, with physicians more experienced in evaluating patients with suspected or known celiac disease, an overall improvement in the interobserver agreement may be expected<sup>[19]</sup>.

Capsule endoscopy also provides information on how much bowel is involved by the celiac disease, allowing the visualization of portions of the small bowel not accessible by other traditional endoscopic methods. Although precise evaluation of the extent of the affected bowel is not possible, it is, however, possible to determine whether the disease is confined to the duodenum, to the proximal part of the jejunum or whether it extends throughout the whole of the small bowel. Rondonotti *et al*<sup>[19]</sup> showed that 66.6% of patients had an extension of the mucosal changes seen at capsule endoscopy beyond the proximal small bowel, and 11.1% had lesions that involved the small bowel entirely. The significance of the extent of involvement of the small bowel in patients with celiac disease is still unclear. However, available data suggest that there is a trend in correlation between the severity of symptoms and the degree of small bowel involvement<sup>[16,19]</sup>. This could be an interesting and important topic for future research. Moreover, evaluation of the entire small bowel may reveal mucosal changes undetected by traditional endoscopy in case of a "patchy" distribution (Figure 3). Rondonotti *et al*<sup>[19]</sup> reported of a patient who had a normal mucosa at upper GI endoscopy and scalloping of folds in the distal part of duodenum at capsule endoscopy. Authors argued that the normal duodenal histology in this patient was a sampling error and the capsule findings were compatible with a "patchy" distribution of mucosal changes.

Celiac disease may be complicated by a variety of pathologies, including small bowel adenocarcinoma, intestinal T-cell lymphoma and ulcerative jejunitis. These complications are often not identifiable by conventional imaging modalities as they are located beyond the site reachable by traditional endoscopy. Capsule endoscopy has

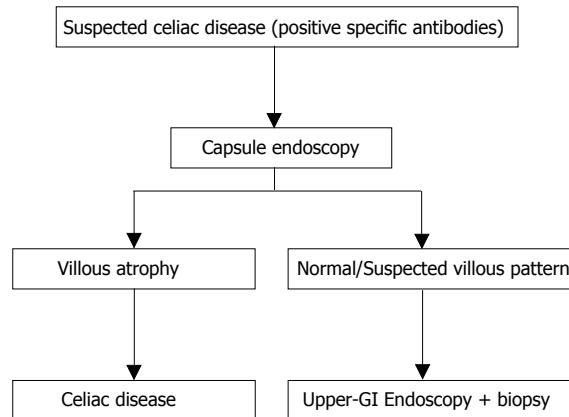
been reported to be able to demonstrate intussusception, ulcerative jejunoileitis, lymphoma and adenocarcinoma in patients with celiac disease<sup>[23-27]</sup>. In a series of 47 celiac patients with a high risk of complication (persistent unexplained abdominal pain, weight loss, history of small bowel neoplasia, long-standing celiac disease, positive faecal occult blood test or iron deficiency anaemia unresponsive to iron supplementation), lesions were detected in about 50% of cases<sup>[20]</sup>. These data support the role of capsule endoscopy in patients who have complicated disease, who present alarm symptoms or who do not respond to a gluten-free diet<sup>[28]</sup>. In this group of patients, capsule endoscopy should be promptly performed as it avoids several unnecessary diagnostic tests, it permits to visualize small bowel complications that generally are diagnosed late, and it allows initiation of specific therapies.

Unsuspected celiac disease can also be diagnosed during capsule endoscopy performed for other indications including abdominal pain, gastrointestinal bleeding, and dyspepsia<sup>[22,29,30]</sup>. In these cases, findings evocative for celiac disease should suggest the performance of additional testing to rule out celiac disease. For this reason, recognition of endoscopic markers for celiac disease and villous atrophy is mandatory for physicians who perform capsule endoscopy.

Data presented in the literature are interesting and they make us optimistic about the role of capsule endoscopy in the evaluation of patients with celiac disease. However, some issues remain still open and need to be clarified. In all the studies presented, patients had a high pre-test probability (EMA and/or t-TG positive) of having celiac disease. This may provide an over estimation of the performance of capsule endoscopy in the detection of endoscopic markers and villous atrophy. A further limitation is represented by patients with less severe histological changes (Marsh I and II). In fact, it is demonstrated that capsule endoscopy is able to detect the majority of Marsh III lesions, which are associated with evident mucosal abnormalities; however, it may not distinguish patients with Marsh I and II lesions as they may have a normal villous pattern. Furthermore, the role of capsule endoscopy in screening or surveillance for malignancies in patients with celiac disease should be clarified. It is unclear what group of patients should be screened or should undergo surveillance to detect small bowel malignancies<sup>[23]</sup>. Finally, it should be clarified whether capsule endoscopy could play a role in diagnosing celiac disease in patients with positive serologic tests and negative biopsies.

## FUTURE PERSPECTIVES

Recently, new endoscopic approaches and technologies that allow a direct visualization of the duodenal villous structure with high accuracy have been proposed<sup>[11,13,31,32]</sup>. Using these new procedures, it is possible to detect the presence of the intestinal villi or to suggest their absence. These observations led the Authors to propose a new diagnostic approach to celiac disease avoiding the biopsy



**Figure 4** Possible algorithm in the diagnostic work-up of celiac disease.

sampling. All these new methods still require "standard" endoscopy which is an invasive procedure, it is not well tolerated by patients and it only reaches the proximal part of the small bowel. Capsule endoscopy offers several advantages over standard endoscopy: it is a non-invasive procedure; it is well tolerated by patients; and it allows the visualization of the entire small bowel. Moreover, the optical system of the capsule allows an 8-fold magnification, providing a good visualization of small bowel villous pattern. Therefore, if a biopsy-avoiding approach using new endoscopic methods (such as the "immersion" technique or high-resolution and magnifying endoscope) seems reasonable, it appears more and more rational to obtain the same information recurring to a non-invasive procedure, that allows to explore all the small bowel. It is our feeling that in the next few years the diagnostic approach to celiac disease will change if larger studies will confirm the results published to date regarding the role of capsule endoscopy in celiac disease (Figure 4). "High risk" patients for celiac disease (positive specific antibodies)<sup>[3]</sup> will avoid traditional endoscopy and undergo capsule endoscopy directly; only those patients with a normal or suspected villous pattern at capsule endoscopy will undergo standard upper GI endoscopy with duodenal biopsy. Several studies are needed to confirm these new approaches.

## CONCLUSION

The essential requirement for the diagnosis of celiac disease is the histopathologic demonstration of villous atrophy. For this reason, endoscopy plays a critical role as it permits to obtain duodenal specimens. Video capsule endoscopy provides good quality images of the small bowel mucosa, including well-defined villous pattern in patients with celiac disease. At present, capsule endoscopy may be an alternative to traditional endoscopy and duodenal biopsy in patients with suspected celiac disease who are unable or unwilling to undergo conventional upper GI endoscopy for confirmation of villous atrophy. Capsule endoscopy also provides information on the extent of the small bowel involved, but the meaning of this data is still unknown. Other potential indications for

capsule endoscopy include complications related to celiac disease, refractory patients and long-term surveillance to detect malignancies. Further prospective studies are needed to confirm these preliminary results and to investigate if capsule endoscopy could represent a first line approach in patients with suspected celiac disease.

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## TOPIC HIGHLIGHT

Miguel A Muñoz-Navas, Profesor, Series Editor

# Capsule endoscopy in pediatric patients

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## Abstract

Wireless capsule endoscope (WCE) for the investigation of the small bowel is an approved technique both in adults and children (more than 10 years old). The present review provides data on the indications, diagnostic yield, adverse events and limitations of the WCE technique in children and tries to predict the future of WCE usage in this population of patients.

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## INTRODUCTION

The concept of a wireless capsule endoscope (WCE) for the investigation of the small bowel, was introduced in 2000<sup>[1]</sup>, soon to be followed by a commercially available product both in Europe and the USA<sup>[2]</sup>.

Soon after its first reports in adult patients, the advantages of this imaging technique turned WCE into an experimental and later an approved technique in the

diagnostic arsenal of pediatric gastroenterology (from the age of 10 years old) worldwide.

The present review will provide data on the indications, diagnostic yield, adverse events and limitations of PillCam SB technique in children and try to predict the future of WCE. An electronic search of MEDLINE was done (up to May 15, 2007), limiting full citations to papers published in English while reviewing abstracts in any language.

## PEDIATRIC PATIENTS AND WCE

### Age range

In 2001, the Food and Drug Administration approved WCE, then referred to as M2A, and now PillCam SB (Given Imaging, Yoqneam, Israel) as an adjunct tool for the evaluation of small-intestinal disorders and later (2003) as a first line modality for the evaluation of small-intestinal disorders. In 2004, WCE was approved as a diagnostic tool for children not younger than 10 years of age. As will be discussed later, the main limitation in children is the need to swallow this 27 mm × 11 mm relatively large pill. Using any of the traditional endoscopy assisted methods of ingestion, successful WCE examinations were reported in children as young as 2.5 years and 3 years of age<sup>[3,4]</sup>. Interestingly, in a recent report from China, 6 out of 16 pediatric studies (37%), were done in children younger the 10 years<sup>[5]</sup>.

### Clinical indications

The literature on the use of WCE in children is limited. In adults, the main indications include obscure gastrointestinal bleeding (OGIB), iron deficiency anemia, suspected Crohn's disease, small intestinal tumors and refractory celiac disease. Nevertheless, any small bowel disease/pathology manifested beyond the reach of the endoscope might be an indication for WCE. Similarly, in children, the main indications include OGIB and suspected Crohn's disease, though many other indications for the use of WCE were described and are summarized in Table 1. In this review we evaluated the available literature on the use of WCE in children on these two main topics (suspected intestinal inflammation and OGIB) and mention some others.

### Intestinal inflammation Crohn's disease

There are 5 studies in the pediatric literature looking at suspected Crohn's disease. A prospective pediatric study

evaluating small bowel pathologies<sup>[6]</sup> found that, among 10 out of 20 patients, multiple lesions consistent with Crohn's disease were found using PillCam SB. Not less important, small-bowel Crohn's disease was ruled out in 8 patients. In another study<sup>[7]</sup>, WCE was used in 12 patients with suspected Crohn's disease not diagnosed by gastroscopy, colonoscopy, and small-bowel follow-through examinations that were carried out in all of the patients. Here too, WCE identified lesions suggestive of Crohn's disease in seven of the 12 (58.3%), the majority of the lesions being in the ileum. In a prospective cohort of children<sup>[8]</sup>, 16 patients with suspected small bowel Crohn's disease (10 newly diagnosed; 6 known cases), underwent WCE which was compared with standard investigation. All of the patients had preceding upper gastrointestinal endoscopy and ileocolonoscopy, and the majority had a barium meal and follow-through. In all patients with CD who had successful WCE studies (12/16), small bowel disease was identified (11/12 active disease, 1/12 chronic disease) by PillCam SB. In this study, ileocolonoscopy and WCE complemented each other with respect to the extent of the disease. In a retrospective review of 46 WCE studies in children<sup>[9]</sup> (a denominator was not provided), among 9 children with newly diagnosed small intestinal Crohn's disease, seven (78%) had their treatment changed after WCE was done. Finally, PillCam SB was able to suggest the diagnosis of Crohn's disease in 2 adolescents with abdominal pain, protein loosing enteropathy and anemia with negative radiologic and endoscopic evaluation<sup>[10]</sup>.

In accordance with adult studies and some of the studies cited, the North American Society for Gastroenterology Hepatology and Nutrition<sup>[11]</sup> concluded that WCE is increasingly being used in the detection of obscure small bowel lesions and now has a proven role in the identification of Crohn's disease of the small intestine. It was concluded that the sensitivity of WCE at identifying small bowel ulceration or stricture appears to be superior to conventional barium radiography and enteroclysis, but this conclusion was made based on adult literature<sup>[12,13]</sup>.

From adult studies, it is clear that WCE may have other important roles such as the assessment of mucosal healing of the small intestine, defining the extent of disease and providing evidence for distinction between Crohn's and UC in patients with indeterminate colitis<sup>[2]</sup>.

### Celiac disease

Endoscopy with intestinal mucosa biopsies is still considered the gold standard in diagnosing celiac disease. However, in adults, there is mounting evidence that WCE may be useful in the evaluation of patients with already diagnosed celiac disease<sup>[14]</sup>. In a recent study, in adults, evaluating the role of WCE in the diagnosis of celiac disease, findings regarding the degree of intestinal mucosal atrophy showed only moderate agreement with the histologic pattern<sup>[15]</sup>. The authors concluded that WCE cannot be an alternative to biopsy, but a duodenal biopsy should be done when an atrophic mucosal pattern is observed in patients undergoing WCE for other

**Table 1 Pediatric indications for the use of WCE**

Pediatric indications for the use of WCE
Intestinal inflammation
Crohn's disease
Celiac disease
Occult or obscure intestinal bleeding
Vascular malformations
Vasculitis (Henoch-Schönlein Purpura)
Meckel's diverticulum
Protein-losing enteropathies
Intestinal lymphangiectasia
Miscellaneous
Peutz-Jeghers syndrome
Familial and nonfamilial polyposis
Eosinophilic enteropathy
Food allergy
Mucosal injury
Drugs
Chemotherapy
Radiotherapy
Graft versus host disease
Malignancy
Chronic abdominal pain

reasons. In adults, a consensus expert panel suggested that WC endoscopists should be able to recognize celiac disease, and for the time being, WCE might be an option in patients unable or unwilling to undergo conventional upper GI endoscopy<sup>[16]</sup>. Data in children are lacking to evaluate and apply these statements in children.

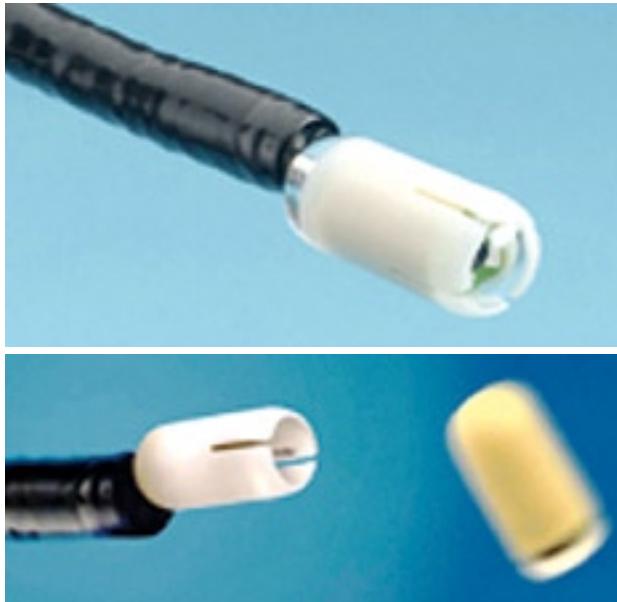
### Obscure gastrointestinal bleeding and other sources of bleeding

WCE is the imaging method of choice in adults with OGIB<sup>[17]</sup>. In a cohort of patients from Canada, among four patients with obscure bleeding, WCE confirmed a diagnosis of vascular malformations in three. In that study, WCE was more accurate identifying the precise source of bleeding compared with angiography<sup>[6]</sup>. In another study from England<sup>[8]</sup> a possible source of bleeding was identified in all 6 patients suffering from chronic anemia and OGIB. In that study, WCE had a higher diagnostic yield compared to a combination of gastroduodenoscopy and ileocolonoscopy.

WCE was reported to be helpful in evaluating and directing treatment options in chronic Henoch-Schonlein Purpura vasculitis<sup>[18]</sup>. Similarly, it has been found helpful in monitoring the effect of therapy in patients with blue rubber bleb nevus syndrome<sup>[19]</sup>. Other miscellaneous diagnoses of PillCam SB are bleeding varices, angiodysplastic lesions, jejunal hemangioma, Meckel's diverticulum and bleeding lymphonodular hyperplasia<sup>[20-23]</sup>.

### Miscellaneous

As reviewed recently<sup>[20]</sup>, WCE is used to diagnose and survey small bowel polyps in children with familial polyposis and those with Peutz-Jeghers syndrome. It may also be used to diagnose malignancies<sup>[24]</sup>, intestinal lymphangiectasia<sup>[25]</sup>, as well increasing number of entities summarized in Table 1. It is clear that with time,



**Figure 1** An introducing device for the capsule endoscope.

many of these entities will become more established indications for performing WCE. Future studies should delineate the role of WCE compared to other imaging modalities for some of these entities.

#### Advantages and limitations

WCE is clearly advantageous for evaluating small bowel pathologies when the child is able to swallow the capsule as it is a procedure that is free of anesthesia and its risks. Often, we are able to select those patients who will readily swallow the capsule (using candies of increasing sizes) and train some who were afraid to swallow big candies<sup>[26]</sup>. When the patient can not swallow the capsule due to any reason (including swallowing disorders, dysphagia, gastroparesis) the capsule can be safely introduced to the duodenum using various techniques and a standard endoscope (Figure 1)<sup>[20,27]</sup>.

The main adverse event when using the capsule is capsule retention mainly due to strictures. Studies in adults have demonstrated the usefulness of using a Patency capsule (see accompanying article) and predicting the uneventful passage of the capsule using a Patency capsule has also been reported in children<sup>[6, 20]</sup>. In adults, capsule retention was reported in 13% (95% CI, 5.6%-28%) of patients with known CD, but only in 1.6% (95% CI, 0.2%-10%) with suspected Crohn's disease<sup>[28]</sup>. Capsule retention occurred in children, but not in adults with suspected Crohn's disease. In a cohort of 45 children undergoing WCE, nine subjects (20%) had adverse events<sup>[19]</sup>. Of these patients, five had delayed passage from the stomach with 2 patients requiring endoscopic retrieval and 4 had delayed passage from the small intestine (more than 5 d). Three of the 4 patients with intestinal retention underwent surgery (one underwent ileocolic resection 2 mo later) and one responded to steroids. In the study from Canada<sup>[6]</sup>, all 30 capsule studies were well tolerated, although 1 capsule was retained owing to an inflammatory stenosis. The capsule was eventually ex-



**Figure 2** A device used to introduce the capsule endoscope into the stomach.

peled after corticosteroid therapy. No capsule retention occurred in a Chinese series of 16 patients<sup>[5]</sup>. Figure 2 shows a device used to introduce the capsule endoscope to the stomach.

#### CONCLUSION

The North American Society for Gastroenterology Hepatology and Nutrition recently stated that regarding WCE in Crohn's disease, "Drawbacks of WCE include the cost of the test, as well as the potential risk of capsule impaction in strictured areas of the small bowel. Future studies may determine whether capsule endoscopy should be performed as a routine examination on new-onset patients with colitis and normal contrast studies. At this time, we recommend that this test be used primarily when CD of the small bowel is strongly suspected but cannot be documented by other modalities"<sup>[11]</sup>.

Thus, in summary, WCE is a promising diagnostic tool for small bowel imaging both in adults and children. A smaller capsule may allow younger children to use it without the need of traditional endoscopy.

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## COLORECTAL CANCER

# VEGF-D expression correlates with colorectal cancer aggressiveness and is downregulated by cetuximab

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## Abstract

**AIM:** To gain mechanistic insights into the role played by epidermal growth factor receptor (EGFR) in the regulation of vascular endothelial growth factors (VEGFs) in colorectal cancer (CRC).

**METHODS:** The impact of high-level expression of the growth factor receptors EGFR and VEGF receptor (VEGFR)3 and the VEGFR3 ligands VEGF-C and VEGF-D on disease progression and prognosis in human CRC was investigated in 108 patients using immunohistochemistry. Furthermore, the expression of the lymphangiogenic factors in response to the modulation of EGFR signalling by the EGFR-targeted monoclonal antibody cetuximab was investigated at the mRNA and protein level in human SW480 and SW620 CRC cell lines and a mouse xenograft model.

**RESULTS:** Human CRC specimens and cell lines displayed EGFR, VEGF-C and VEGF-D expression with varying intensities. VEGF-C expression was associated with histological grade. Strong expression of VEGF-D was significantly associated with lymph node metastases and linked to a trend for decreased survival in lymph node-positive patients. EGFR blockade with ce-

tuximab resulted in a significant decrease of VEGF-D expression *in vitro* and *in vivo*.

**CONCLUSION:** In conclusion, the expression of VEGF-D in colorectal tumours is significantly associated with lymphatic involvement in CRC patients and such expression might be blocked effectively by cetuximab.

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**Key words:** Human colorectal cancer; Lymphangiogenesis; Vascular endothelial growth factor-C; Vascular endothelial growth factor-D; Epidermal growth factor receptor

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## INTRODUCTION

Globally, colorectal cancer (CRC) is one of the three most commonly diagnosed malignancies<sup>[1]</sup>. For patients with metastatic disease, systemic cytotoxic chemotherapy has been shown to clearly improve survival<sup>[2-5]</sup>. More recently, the addition of therapeutic antibodies including cetuximab<sup>[6-8]</sup> and bevacizumab<sup>[9,10]</sup> to such cytotoxic regimens has been shown to further improve outcomes in first- and second-line settings.

The mode of action of the new therapeutic antibody cetuximab is thought to be based primarily upon perturbation of epidermal growth factor receptor (EGFR)-ligand interactions<sup>[11]</sup>. Binding of cetuximab blocks EGFR-associated cellular signal transduction cascades, which govern processes such as tumour cell survival, proliferation, invasion and metastasis<sup>[12-16]</sup>. Since high-level expression of the EGFR gene has been associated with reduced survival in a range of malignancies, targeting growth factor receptor signalling

cascades is a promising anticancer strategy<sup>[17-24]</sup>. Even more, an additional mode of action of cetuximab has been suggested which relates to tumour cell binding inducing an antibody-dependent cell-mediated cytotoxicity reaction<sup>[25,26]</sup>.

However, even if a number of such molecular tumour characteristics appeared to be associated with the antitumor efficacy of EGFR-targeted agents<sup>[27]</sup>, it remains a matter of debate as to whether the intensity or extent of immunohistochemical detection of EGFR expression in the tumour correlate with prognosis and response to EGFR-targeted agents<sup>[28,29]</sup>.

The hypothesis that growth and spread of tumours are dependent on their vascular and lymphatic systems was proposed several decades ago<sup>[30]</sup>. Interest in this concept has recently been rekindled following the molecular identification of regulators of (lymph-) angiogenesis such as the vascular endothelial growth factor (VEGF) family, including the ligands VEGF-A, -B, -C and -D, and the VEGF receptors VEGFR1 (FLT1), VEGFR2 (KDR) and VEGFR3 (FLT4)<sup>[31]</sup> and their clinical utilization by recombinant strategies for targeting angiogenesis, such as the anti-VEGFA antibody, bevacizumab<sup>[9]</sup> and the decoy receptor, VEGF Trap<sup>[32]</sup>. Interestingly, VEGF-C and VEGF-D, signalling through VEGFR3, have been identified as key regulators of lymphangiogenesis<sup>[33-36]</sup>. Data from *in vitro* and murine tumour models further support the key role of VEGF-C and VEGF-D in malignancy. For many tumour types, clinical studies have revealed a correlation between VEGF-C, VEGF-D and VEGFR3 expression and lymphatic spread, tissue invasion or poor prognosis<sup>[37-41]</sup>. However, in other studies, clear associations were not identified<sup>[42,43]</sup> or low levels of VEGF-D were correlated with an increased risk of metastasis and reduced survival<sup>[44]</sup>. Similar data have also been reported for CRC. In one study, VEGF-C and VEGF-D expression correlated with the tumour invasion, lymphatic and venous involvement, lymph node metastasis and liver metastasis, and reduced survival time<sup>[45]</sup>. A second study also reported that high-grade VEGF-D expression was associated with lymphatic involvement and poor patient survival<sup>[46]</sup>, while a third confirmed that VEGF-D expression correlated with the depth of tumour invasion, lymph node metastasis and reduced survival time<sup>[47]</sup>. However, in other analyses VEGF-D expression at the mRNA-level was reported to be downregulated in CRCs with lymphatic spread<sup>[48]</sup> and appeared to be lower at the leading edge of tumours in which lymphatic vessels were present<sup>[49]</sup>.

Given that lymphangiogenesis is increasingly recognized as a critical component of tumourigenesis and that EGFR signalling, a key regulator of tumourigenesis in CRC, possibly acts to some extent through regulation of VEGF-C and VEGF-D expression, we evaluated the co-expression profiles of EGFR, VEGF-C and VEGF-D in human CRC specimens. Results were correlated with the patients' clinicopathological parameters and survival. Furthermore, in order to gain mechanistic insights into the role played by EGFR in the regulation of VEGF-D in colorectal cancer, we analyzed the effect of cetuximab *in*

*vitro* and *in vivo* on the expression of VEGF-D in SW480 and SW620 human colon cancer cell and xenograft models of CRC. We thus showed that expression of VEGF-D is prognostically relevant in CRC and for the first time provided experimental evidence that EGFR-targeted antitumor therapy exerts its effect in part through suppressing lymphangiogenesis by downregulating VEGF-D.

## MATERIALS AND METHODS

### Tissue samples and patient characteristics

All tissues investigated in this study were obtained from patients ( $n = 108$ ) who underwent colectomy between 1995 and 2003 at the Department of Abdominal Surgery, University Hospital Mainz, Germany. Written informed consent for experimental immunohistochemistry was obtained from all patients before analysis. Expression of EGFR was analyzed in all patients, with assessment of VEGF-C and VEGF-D conducted in 102 cases and 104 cases, respectively, because of limited availability of tumour material.

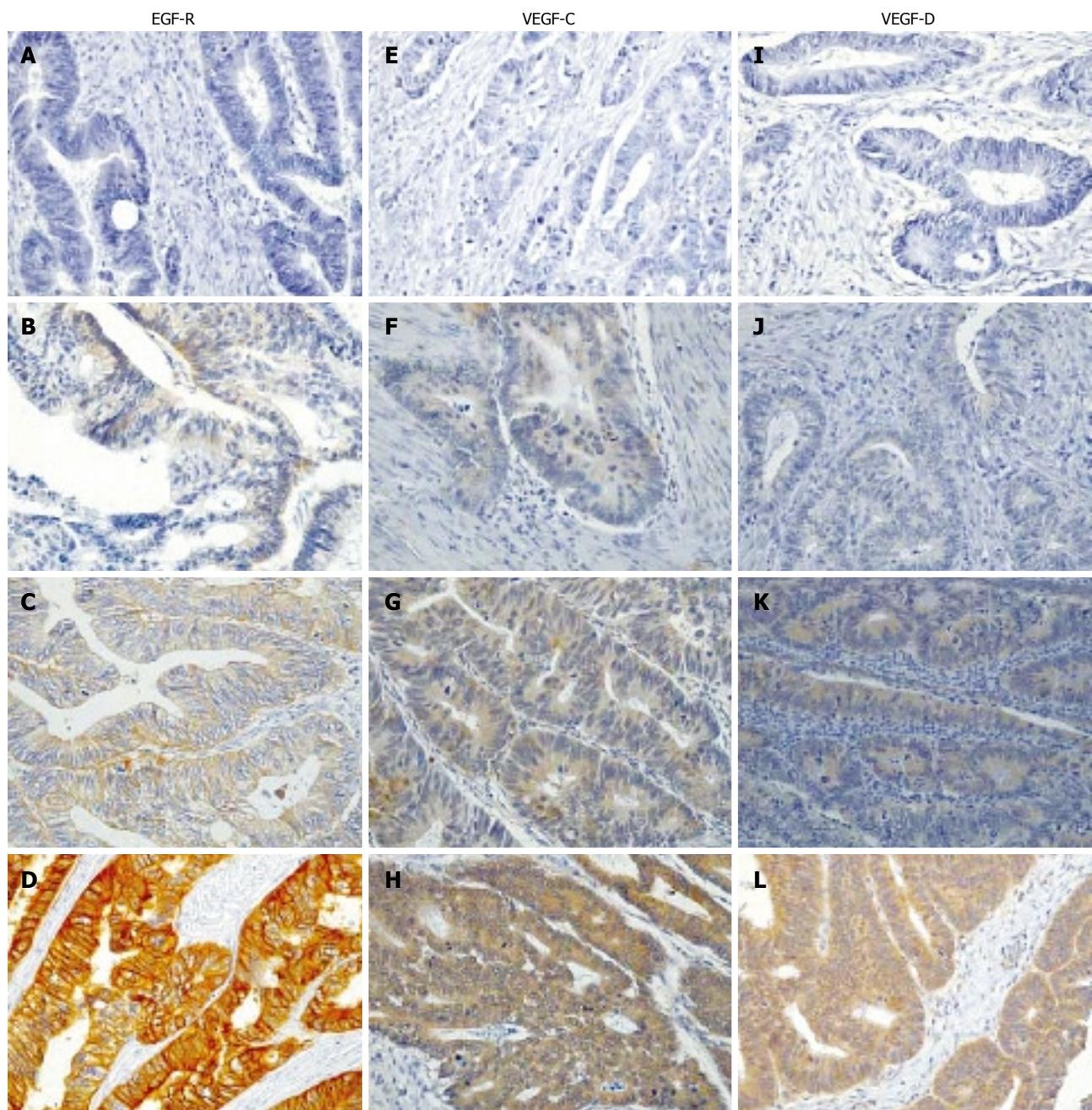
Patient age at the time of primary surgery ranged from 36.2 years to 83.1 years ( $63.6 \pm 10.45$  years). Seven patients were lost to follow up and were therefore censored at the time of last contact ( $34.86 \pm 4.18$  mo). Staging and diagnosis of CRC was assessed according to the World Health Organization classification and the TNM classification as set out by the International Union Against Cancer [Union International Contre le Cancer (UICC)]. After resection, patients were followed up every 6 mo. Patients with synchronous or metachronous metastasis underwent additional restaging every 3 mo during chemotherapy.

### Immunohistochemical (IHC) staining

Formalin-fixed paraffin-embedded tissues of patients with CRC from the Department of Pathology, University Hospital Mainz, Germany, were used in this study. Tissue sections (4  $\mu$ m) were cut from these blocks and used for IHC staining. All tissue sections were deparaffinized in xylene and rehydrated in a graded ethanol series.

Staining for EGFR was performed using the commercially available EGFR pharmDx kit (DakoCytomation, Carpinteria, CA, USA), which includes the pharmDx mouse anti-EGFR monoclonal antibody (clone 2-18C9), a negative control reagent (a mouse monoclonal antibody for an enzyme that is not expressed in mammalian tissue), and positive and negative control cancer cell preparations (CAMA-1 breast cancer and HT29 colon cancer cell lines). IHC staining was performed according to the manufacturer's instructions.

Staining for VEGF-C and VEGF-D was carried out following antigen retrieval. Sections were heated in citrate buffer and then cooled for 20 min. Endogenous peroxidase was blocked in 3% hydrogen peroxide in methanol for 15 min. To block nonspecific binding, prior to incubation with the primary antibody, tissue sections were incubated with serum-free DAKO Antigen-Block for 30 min. Primary antibodies specific for VEGF-C



**Figure 1** Immunohistochemical analysis of the expression levels of EGFR (A-D), VEGF-C (E-H) and VEGF-D (I-L) in human colorectal cancer sections ( $\times 400$ ). A, E and I: No expression; B, F and J: Weak expression; C, G and K: Moderate expression; D, H and L: Strong expression.

(sc-9047, Santa Cruz Biotechnology Inc. CA, USA) and VEGF-D (sc-13085, Santa Cruz Biotechnology Inc.) and were diluted 1:50 in DAKO ChemMate antibody diluent and sections were incubated for 16 h at  $4^{\circ}\text{C}$  (VEGF-C) or 2 h at room temperature (VEGF-D). After incubation with an anti-rabbit secondary antibody for 30 min, bound complex was visualized by using diaminobenzidine (ChemMateTM DAKO EnVisionTM Detection Kit). Sections were counterstained with Mayer's hematoxylin and mounted. Between all incubations, sections were washed in phosphate-buffered saline (PBS). Foetal kidney was used as a positive control. Negative controls were prepared by omitting primary antibody from the process (data not shown).

#### Evaluation of immunostaining

Immunostaining was independently evaluated by 4 authors who were blinded to patient outcome and all clinicopathological findings. EGFR-staining was interpreted according to standard parameters (EGFR pharmDx™ Interpretation Manual, DAKO). The staining intensities (Figure 1) were scored as negative, weak, moderate or strong. To unequivocally categorize cases into two groups, each sample was defined as EGFR-negative or EGFR-positive when  $< 1\%$  or  $\geq 1\%$  of the tumour cells respectively showed an EGFR-immunospecific membranous brown staining.

Staining results for VEGF-C and VEGF-D were similarly classified by estimating both the staining intensity

and the percentage of epithelial cells showing specific immunoreactivity. Staining intensities were not always homogeneous across individual tumour samples (as shown in Figure 1). All results showing more than 25% of tumour cells stained (with weak to strong positivity) were considered to represent biologically relevant levels of expression of these proteins and, for the purposes of statistical analysis, were counted as positive results.

### **Cell lines**

The colon carcinoma cell lines SW480 and SW620 were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI 1640 media (Invitrogen, Carlsbad, CA, USA), supplemented with 10% foetal calf serum (FCS; PAA Laboratories, Pasching, Austria). Cells were cultured at 37°C in a 5% CO<sub>2</sub> atmosphere and passaged routinely using Trypsin-ethylenediaminetetraacetic acid (PAA Laboratories) treatment.

### **Cetuximab**

Clinical grade anti-EGFR monoclonal antibody cetuximab (Erbitux®) was supplied by Merck KGaA (Darmstadt) at a concentration of 2 mg/mL in a buffer consisting of 10 mmol/L sodium phosphate and 145 mmol/L sodium chloride at pH 7.

### **Stimulation assay**

SW480 and SW620 cells were seeded at  $3 \times 10^5$  cells/well in a 6-well tissue culture plate with media containing 10% FCS. After 24 h, cells were starved by incubation in media with a reduced FCS level (0.5%) for an additional 24 h. Afterwards, cells were incubated in medium plus 0.5% FCS supplemented either with 5 ng/mL epidermal growth factor (EGF, Sigma Chemical Co., St. Louis, MO, USA) for 20 min followed by 20 µg/mL cetuximab, or alternatively, the same medium supplemented with one or other of these substances. After culturing for another 24 h, monolayers were washed with icecold PBS, centrifuged and pellets were frozen for RNA isolation. The cell culture experiments were independently repeated at least two times.

### **RNA isolation and quantitative real time RT-PCR**

RNA isolation was performed using the RNeasy Kit according to the manufacturer's recommendations (Qiagen, Hilden, Germany). Transcription of the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), VEGF-D and VEGFR3 was analyzed by a one-step reverse transcriptase-polymerase chain reaction (RT-PCR) using a LightCycler 2.0 system (Roche). RT-PCR was performed with 0.1 µg of RNA in a 35 cycle reaction (20 µL total volume; QuantiTect SYBRGreen RT-PCR, Qiagen) according to the recommendations of the manufacturer. All RT-PCR reactions were done in 2 replicates. All PCRs were established with an exponential phase efficiency of 2 to guarantee that the data were comparable. The evaluation of the expression of the target genes was performed relative to the expression of GAPDH. Control and test

samples in the EGF/cetuximab and xenograft analyses were compared using the  $\Delta\Delta Ct$  approach ( $\Delta\Delta Ct = 2^{-(Ct_{target\ gene\ control} - Ct_{GAPDH\ control}) - (Ct_{target\ gene\ test\ condition} - Ct_{GAPDH\ test\ condition})}$ ). From this formula, for the test compared with the control condition, a  $\Delta\Delta Ct$  over 1 indicated an increase of a target gene expression and a  $\Delta\Delta Ct$  of less than 1 indicated a lower expression of the target gene relative to the housekeeping gene GAPDH.

RT-PCR primers used were: GAPDH, forward: 5'-CC CATCACCATCTCCAGGAGCG-3' and reverse 5'-CA TGCCAGTGAGCTTCCCGITCA-3' (476 bp product); EGFR, forward: 5'-TCTCAGCAACATGTCGATGGA-3' and reverse 5'- GCACTGTATGCACTCAGAGTT-3' (92 bp product), VEGF-D, forward: 5'-GTATGGACTCTCGCTCAGCAT-3' and reverse: 5'-AGGCTCTCTCATGCAACAG-3' (225 bp product), VEGFR3, forward and reverse; QuantiTect Primer Assay: FLT4 (VEGFR3, 127 bp product, Qiagen). Cycle conditions of the one-step real time LightCycler RT-PCRs were as follows: for reverse transcription, 20 min at 50°C. The subsequent PCR reaction was characterized by: initial denaturation (15 min at 95°C) followed by the respective number of cycles (GAPDH: 25, VEGF-D: 35, VEGFR3: 40, EGFR: 35) of: denaturation (15 s at 94°C), annealing (25 s: GAPDH; 63°C, VEGF-D; 61°C, VEGFR3; 55°C, EGFR; 61°C) and elongation (35 s at 72°C). After the last cycle, a melting curve was plotted to confirm the amplification of a single specific RT-PCR product.

### **Western blotting**

SW480 and SW620 cells were plated at  $2.5 \times 10^6$  cells/well in 6 wells. Cells were harvested and lysed in RIPA buffer<sup>[50]</sup>. For EGFR, VEGF-D, VEGFR3 and α-tubulin analysis, 50 µg of cleared lysates were separated on 10.0% sodium dodecyl sulphatepolyacrylamide gels, blotted to nitrocellulose transfer membranes (Schleicher & Schuell) and blocked for 1 h in 5% non-fat dry milk, incubated with a specific primary antibody: antihuman EGFR sc-03, antihuman VEGF-D sc-13085, VEGFR3 sc-321 (Santa Cruz Biotechnology Inc: all antibodies were diluted 1:200 in 5% bovine serum albumin) or anti-α-tubulin (Sigma: diluted 1:10000 in 5% non-fat dry milk). Detection of bound antibody was performed using a peroxidase-conjugated secondary goat anti-rabbit antibody (sc-2030; Santa Cruz Biotechnology Inc.) diluted 1:2000 in 5% non-fat dry milk and an ECL chemiluminescence detection kit (Perkin Elmer).

### **Animals**

Female NOD/SCID -/- mice were purchased from the central animal facility (ZVTE, University of Mainz, Germany). The mice were maintained in a laminar airflow cabinet under pathogen-free conditions and used at 7-10 wk of age. Mice were housed in microisolator cages with laboratory chow.

### **Treatment of subcutaneous colorectal carcinoma xenografts**

Colon carcinoma tumours were established as xenografts

by injecting  $1 \times 10^7$  SW480 cells, mixed in PBS : medium (1:1), subcutaneously into the left flank of eight NOD/SCID -/- mice. Ten days after cell injection, all mice bore a tumour with a minimum diameter of 4 mm. The mice were randomized into two groups of four animals. They were treated with either saline or cetuximab at 1 mg/dose every three days. Cetuximab and the saline placebo were administered intraperitoneally at a constant volume of 0.5 mL/injection. Treated animals were checked daily. After 5 wk of treatment, tumours were isolated and processed with a disperger to enhance subsequent RNA isolation with the RNeasy Kit (Qiagen, Hilden, Germany).

### Statistical analysis

The association of staining intensity with clinicopathological patterns was assessed with the  $\chi^2$  test and with the unpaired Student *t*-test, where appropriate. Differences in migration were evaluated with the unpaired Student's *t*-test. Survival rates were visualized applying the Kaplan-Meier curves and log rank test. *In vitro* and *in vivo* real time gene expression medians were compared using Mann-Whitney *U* test.  $P < 0.05$  was considered significant and  $P < 0.001$  highly significant in all statistical analyses.

## RESULTS

### Patient profiles and tumour characteristics

Disease characteristics for the group of 108 patients selected are representative of CRC patient populations in industrialized countries, except for a lower percentage of T3 cancers (Table 1). The mean age of the patient cohort at the time of primary surgical intervention was 63.8 years (SD, 10.45 years). Tumour stage was distributed as follows: UICC stage I was found in 14%, stage III in approximately a fifth and stages II, and IV, each in approximately a third of all patients (Table 1). The most common histopathological grading was pG2, which was reported for nearly 80% of cases. Approximately half of all patients had positive lymph node status. The UICC stage dependent survival rates after 3 years were 100% for stage I, 92% for stage II, 78% for stage III and 44% for stage IV, which were similar to those reported for other large CRC population series<sup>[51]</sup>. Expression was studied in specimens taken from all 108 CRC patients.

### Expression of EGFR, VEGF-C and VEGF-D in CRC specimens

The expression of EGFR, which could be assessed by IHC staining for all 108 samples, exhibited a predominantly membranous subcellular localization. In a few specimens, an additional weak cytoplasmic localization was found, which was not scored as indicative of positive staining for EGFR (Figure 1 A-D). EGFR expression in CRC specimens was distributed as follows: in 59 cases (54.6%), the specimen showed no positive staining for EGFR, while in 49 patients (45.4%) the specimen showed a positive membranous staining, including 31 patients (28.7%) where staining

**Table 1 Baseline characteristics of the colorectal cancer patients ( $n = 108$ )**

	<i>n</i>	%
UICC-stage		
I	15	13.9
II	36	33.3
III	23	21.3
IV	34	31.5
Grading		
G 1	4	3.7
G 2	85	78.7
G 3	17	15.7
G 4	2	1.9
Lymph node metastasis		
pN 0	53	49.1
pN 1	21	19.4
pN 2	34	31.5

was weakly positive and 9 patients (8.3%) each with moderately or strongly positive staining. No statistically significant correlation was identified between EGFR staining and the UICC stage, grading, tumour invasion or lymph node metastasis (Table 2). Comparison with the histopathological grading of the tumour cells however showed a trend towards the expression of EGFR in less well differentiated lesions. Thus, the prognostic value of IHC-determined EGFR expression status in relation to the prediction of poor survival<sup>[52-56]</sup> could not be confirmed in the current CRC population, again arguing to successfully integrate the anti-EGFR monoclonal antibody cetuximab into therapeutic regimens for patients whose tumours do not appear to express EGFR<sup>[57,58]</sup>.

The staining for VEGF-C and VEGF-D was predominantly cytoplasmic (Figure 1 E-H and I-L, respectively). VEGF-C expression could be assessed in 102 specimens, with 74 specimens (68.5%) staining positive for VEGF-C and 28 specimens (25.9%) being negative. Twenty-five percent of the G1 tumours ( $n = 4$ ) were positive, while 70.6% of G2 tumours, 70.6% of G3 tumours, and 50.0% of G4 graded tumours were positive for VEGF-C. Thus even if statistically significant (Table 2) but only 4 patients were G1, a clinical clear cut comparison of well versus not well differentiated tumours may not be done. However, another finding was the borderline significant correlation between tumour invasion and VEGF-C expression ( $P = 0.050$ ). Only 11 (55.0%) of the pT1 and pT2 graded tumours were positive for VEGF-C compared with 63 (76.8%) of the tumours with deeper invasion. No statistically significant correlation was apparent with the other clinicopathological parameters including UICC stage and lymph node status (Table 2).

VEGF-D expression was analyzed in 104 available specimens, with 70 (64.8%) staining positive and 34 (31.5%) staining negative. Interestingly, VEGF-D staining was associated with UICC tumour stage ( $P = 0.048$ ). Nine of 14 (64.3%) specimens from patients with UICC stage I tumours stained positive, while 27 (77.1%) of the UICC stage II, 9 (42.9%) of the UICC stage III and 25 (73.5%) of the UICC stage IV tumours stained positive for VEGF-D. No significant association

**Table 2** Correlation of expression levels of EGFR, VEGF-C and VEGF-D with tumour and patient characteristics

	EGFR				VEGF-C				VEGF-D			
	Total	+ ve	% <sup>1</sup>	P	Total	+ ve	% <sup>1</sup>	P	Total	+ ve	% <sup>1</sup>	P
Stage (UICC)				0.197				0.220				0.048
I	15	6	40.0		14	7	50.0		14	9	64.3	
II	36	14	38.9		34	26	76.5		35	27	77.1	
III	23	15	65.2		20	16	80.0		21	9	42.9	
IV	34	14	41.2		34	25	73.5		34	25	73.5	
Differentiation grading				0.063				0.030				0.066
Well	4	0	0		4	1	25.0		4	1	25.0	
Not well	104	49	47.1		98	73	74.5		100	69	69.0	
Tumour invasion (TNM)				0.797				0.050				0.438
T1/T2	21	9	42.9		20	11	55.0		20	12	60.0	
T3/T4	87	40	46.0		82	63	76.8		84	58	69.0	
Lymph node metastases				0.283				0.600				0.025
1 to ≤ 6	37	20	54.1		35	27	77.1		36	18	50.0	
≥ 7	18	9	50.0		17	13	76.5		17	14	82.4	
Age				0.837				0.813				1.0
≤ 60	35	15	42.9		33	25	75.8		33	22	66.7	
≥ 61	73	34	43.6		69	49	71.0		71	48	67.6	

<sup>1</sup>Percentages relate to number of positive tumours out of total number of cases in the subclass.

was found with several other parameters including grading, tumour invasion and pN status (Table 2). Again, the comparative analyses of the combined VEGF-C and VEGF-D co-expression with well *versus* not well differentiated tumours were statistically significant ( $P = 0.022$ ), but possibly not clinically clear cut significant due to the low patient number analysed.

However, when VEGF-D staining was analyzed in relation to the number of tumour positive lymph nodes, there was a statistically significant overall association between the number of lymph node metastases and VEGF-D positivity of the primary tumour ( $P = 0.025$ ). The patient cohort was stratified into patients with no lymph node metastases ( $n = 51$ ), with up to 6 lymph node metastases ( $n = 36$ ), and with more than 6 lymph node metastases ( $n = 17$ ). Among those from patients with up to 6 lymph node metastases, 18 (50.0%) stained positive for VEGF-D, and among the patients with more than 6 lymph node metastases, 14 (82.4%) stained positive for VEGF-D.

When VEGF-D staining was analyzed in relation to the survival of lymph node positive patients, there was a trend ( $P = 0.067$ ) between the VEGF-D positive and negative groups (Figure 2). The patients with lymph node metastases whose tumours were VEGF-D positive had a 30% lower chance of survival at 36 mo in comparison to the patients with VEGF-D negative tumours. Kaplan Meier analysis also indicated highly statistically significant correlations between the number of lymph node metastases and UICC stage and overall survival (in both cases, log rank test  $P < 0.001$ ; Figure 2). It was also noteworthy that in several specimens, VEGF-C and VEGF-D were strongly expressed at the tumour invasion front (data not shown). Consistently, the further comparison of VEGF-C and VEGF-D combined staining profiles in relation to survival, double VEGF-C and VEGF-D positive patients had a trend to an unfavorable 5 years prognosis compared

to the negative group (log rank test  $P = 0.2$ ), whereas triple EGFR, VEGF-C and VEGF-D positivity did not influence survival in these two cohorts (data not shown).

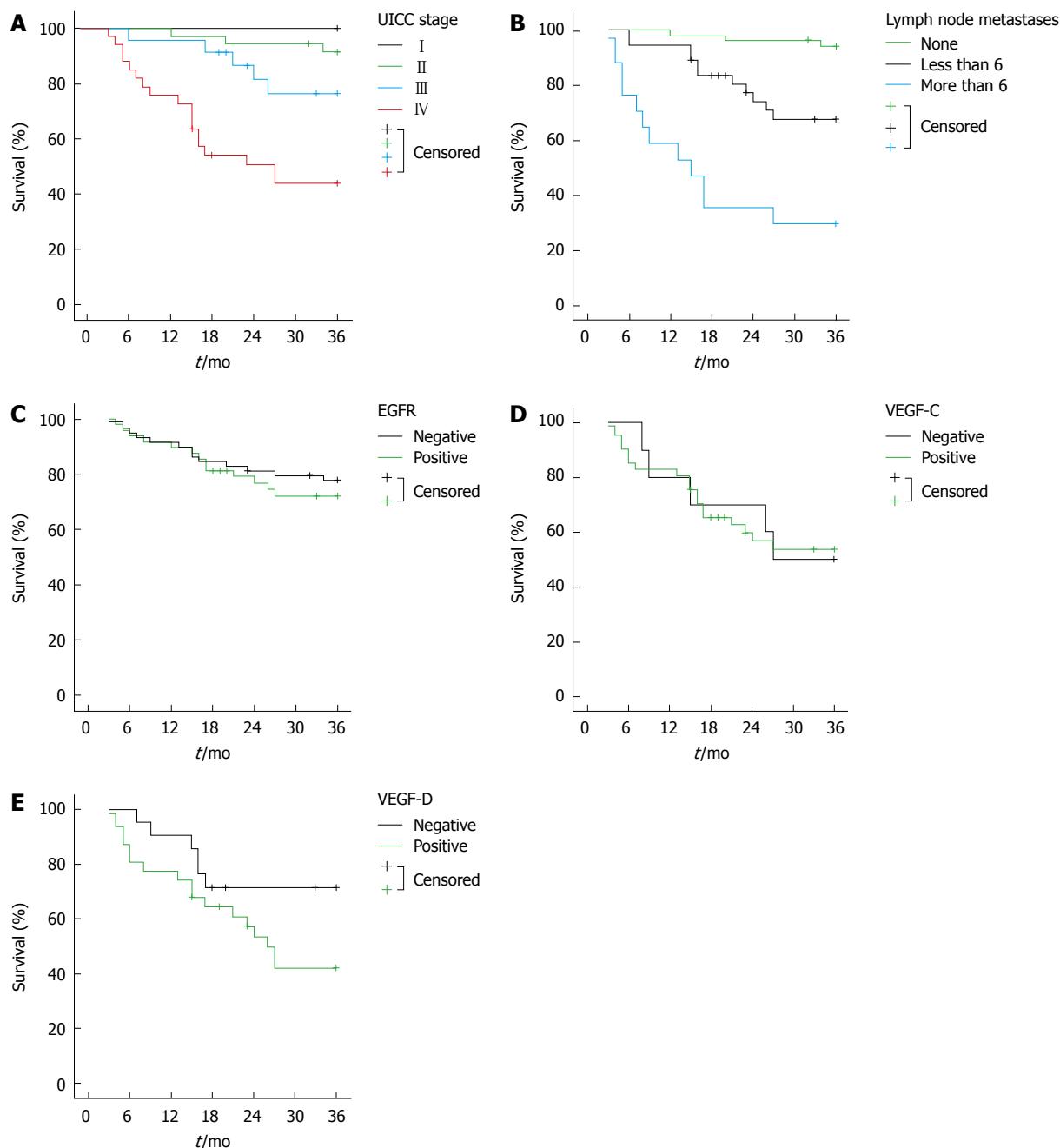
#### **EGFR, VEGF-C, VEGF-D and VEGFR3 expression in colorectal carcinoma cell lines**

Real time RT-PCR assays and Western blotting were used to measure transcript and protein levels in experimental analyses. Whereas VEGF-D transcripts and protein were expressed at moderate levels in both SW480 and SW620 CRC cell lines, a similar analysis of expression of EGFR and VEGFR3 yielded varying results. EGFR mRNA and protein levels were high in SW480 and markedly lower in SW620 cells. Though mRNA levels of VEGFR3 were high in SW480 and moderate in SW620, the protein levels of VEGFR3 were also higher in SW480 than in SW620 cells (Figure 3).

#### **Effect of cetuximab in CRC cell lines and a xenograft model**

In order to analyze the effect of cetuximab on VEGF-D and VEGFR3 transcription, SW480 cells were treated for 24 h with either EGF or cetuximab alone or a combination of these two agents. Incubation of the cells with EGF was associated with a marked relative increase in VEGF-D and VEGFR3 mRNA levels compared with control (0.5% FCS) values. Conversely, incubation with EGF and cetuximab simultaneously resulted in a highly statistically significant ( $P = 0.004$ ) suppression of this inductive effect, for both genes (Figure 4A). There were similar highly statistically significant ( $P = 0.004$ ) differences in the levels of VEGF-D and VEGFR3 expression in the EGF *versus* cetuximab treated cells. Incubation with cetuximab alone also resulted in a decreased level of VEGF-D mRNA compared to the control. This effect was not seen for VEGFR3. In the EGFR negative cell line SW620 no such effects were detectable (data not shown).

In the *in vivo* xenograft model, treatment with either



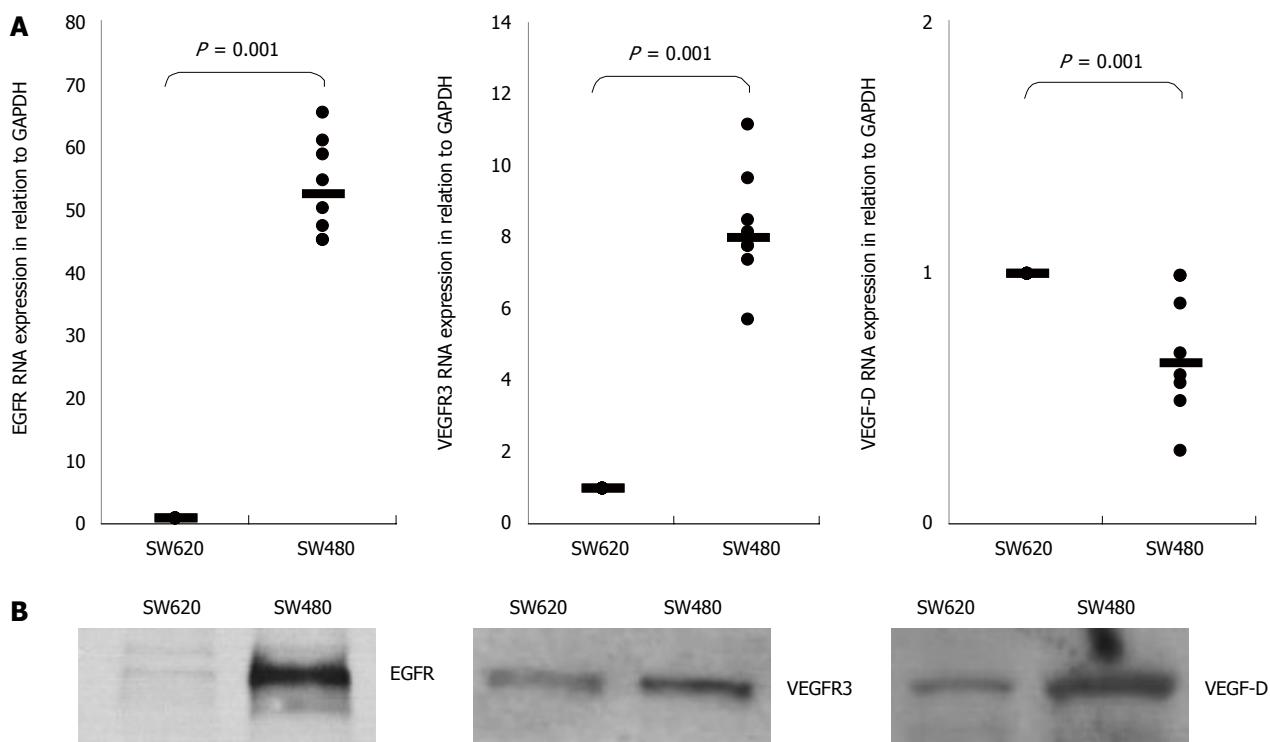
**Figure 2** Kaplan Meier survival analyses of colorectal cancer subgroups in relation to baseline disease characteristics and EGFR, VEGF-C or VEGF-D expression. **A-C:** Survival analysis for all patients in relation to UICC stage ( $P < 0.001$ ), the number of metastases ( $P < 0.001$ ) and EGFR staining ( $P = 0.546$ ), respectively; **D** and **E:** The 3 years survival in patients with lymph node metastasis in relation, respectively, to the expression of VEGF-C ( $P = 0.967$ ) and VEGF-D ( $P = 0.067$ ).

cetuximab or saline placebo was administered over a period of 28 d. After this period, the mRNA level of VEGF-D was highly statistically significantly ( $P = 0.004$ ) reduced in the tumour tissue of mice which had received cetuximab compared with those that had received saline. No such effect could be detected for VEGFR3 ( $P = 0.577$ , Figure 4B).

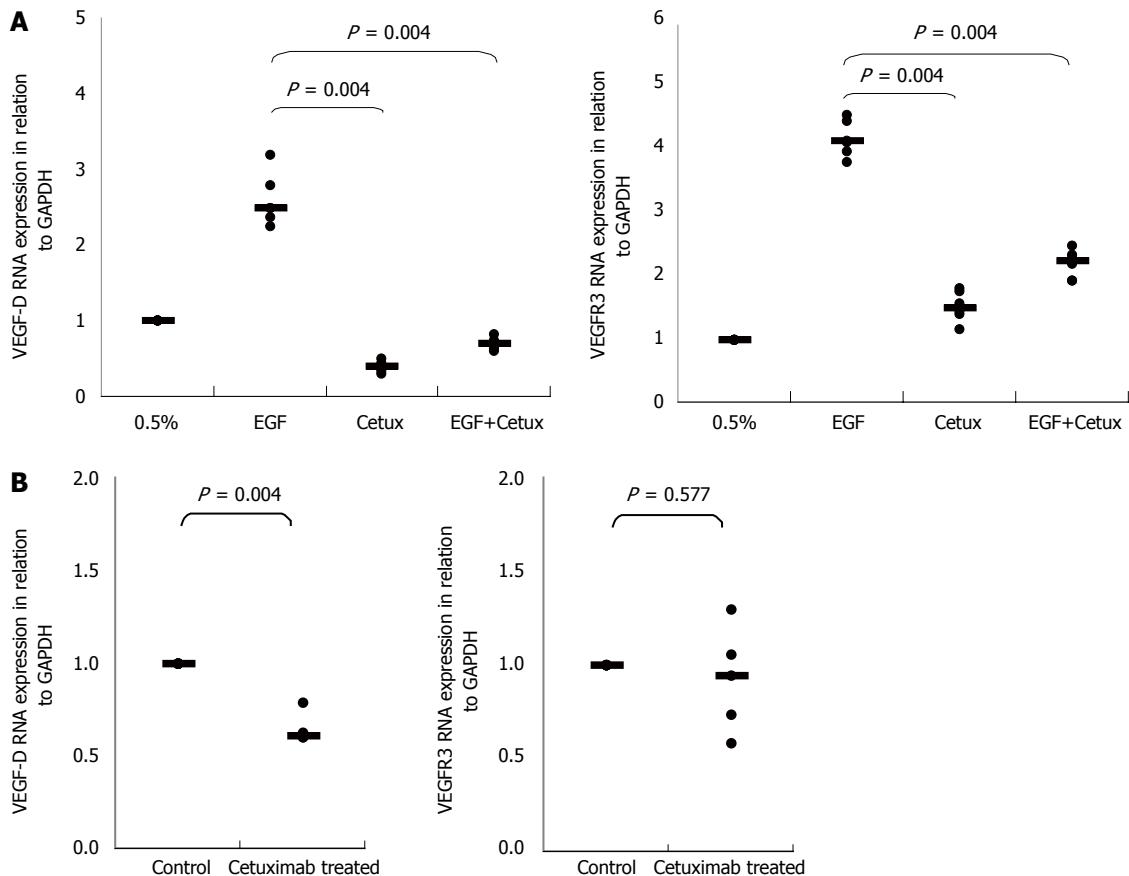
## DISCUSSION

A significant proportion of patients with advanced but non-metastatic CRC which has seemingly been curatively resected experience disease recurrence<sup>[52,53]</sup>. In order to identify high-risk patients at an early stage, it

is important to understand the molecular mechanisms behind the behaviour of these tumour types<sup>[54,55]</sup>. Herein, activation of EGFR-mediated signalling cascades has been identified in promotion of cell proliferation, malignant transformation, angiogenesis and metastatic dissemination<sup>[54,56]</sup>. In addition, lymphangiogenesis, mediated through tumour-derived VEGF-C and VEGF-D, gained attention in relation to the facilitation of lymph node metastasis and tumour spread<sup>[37,39,40,57]</sup>. Recent data from clinical studies suggest that VEGF-C and/or VEGF-D expression in tumour tissue might be prognostic factors for lymphatic spread, tumour invasion and/or poor prognosis in a variety of cancers including gastric, colorectal, endometrial and breast<sup>[37,39,40,45,46]</sup>.



**Figure 3** Quantitative EGFR, VEGFR3 and VEGF-D mRNA expression (A) and Western blot analysis (B) of untreated SW480 and SW620. The  $\alpha$ -tubulin signal served as a control to confirm the loading of equivalent amounts of protein per track.



**Figure 4** Effect of cetuximab on (A) the expression of VEGF-D and VEGFR3 in SW480 cells cultured in the presence and absence of EGF and (B) the *in vivo* levels of VEGF-D and VEGFR3 in a NOD/SCID mouse xenograft model.

To our knowledge, this is the first study analyzing concurrently the expression profiles of EGFR and the

lymphangiogenic ligands VEGF-C and VEGF-D in a large series of human CRC specimens. With a relatively

low patient numbers used for well differentiated tumors, the statistical correlations of this current study between the histopathological grading and VEGF-C, but not VEGF-D expression levels may only be assumed to hold true for a larger clinical setting. However most interestingly, the comparison of the expression profile of VEGF-D in the colorectal tumour series with clinicopathological parameters revealed a significant association between VEGF-D and the number of lymph node metastasis. Moreover, we show with a clear trend that patients in a pN+ setting and positive VEGF-D expression define a subgroup with shorter survival. These data are consistent with previous analyses in a range of other cancers. Furthermore, they are in agreement with the established clinical observation that lymphatic dissemination in particular the number of metastatic lymph nodes is closely related to the clinical outcome/prognosis of patients with CRC<sup>[59]</sup>.

Signal transduction *via* the ligands VEGF-C and -D and VEGFR3 triggers lymphatic endothelial cell growth and migration<sup>[34-36]</sup>. Thus, it is noteworthy that in our specimens VEGF-C and VEGF-D were also strongly expressed at the tumour invasion front. We have previously shown that VEGFR3 is expressed in 67% of primary gastric cancers<sup>[60]</sup>. Data on animal models suggest that VEGF-C/VEGF-D/VEGFR3 signalling can promote tumour lymphangiogenesis and the metastatic spread of tumour cells<sup>[35,61-63]</sup>. Indeed, it has been suggested that primary tumours may prepare their future metastasis site by producing lymphangiogenic factors that mediate their efficient transport to the sentinel node<sup>[64]</sup>. Data from Jia *et al* suggested that VEGF-C expression may induce lymphangiogenesis in CRC and as a result, tumour cells could perhaps enter lymphatic vessels more easily<sup>[57,65]</sup>. These processes could be blocked by inhibition of VEGFR3 signalling by systemic delivery of a soluble VEGFR3-immunoglobulin fusion protein. However, lymph node metastasis was not suppressed if such treatment was started later, after tumour cells had already disseminated, suggesting that tumour cell entry into the lymphatic vessels is a key step during the metastatic process<sup>[66]</sup>. Consistently with these findings, our double VEGF-C and VEGF-D positive patients had unfavorable survival prognosis compared to the negative groups in the Kaplan-Meier analysis, again arguing for a large analysis of these markers in colorectal cancer patients with chemotherapy and/or cetuximab-based adjuvant therapy.

We further explored whether EGFR blockade influenced the expression of VEGF-D and VEGFR3 using *in vitro* and *in vivo* models. While treatment of the EGFR-expressing CRC cell line SW480 with EGF resulted in an increase in the transcript levels of both genes, there was a highly significant reduction in those induced mRNA levels when cetuximab was added. These data suggest that cetuximab, by blocking EGFR-associated signalling, might act as an inhibitor of VEGF-D expression, and consequently, of lymphangiogenesis. In contrast with an earlier report of another EGFR-specific antibody (ICR62)

stimulating VEGF-D<sup>[67]</sup>, cetuximab blocks additionally the production of pro-angiogenic factors such as VEGF and IL-8<sup>[12-14,68-70]</sup>. Although complete inhibition of VEGF-D expression was not achieved in our model systems the effect of cetuximab was nevertheless dramatic. In a therapeutic context, cetuximab, might also therefore induce a clinically-relevant reduction in tumour lymphangiogenesis. Given that the induction of lymphangiogenesis appears to be one of the early events in the progression of cancer<sup>[57]</sup>, a therapy targeting such processes would have a clear role in (neo-)adjuvant chemopreventive settings. Further preclinical and clinical studies are therefore warranted to explore these issues.

In conclusion, immunohistochemical staining of VEGF-D coupled with the examination of lymph node status may aid in the definition of patient subpopulations with more aggressive tumours. A cetuximab-based adjuvant therapy might then improve overall survival in these patients. Still, the link between prognostic markers and response to a certain treatment remains elusive. However, the new data in the current paper suggest that the inhibition of VEGF-D signalling might contribute to the widely demonstrated clinical activity of cetuximab in the treatment of CRC.

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## COMMENTS

### Background

Since the use of new biologic agents, such as epidermal growth factor receptor (EGFR)-targeted agents may improve our current therapeutic approaches for advanced human colorectal cancer (CRC) which seeds quite often metastatic tumor cells in the lymphatic glands, is of high interest to analyse prognostic and predictive expression markers for lymphangiogenic tumor spread, particularly in view of the possible modulation of these factors by the EGFR-targeted agents cetuximab and panitumumab.

### Research frontiers

Our clinical human colon cancer specimens displayed these markers EGFR, vascular endothelial growth factor (VEGF)-C and VEGF-D expression. Strong expression levels of VEGF-D were associated with lymph node metastasis and linked to decreased survival in lymph node-positive colon cancer patients. In tumor models, the new antibody for EGFR blockade cetuximab resulted in a highly significant decrease of VEGF-D expression.

### Innovations and breakthroughs

The lymphangiogenic marker VEGF-D thus associated with lymph node metastasis and is linked to a decreased survival in lymph node-positive patients. The EGFR blockade with cetuximab resulted in a significant decrease of VEGF-D expression, particularly favouring these EGFR-targeted agents as treatment options of lymph node-positive colorectal cancer.

### Applications

Patients with advanced lymph node-positive colorectal cancer might better be

selected as well as better be treated in the near future.

#### Peer review

Immunohistochemical staining of VEGF-D coupled with the examination of lymph node status may define patient subpopulations of with more aggressive colorectal cancers. Since the EGFR blockade with cetuximab resulted in a significant decrease of VEGF-D, EGFR-targeted agents may improve the overall survival, particularly as a new treatment option of lymph node-positive colorectal cancer.

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BASIC RESEARCH

# Evaluation of mannitol effect in patients with acute hepatic failure and acute-on-chronic liver failure using conventional MRI, diffusion tensor imaging and *in-vivo* proton MR spectroscopy

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**METHODS:** Five patients each with ALF and ACLF in grade 3 or 4 hepatic encephalopathy and with clinical signs of raised intracranial pressure were studied along with five healthy volunteers. After baseline MRI, an intravenous bolus of 20% mannitol solution was given over 10 min in controls as well as in patients with ALF and ACLF. Repeat MRI for the same position was acquired 30 min after completing the mannitol injection.

**RESULTS:** No statistically significant difference was observed between controls and patients with ALF and ACLF in metabolite ratios, DTI metrics and brain volume or CSF volume following 45 min of mannitol infusion. There was no change in clinical status at the end of post-mannitol imaging.

**CONCLUSION:** The osmotic effect of mannitol did not result in significant reduction of brain water content, alteration in metabolite ratios or any change in the clinical status of these patients during or within 45 min of mannitol infusion.

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**Key words:** Mannitol; Acute liver failure; Acute-on-chronic liver failure; Proton MR spectroscopy; Diffusion tensor imaging

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## Abstract

**AIM:** To evaluate the effect of an intravenous bolus of mannitol in altering brain metabolites, brain water content, brain parenchyma volume, cerebrospinal fluid (CSF) volume and clinical signs in controls and in patients with acute liver failure (ALF) and acute-on-chronic liver failure (ACLF), by comparing changes in conventional magnetic resonance imaging (MRI), *in vivo* proton magnetic resonance spectroscopy (PMRS) and diffusion tensor imaging (DTI) before and after its infusion.

## INTRODUCTION

Liver failure is a life-threatening condition following

severe hepatocellular injury that affects many other organ systems, most notably the brain and kidneys<sup>[1]</sup>. Liver failure may develop in the absence of pre-existing liver disease i.e. acute liver failure (ALF) or when an acute insult is superimposed on a chronic liver disorder [acute-on-chronic liver failure (ACLF)<sup>[2,3]</sup>]. Cerebral edema leading to raised intracranial pressure (ICP) is known to be a major complication in patients with ALF<sup>[4]</sup>. The pathophysiological mechanisms underlying development of cerebral edema and ICP in ALF are multifactorial<sup>[5,6]</sup>. Though the occurrence and nature of cerebral edema in ACLF is not well studied, anecdotal evidence from patients with ACLF suggests that hyponatremia is implicit in the progression to cerebral edema<sup>[7]</sup>.

Presently, osmotic therapy is widely used to control cerebral edema and raised ICP. Mannitol is the most frequently used osmotically active agent in clinical practice<sup>[8]</sup>. It acts by increasing blood osmolarity, thereby establishing a concentration gradient across the intact blood-brain barrier (BBB) that forces movement of water from the edematous brain tissue to the intravascular space<sup>[9]</sup>. In controlled trials, mannitol has been shown to decrease the raised ICP level in ALF patients and is associated with improved survival<sup>[10]</sup>. Other experimental studies have suggested that mannitol infusion reduces the water content and volume of normal, but not of infarcted brain tissue<sup>[11,12]</sup>.

Noninvasive imaging techniques such as magnetic resonance imaging (MRI) and computed tomography (CT) have been used to evaluate the brain water content changes after mannitol administration. Increased brain density of both edematous peritumoral white matter as well as normal gray and white matter on CT scans has been attributed to mannitol induced reduction in brain water content in patients with cerebral tumors<sup>[13]</sup>. Similarly, MR scans in patients with cerebral tumors detect reduced longitudinal relaxation time (T1) in edematous peritumoral white matter after mannitol infusion, which correlates with decrease in water content<sup>[14]</sup>.

Newer MR imaging techniques such as proton magnetic resonance spectroscopy (PMRS) and diffusion tensor imaging (DTI) are considered to be more sensitive and accurate in assessing the changes in brain metabolites and brain water content within the intracellular and extracellular compartments<sup>[15]</sup>. Changes in brain osmolytes detected by the PMRS are indirect or proxy evidence for cerebral edema. PMRS changes (i.e. reduction of choline, myoinositol and N-acetyl aspartate peaks and increase in Gln-Glx peak) are well-known in liver cirrhosis and chronic liver failure, conditions where there is sufficient time for organic osmolytes to compensate for increased intracellular osmolarity caused by astrocytic accumulation of glutamine<sup>[16]</sup>. PMRS changes in ALF are not well documented and are unlikely to be so clear-cut since rapid onset of the syndrome does not allow sufficient time for compensatory mechanisms to counterbalance rapid changes in osmolarity due to rapid and massive accumulation of astrocytic glutamine in this condition<sup>[17]</sup>. In patients with hyponatremia,

PMRS has revealed significantly lower levels of the organic osmolyte myoinositol which would normally compensate for the rising intracellular glutamine seen with acute liver injury<sup>[18]</sup>.

DTI is a technique that is able to reveal and quantify the direction of water mobility in tissues, which in white matter is normally anisotropic because of axonal fibers running in parallel. The two commonly used DTI metrics are fractional anisotropy (FA) and mean diffusivity (MD). FA reflects the degree of anisotropy due to the hindrance of water molecules mobility caused by various physical barriers. MD is a measure of tissue water diffusivity and is dependent upon interactions between water molecules and the structural components at cellular and subcellular level<sup>[19]</sup>.

To the best of our knowledge, this is the first study evaluating the effect of mannitol on brain water content using DTI and PMRS in controls as well as in patients with ALF and ACLF. We hypothesize that the effect of bolus infusion of mannitol in reducing brain water content will be reflected by changes measured by conventional MRI, *in-vivo* PMRS and DTI. Since the peak effect of a single bolus in mobilizing fluid from tissues to the intravascular compartment according to the osmotic gradient is observed at about 45 min after infusion<sup>[14]</sup>, it was decided to evaluate the effect of intravenous bolus of mannitol during the baseline MRI scan (pre-mannitol study) and after the completion of mannitol administration (post-mannitol study). The parameters compared between controls and patients, before and after mannitol infusion were (1) relative metabolite alterations in the right parietal region using *in-vivo* PMRS, (2) brain water content using DTI metrics, and (3) changes in the brain parenchyma volume as well as cerebrospinal fluid (CSF) volume. Changes were further correlated with the clinical outcome.

## MATERIALS AND METHODS

### **Patients**

In this study, we have included 5 ALF patients (3 males, 2 females; range, 14–46 years), and 5 ACLF patients (4 males, 1 female; range, 24–48 years). The patients with diagnosis of ALF and ACLF were admitted to the Gastroenterology intensive care ward and those who were clinically stable underwent imaging protocol. ALF was diagnosed in the presence of jaundice and encephalopathy with jaundice-to-encephalopathy interval being < 4 wk, and in the absence of clinical and radiological evidence of cirrhosis<sup>[20]</sup>. ACLF was diagnosed when there was acute hepatitis defined by abrupt (<4 wk) rise in serum bilirubin to  $\geq 10$  mg/dL and ALT to  $\geq 5$  times of normal ( $\geq 200$  IU/L) on a patient with clinical, biochemical or ultrasonographic evidence of chronic liver disease (ascites, nodular and irregular liver, patent portal vein and portosystemic collaterals). Upper gastrointestinal endoscopy was done in the patients who recovered from encephalopathy to look for portal hypertension (esophageal varices  $\geq$  grade II) as evidence of chronic liver disease. The

etiology, baseline clinical profile and biochemical profile of ALF has been shown in Tables 1 and 2, respectively. All the patients had acute viral hepatitis; none of them had drug induced hepatitis. The etiology of acute and chronic liver disease, baseline clinical profile and biochemical profile in ACLF patients has been shown in Tables 3 and 4, respectively. None of these patients had history of prior neurological illness. Patients with history of significant alcohol intake over the previous six months were excluded since de-compensation could have been related to recent alcohol intake. Patients with ultrasonographic evidence of a mass lesion in the liver (hepatocellular carcinoma), portal vein thrombosis or biliary obstruction, or suspected sepsis at presentation were excluded, since the liver decompensation could have been related to these factors. Serum specimens were obtained from the study subjects and stored at -70°C until analysis. Etiological tests for cause of both acute hepatitis and chronic liver disease were done in patients with ALF and ACLF. These included IgM anti-hepatitis E virus, IgM anti-Hepatitis A virus (HAV), HBsAg, IgM anti-HAV, anti-HCV, antinuclear antibodies, anti smooth muscle antibodies, anti LKM, anti-mitochondrial antibodies, serum ferritin, and Wilson's disease work-up.

### **Healthy controls**

Five healthy controls (3 males, 2 females; range, 18-50 years) were also included in this study, who had no history of neurological or psychiatric illness, alcohol or drug abuse, and head injury or liver disease.

### **Management**

Close monitoring and meticulous optimization of all metabolic derangements identified were done in all patients. Oxygenation was continuously monitored in each patient using pulse oximetry. Blood glucose level was monitored 6-hourly using a glucometer. Vital parameters, neurologic status and signs of raised ICP were checked 4-hourly. Raised ICP was diagnosed in presence of decerebrate posturing alone or, when two out of four of the following criteria were met, i.e. hypertension (supine blood pressure > 150/90 mmHg), bradycardia (pulse rate < 10/min for the expected pulse rate for the given body temperature), pupillary changes and neurogenic hyperventilation (hyperventilation in absence of metabolic or respiratory cause)<sup>[21]</sup>. ICP recordings were not done in any of these patients. However, the diagnosis of raised ICP was based on the clinical signs, imaging features of cytotoxic and interstitial edema on DTI. Sedation or muscle paralyzing agents were not used. We did not treat seizures prophylactically nor looked for subclinical seizures as prophylactic therapy for seizures as well as monitoring by EEG is not recommended in the management of ALF<sup>[22,23]</sup>. Arterial ammonia was measured in heparinized plasma by enzymatic method (RANDOX lab Ltd, UK) immediately before or within 6 h of imaging. Prior to infusion of mannitol infusion any metabolic derangement identified was corrected and all patients had been receiving standard anti-coma measures as well

as other supportive measures such as head end elevation, oxygen supplementation, dextrose infusion to maintain normoglycemia. We did not hyperventilate our patients because this is not an established method for decreasing raised ICP, and our second aim was to see the effect of mannitol so we tried to avoid all other methods of decreasing ICP mentioned in literature<sup>[23,24]</sup>.

An intravenous bolus of 20% mannitol solution (1 g/kg body weight) was given in controls as well as patients with ALF and ACLF after a baseline MRI scan (pre-mannitol study) for 10 min. After 30 min of mannitol injection, repeat MRI (post-mannitol study) for the same position was acquired. In post-mannitol study, both conventional T2-weighted and T1-weighted spin-echo (SE) imaging took a total time of 2.14 min and 1.34 min respectively, *in vivo* PMRS was done in 3.45 min and DTI data was acquired after 9.36 min and therefore the effect of mannitol was seen between 30-46 min (including time). The head was strapped to prevent any artifacts related to motion. The study protocol was approved by the institutional Ethics Committee and written informed consent was obtained from each subject or the nearest kin after explaining the nature of investigation to be carried out.

### **MRI protocol**

Imaging was performed on a 1.5 tesla MRI scanner (Signa Lx Echo speed plus, General Electric Healthcare Technologies, Milwaukee, WI) equipped with an actively-shielded whole body magnetic field gradient set (allowing up to 33 mT/m) equipped with a quadrature birdcage receive and transmit radio frequency head coil. The routine imaging studies included the following: fast spin echo (FSE) T2-weighted images with repetition time (TR)/echo time (TE)/number of excitations (NEX) = 6000 ms/85 ms/4, and T1-weighted SE images with TR/TE/NEX = 1000 ms/14 ms/2. A total of 36 contiguous 3 mm thick axial sections were acquired with a 240 mm × 240 mm field of view (FOV) and image matrix of 256 × 256.

### **In vivo proton MR spectroscopy**

The controls, ALF and ACLF patients underwent *in vivo* PMRS during the pre-mannitol study and post-mannitol study after the completion of intravenous mannitol administration to determine the changes in the brain metabolites. Spectra were obtained by using a water suppressed localized single voxel SE sequence with TR/TE = 3000 ms/35 ms. A voxel of 2 cm × 2 cm × 2 cm was located mainly in the right parietal region of the brain<sup>[25]</sup> in all the cases, containing part of white matter and gray matter (putamen and caudate nucleus) (Figure 1A). After global shimming, voxel shimming was performed, and a full width at half maximum of 4-6 Hz was achieved in all the cases. For evaluation and quantification of all individual spectra, the LC-Model software package (Version 6.0; Stephen Provencher, Ontario, Canada) was used for processing the MRS data. The process of determining peak intensities of the different metabolites is described in detail elsewhere<sup>[26]</sup>.

The metabolite ratios of N-acetylaspartate (NAA), choline (Cho), glutamine (Gln), glutamine/glutamate (Glx), and myoinositol (mI) were calculated with respect to creatine (Cr).

### **DTI protocol**

DTI data were acquired using a single-shot echo-planar dual SE sequence with ramp sampling<sup>[27]</sup>. The diffusion-weighted acquisition parameters were: b-factor = 0 s/mm<sup>2</sup>, 1000 s/mm<sup>2</sup>, slice thickness = 3 mm with no gap, number of slices = 34-38, FOV = 240 mm × 240 mm, TR = 8 s, TE = 100 ms and NEX = 8. The acquisition matrix was 128 × 80 and the homodyne algorithm was used to construct the  $\kappa$ -space data to 128 × 128 and zero-filled to generate an image matrix of 256 × 256. The diffusion tensor encoding used was the balanced rotationally invariant<sup>[28]</sup> dodecahedral scheme with 10 uniformly distributed directions over the unit hemisphere.

### **DTI data processing and analysis**

The magnitude averaged data were transferred to a workstation for further analysis. In general, the DTI data analysis involves three major steps: pre-processing, processing, and post-processing.

### **Data pre-processing**

The data were distortion corrected for shear, scale, rotation, and translation using the Automated Image and Registration package<sup>[29]</sup>. The removal of scalp to isolate the brain in the collected raw images was done, in all the cases, by an automated stripping procedure<sup>[30]</sup>. Subsequent DTI processing did not require any filtering, as justified by the absence of unprocessed voxels.

### **Data processing**

The distortion corrected data were then interpolated to attain isotropic voxels and decoded to obtain the tensor field for each voxel. The tensor field data were then diagonalized using the analytical diagonalization method<sup>[31]</sup> to obtain the eigenvalues ( $\lambda_1$ ,  $\lambda_2$  and  $\lambda_3$ ) and the three orthonormal eigenvectors ( $e_1$ ,  $e_2$  and  $e_3$ ). The orthogonality of the computed eigenvectors and the correctness of the eigenvalues were checked using random sampling at a number of voxels. The correctness observed was up to an order of 10<sup>-17</sup>, indicating that no iterative refinement of the computed eigenvalues/vectors was needed. The tensor field data was then used to compute the DTI metrics such as mean diffusivity (Equation 1) and fractional anisotropy (Equation 2) for each voxel.

$$MD = (\lambda_1 + \lambda_2 + \lambda_3)/3 \quad \text{Equation 1}$$

$$FA(\lambda_1, \lambda_2, \lambda_3) = \text{Sqrt}\{[(\lambda_1 - \lambda_2)^2 (\lambda_2 - \lambda_3)^2 (\lambda_1 - \lambda_3)^2]/2(\lambda_1^2 + \lambda_2^2 + \lambda_3^2)\} \quad \text{Equation 2}$$

### **Data post-processing and quantification**

JAVA based software was used to calculate various DTI derived metrics (MD and FA)<sup>[32]</sup>. To show homogenous distribution of metabolites within the brain, we selected major white matter and deep gray matter regions for region-of-interest(s) (ROI's) analysis, because it

has been reported in the previous studies that the changes are widespread in both the gray and the white matter in patients with hepatic encephalopathy<sup>[33]</sup>. For quantitative analysis, the DTI derived FA and MD maps were displayed and overlaid on images with different contrasts to facilitate the ROI's placement. Elliptical and/or rectangular ROI's varying from 2 × 2 and 6 × 6 pixels were placed on right and left anterior (ALIC) and posterior (PLIC) limb of internal capsule, right and left caudate nuclei (CN), right and left putamen (P), right and left thalamus (TH), right and left periventricular white matter of frontal (FWM) and occipital (OWM) lobes, right and left cingulum and corpus callosum (CC) at the level of third ventricle for DTI metrics quantification in these controls as well as patients (Figure 1E). Rectangular ROI's were also placed at the level of frontal cortex, parietal cortex and occipital cortex to quantify the various DTI metrics.

The total brain volume changes (brain parenchyma as well as CSF volume) before and after the intravenous infusion of mannitol was also measured in all subjects on the T2-weighted images using the in-house JAVA based software.

### **Statistical analysis**

Before statistical analysis, left and right measurements for all regions were pooled together. Mann-Whitney rank sum test was performed between the pre-mannitol study and post-mannitol study to see the statistical significant difference in the metabolite ratios (with respect to Cr), DTI metrics (FA and MD), brain parenchyma volume as well as CSF volume for controls ( $n = 5$ ), ALF ( $n = 5$ ) and ACLF ( $n = 5$ ) patients. A P value of less than or equal to 0.05 was considered to be statistically significant. All the statistical analyses were performed using the statistical package for social sciences (SPSS, V12, Inc, Chicago, USA).

## **RESULTS**

### **Imaging findings**

All patients in ALF and ACLF groups had grade 3 or grade 4 hepatic encephalopathy when they were subjected to MRI study after excluding all the metabolic factors which might contribute to altered mental state. All of these patients had imaging features of cytotoxic and interstitial edema on DTI suggestive of raised ICP. These patients were then treated with mannitol infusion and repeat MRI for the same position was acquired as shown in Tables 5-7, respectively. We compared the clinical signs and MR changes in both group of patients as well as controls pre and post mannitol infusion. None of the patients with ALF and ACLF showed significant clinical improvement in terms of the grade of encephalopathy and appearance of defined clinical signs of cerebral edema and MR findings.

### **In vivo MR spectroscopy**

The mean metabolites ratios calculated for the controls, ALF patients and ACLF patients are shown in Table 5. In all these subjects, no statistical significant difference

**Table 1** Clinical profile of acute liver failure patients

Case	Age/Sex	Etiology	Grade of HE	Jaundice-HE interval (d)	HE duration at presentation (d)	Clinical features of raised ICP				
						DP	HT	B	PC	NH
1	24/M	Hepatitis E	4	7	2	+	+	-	-	+
2	14/M	Hepatitis B	4	8	2	+	+	-	+	+
3	24/F	Hepatitis B	4	23	2	+	+	+	+	+
4	46/F	Hepatitis E	4	2	2	+	+	+	+	+
5	25/F	Hepatitis E	3	2	1	+	+	-	-	-

HE: Hepatic encephalopathy; DP: Decerebrate posture; HT: Hypertension; B: Bradycardia; PC: Pupillary changes; NH: Neurogenic hyperventilation.

**Table 2** Biochemical profile of acute liver failure patients

Case	Serum bilirubin (mg/dL)	ALT (U/L)	AST (U/L)	ALP (U/L)	INR	Serum sodium (mmol/L)	Serum potassium (mmol/L)	Serum creatinine (mg/dL)	Blood sugar (mg/dL, range)	Blood ammonia (μmol/dL)
1	13.3	242	349	134	1.8	142	3.8	1.2	110-186	
2	26.4	1020	920	120	8.2	146	4.7	1.2	130-194	438
3	28.6	206	156	140	6.8	140	4.9	0.6	98-178	150
4	15.3	1688	276	210	5.5	145	4.3	0.6	130-188	265
5	21.5	232	142	146	2.8	143	3.6	0.4	88-176	182

ALT: Alanine transaminase; AST: Aspartate transaminase; ALP: Alkaline phosphatase; INR: International normalized ratio.

**Table 3** Clinical profile of acute-on-chronic liver failure patients

Case	Age/Sex	Etiology		Grade of HE	Jaundice-HE interval (d)	HE duration at presentation (d)	Clinical features of raised ICP				
		Acute	Chronic				DP	HT	B	PC	NH
1	48/M	Hepatitis E	Cryptogenic	3	17	2	+	+	-	-	+
2	48/M	Hepatitis E	Chronic Hepatitis B	4	15	3	+	+	-	+	+
3	24/F	Hepatitis E	Autoimmune	3	22	1	+	-	-	-	+
4	45/F	Hepatitis E	Chronic Hepatitis C	3	13	2	+	+	-	+	+
5	28/F	Hepatitis E	Chronic Hepatitis B	3	7	2	+	+	-	-	+

HE: Hepatic encephalopathy; DP: Decerebrate posture; HT: Hypertension; B: Bradycardia; PC: Pupillary changes; NH: Neurogenic hyperventilation.

**Table 4** Biochemical profile of acute-on-chronic liver failure patients

Case	Serum bilirubin (mg/dL)	ALT (U/L)	AST (U/L)	SAP (U/L)	INR	Serum sodium (mmol/L)	Serum potassium (mmol/L)	Serum creatinine (mg/dL)	Blood sugar (mg/dL, range)	Blood ammonia (μmole/dL)	Child score	MELD score
1	18.8	346	274	148	2.6	143	3.7	1.2	130-194	180	12/15	30
2	17.2	254	142	165	2.4	144	4.9	0.9	98-210	280	13/15	29
3	25.7	636	524	164	3.7	136	4.5	0.5	98-166	261	13/15	33
4	25.1	240	320	97	3.9	145	3.9	0.9	108-210	310	14/15	34
5	10.2	279	151	79	2.25	137	4.3	0.8	89-189	210	13/15	24

ALT: Alanine transaminase; AST: Aspartate transaminase; SAP: Serum alkaline phosphatase; INR: International normalized ratio; MELD: Modified end stage liver disease.

was observed in the ratios of NAA/Cr, Cho/Cr, Gln/Cr, Glx/Cr, and mI/Cr between the pre-mannitol study (Figure 1B) and post-mannitol study (Figure 2B).

#### Changes in DTI metrics

The mean MD and FA values extracted from ROI's in controls, ALF patients and ACLF patients are summarized in Tables 6 and 7, respectively. None of the DTI metrics showed any significant difference between the pre-mannitol study (Figure 1C and D) and post-mannitol study (Figure 2C and D) in controls as well as ALF and ACLF patients.

#### Changes in total brain volume

In all subjects, no significant change was found in both brain parenchyma volume as well as CSF volume before mannitol administration and at 45 min after the completion of mannitol infusion (Table 8).

#### DISCUSSION

In case of patients with abnormal liver function or liver failure, detoxification of ammonia into glutamine by glutamine synthetase occurs in the brain astrocytes<sup>[34,35]</sup>. It has been reported that the facilitated transport system

**Table 5** Peak integrals relative to those of creatine (mean  $\pm$  SD) in right parietal white and gray matter in healthy controls, acute liver failure (ALF) patients and acute-on-chronic liver failure (ACLF) patients before the intravenous infusion of mannitol (pre-mannitol study) and after receiving mannitol (post-mannitol study)

Metabolites	Study								
	Control ( $n = 5$ )			ALF patient ( $n = 5$ )			ACLF patient ( $n = 5$ )		
	Pre-mannitol	Post-mannitol	P	Pre-mannitol	Post-mannitol	P	Pre-mannitol	Post-mannitol	P
NAA/Cr	1.25 $\pm$ 0.16	1.23 $\pm$ 0.17	0.92	1.20 $\pm$ 0.13	1.27 $\pm$ 0.32	0.75	0.90 $\pm$ 0.35	0.58 $\pm$ 0.29	0.18
Cho/Cr	0.24 $\pm$ 0.06	0.24 $\pm$ 0.08	0.75	0.18 $\pm$ 0.09	0.16 $\pm$ 0.07	0.47	0.20 $\pm$ 0.05	0.20 $\pm$ 0.03	0.75
Gln/Cr	1.44 $\pm$ 1.06	1.18 $\pm$ 0.94	0.35	3.04 $\pm$ 1.32	2.52 $\pm$ 1.56	0.25	1.44 $\pm$ 0.30	2.42 $\pm$ 1.44	0.60
Glx/Cr	2.84 $\pm$ 0.98	2.70 $\pm$ 1.21	0.60	4.46 $\pm$ 1.48	4.38 $\pm$ 1.61	0.92	2.85 $\pm$ 0.70	3.85 $\pm$ 2.13	0.35
mI/Cr	0.57 $\pm$ 0.48	0.49 $\pm$ 0.34	0.92	0.56 $\pm$ 0.15	0.47 $\pm$ 0.16	0.18	0.41 $\pm$ 0.17	0.89 $\pm$ 1.14	0.81

Cr: Creatine; NAA: N-acetylaspartate; Cho: Choline; Gln: Glutamine; Glx: Glutamine/glutamate; mI: Myoinositol.

**Table 6** Summary of fractional anisotropy values (mean  $\pm$  SD) from different white and gray matter regions in controls, patients with acute liver failure (ALF) and acute-on-chronic liver failure (ACLF) before the intravenous infusion of mannitol (pre-mannitol study) and after receiving mannitol (post-mannitol study)

Region	Study								
	Control ( $n = 5$ )			ALF patient ( $n = 5$ )			ACLF patient ( $n = 5$ )		
	Pre-mannitol	Post-mannitol	P	Pre-mannitol	Post-mannitol	P	Pre-mannitol	Post-mannitol	P
ALIC	0.36 $\pm$ 0.05	0.37 $\pm$ 0.04	0.60	0.30 $\pm$ 0.03	0.30 $\pm$ 0.02	0.47	0.32 $\pm$ 0.02	0.33 $\pm$ 0.02	0.56
PLIC	0.49 $\pm$ 0.05	0.48 $\pm$ 0.05	0.60	0.33 $\pm$ 0.02	0.34 $\pm$ 0.01	0.12	0.45 $\pm$ 0.03	0.40 $\pm$ 0.03	0.08
CN	0.11 $\pm$ 0.00	0.11 $\pm$ 0.00	0.47	0.09 $\pm$ 0.01	0.09 $\pm$ 0.01	0.60	0.08 $\pm$ 0.01	0.09 $\pm$ 0.01	0.56
P	0.09 $\pm$ 0.01	0.09 $\pm$ 0.01	0.92	0.06 $\pm$ 0.01	0.07 $\pm$ 0.01	0.92	0.06 $\pm$ 0.02	0.08 $\pm$ 0.01	0.15
TH	0.15 $\pm$ 0.01	0.14 $\pm$ 0.01	0.12	0.12 $\pm$ 0.00	0.12 $\pm$ 0.01	0.92	0.14 $\pm$ 0.01	0.15 $\pm$ 0.03	0.77
FWM	0.31 $\pm$ 0.03	0.30 $\pm$ 0.03	0.92	0.30 $\pm$ 0.03	0.32 $\pm$ 0.07	0.92	0.32 $\pm$ 0.04	0.36 $\pm$ 0.07	0.56
OWM	0.38 $\pm$ 0.06	0.38 $\pm$ 0.07	0.92	0.38 $\pm$ 0.04	0.37 $\pm$ 0.05	0.92	0.35 $\pm$ 0.04	0.31 $\pm$ 0.02	0.15
CC	0.53 $\pm$ 0.04	0.54 $\pm$ 0.06	0.92	0.44 $\pm$ 0.06	0.45 $\pm$ 0.02	0.35	0.50 $\pm$ 0.04	0.51 $\pm$ 0.05	0.77
Cingulum	0.36 $\pm$ 0.03	0.37 $\pm$ 0.04	0.75	0.44 $\pm$ 0.07	0.41 $\pm$ 0.03	0.25	0.38 $\pm$ 0.03	0.36 $\pm$ 0.03	0.56
Frontal cortex	0.12 $\pm$ 0.01	0.11 $\pm$ 0.00	0.47	0.12 $\pm$ 0.04	0.12 $\pm$ 0.04	0.60	0.14 $\pm$ 0.02	0.11 $\pm$ 0.02	0.08
Parietal cortex	0.12 $\pm$ 0.01	0.12 $\pm$ 0.01	0.75	0.11 $\pm$ 0.01	0.10 $\pm$ 0.01	0.75	0.14 $\pm$ 0.01	0.11 $\pm$ 0.03	0.39
Occipital cortex	0.11 $\pm$ 0.02	0.12 $\pm$ 0.03	0.47	0.14 $\pm$ 0.05	0.10 $\pm$ 0.03	0.25	0.10 $\pm$ 0.05	0.13 $\pm$ 0.03	0.39

ALIC: Anterior limb of internal capsule; PLIC: Posterior limb of internal capsule; CN: Caudate nuclei; P: Putamen; TH: Thalamus; FWM: Frontal white matter; OWM: Occipital white matter; CC: Corpus callosum.

operates to maintain the normal level of the nitrogen-rich osmolytes<sup>[36]</sup>. This process is energy dependent and requires normal metabolic conditions to operate. However, in case of patients with liver failure such a favorable milieu is not present. This suggests the increased concentration of nitrogen-rich compounds in brain of patients with liver failure, as also reported in previous studies<sup>[37,38]</sup>. It has been reported that the increased concentration of glutamine in these patients is associated with increased brain water content resulting in cerebral edema<sup>[39,40]</sup>. The use of hypertonic solutions for pulling out water initially from extracellular space and eventually from the intracellular compartment, along with clinical improvements in these patients, has been reported<sup>[41]</sup>.

In the present study, no significant change in the relative concentration of various metabolites in controls, ALF and ACLF patients after mannitol administration was observed, and this finding was associated with no change in the brain water content as well as in the clinical condition of patients. It has been reported that decreased myoinositol concentration is associated with compensatory response to the osmotic gradient induced by the high level of glutamine<sup>[17]</sup>. In case of controls and ALF patients, no significant decrease in mI/Cr

ratio after mannitol infusion suggests that the osmotic gradient due to mannitol might not be able to shift the myoinositol osmolyte across the BBB to compensate the intracellular osmolarity caused by accumulation of astrocytic glutamine. In case of ACLF patients, the slight increase in Gln/Cr, Glx/Cr and mI/Cr was seen after the infusion of mannitol; however, it did not reach the level of statistical significance. This further confirms that the osmotic gradient due to mannitol is not able to influence the efflux of organic osmolytes across the BBB. The lack of significant change in PMRS findings may be due to the fact that this was an acute study in which the pre- and post-mannitol studies were spaced 45 min apart. It is well known that the proxy changes of cerebral edema picked up by PMRS (i.e. depletion of choline, myoinositol, N-acetyl aspartate) are due to osmolyte shifts that occur over a prolonged timeframe and not in minutes<sup>[42,43]</sup>.

We did not find any significant change in the DTI metrics (FA and MD) in controls as well as in patients with ALF and ACLF at 45 min after the completion of mannitol administration. In controls, insignificant change in either FA or MD values in normal brain tissues (white matter and gray matter) suggest that mannitol has no effect on the microstructural integrity and brain water

**Table 7 Summary of mean diffusivity values (mean  $\pm$  SD) in units of  $10^{-3}$  mm $^2$ /s from different white and gray matter regions in controls, patients with acute liver failure (ALF) and acute-on-chronic liver failure (ACLF) before the intravenous infusion of mannitol (pre-mannitol study) and after receiving mannitol (post-mannitol study)**

Region	Study								
	Control (n = 5)			ALF patient (n = 5)			ACLF patient (n = 5)		
	Pre-mannitol	Post-mannitol	P	Pre-mannitol	Post-mannitol	P	Pre-mannitol	Post-mannitol	P
ALIC	0.76 $\pm$ 0.07	0.76 $\pm$ 0.05	0.60	0.68 $\pm$ 0.02	0.68 $\pm$ 0.02	0.75	0.72 $\pm$ 0.02	0.71 $\pm$ 0.08	0.56
PLIC	0.74 $\pm$ 0.06	0.75 $\pm$ 0.05	0.75	0.67 $\pm$ 0.03	0.67 $\pm$ 0.02	0.92	0.65 $\pm$ 0.03	0.65 $\pm$ 0.04	0.77
CN	0.73 $\pm$ 0.02	0.74 $\pm$ 0.02	0.75	0.68 $\pm$ 0.01	0.69 $\pm$ 0.02	0.92	0.69 $\pm$ 0.02	0.69 $\pm$ 0.09	0.25
P	0.69 $\pm$ 0.01	0.70 $\pm$ 0.06	0.25	0.64 $\pm$ 0.03	0.63 $\pm$ 0.01	0.47	0.69 $\pm$ 0.02	0.69 $\pm$ 0.06	0.39
TH	0.72 $\pm$ 0.03	0.71 $\pm$ 0.03	0.92	0.66 $\pm$ 0.02	0.66 $\pm$ 0.04	0.92	0.71 $\pm$ 0.02	0.74 $\pm$ 0.05	0.56
FWM	0.77 $\pm$ 0.06	0.76 $\pm$ 0.07	0.75	0.67 $\pm$ 0.01	0.66 $\pm$ 0.05	0.92	0.65 $\pm$ 0.02	0.66 $\pm$ 0.13	0.25
OWM	0.76 $\pm$ 0.05	0.76 $\pm$ 0.07	0.92	0.66 $\pm$ 0.03	0.66 $\pm$ 0.05	0.75	0.76 $\pm$ 0.11	0.68 $\pm$ 0.05	0.56
CC	0.74 $\pm$ 0.07	0.74 $\pm$ 0.07	0.92	0.74 $\pm$ 0.09	0.74 $\pm$ 0.08	0.92	0.77 $\pm$ 0.09	0.77 $\pm$ 0.09	0.77
Cingulum	0.72 $\pm$ 0.05	0.72 $\pm$ 0.09	0.75	0.72 $\pm$ 0.10	0.72 $\pm$ 0.04	0.75	0.73 $\pm$ 0.03	0.70 $\pm$ 0.06	0.56
Frontal cortex	0.69 $\pm$ 0.07	0.69 $\pm$ 0.04	0.92	0.68 $\pm$ 0.04	0.68 $\pm$ 0.05	0.92	0.65 $\pm$ 0.05	0.70 $\pm$ 0.03	0.08
Parietal cortex	0.68 $\pm$ 0.06	0.68 $\pm$ 0.05	0.92	0.68 $\pm$ 0.01	0.68 $\pm$ 0.03	0.47	0.69 $\pm$ 0.04	0.63 $\pm$ 0.04	0.08
Occipital cortex	0.67 $\pm$ 0.07	0.67 $\pm$ 0.06	0.92	0.66 $\pm$ 0.02	0.66 $\pm$ 0.05	0.92	0.69 $\pm$ 0.06	0.62 $\pm$ 0.03	0.15

ALIC: Anterior limb of internal capsule; PLIC: Posterior limb of internal capsule; CN: Caudate nuclei; P: Putamen; TH: Thalamus; FWM: Frontal white matter; OWM: Occipital white matter; CC: Corpus callosum.

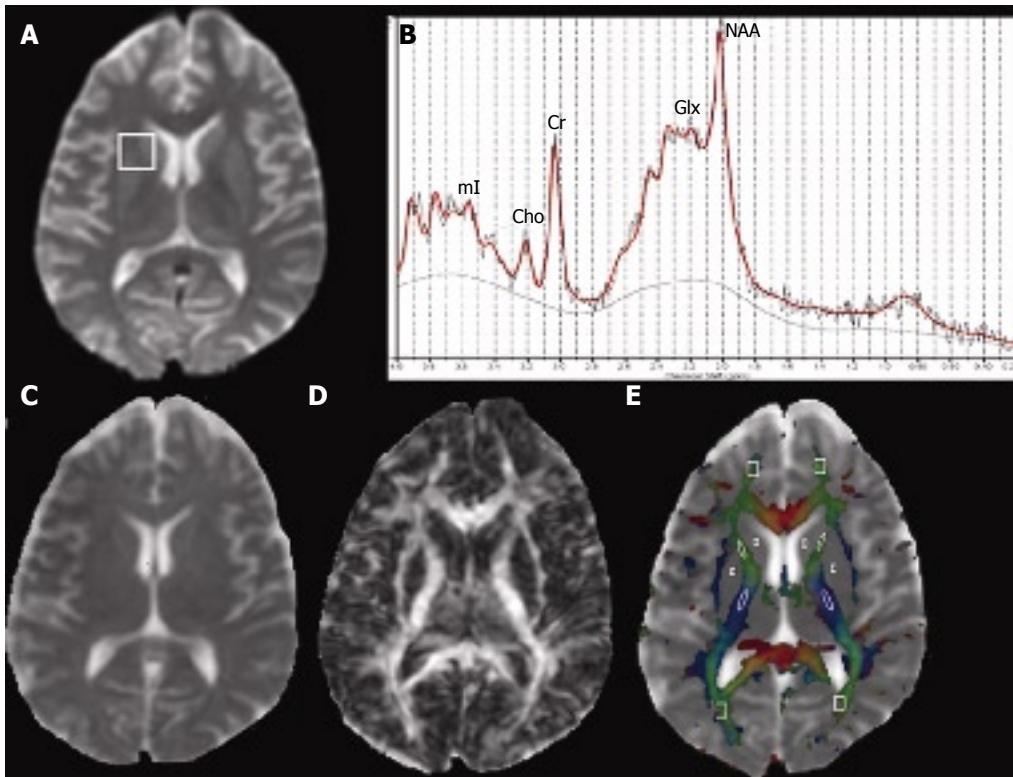
**Table 8 Change in brain volume and cerebrospinal fluid (CSF) volume values (mean  $\pm$  SD) in units of cubic centimeter from different white and gray matter regions in controls, patients with acute liver failure (ALF) and acute-on-chronic liver failure (ACLF) before the intravenous infusion of mannitol (pre-mannitol study) and after receiving mannitol (post-mannitol study)**

Volume	Study								
	Control (n = 5)			ALF Patient (n = 5)			ACLF Patient (n = 5)		
	Pre-mannitol	Post-mannitol	P	Pre-mannitol	Post-mannitol	P	Pre-mannitol	Post-mannitol	P
Brain volume	1303.47 $\pm$ 54.95	1308.88 $\pm$ 64.95	0.75	1130.76 $\pm$ 86.73	1162.39 $\pm$ 77.75	0.47	1096.85 $\pm$ 79.55	1076.90 $\pm$ 71.63	0.60
CSF volume	84.05 $\pm$ 4.88	85.10 $\pm$ 6.46	0.60	73.77 $\pm$ 19.72	73.27 $\pm$ 19.53	0.47	76.77 $\pm$ 18.68	83.84 $\pm$ 22.09	0.12

content. This finding is in line with the previous study, which showed no change in the brain water content in normal white matter and cortex of patients with cerebral tumors<sup>[14]</sup>. The possible explanation could be due to the high hydraulic resistance of the capillaries in the normal brain tissues, and therefore mannitol might not be able to withdraw water osmotically from normal brain tissues<sup>[14]</sup>. Cascino *et al* have shown that the increased brain density of both edematous peritumoral white matter and normal gray and white matter on CT is related to the mannitol induced reduction in brain water content in patients with cerebral tumors<sup>[13]</sup>. They found that the maximum changes occurred after 36 min of mannitol infusion<sup>[13]</sup>. Bell *et al* showed that mannitol significantly reduced longitudinal relaxation time (T1) of oedematous peritumoral white matter and tumor tissue but did not have any significant effect on the normal white matter or cortex<sup>[14]</sup>. The maximum decrease in brain water content was found after 30 min of mannitol infusion, which was associated with the reduction in T1 values<sup>[14]</sup>.

The effect of mannitol in patients with liver failure depends on the nature of cerebral edema. It has been reported that the nature of cerebral edema in case of ALF is predominantly cytotoxic along with some interstitial component<sup>[44]</sup>. However, the nature of cerebral edema in ACLF has yet to be described. The result from unpublished data suggests the predominant interstitial component of edema in ACLF<sup>[45]</sup>. In a

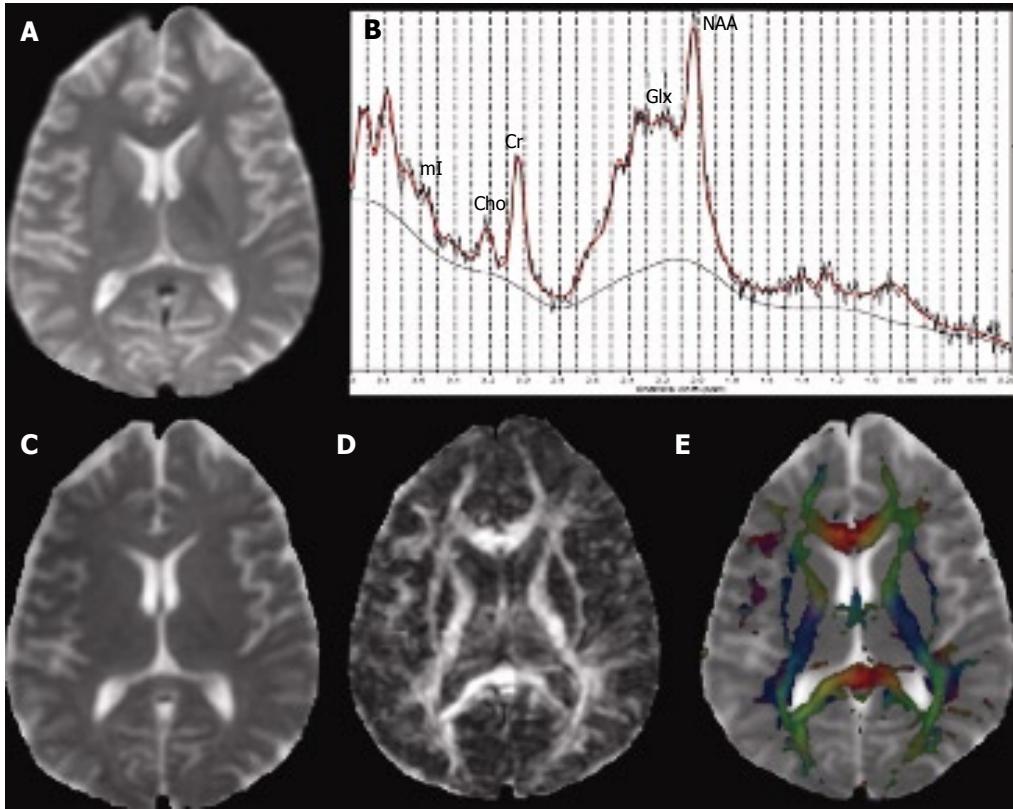
previous study, the reduction in brain water content has been reported after mannitol administration in ALF patients by the observed decrease in ICP, as well as reversal of the clinical signs and concluded that the operative edema is of the cytotoxic type that resolved after mannitol treatment<sup>[10]</sup>. However, in the current study no change in the FA and MD values in patients with ALF and ACLF suggests any effect of mannitol on the brain water content along with the microstructural integrity. The BBB is known to be disrupted in both conditions of ALF and ACLF resulting in the interstitial component of cerebral edema. The tight endothelial junctions of the BBB open in both these conditions; however, the extent to which these endothelial junctions of BBB open is different. Hartwell *et al* also have reported no significant change in the water content of the edematous white matter after mannitol infusion in cats. This finding was explained based on the changes in the BBB that extended beyond the region of central necrosis induced by the cold lesion, which may have resulted in the disturbance of osmotic gradient<sup>[12]</sup>. In our study, no change in the brain water content as depicted by no change in the MD values in ALF and ACLF patients after mannitol infusion can be explained on the basis of the disruption of BBB due to the presence of interstitial component of cerebral edema. This may affect the mannitol induced osmotic gradient across the BBB and result in no withdrawal of water from the edematous brain tissue to the intravascular space.



**Figure 1** Conventional magnetic resonance imaging (MRI) and diffusion tensor imaging (DTI) were performed at three days after the onset of encephalopathy in a 29-year-old man with acute liver failure (ALF) before the mannitol administration. **A:** Axial T2-weighted imaging at the level of third ventricle does not show any visible abnormality; **B:** Localized proton spectrum from  $2\text{ cm} \times 2\text{ cm} \times 2\text{ cm}$  voxel placed on right parietal region (**A**) shows the metabolite ratios with respect to Cr (NAA/Cr = 1.35, Cho/Cr = 0.34, Gln/Cr = 2.57, Glx/Cr = 4.12, mI/Cr = 0.45); **C:** Mean diffusivity (MD) map, [anterior limb of internal capsule (ALIC) = 0.68, posterior limb of internal capsule (PLIC) = 0.65, caudate nuclei (CN) = 0.67, putamen (P) = 0.60, thalamus (TH) = 0.66, frontal white matter (FWM) = 0.66, occipital white matter (OWM) = 0.67, corpus callosum (CC) = 0.60, cingulum = 0.70, frontal cortex = 0.63, parietal cortex = 0.70, occipital cortex = 0.63]; **D:** Fractional anisotropy (FA) map, (ALIC = 0.32, PLIC = 0.34, CN = 0.09, putamen = 0.08, thalamus = 0.11, FWM = 0.33, OWM = 0.38, CC = 0.53, cingulum = 0.45, frontal cortex = 0.16, parietal cortex = 0.10, occipital cortex = 0.12). The cut off value for the color-coded FA for display is kept at 0.2 (**E**) above which the color-coded regions reflect the white matter only [red (right-left), green (anterior-posterior), and blue (superior-inferior)]. Cho: Choline; Cr: Creatine; Gln: Glutamine; Glx: Glutamine/glutamate; mI: Myoinositol; NAA: N-acetylaspartate.

Our above results are further supported by no significant change in the brain parenchyma volume and CSF volume in controls and patients with ALF and ACLF. In our study, we have explained the action of mannitol only on the basis of the osmotic gradient across the BBB, which is known to affect the brain volume of the normal tissue<sup>[11]</sup>. The cerebral blood flow is an important hemodynamic parameter that is shown to be reduced after mannitol administration<sup>[46,47]</sup>. Although in our study we do rule out the possibility of an osmotic gradient across the BBB, other vascular factors such as changes in cerebral blood flow, blood viscosity and oxygen delivery at the tissue level might be responsible for the effects of mannitol<sup>[12]</sup>. However, in our patients we did not quantify the cerebral blood flow and ICP. The use of ICP monitoring in ALF is a subject of ongoing debate. ICP monitoring is used variably across the world, with some centers not considering it useful and other using it regularly. In our study and, in fact, in most of the centers in our country direct ICP measurement is not used. The clinical signs used in our study are the reliable clinical signs of raised ICP, provided that other causes like brain hemorrhage or intracranial space occupying lesion which may cause raised ICP are excluded. However, these clinical signs are

not uniformly present in all cases, but if at all present these signs are suggestive of raised ICP. In our study, all the patients had these clinical signs. In a landmark study, Canalese *et al* measured cerebral edema by the presence of defined clinical signs as well as continuous monitoring of ICP in two different groups in their study<sup>[10]</sup>. Among the patients who received mannitol, cerebral edema was considered to have developed in 17 patients, in nine on evidence from continuous intracranial monitoring and in eight on the basis of clinical signs<sup>[10]</sup>. In those patients who died, ICP was monitored with either clinical signs of cerebral edema or direct measurement; when brain autopsy was done, a close correlation was found between the evidence of cerebral edema in life and findings at the autopsy of brain, whereas there was no evidence of correlation in the four other patients whose ICP was not raised and who had no clinical features of cerebral edema<sup>[10]</sup>. Acharya *et al* in their study of clinical profile of ALF patients and predictors of mortality from tropical country had also used similar clinical signs to monitor cerebral edema<sup>[21]</sup>. The AASLD guidelines for management of ALF mentioned ICP monitoring either by direct measurement or by obvious clinical signs<sup>[23]</sup>. Stravitz *et al* reported that there are insufficient data to recommend ICP monitor placement in all patients with



**Figure 2** Repeat MRI and DTI were performed after 30 min of mannitol infusion on the same ALF patient as in Figure 1 to look for any mannitol effect on imaging. In post-mannitol study, the effect of mannitol was quantified between 30–46 min. **A:** Axial T2-weighted image; **B:** Localized proton spectrum from the same region as in Figure 1A shows NAA/Cr = 1.83, Cho/Cr = 0.29, Gln/Cr = 2.60, Glx/Cr = 4.57, mI/Cr = 0.32; **C:** Mean diffusivity (MD) map, [anterior limb of internal capsule (ALIC) = 0.67, posterior limb of internal capsule (PLIC) = 0.70, caudate nuclei (CN) = 0.70, putamen (P) = 0.63, thalamus (TH) = 0.62, frontal white matter (FWM) = 0.66, occipital white matter (OWM) = 0.60, corpus callosum (CC) = 0.70, cingulum = 0.72, frontal cortex = 0.69, parietal cortex = 0.66, occipital cortex = 0.62]; **D:** Fractional anisotropy (FA) map, (ALIC = 0.32, PLIC = 0.34, CN = 0.08, putamen = 0.06, thalamus = 0.13, FWM = 0.30, OWM = 0.43, CC = 0.46, cingulum = 0.40, frontal cortex = 0.10, parietal cortex = 0.12, occipital cortex = 0.08) at the level of third ventricle. **E:** There is no significant change in the MD and FA values, and metabolite ratios mentioned here compared to what is mentioned in Figure 1. Cho: Choline; Cr: Creatine; Gln: Glutamine; Glx: Glutamine/glutamate; mI: Myoinositol; NAA: N-acetylaspartate.

ALF; however, according to these authors all patients who are candidate for orthotopic liver transplantation (OLT) should undergo ICP monitoring<sup>[20]</sup>. In our study, all the patients were managed conservatively and none had finances for OLT. Nevertheless, it is true without ICP monitoring we can not quantify ICP, and this is one of limitations of our study since we did not measure ICP. However, the monitoring of response can be done by improvement of clinical signs or reduction of ICP, and obviously reduction in ICP would have been the best way to see the amount of response. There is no relationship of dosing of mannitol according to the severity of ICP. AASLD guidelines suggested the main role of ICP monitoring to detect early rise in intracranial pressure even before the development of clinical signs, and ICP should be maintained below 20–25 mmHg if possible. So once clinical features of raised ICP appear, it means intracranial pressure has significantly raised<sup>[23]</sup>.

The absence of changes in the clinical status of these patients further supports that there is likely no significant effect of mannitol on their clinical management in this time window. However, the effect of mannitol over days is more difficult to assess due to other co-variates used for its management that might also contribute for the survival of some of these patients.

The osmotic effect of mannitol in the reduction of brain water content does not have any significant immediate effect on the clinical status in ALF and ACLF patients. The effect of other vascular factors that may alter the ICP indirectly will be the subject for future study to assess the mannitol effect using other noninvasive techniques such as perfusion imaging.

This is a first pilot study to see the direct effect of mannitol in ALF and ACLF patients. The conclusion of our study is that mannitol does not have an early effect (single dose effect seen in 45 min). However, we cannot conclude by saying that mannitol does not have any role in the management of ALF and ACLF patients, as previous studies<sup>[10]</sup> have shown that average response to mannitol therapy comes after 3 doses. Therefore, from our study, we cannot comment upon the delayed effect of mannitol with multiple doses. We plan to study the delayed effect of mannitol with more than one dose of mannitol in future.

## COMMENTS

### Background

Cerebral edema plays a major role in the outcome of both acute liver failure (ALF) and acute-on-chronic liver failure (ACLF). Bolus intravenous infusion of

mannitol has been widely used to treat episodes of raised intracranial pressure (ICP) in these conditions. However, there are no data available regarding the effect of mannitol infusions on brain water content in ALF and ACLF, using sensitive imaging techniques.

### Research frontiers

This is a pilot study to see the acute effect of mannitol in ALF and ACLF patients. The conclusion of our study is that mannitol does not have an early effect (single dose effect seen in 45 min). However, we cannot conclude by saying that mannitol does not have any role in the management of ALF and ACLF patients, as previous studies have shown that average response to mannitol therapy comes after three doses of mannitol. Therefore, from our study, we cannot comment upon the late effect of mannitol with multiple doses. The osmotic effect of mannitol in the reduction of brain water content does not have any significant immediate effect on the clinical status in ALF and ACLF patients. The effect of other vascular factors that may alter the ICP indirectly will be the subject for future study to assess the mannitol effect using other noninvasive techniques such as perfusion imaging.

### Innovations and breakthroughs

This is the first study evaluating the effect of mannitol on brain water content using diffusion tensor imaging (DTI) and proton magnetic resonance spectroscopy (PMRS) in controls as well as in patients with ALF and ACLF. We hypothesize that the effect of bolus infusion of mannitol in reducing brain water content will be reflected by changes measured by conventional magnetic resonance effect (MRI), *in vivo* PMRS and DTI. Since the peak effect of a single bolus in mobilizing fluid from tissues to the intravascular compartment according to the osmotic gradient is observed at about 45 min after infusion, it was decided to evaluate the effect of intravenous bolus of mannitol during the baseline MRI scan (pre-mannitol study) and after the completion of mannitol administration (post-mannitol study).

### Applications

Conventional MRI, DTI and PMRS can be used as a diagnostic modality to demonstrate the raised ICP in ALF and ACLF patients noninvasively. The purpose of this paper is to establish whether mannitol has any role in the reduction of brain water content, alteration in metabolite ratios or any change in the clinical status of ALF and ACLF patients during or within 45 min of mannitol infusion, and to know about the exact duration and dose of mannitol to show response after therapy.

### Peer review

This is the first study evaluating the effect of mannitol on brain water content using DTI and PMRS in controls as well as in patients with ALF and ACLF. The osmotic effect of mannitol in the reduction of brain water content does not have any significant immediate effect on the clinical status in ALF and ACLF patients. The effect of other vascular factors that may alter the ICP indirectly will be the subject for future study to assess the mannitol effect using other noninvasive techniques such as perfusion imaging.

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# Immunohistochemical localization of glutathione S-transferase-pi in human colorectal polyps

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adenoma and the highest levels found in high-grade adenoma. GST-pi was located mainly in undifferentiated epithelial cells. GST-pi positive particles were found in the cytoplasm and especially in the nucleus adjacent to the nuclear membrane of these cells.

**CONCLUSION:** The overexpression of GST-pi in mild-grade adenomas with significant subcellular changes and in the majority of high-grade dysplasia adenoma suggests that this might be related to the carcinogenetic proceeding. Immunohistochemical localization of GST-pi in combination with ultrastructural changes indicate that GST-pi might be a sensitive agent for the detection of preneoplastic transformations in adenoma.

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**Key words:** Glutathione S-transferase-pi; Colorectal polyps; Adenoma; Electron microscopy; Peroxidase anti-peroxidase method; Immunogold method

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## Abstract

**AIM:** To investigate the distribution of the placental form of glutathione-S-transferase (GST) in colon polyps in order to evaluate the role of GST-pi in these tissues.

**METHODS:** Sixteen polyp tissues removed at colonoscopy were examined. Tissues were investigated histologically and ultrastructurally. GST-pi expression was also analysed immunohistochemically, using peroxidase anti-peroxidase (PAP) method and immunogold labelling method, for light and electron microscope respectively.

**RESULTS:** All polyp tissues examined were adenoma of low, mild and high- grade dysplasia as shown in the histopathological reports. Nevertheless, the examination of the above specimens with electron microscope revealed that 3 of 9 adenoma of mild dysplasia had ultrastructural features similar to high-grade dysplasia adenoma. GST-pi was variably expressed in adenoma, with the lowest relative levels occurring in low-grade

## INTRODUCTION

Glutathione S-transferases (GST) are a family of enzymes that play an important role in the prevention of cancer by detoxifying numerous potentially carcinogenic compounds<sup>[1,2]</sup>. In this respect high tissue levels of GST's are protective against cancer. In preneoplastic cells as in neoplastic cells, specific molecular forms of GST are known to be expressed and have been known to participate in their resistance to drugs. GSTs are present in most epithelial tissues of the human gastrointestinal tract, as it is an important site of contact with compounds from food, drugs or medication<sup>[3,4]</sup>. The cytoplasmic GSTs have been grouped into four main classes, each with a different tissue-specific expression<sup>[5]</sup>. Significant amounts of the class pi GST were expressed in the majority of human

tumors and human tumor cell lines<sup>[6]</sup>. Recent studies have shown increased levels of human placental glutathione S-transferase (GST-pi) in different tumors of gastrointestinal tract as well as in precancerous lesions<sup>[5,7,8]</sup>. GST-pi was significantly increased in proliferative hepatic nodules induced by chemical carcinogen and in well-differentiated carcinoma<sup>[9]</sup>. Studies have shown that GST-pi is expressed highly in neoplasms and could be regarded as a tumor marker<sup>[10-12]</sup>.

There are immunohistochemical studies about colorectal carcinoma, suggesting that GST-pi is located in cancer cells<sup>[13,14]</sup>.

As the description of the immunohistochemical and the immunoelectron microscopic localization of GST-pi in human polyps is not available, we performed a combined study to examine the distribution of GST-pi in these tissues.

## MATERIALS AND METHODS

### Materials

In this study, 16 polyp tissues were removed at colonoscopy from the Department of Gastroenterology of the Regional Anticancer-Oncologic Hospital of Athens "Agios Savvas". Paraffin-sections were obtained from all tissues and examined by pathologists. When histological grading was performed, all polyp tissues were adenoma of different stage of dysplasia (3 of low-grade, 9 of mild-grade and 4 of high-grade).

### Methods

**Specimen preparation for light microscopy:** Samples for light immunohistochemical study were immediately placed in ice-cold saline in the endoscopic room. In the laboratory, the tissue was washed free of blood with ice-cold saline, frozen in liquid nitrogen and kept at -80°C until further use. The specimens were cut in frozen sections (6 µm).

**Electron microscopy:** Shortly after colonoscopy, tissues for electron microscopy were fixed in 2.5% glutaraldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.3) for 1 h at room temperature, and postfixed with 1% osmium tetroxide for 1 h at room temperature. All samples were then dehydrated *via* graded alcohol (25%, 30%, 50%, 70%, 90%, and 100%) and propylene oxide and embedded in Araldite resin. Semi-thin sections (1 µm) were cut, stained with toluidine blue, and examined under light microscope. Silver sections (500 Å) were collected on uncoated copper grids for ultrastructural observation and on uncoated nickel grids for immunolabeling. These sections were further stained with uranyl acetate and lead citrate and examined with a C-100 Phillips Electron Microscope.

**Peroxidase anti-peroxidase (PAP) method for light microscopy:** Sections on gelatin coated slides were treated in 0.3% H<sub>2</sub>O<sub>2</sub>. Following incubation for 30 min in a blocker solution containing 1/30 normal goat serum in phosphate buffered saline (PBS), sections

were immunostained with antiserum to glutathione-S-transferase-pi (Dako Company). Then PAP was applied and diluted in bovine serum albumin (BSA) and PBS 1/400 for 30 min. Finally, the sections were incubated for 5 min in a solution containing diaminobenzidine (DAB), H<sub>2</sub>O<sub>2</sub> and PBS.

Following that, the slides were rinsed twice with tap water and counterstained in hematoxyline for 2 min. They then were visualized using an optical microscope and were photographed using a Nikon Coolpix 3100.

### Immunogold labeling for electron microscopy:

All steps except for incubation with antibodies were carried out at room temperature. Ultra-thin sections in silver uncoated grids were treated in 8% H<sub>2</sub>O<sub>2</sub> for 8 min and etched with saturated aqueous sodium metaperiodate for 30 min. After incubating for 30 min. in a blocker solution containing 5% normal goat serum in PBS, sections were immunostained with antiserum to glutathione S-transferase-pi. The rabbit monoclonal anti S-transferase pi antiserum (Dako Company) was used at a dilution of 1:20 to 1:40. The incubations were carried out at 4°C, overnight. Following several washings in BSA, immunoreactivity was visualized by incubating the sections in 20 nm colloidal gold-labeled goat anti-rabbit IgG (British BioCell International, Cardiff, UK). The specificity of the immunolabeling was assessed by incubation of the sections with non-immune serum. When we performed the appropriate negative controls, we followed the same procedure described above except for incubation in the absence of primary antibody after the treatment with non-immunoreactive goat serum. After counterstaining with uranyl acetate and lead citrate, the sections were viewed under the Philips C100 Electron Microscope.

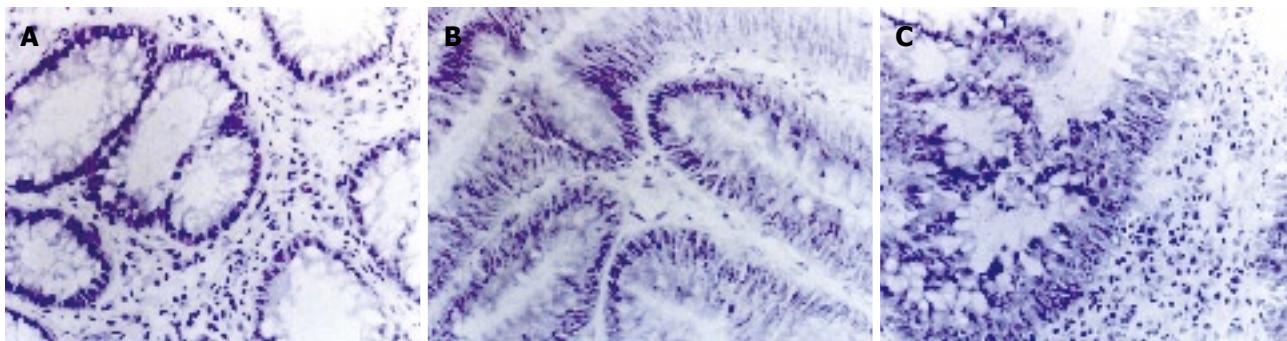
## RESULTS

### Light microscopy

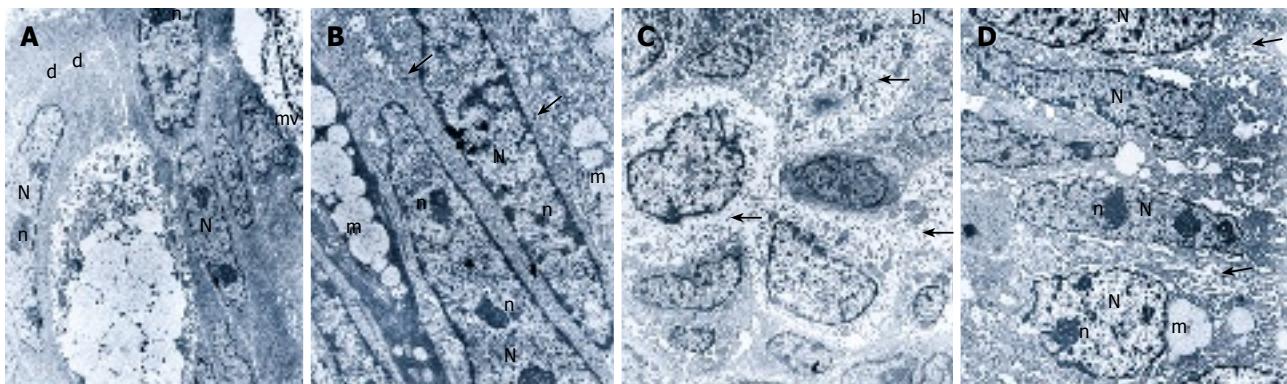
Histological examination of the tissues demonstrated that all were colorectal adenomas of low-grade dysplasia to high-grade dysplasia. Morphologically, adenomas showed abnormalities in epithelial cells that agree with the histopathological reports. Low-grade dysplasia adenoma is characterized by tall epithelial cells with elongated and hyperchromatic nuclei (Figure 1A). In mild-grade dysplasia adenoma, crypt architecture tends to be distorted. Nuclei are crowded, hyperchromatic and may be stratified near the base of the crypts without reaching the lumen (Figure 1B). High-grade dysplasia adenoma is characterized by a true nuclear stratification and a back-to-back pattern, and nuclei extend all the way to the lumen (Figure 1C).

### Electron microscopy

All cases were examined under the electron microscope. At least four specimens from each case were examined and the most representative block was used for electron microscopic examination. The relative number of each cell type in the low, mild and high-grade dysplasia varied. Low-grade dysplasia tissues closely mimicked those of normal colorectal mucosa but goblet cells



**Figure 1** Sections of human colorectal adenoma (HE,  $\times 150$ ). **A:** Low-grade dysplasia; **B:** Mild-grade dysplasia; **C:** High-grade dysplasia.



**Figure 2** Transmission electron micrograph, routine double staining with uranyl acetate and lead citrate. Nucleus (N), nucleoli (n), mucus granules (m), microvilli (mv). **A:** From a low-grade dysplasia adenoma ( $\times 5900$ ), desmosomes (d); **B:** From a mild-grade dysplasia adenoma, desmosomes (arrows); **C:** From a mild-grade dysplasia tissue ( $\times 11700$ ), free polyribosomes (arrows), basal lamina (bl); **D:** From a high-grade dysplasia adenoma ( $\times 5900$ ), intracellular junctions (arrows).

were fewer and nuclei were elongated. The luminal surface of epithelial cells exhibited microvilli with core rootlets and glycocalyxal bodies (Figure 2A). Mild-grade dysplasia tissues contained not only fewer but also incompletely differentiated goblet cells. In mild-grade dysplasia, we observed an increased nuclear cytoplasm ratio. The nuclei were large, elongated with peripheral (around the nuclear membranes) aggregation of heterochromatin, and nucleoli were increased in number. There were no significant changes in intercellular relationships. The junctions, especially desmosomes, were well developed (Figure 2B). In 3 out of 9 tissues histologically characterized as mild-grade dysplasia, we observed more significant subcellular changes. Almost every cell was undifferentiated with an abundance of free polyribosomes. Intercellular junctions appeared poorly differentiated. Nevertheless, the basal lamina was reduplicated (Figure 2C). In high-grade dysplasia tissues, intercellular junctions were slightly opened and in many cases we could not confirm cell borders. Epithelial cells and especially goblet cells were undifferentiated. The most significant ultrastructural change occurred in the nucleus. Nuclei were elongated or round with prominent nucleoli. Nucleoli were also increased in number and were marginated near the nucleus periphery (Figure 2D).

#### Immunohistochemistry for light microscopy

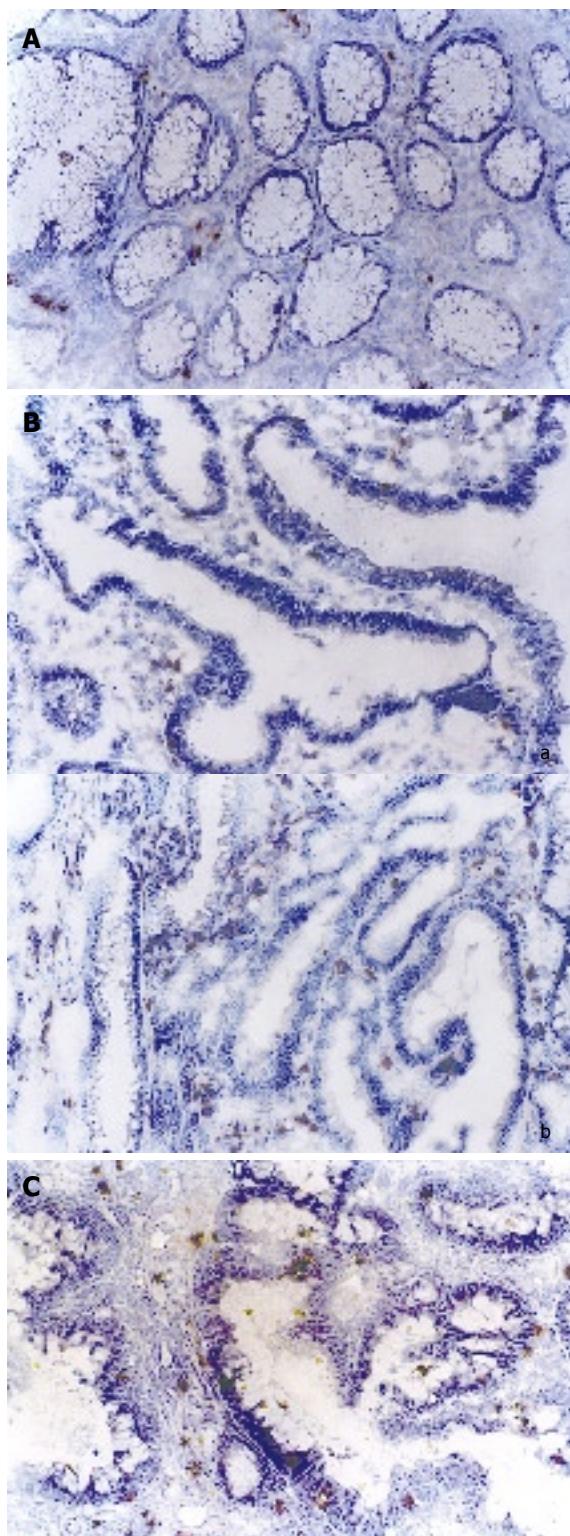
The examined colorectal adenoma tissues exhibited positive immunoreaction to glutathione S-transferase-

**Table 1** Intensity of staining for GST-pi in adenoma and adenocarcinoma colon tissues

Adenoma	Dysplasia	Intensity of staining for GST-pi
1	Low-grade	Moderate
2	Low-grade	Weak
3	Low-grade	Weak
4	Mild-grade	Moderate
5	Mild-grade	Weak
6	Mild-grade	Moderate
7	Mild-grade <sup>1</sup>	Strong
8	Mild-grade <sup>1</sup>	Strong
9	Mild-grade <sup>1</sup>	Strong
10	Mild-grade	Moderate
11	Mild-grade	Moderate
12	Mild-grade	Moderate
13	High-grade	Strong
14	High-grade	Strong
15	High-grade	Strong
16	High-grade	Moderate

<sup>1</sup>Mild-grade dysplasia adenoma tissues with more significant ultrastructural changes.

pi with different density, which is expressed as weak, moderate and strong (Table 1). Low-grade dysplasia adenoma showed mainly a weak immunoreactivity to GST-pi (Figure 3A). Five out of nine adenoma of mild-grade dysplasia immunoreacted moderately and three out of nine showed a strong immunoreaction (Figure 3B), respectively. Three out of four adenoma of high-grade

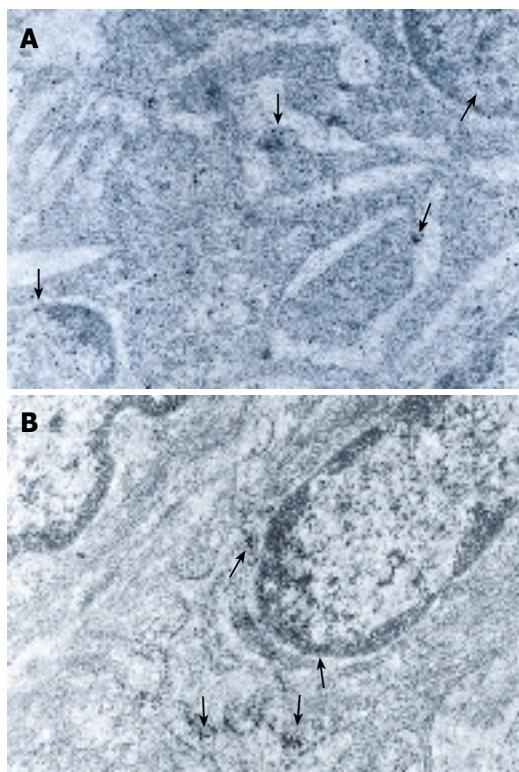


**Figure 3** GST-pi positive cells (HE,  $\times 100$ ). **A:** In low-grade dysplasia colorectal adenoma; **B:** In mild-grade dysplasia colorectal adenoma, moderate immunoreactivity (**a**) and strong immunoreactivity (**b**); **C:** In high-grade dysplasia colorectal adenoma.

dysplasia showed a strong immunoreactivity to GST-pi (Figure 3C). In all the above specimens, GST-pi was positive in epithelial cells and lamina propria cells.

#### Electron Immunocytochemistry

In low and mild-grade dysplasia tissues, GST-pi was



**Figure 4** **A:** Electron micrograph from a mild-grade dysplasia adenoma showing GST-pi positive particles located in ribosomes and nucleus (arrows,  $\times 42000$ ); **B:** Ultrathin section from a high-grade dysplasia adenoma showing GST-pi positive particles located in cytoplasmic membranes and nuclear membrane (with uranylacetate and lead citrate arrows,  $\times 57000$ ).

located in the cytoplasm, mainly ribosomes, and nucleus adjacent to nuclear membrane, and the intensity of the immunostaining was moderate (Figure 4A). In histologically and ultrastructurally high-grade dysplasia cases, GST-pi was located mainly in undifferentiated epithelial cells. GST-pi positive particles were found in membranes of the cytoplasm and especially the nucleus adjacent to the nuclear membrane of these cells. The positive particles accumulated in lumps and the intensity of the immunostaining was strong (Figure 4B).

#### DISCUSSION

Investigations revealed that GST-pi was widely distributed in the human gastrointestinal tract<sup>[15-17]</sup>. GST-pi may be over expressed in an early phase of malignant transformation in premalignant and malignant cells<sup>[18,19]</sup>. Nevertheless, the description of the immunohistochemical and immunoelectron microscopic localization of GST-pi in human polyps is not available. In this respect, we performed a combined study to explore the distribution of GST-pi in these tissues.

We combined the power of morphologic evaluation that is obtained through the use of light and electron microscopy with the detection of GST-pi in tissue and in subcellular structures of colorectal polyp tissues. We believe it was necessary for the correct assessment of changes associated with premalignant transformation in these tissues.

Our study revealed that the immunoreaction of GST-pi was in specific cell-types in adenoma tissues. The location of GST-pi was mainly in epithelial cells of the crypts. Immunoreaction was also found in lamina propria cells, where the positive staining was located in lymphocytes and phagocytes. Endocrine cells were negative. Electron microscope immunohistochemistry revealed that between different cell types the highest intensity stain was in the columnar epithelial cells.

Other investigators have shown cell-type specific expression of the isoenzyme in the human gastrointestinal tract<sup>[15,20]</sup> in normal<sup>[16]</sup> and cancerous tissues<sup>[5]</sup>. This expression of GST pi has been associated with the progression of cancer after exposure to carcinogens<sup>[21]</sup>. In other studies, GST-pi is regarded as a marker in evaluating the effect of tumorectomy or in predicting the drug resistance of tumor cells<sup>[22-24]</sup>.

Our investigation revealed that the immunoreaction varied from weak to strong, according to the degree of adenoma and the ultrastructural changes. The different density of GST-pi immunoreaction was weak in low-grade dysplasia to strong in high-grade dysplasia. There was an exception in this rule. We observed significant ultrastructural changes in some cases histologically characterized as mild-grade dysplasia. These cases, as high-grade dysplasia cases, revealed a contrast with the pattern of progressive differentiation seen in low-grade dysplasia tissues. We found immature undifferentiated cells showing a fast growing population, with many free polyribosomes and enlarged nucleus and nucleoli increased in size and number, showing increased protein synthetic activity. These cases also exhibited strong GST-pi immunoreactivity similar to those of high-grade dysplasia.

In our study, immunostaining was also observed in specific cellular structures. We found that GST-pi was located in the cytoplasm and especially in the nucleus adjacent to the nuclear membrane of colorectal dysplasia cells. This nuclear staining of GST-pi as found here also has been investigated in other studies. It has been reported previously that GST-pi was located in healthy and diseased stomach<sup>[25]</sup>, esophagus<sup>[19,26]</sup> and uterine cervix<sup>[27]</sup>. It has been postulated that nuclear GSTs are involved in RNA processing<sup>[28]</sup>. GST-pi also may induce apoptosis in premalignant cases and may play a pivotal role in early colon carcinogenesis. Other studies revealed lower percentages of apoptotic cells in premalignant cases than in healthy epithelium<sup>[29]</sup>.

Glutathione-pi analog has been synthesized recently. Clinical trials have shown that GST-pi inhibitor induces the apoptosis in the precancerous lesions. It is expected to be promising in future carcinogenesis preventive medicine<sup>[30,31]</sup>.

The combination of immunohistochemical and ultrastructural analysis of GST-pi in polyp tissues and the variation of the isoenzyme expression in adenoma of different degree of dysplasia ultimately may lead to a better understanding of its role in carcinogenesis. These findings also may contribute to a better treatment of colorectal polyps in the future.

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## COMMENTS

### Background

Placental Glutathione S-transferase (GST-pi) is an enzyme that plays an important role in the removal of toxic, probably carcinogenic, agents from the cell. GST-pi is extremely increased in colon adenocarcinoma in comparison to normal samples. The authors performed a combined study to explore the distribution of GST-pi in colon polyps, as they had no data for these tissues. They used the power of morphologic evaluation that is obtained through the use of light and electron microscopy with the detection of GST-pi in tissue and in subcellular structures of colorectal polyp tissues.

### Research frontiers

Clinical outcome of colon polyps is under consideration. Human placental GST-pi concentrations are increased in colon tumors even in premature stages, as well as in precancerous lesions. GST-pi protects cancer cells from cytostatic compounds and thereby apoptosis. The increased GST-pi levels contribute to the relatively high resistance to anti-cancer drugs, such as mitomycin C.

### Innovations and breakthroughs

Investigations revealed that GST-pi was widely distributed in the human gastrointestinal tract. GST-pi may be over-expressed in an early phase of malignant transformation in premalignant and malignant cells. Nevertheless, the description of the immunohistochemical and the immunolectron microscopical localization of GST-pi in human polyps was not available.

### Applications

The combination of immunohistochemical and ultrastructural analysis of GST-pi in polyp tissues and the variation of the isoenzyme expression in adenoma of different degree of dysplasia ultimately may lead to a better understanding of its role in carcinogenesis. These findings also may contribute to a better treatment of colorectal polyps in the future, as Glutathione-pi analog has been synthesized recently. Clinical trials have shown that GST-pi inhibitor induces the apoptosis in the precancerous lesions. It is expected to become promising in future carcinogenesis preventive factors.

### Peer review

This is an interesting study. Authors give valuable data for colon polyps. Immunohistochemical localization of GST-pi in combination with ultrastructural changes indicates that GST-pi might be a sensitive agent for the detection of preneoplastic transformations in colon adenoma tissues.

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RAPID COMMUNICATION

# Endoscopic treatment of biliary complications after liver transplantation

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**CONCLUSION:** Although ERCP is quite an effective mode of managing post-transplant bile duct complications, a significant number of patients need other types of approach. Further prospective studies are necessary in order to establish whether other endoscopic protocols or new devices, could improve the current results.

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**Key words:** Endoscopic retrograde cholangiopancreatography; Biliary complication; Liver transplant; Benign stenosis; Biliary leak

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## Abstract

**AIM:** To evaluate the efficacy of endoscopic treatment in patients who undergo OLTx or LRLTx and develop biliary complications.

**METHODS:** This is a prospective, observational study of patients who developed biliary complications, after OLTx and LRLTx, with duct-to-duct anastomosis performed between June 2003 and June 2007. Endoscopic Retrograde Cholangiopancreatography (ERCP) was considered unsuccessful when there was evidence of continuous bile leakage despite endoscopic stent placement, or persistence of stenosis after 1 year, despite multiple dilatation and stent placement. When the ERCP failed, a percutaneous trans-hepatic approach (PTC) or surgery was adopted.

**RESULTS:** From June 2003 to June 2007, 261 adult patients were transplanted in our institute, 68 from living donors and 193 from cadaveric donors. In the OLTx group the rate of complications was 37.3%, while in the LRLTx group was 64.7%. The rate of ERCP failure was 19.4% in the OLTx group and 38.6% in LRLTx group. In OLTx group, 1 patient was re-transplanted and 8 patients died. In the LRLTx group, 2 patients underwent OLTx and 8 patients died. The follow-up was  $23.3 \pm 13.13$  mo and  $21.02 \pm 14.10$  mo, respectively.

## INTRODUCTION

Biliary complications are the most frequent problem after liver transplantation. The available data show that the rate of biliary complications in transplant recipients ranges between 8% and 20%. This rate of complications is higher for living-related liver transplantation (LRLTx) vs orthotopic liver transplantation (OLTx)<sup>[1,2]</sup>. Biliary complications may include: anastomotic stricture, biliary leaks, stones or debris and Oddi dysfunction. Often patients develop more than one complication<sup>[3]</sup>. Whether the rate of biliary complications is lower in patients with the duct-to-duct anastomosis than with choledocojejunum anastomosis is unknown. However, the duct-to-duct anastomosis is usually preferred because it can easily be reached with an endoscopic procedure, which the published data has proven to be very effective<sup>[1]</sup>. Early diagnosis is crucial, as the treatment may improve graft function and help to avoid repeated surgery. Patients often show unspecific symptoms, such as fever or anorexia, even without pain<sup>[3]</sup>. More often patients are asymptomatic but have high liver functions test (LFT) values and/or bilirubin levels. Abdominal ultrasonography often shows no biliary dilatation, while

liver biopsy (used in many centers) does not appear to be conclusive in this type of patient<sup>[4]</sup>. Interpreting the histologic findings of bile duct damage and bile flow impairment can be confusing. Typical findings of extra-hepatic bile duct obstruction include portal edema, proliferation of ducts and ductules, neutrophilic cholangitis, and hepatocanicular cholestasis. However, concomitant histologic findings of cellular rejection from associated biliary stasis or infection can confuse the primary diagnosis and lead to a misdiagnosis of rejection<sup>[4]</sup>. When comparing histologic features from patients with and without biliary strictures, Campbell *et al*<sup>[5]</sup> found that cholangitis was the only biopsy feature significantly associated with a documented stricture. Therefore, a low threshold to obtain an early cholangiogram should exist. Magnetic Resonance Cholangiopancreatography (MRCP) has shown a 93% diagnostic accuracy, with sensitivity and specificity over 90% and a PPV equal to 86%<sup>[6]</sup>. Endoscopic Retrograde Cholangiopancreatography (ERCP) remains the gold standard when suspicion of biliary complications is high, and allows a direct approach to interventional procedures<sup>[3]</sup>. Some studies have already been published on the endoscopic treatment of biliary complications, and show a success rate of approximately 70%-80% of cases<sup>[6-10]</sup>.

The aim of this study was to evaluate the efficacy of endoscopic treatment in patients who undergo OLTx or LRLTx and develop biliary complications.

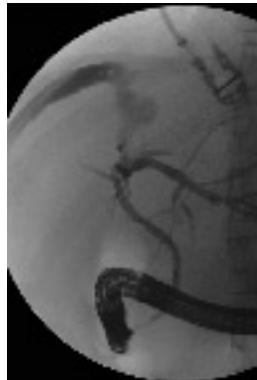
## MATERIALS AND METHODS

This is a prospective, observational study of patients who developed biliary complications after cadaveric and living-related liver transplants (OLTx and LRLTx) with duct-to-duct anastomosis between June 2003 and June 2007.

To overcome the gap between the number of donations and number of patients listed for OLTx, our institute decided to accept "extended criteria donors" (ECD). An ECD was defined as: age > 60 years; macrovesicular steatosis > 30%; prolonged intensive care unit stay (> 7 d); hemodynamic risk factors, including under this term the following conditions: prolonged hypotension (systolic blood pressure < 60 mmHg for more than 2 h); use of dopamine > 10 mcg/kg per min for more than 6 h to sustain blood pressure; need for 2 inotropic drugs to sustain donor blood pressure for more than 6 h; cold ischemic time > 12 h; and hypernatraemia (Na peak > 160 Meq/L) before aortic cross clamp.

With regard to the diagnosis of biliary complications, abdominal ultrasonography with Doppler was routinely performed in order to rule out vascular alterations, but indication for ERCP was based on increased LFT values and MRCP for the OLTx group. In the LRLTx group all patients had a T-tube insertion during the transplant, so the diagnosis of biliary complications was made on T-tube cholangiogram.

Separate databases were created for patients who



**Figure 1** Endoscopic retrograde cholangiography shows biliary fistula at the anastomosis.



**Figure 2** **A:** Endoscopic retrograde cholangiography shows anastomotic stenosis; **B:** Pneumatic dilatation on anastomotic stricture.



**Figure 3** Endoscopic retrograde cholangiography shows lack of contrast flow through the papilla, and dilatation of the choledochus.

underwent OLTx and LRLTx. For each patient, the following data were recorded: demographic information and clinical data, type of biliary complication, time of onset, type of endoscopic treatment, number of procedures received, recovery-time, need for other treatment, surgery or re-OLTx, and final outcome.

All biliary complications were initially treated with standard ERCP, performing the conventional procedure: sphincterotomy plus stent placement for biliary leak (Figure 1) and progressive pneumatic dilatation, and double stent placement for stricture (Figure 2). Sphincterotomy alone for Oddi dysfunction, diagnosed as a lack of biliary and contrast flow through the papilla and dilatation of the choledochus (manometry of Oddi is not available at our Institute, Figure 3); and finally, stone removal with Fogarty balloon or Dormia for stones. For cases requiring more than one

**Table 1** Biliary complications

	<b>OLTx (72/193)</b>	<b>LRLTx (44/68)</b>
Stenosis	55	20
Leak	5	12
Stenosis + leak	4	6
Oddi dysfunction	6	5
Stenosis + stones	1	1
Biliary sludge	1	0
Time of onset (mo)	5.1 (SD, 7.1)	3.7 (SD, 3.97)

procedure, ERCPs were repeated every 3 mo. ERCP was considered unsuccessful when biliary damage was not resolved despite an adequate endoscopic treatment: more precisely, when there was evidence of continuous bile leakage despite endoscopic stent placement, and/or persistence of stenosis, after 1 year, despite multiple dilatation and stent placement. When the endoscopic treatment failed, a percutaneous transhepatic approach (PTC) or surgery, with Roux-en-Y choledochojejunostomy, was adopted.

## RESULTS

### **Patients**

From June 2003 to June 2007, 261 adult patients were transplanted in our institute, 68 from living donors and 193 from cadaveric donors (2 were re-transplants after LRLTx failure). In the OLTx group with duct-to-duct anastomosis, 72/193 (37.3%) had biliary complications, while the rate in the LRLTx group was 44/68 (64.7%, Table 1). The follow-up was  $22.18 \pm 13.6$  mo.

### **OLTx group**

The highest rate of complications, 78.6%, was observed during the first 6 mo after transplantation. Biliary leaks occurred in 9 (12% of all complications) patients: 5 with leak alone and 4 with an associated anastomotic stricture. All biliary leaks occurred at the anastomosis, and were diagnosed within 3 mo of transplant. Of the 5 patients with leaks alone, 2 recovered well after endoscopy (within 3 mo), 2 required PTC, and 1 is still in follow-up. Of the 4 patients with leaks and stenosis, 1 recovered after 3 ERCPs (6 mo), 1 needed percutaneous stent placement, and 2 are in follow-up.

Anastomotic stenosis occurred in 60 patients (80% of all complications): 4 of them with biliary leak and 1 with associated biliary stones. In 45/60 patients (75%) the diagnosis was done within 6 mo. Of the 55 patients with stenosis alone, 27 recovered well with endoscopic treatment within 1 year, while 2 recovered after 18 mo (success rate, 52.7%). Seventeen patients are still in follow-up. Eleven patients required further treatment: 10 required PTC (2 required subsequent surgery), while 1 patient was treated directly with surgical anastomosis reconstruction. The patient with biliary stenosis and stones recovered after a single ERCP with dilatation and stone removal. The patients with biliary stenosis and leak are described above.

One patient presented biliary sludge at 1 mo after

**Table 2** Results and final outcome

	<b>OLTx (n = 72)</b>	<b>LRLTx (n = 44)</b>
Number of procedures	3.2 (SD, 2.4)	3.5 (SD, 2.9)
Success rate (%)	80.56	61.37
Mean follow-up (mo)	23.34 (SD, 13.13)	21.02 (SD, 14.10)
PTC	13	15
Surgery	3	6
RE-OLTx	1	2
Death	8	8

transplant, which was solved with an ERCP session with sphincterotomy and stone removal.

Six patients had Oddi dysfunction after an average of 4 mo post-transplant. These patients were treated with sphincterotomy and recovered after one procedure. Three patients with high levels of cholestatic index and MRI positive for anastomosis stricture showed a normal cholangiography, so no therapy was undertaken.

Finally, in the OLTx group, we observed 14 ERCP failures (19.4%): 13 patients needed PTC with internal-external stent placement (2 of them required surgery), and 1 underwent surgical biliary anastomosis reconstruction. The mean follow-up was 23.3 mo (4–52 mo). During the follow-up, 1 patient was re-transplanted due to graft failure. Eight patients died during follow-up (Table 2).

### **LRLTx group**

The highest rate of complications (79%) was observed in the LRLTx group during the first 6 mo after transplant. Biliary leaks occurred in 18 patients (40.9% of all complications). In 6 patients the leak was associated with anastomotic stricture. All leakages were observed at anastomosis, and all were diagnosed within 2 mo of transplantation. With regard to the 12 patients with leak alone, 3 recovered with ERCP after 1 mo, 3 mo and 4 mo, respectively, (average of 2.3 procedures). Of the 8 patients who did not recover despite the endoscopic treatment, 7 were treated with PTC (3 required subsequent surgery), and one was directly treated surgically due a large leak. The last patient treated by ERCP is still in follow-up. Of the 6 patients with leak and anastomosis stenosis, 4 are still in follow-up, 1 recovered with percutaneous approach and 1 surgically. None of the patients in this subgroup recovered after ERCP.

Anastomotic stenosis occurred in 27 patients: 1 patient had associated stones. Six patients had associated leak (discussed above). Anastomotic strictures occurred in 61.3% of patients with biliary complications. Of the 20 patients with anastomotic stricture, 9 recovered with the endoscopic treatment alone after a mean of 3.4 procedures (45% success rate); 5 patients are still in follow-up; and 6 required a percutaneous approach. The patient with stenosis and stones did not recover despite 7 ERCPs, and was treated successfully with a percutaneous approach. Five patients had Oddi dysfunction and recovered after 1 ERCP with sphincterotomy.

Finally, in the LRLTx group we observed 17 patients in whom the ERCP failed (38.6%): 15 were treated with

PTC (4 requiring subsequent surgical treatment), and 2 were sent directly to surgery after ERCP failure. The follow-up was  $21.02 \pm 14.10$  mo, with a maximum of 59 mo and a minimum of 3 mo. During the follow-up, 2 patients underwent OLTx due to graft failure. Eight patients died (Table 2).

## DISCUSSION

This report shows the results and follow-up of a large cohort of patients who developed biliary complications after liver transplantation (from both cadaveric and living donors) and were treated with ERCP. As previously reported, even in our transplant recipient population, biliary complications are very frequent. Moreover, complications occur more frequently in the LRLTx than in the OLTx recipients. The high rate observed in our OLTx group may be explained by the broad use of extended donor criteria (EDC) at our institute: 46.1% (89/193) of OLTx recipients received a marginal allograft. In the sub-group of patients receiving marginal livers the rate of biliary complications was higher (41.6%) than in non-marginal graft recipients (33.6%), though this difference was not statistically significant.

The extensive use of LFT and MRI for diagnosis of biliary disease results in a correct indication for ERCP: no false negative and 3 false positive (with normal cholangiography at ERCP) were observed. Most of the complications (about 80%) occurred within 6 mo of transplant (early complications); the most frequent was the anastomotic stricture, even if biliary leaks accounted for approximately 40% of all complications in the LRLTx group.

In this series, biliary complications were initially treated with a standard ERCP, using the conventional procedures: sphincterotomy with stent placement for biliary leak, and with progressive pneumatic dilatation, and double stents placement for stricture; sphincterotomy alone for Oddi dysfunction and stone removal with Fogarty balloon for stones. In the cases for which more than one session was required, the ERCPs were repeated every 3 mo (as maximum). The evidence of continuous bile leakage despite the endoscopic stent placement, or the persistence of stenosis after 1 year despite multiple dilatation and stent placement, was considered criteria for switching to another type of treatment: PTC as first choice, or surgery with biliary anastomosis reconstruction if the PTC approach failed or in the event of a very large leak. This approach led to a higher success rate in the OLTx group than in the LRLTx group: the ERCP was successful in 80.56% of the OLTx recipients, vs 61.37% for the LRLTx group. If we analyze the complications separately, the success rate for the treatment of anastomotic stenosis was the same in the both groups, but the success rate for the leak treatment was lower in the LRLTx group. The mean follow-up was long enough to allow us to view these data as reliable ( $22.18 \pm 13.6$  mo).

In conclusion, our data confirmed that, although ERCP is a quite effective mode of managing post-

transplant bile duct complications, a significant number of patients need other types of approach. Therefore, different techniques should be considered in order to improve the results. For instance, large, fully-covered metallic stents could improve the results for biliary leakage, and, in patients with stenosis, reduce the number of dilatation sessions. Partially covered metal stents have been employed in an effort to reduce stricture recurrence and to maintain duct patency; these stents are effective in the initial treatment of stricture but long term success is limited due to problems with stent patency<sup>[11]</sup>. The use of fully covered metal stents (removable) for leaks and strictures has not been tested yet, so further prospective studies are necessary in order to establish whether these new devices could improve the current results.

## COMMENTS

### Background

Biliary complications are the most frequent problem after liver transplantation. This rate of complications is higher for living-related liver transplantation (LRLTx) vs orthotopic liver transplantation (OLTx). Early diagnosis is crucial, as the treatment may improve graft function and help to avoid repeated surgery. Some studies have already been published on the endoscopic treatment of biliary complications and show a success rate of approximately 70%-80% of cases. But no standardized treatments in a long follow up were tested.

### Research frontiers

Although our data confirmed that Endoscopic Retrograde Cholangiopancreatography (ERCP) is a quite effective mode of managing post-transplant bile duct complications in a long follow up, a significant number of patients need other types of approach. Therefore, different techniques should be considered in order to improve the results. Partially covered metal stents have been employed in an effort to reduce stricture recurrence in benign stenosis and to maintain duct patency, but these stents are effective only in the initial treatment of strictures and the long term success is limited due to problems with stent patency. For instance, large, fully-covered metallic stents (removable) could improve the results for biliary leakage, and in patients with stenosis, reduce the number of dilatation sessions. The use of fully covered metal stents for leaks and strictures has not been tested yet, so in our opinion further prospective studies are required in order to establish whether these new devices, could improve the current results.

### Innovations and breakthroughs

This is a large prospective study on transplanted patients (both from cadaveric and live donors), in which the endoscopic treatments and the criteria of endoscopic therapy failure were well defined and standardized. The mean follow-up is long enough to allow us to view these data as reliable.

### Applications

Management of patients who underwent liver transplant from cadaveric and living donors, with duct-to-duct biliary anastomosis, who develop biliary complications.

### Peer review

This paper shows the long term results of a large cohort of patients treated with a well standardized endoscopic therapy; it clearly defines the criteria of endoscopic treatment failure with the appropriate timing to shift for other techniques. The long follow up allows the readers to consider the consistency of the results.

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RAPID COMMUNICATION

## Pituitary hormone circadian rhythm alterations in cirrhosis patients with subclinical hepatic encephalopathy

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### Abstract

**AIM:** To analyze pituitary hormone and melatonin circadian rhythms, and to correlate hormonal alterations with clinical performance, hepatic disease severity and diagnostic tests used for the detection of hepatic encephalopathy in cirrhosis.

**METHODS:** Twenty-six patients with cirrhosis were enrolled in the study. Thirteen patients hospitalized for systemic diseases not affecting the liver were included as controls. Liver disease severity was assessed by the Child-Pugh score. All patients underwent detailed neurological assessment, electroencephalogram (EEG), brain magnetic resonance imaging (MRI), assays of pituitary hormone, cortisol and melatonin, and complete blood chemistry evaluation.

**RESULTS:** Pituitary hormone and melatonin circadian patterns were altered in cirrhosis patients without clinical encephalopathy. Circadian hormone alterations were different in cirrhosis patients compared with controls. Although cortisol secretion was not altered in any patient with cirrhosis, the basal cortisol levels were low

and correlated with EEG and brain MRI abnormalities. Melatonin was the only hormone associated with the severity of liver insufficiency.

**CONCLUSION:** Abnormal pituitary hormone and melatonin circadian patterns are present in cirrhosis before the development of hepatic encephalopathy. These abnormalities may be early indicators of impending hepatic encephalopathy. Factors affecting the human biologic clock at the early stages of liver insufficiency require further study.

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**Key words:** Liver cirrhosis; Minimal hepatic encephalopathy; Circadian rhythms; Melatonin; Pituitary hormones

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Velissaris D, Karanikolas M, Kalogeropoulos A, Solomou E, Polychronopoulos P, Thomopoulos K, Labropoulou-Karatza C. Pituitary hormone circadian rhythm alterations in cirrhosis patients with subclinical hepatic encephalopathy. *World J Gastroenterol* 2008; 14(26): 4190-4195 Available from: URL: <http://www.wjnet.com/1007-9327/14/4190.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4190>

### INTRODUCTION

Hepatic encephalopathy, a major complication of cirrhosis, is a clinical syndrome characterized by mental status changes in patients with severe hepatic insufficiency. By contrast, the term "Minimal Hepatic Encephalopathy", also known as subclinical hepatic encephalopathy (SHE) describes disturbances of several biological functions, including sleep and activities of daily living, in the absence of clinical neurologic symptoms<sup>[1-3]</sup>. Hormonal disorders and circadian rhythm abnormalities are often associated with liver disease<sup>[4]</sup>, and the severity of these disorders is related to liver disease severity and duration. The role of melatonin is critical, as diurnal melatonin rhythm disrupts

tion may significantly contribute to circadian function alterations<sup>[5]</sup>.

The main goal of the present study was to evaluate the circadian hormone secretion profile in cirrhosis patients without hepatic encephalopathy. Specifically, the study was designed to analyze the circadian rhythm of pituitary hormone, serum cortisol and melatonin, and correlate the hormone levels and 24-h hormone secretion abnormalities with brain magnetic resonance imaging (MRI) and electroencephalogram (EEG), which are used for the diagnosis of encephalopathy. In addition, the study also assessed the correlation between circadian hormone rhythm and the severity of hepatic disease as measured by the Child-Pugh score.

## MATERIALS AND METHODS

### Study design

This was an observational study conducted at the University Hospital of Patras, Greece, in the years 2005-2006.

### Patient recruitment

Twenty-six patients with cirrhosis were enrolled in the study. In addition, 13 patients hospitalized for various chronic diseases without liver or central nervous system (CNS) involvement were included as controls. We chose not to have a healthy control group, because hormone patterns in healthy people have been described in detail. Inclusion criteria were: age 35-75 years, abstinence from alcohol for at least 6 mo, cirrhosis confirmed by liver biopsy, and regular follow-up in our Liver Outpatient Clinic. Exclusion criteria were signs or symptoms of encephalopathy, any CNS or endocrine disease, use of medications with CNS effects, and illegal substance abuse.

Mean age was  $64.6 \pm 9.5$  years in cirrhosis patients and  $67.8 \pm 10.8$  years in controls. There were no significant differences between men and women. The etiology of disease in the two study groups is shown in Table 1.

The study was approved by the Institution Ethics Committee, and a written informed consent was obtained from all patients.

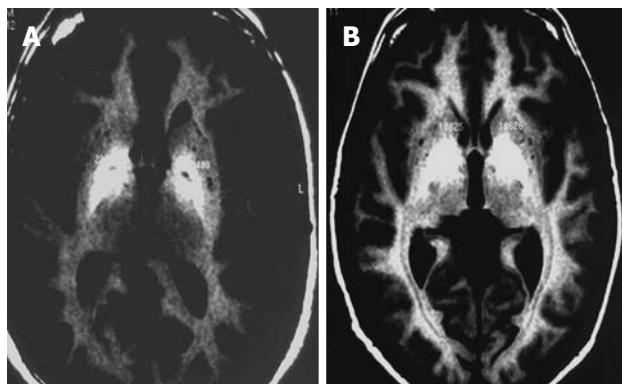
All study participants underwent comprehensive biochemical and clinical evaluation. The severity of cirrhosis was assessed by the Child-Pugh classification: 22 patients were Child-Pugh A (16 with score 5, and 6 with score 6), and 4 patients were Child-Pugh B (1 with score 7, 2 with score 8 and 1 with score 9). There was no patient with Child-Pugh class C.

### Hormone assays

Blood samples for cortisol, melatonin, prolactin and TSH levels were drawn at 09.00, 14.00 and 21.00 h in an attempt to make inferences about circadian hormone secretion patterns. Prolactin, TSH and cortisol levels were measured by the electrochemiluminescence technique (Elecys 2010 ROCHE, Roche Diagnostics GmbH D-68298 Manheim), while melatonin levels were determined using a radio-immunoassay method (Biosource, rue de l'Industrie-B-1400 Nivelles, Catalog Nr. KIPLO800).

**Table 1 Diagnostic data on patients with cirrhosis and control patients**

Cirrhosis etiology	Patient number	Controls	
		Admission diagnosis	Patient number
Alcohol	13	COPD	5
HBV infection	9	Cancer (no liver or CNS metastasis)	4
HCV infection	1	Ulcerative colitis	3
Alcohol + HBV	1	Crohn's disease	1
Alcohol + HCV	1		
Unknown	1		



**Figure 1** T1 MRI of basal ganglia in cirrhosis: mild (grade 1) signal increase (A) and severe (grade 2) signal increase (B).

### Brain MRI

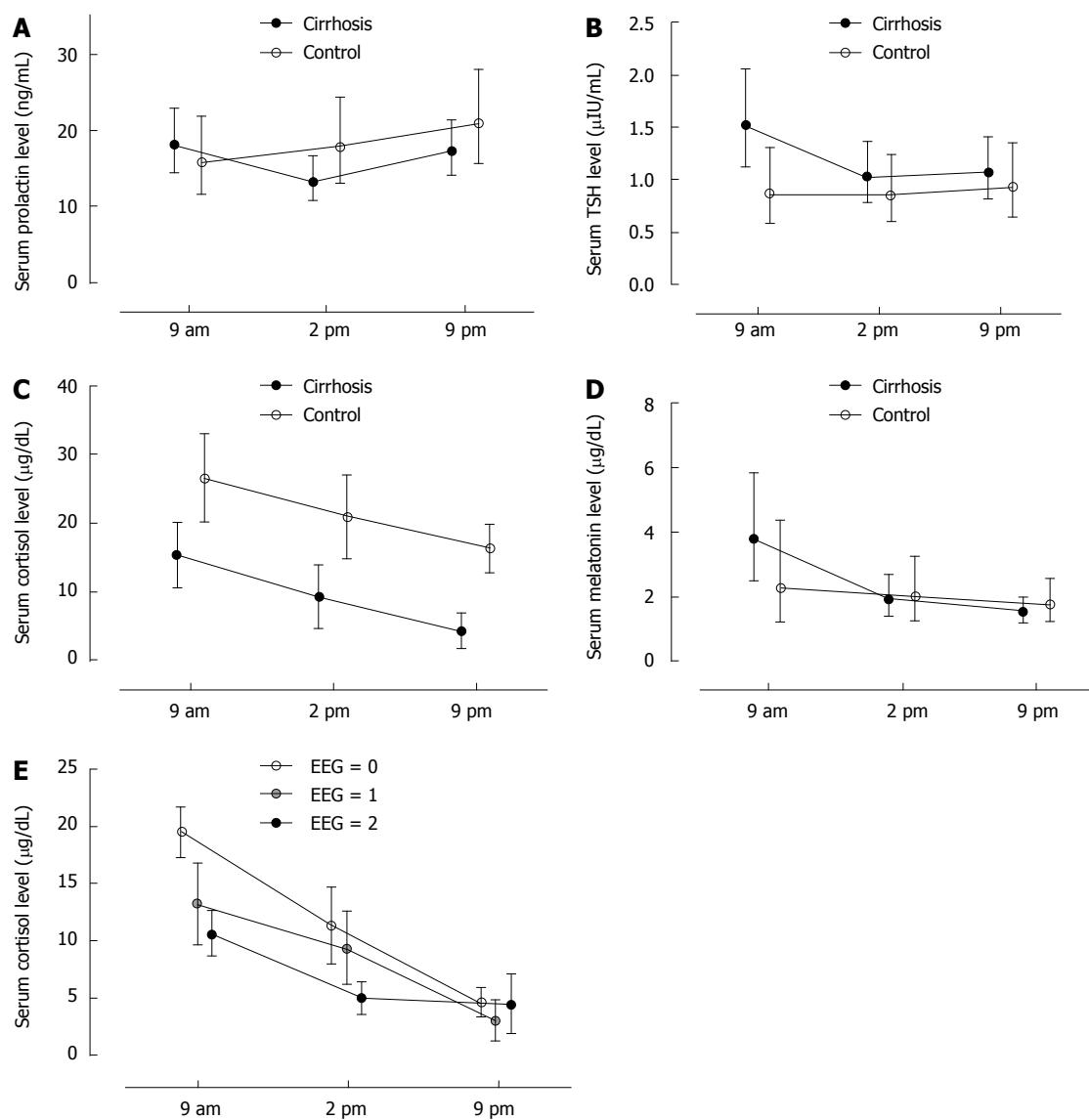
Brain MRI was performed without contrast with a 1 Tesla Gyroscan Intera MRI scanner using a head coil. Transverse and coronal sections were obtained with T1 sequences. Basal ganglia MRI signal was evaluated and compared with adjacent brain white matter MRI signal. The regions of interest (ROI) in the globus pallidus were defined bilaterally in axial and coronal images, whereby each ROI included a predetermined number of pixels. Quantitative image analysis was done by calculating the mean signal intensity for each ROI. MRI signal was classified as Grade 0 = no alterations, Grade 1 = mild alterations (Figure 1A) or Grade 2 = severe alterations (Figure 1B).

### Neurologic assessment

All patients underwent comprehensive clinical neurologic examination with emphasis on cortical function assessment. An awake 16-channel digital EEG was obtained with a standard 10-20 scalp electrode system. Abnormal EEG findings were classified as specific (epileptiform or paroxysmal) or nonspecific (theta and delta waves in various combinations) disturbances. Nonspecific disturbances were further classified as mild, moderate or severe.

### Statistical analysis

All statistical analysis was done with SPSS (Chicago, Illinois, USA) version 12 for Windows. Continuous data are presented as mean  $\pm$  SD. The Student's *t*-test was used to compare means and the Fischer's exact test to compare



**Figure 2** A: Prolactin levels in cirrhosis patients and controls; B: TSH levels in cirrhosis patients and controls; C: Cortisol levels in cirrhosis patients and controls; D: Melatonin levels in cirrhosis patients and controls; E: Cortisol and severity of EEG abnormalities.

proportions. Correlations between continuous variables were evaluated with Pearson's correlation coefficient. Hormone assay results were analyzed with repeated measures ANOVA on the log of measured values. The Student-Neumann-Keuls test was used for post-hoc multiple comparisons.  $P < 0.05$  was considered statistically significant for all tests.

## RESULTS

### Physical exam

All cirrhosis patients had normal muscle tone, normal tendon reflexes and no flapping tremors. Ascites was present in 2 patients, splenomegaly in 14 and endoscopically documented esophageal varices in 8 patients. Neurological evaluation did not reveal any abnormalities in the control subjects.

### Liver function tests and hormone assays

Baseline liver function tests showed minimal hepatic insufficiency without evidence of active liver disease (Table 2).

Hormone concentrations at 09.00 h, 14.00 h and

**Table 2 Baseline biochemical data (mean  $\pm$  SD)**

Parameter	Cirrhosis	Control	P
Bilirubin (mg/dL)	$1.44 \pm 0.95$	$0.89 \pm 0.24$	< 0.01
Albumin (g/dL)	$4.0 \pm 0.5$	$3.7 \pm 0.9$	
Globulin (g/dL)	$3.9 \pm 0.7$	$3.8 \pm 0.5$	
PT (s)	$14.4 \pm 1.8$	$11.4 \pm 0.8$	< 0.001
SGOT (U/L)	$49 \pm 31$	$42 \pm 34$	

21.00 h are presented in Figure 2A-D.

Prolactin levels did not show significant variations during the day in the controls. By contrast, prolactin levels were significantly ( $P < 0.05$ ) lower at 14.00 compared to 09.00 and 21.00 h in the cirrhosis group.

Morning TSH levels were significantly higher ( $P < 0.05$ ) in cirrhosis patients compared to controls. Within the cirrhosis group, TSH levels were significantly higher ( $P < 0.001$ ) at 9.00 h compared to 14.00 h and 21.00 h.

Cortisol levels were significantly lower ( $P < 0.001$ ) at all times in cirrhosis patients compared to controls. However, the circadian cortisol secretion pattern was not altered compared to the pattern described in healthy

**Table 3** MRI abnormalities and child scores n (%)

Child score	A5 (n = 16)	A6 (n = 6)	B7 (n = 1)	B8 (n = 2)	B9 (n = 1)	C (n = 0)
Severity of brain MRI abnormalities	0	8 (50)	-	-	-	-
	1	5 (31.20)	5 (83)	1 (100)	1 (50)	-
	2	3 (18.80)	1 (17)	-	1 (50)	1 (100)

MRI abnormality Grading: 0 = no abnormalities, 1 = mild abnormalities, 2 = severe abnormalities.

individuals. Both the study groups (cirrhosis patients and controls) demonstrated a significant ( $P < 0.05$ ) trend of decreasing cortisol levels from morning to night.

Melatonin levels were higher at 9.00 h compared to 14.00 h ( $P < 0.05$ ) and 21.00 h ( $P < 0.01$ ) in cirrhosis patients, whereas there was no such pattern in controls. The main difference between cirrhosis patients and controls was higher morning melatonin levels in patients with cirrhosis.

### Brain MRI

Brain MRI was abnormal in 18 of 26 cirrhosis patients, with high bilateral symmetrical signal intensity on T-1 images in the globus pallidus, the putamen, or both. The mean basal ganglia signal intensity was  $1093.4 \pm 171.8$  units on T-1 images. We did not find any brain MRI abnormalities in controls. The MRI abnormalities and Child Scores are presented in Table 3.

### EEG

EEG was performed in 22 of 26 cirrhosis patients and demonstrated nonspecific disturbances in 11 (50%) patients. Disturbances consisted of theta or delta waves, and were graded as mild (7 patients), moderate (3 patients), or severe (1 patient). We did not find epileptiform discharges in any patient. Cirrhosis patients with abnormal EEG had significantly ( $P < 0.05$ ) higher mean basal ganglia MRI signal intensity ( $1151.1 \pm 177.8$  units) compared to those with normal EEG ( $1014 \pm 135.3$  units).

### Hormone profile and MRI

We did not find any correlation between melatonin, TSH and prolactin levels with MRI abnormalities. However, serum cortisol levels showed significant ( $P < 0.005$ ) association with brain MRI abnormalities; the 9.00 ( $P < 0.04$ ) and 14.00 h ( $P < 0.01$ ) cortisol levels correlated with the severity of MRI disturbances.

### Hormone profile and cirrhosis severity

We did not find any association between prolactin or TSH levels and cirrhosis severity as measured by the Child-Pugh score. However, there was an association between cortisol and melatonin levels in patients with Child-Pugh score of 5 (16 patients) compared with those with score  $> 5$  (10 patients). Specifically, patients with Child score  $> 5$  manifested impaired circadian cortisol variation ( $P < 0.05$ ) and significantly lower morning cortisol levels ( $P < 0.01$ ) compared to those with Child score of 5 (Figure 2E).

The evening melatonin levels were significantly ( $P$

$< 0.04$ ) lower in cirrhosis patients with Child-Pugh score  $> 5$  (Group 2) compared to those with Child-Pugh score of 5 (Group 1).

### Hormone profile and EEG

We did not observe any association between prolactin, TSH, or melatonin levels and the severity of EEG disturbances (quantified as 0 = no EEG abnormalities, 1 = mild EEG abnormalities, 2 = severe EEG abnormalities). However, cortisol levels were higher in cirrhosis patients without EEG abnormalities compared to those with mild ( $P < 0.05$ ) EEG disturbances, and were even higher compared to those with severe ( $P < 0.01$ ) EEG abnormalities. The observed association between cortisol and EEG abnormalities was more pronounced in the morning ( $P < 0.001$ ).

## DISCUSSION

The presence of characteristic brain MRI and EEG disturbances, and hormone abnormalities in cirrhosis patients without hepatic encephalopathy is the main finding of the present study.

Hepatic encephalopathy is a syndrome characterized by abnormal mental status in patients with severe liver disease<sup>[6-8]</sup>. SHE is a milder condition associated with cirrhosis and/or porto-systemic shunts. The diagnosis of SHE is clinically relevant because it may precede the development of overt hepatic encephalopathy. Moreover, the psychomotor deficits in SHE may impair cognitive function and activities of daily living<sup>[2,3]</sup>. SHE diagnosis is based on psychometric tests, EEG and brain MRI. However, since there is no "gold standard" for diagnosing SHE<sup>[1-3,9]</sup>, the prevalence of this condition in cirrhosis has been reported variously as 30% to 84%, possibly due to different diagnostic criteria used in the various studies.

Cirrhosis patients without clinical encephalopathy often demonstrate high basal ganglia MRI T-1 signal intensity, likely due to manganese deposition in the brain<sup>[10-12]</sup>.

EEG is useful in the diagnosis of SHE, as slow (2-5 Hz) high-amplitude frontal lobe waves are characteristic of early hepatic encephalopathy. Although EEG abnormalities are not encephalopathy-specific, abnormal theta and delta wave activity correlates with disease severity<sup>[13]</sup>.

Several biological rhythm abnormalities, including impaired arterial pressure diurnal variation, nocturnal portal pressure rise, melatonin secretion and sleep pattern alterations occur in cirrhosis. The various mechanisms proposed to explain these circadian abnormalities include the following: (a) effect of neurotoxins on the

suprachiasmatic hypothalamic nucleus (SCN), which is the "human biologic clock"<sup>[14,15]</sup>, and (b) elevated morning melatonin levels due to impaired liver melatonin metabolism, causing a circadian clock phase-shift<sup>[16,17]</sup>.

Liver diseases are associated with several hormone disorders, including decreased serum levels of T3, cortisol, testosterone, FSH and insulin, and elevated prolactin concentrations<sup>[18-22]</sup>. In addition, a characteristic high daytime melatonin pattern<sup>[5]</sup> has been described; this may contribute to the sleep-wake cycle disturbances and hormone disorders, as SCN is located in the hypothalamus, which regulates pituitary hormones<sup>[5,23-26]</sup>.

Melatonin may act as an internal circadian body rhythm "synchronizer", and plasma melatonin profile may be a circadian pacemaker marker. Therefore, melatonin rhythm disruption observed in cirrhosis may reflect circadian alterations with significant clinical implications: high daytime melatonin levels can cause an endogenous clock phase-shift and may therefore partly explain the sleep disturbances observed in cirrhosis<sup>[5,27-29]</sup>. Additional factors, unrelated to melatonin but involved in liver failure, such as false neurotransmitters, cerebral amines and cerebral arteriovenous shunts may also contribute to hormonal circadian abnormalities in the early stages of hepatic encephalopathy. Our findings suggest that diurnal melatonin abnormalities correlate with the severity of liver disease in cirrhosis and may be identifiable early, before the development of clinical hepatic encephalopathy.

Prolactin secretion follows a pulsatile pattern, with a characteristic nocturnal rise, but cirrhosis is associated with elevated 24-h prolactin levels and loss of circadian prolactin rhythm<sup>[30-32]</sup>. However, our cirrhosis patients had significant prolactin circadian rhythm disturbances without baseline elevation. The differences between our findings and previous studies<sup>[30,31]</sup> may be explained by patient selection, as we excluded patients with clinical encephalopathy.

A diurnal TSH secretion pattern, with the highest concentrations in late evening and the first hours of nocturnal sleep is well documented in normal subjects. Circadian TSH level variations may be modulated, in part, by a dopaminergic mechanism, which plays a major role in TSH rhythmicity in liver disease<sup>[33-35]</sup>. In our study TSH circadian abnormality was identified in the absence of clinical encephalopathy.

Although impaired cortisol inactivation is well documented in cirrhosis, basal circadian cortisol secretion remains stable<sup>[36,37]</sup>. In the present study, patients with cirrhosis had low 24 h cortisol levels compared to controls.

Our data suggests that cirrhosis patients without encephalopathy have disrupted melatonin, TSH and prolactin circadian cycle, and suppressed 24-h cortisol levels, but the circadian cortisol rhythmicity is unaffected. More importantly, the melatonin abnormalities (lower night levels) are more pronounced in advanced liver failure (Child score > 5). As these findings were not seen in controls, the hormone abnormalities identified in cirrhosis could be specific for liver disease.

We did not find any correlation between melatonin, TSH or prolactin levels with the severity of brain MRI

or EEG abnormalities, but the lack of association may be a type-II error due to the small number of patients. Cortisol levels correlated with brain MRI abnormalities, EEG abnormalities and severity of liver disease (Child score > 5). Melatonin and cortisol abnormalities correlated with severity of liver disease, while TSH and prolactin levels did not. As we could not find any similar findings in the literature, we believe these observations deserve further investigation.

Our cirrhotic patients with SHE had abnormal circadian pituitary hormone secretion and diurnal melatonin cycle. The abnormal hormonal pattern in SHE is different compared to patients with systemic diseases not affecting the liver and healthy individuals. The presence of these abnormalities in cirrhosis without clinical encephalopathy raises the possibility that these patterns may represent indicators of early hepatic encephalopathy.

We believe that originality is the main strength of our study, as there are no published reports on pituitary hormone abnormalities in relation to the severity of hepatic encephalopathy. The main limitations include the study design (observational, no randomization, no power analysis, small patient number), and the attempt to make inferences about circadian patterns from three measurements per day. An additional (fourth) measurement if obtained the following morning could have provided additional insight into the time course of the observed hormone changes. Since the number of statistical comparisons largely exceeded the number of cases and groups, positive findings should be interpreted with caution, because of the possibility of type I error. The inclusion of a healthy control group would have improved the study, and should perhaps be considered in future studies on this subject.

In conclusion, circadian hormone disturbances occur early in cirrhosis and are associated with disease severity. These observations raise the interesting hypothesis that alterations in circadian hormone secretion may be an early sign of impending clinical encephalopathy. This hypothesis has significant clinical implications and therefore, we believe, deserves further investigation.

## COMMENTS

### Background

Hepatic encephalopathy is a neuropsychiatric disorder associated with clinical manifestations ranging from slightly altered mental status to coma. The severity of symptom depends on the severity of liver disease, and the presence of metabolic or infectious complications. The term "Minimal Hepatic Encephalopathy" includes biological disturbances such as that of sleep and daily activities, in the absence of neurologic symptoms. Abnormalities of psychometric tests, electroencephalogram (EEG) and brain magnetic resonance imaging (MRI) may support the diagnosis of Minimal Hepatic Encephalopathy. Hormonal disorders and circadian rhythm abnormalities are often associated with liver disease, and the severity of these disorders is related to the severity and duration of the liver disease. The role of melatonin, a marker of intrinsic circadian pacemaker is critical, since diurnal melatonin rhythm disruption may reflect circadian function alterations leading to disturbances in the daily activities.

### Research frontiers

Circadian pituitary hormone alterations, brain MRI and EEG abnormalities have been described in cirrhosis. However, there is limited data on the relationship of these abnormalities with the severity of cirrhosis and hepatic encephalopathy. This field deserves further study.

### Innovations and breakthroughs

This present study is the first attempt to evaluate circadian hormone abnormalities in relation to EEG, brain MRI, subclinical hepatic encephalopathy (SHE) and the severity of cirrhosis. The main finding is the presence of characteristic brain MRI, EEG and hormone secretion abnormalities in cirrhosis patients with SHE.

### Applications

Our data suggests that melatonin and pituitary hormone circadian rhythm abnormalities are present early in the course of cirrhosis and are associated with the severity of liver disease in patients without clinical encephalopathy. These findings raise the interesting hypothesis that circadian hormone abnormalities may be an early sign of the development of hepatic encephalopathy.

### Terminology

Circadian rhythm means a predictable physiologic fluctuation in a 24 h period; SHE is a clinical entity consisting of mild neuropsychological abnormalities affecting the activities of daily living in cirrhosis patients without clinical encephalopathy.

### Peer review

This is a non-randomized observational study with novel and interesting findings, demonstrating that abnormal pituitary hormone and melatonin circadian patterns are present in cirrhosis patients without hepatic encephalopathy. The manuscript is well written.

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RAPID COMMUNICATION

## Tongue-like Barrett's esophagus is associated with gastroesophageal reflux disease

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was significantly higher in males compared with female subjects. Statistical analysis showed that the prevalence of heartburn and RE were significantly higher in the T type ESEM than in the W type ESEM ( $P < 0.05$ ).

**CONCLUSION:** The T type ESEM was strongly associated with reflux symptoms and RE whereas the W type ESEM was not associated with GERD.

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**Key words:** Tongue-like endoscopically suspected esophageal metaplasia; Dome-like endoscopically suspected esophageal metaplasia; Wave-like endoscopically suspected esophageal metaplasia; Gastroesophageal reflux disease

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### Abstract

**AIM:** To test this hypothesis of barrett esophagus (BE) classified into two types and to further determine if there was any correlation between the shape of endoscopically suspected esophageal metaplasia (ESEM), prevalence of reflux esophagitis (RE) and heartburn.

**METHODS:** A total of 6504 Japanese who underwent endoscopy for their annual stomach check-up were enrolled in this study. BE was detected without histological confirmation that is ESEM. We originally classified cases of ESEM into 3 types based on its shape: Tongue-like (T type), Dome-like (D type) and Wave-like (W type) ESEM. The respective subjects were prospectively asked to complete questionnaires concerning the symptoms of heartburn, dysphagia, and abdominal pain for a one-month period.

**RESULTS:** ESEM was observed in 10.3% of 6504 subjects (ESEM  $< 1$  cm, 9.4%; 1 cm  $\leq$  ESEM  $<$  3 cm, 1.7%; ESEM  $\geq$  3 cm, 0.5%). The frequency of ESEM

### INTRODUCTION

Barrett's esophagus (BE) is considered to be a significant complication of gastroesophageal reflux disease (GERD) due to its association with adenocarcinoma<sup>[1]</sup>. For many years, BE was considered as an acquired disorder that developed as a result of GERD<sup>[2]</sup>. Patients with BE tended to have higher esophageal acid exposure compared with normal subjects, patients with non-erosive reflux disease, or those with erosive esophagitis<sup>[3]</sup>. The age of onset, duration of symptoms, and complications of GERD have been also demonstrated to be markers for increased risk of BE<sup>[4]</sup>. Interestingly, similar clinical risk factors were identified for esophageal

adenocarcinoma<sup>[5]</sup>.

According to Montreal definitions, the term endoscopically suspected esophageal metaplasia (ESEM) describes endoscopic findings consistent with BE that await histological evaluation<sup>[6]</sup>. The epidemiology of ESEM in Japan has been investigated<sup>[7,8]</sup>. It has been reported that the prevalence of long-segment ESEM was estimated to be 0.2%-0.4%<sup>[7]</sup>, which is consistent with reports from several other studies<sup>[8]</sup>. However, there appears to be considerable variation in reports of prevalence of short segment ESEM. The estimated range of short segment ESEM has been reported, varying from 6.0%-20.6%<sup>[7,8]</sup>. It is difficult to compare previous reports on the prevalence of ESEM because of the diverse methodologies used and the different methods of sample selection. However, because of considerable differences in the reported prevalence of short segment ESEM, we hypothesize that short segment ESEM may not always be associated with reflux esophagitis (RE). In fact, there are paradoxical reports in which no association between RE and BE has been reported<sup>[9]</sup>. Although ESEM is generally a complication of GERD, some cases of ESEM, especially the short segment type, have an obscure connection with GERD<sup>[10,11]</sup>. Therefore, ESEM may be best described as having two types: ESEM non-short segment type, which is closely associated with GERD, and ESEM short segment type, which may not be associated with GERD.

The aim of the present study was to determine whether there was a correlation between the shape and localization of ESEM and the prevalence of GERD. We also tested the hypothesis that ESEM might result from replacement of erosions in RE, thus we compared the circumferential involvement by erosions in RE and ESEM.

## MATERIALS AND METHODS

### **Patients**

A total of 160 983 Japanese patients (male/female, 60 774/100 209; mean age,  $61.9 \pm 11.1$ ), who underwent a stomach exam at the Miyagi Cancer Society between January and December 2003, were enrolled in this study. In order to check for gastric cancer, X-ray examinations were performed on all enrolled subjects. In addition, we evaluated disease classifications based on X-ray examinations. There were 15 616 subjects in whom further endoscopic testing was necessary because they were suspected of having gastric cancer. From this group, a total of 6504 subjects (male/female, 3197/3307; mean age  $62.7 \pm 10.7$ ) who underwent further endoscopic testing at the above center between January and December 2003 were enrolled in this study.

### **Endoscopic findings**

At the Miyagi Cancer Society, when endoscopically

examining and photographing the esophageal mucosa, the gastroesophageal junction (GEJ) is always prospectively photographed, and the ventral side of the esophagus is positioned at 12 o'clock, in the top upper part of the photograph. The Miyagi Cancer Society operates a digital filing system for endoscopic images (ScopeReader DCR. Rise Co., Ltd). All digital endoscopic images were independently and retrospectively reviewed by two endoscopists to investigate the presence and localization of RE and ESEM. If there was any inconsistency in the assessment of the digital endoscopic images, a final diagnosis was decided upon by a joint review of the digital endoscopic images. Furthermore, if less than 60% of the esophageal mucosa could be seen in any photograph, that patient was excluded from the analysis. A total of 134 cases were excluded from the analysis because of difficulty in assessment.

### **ESEM**

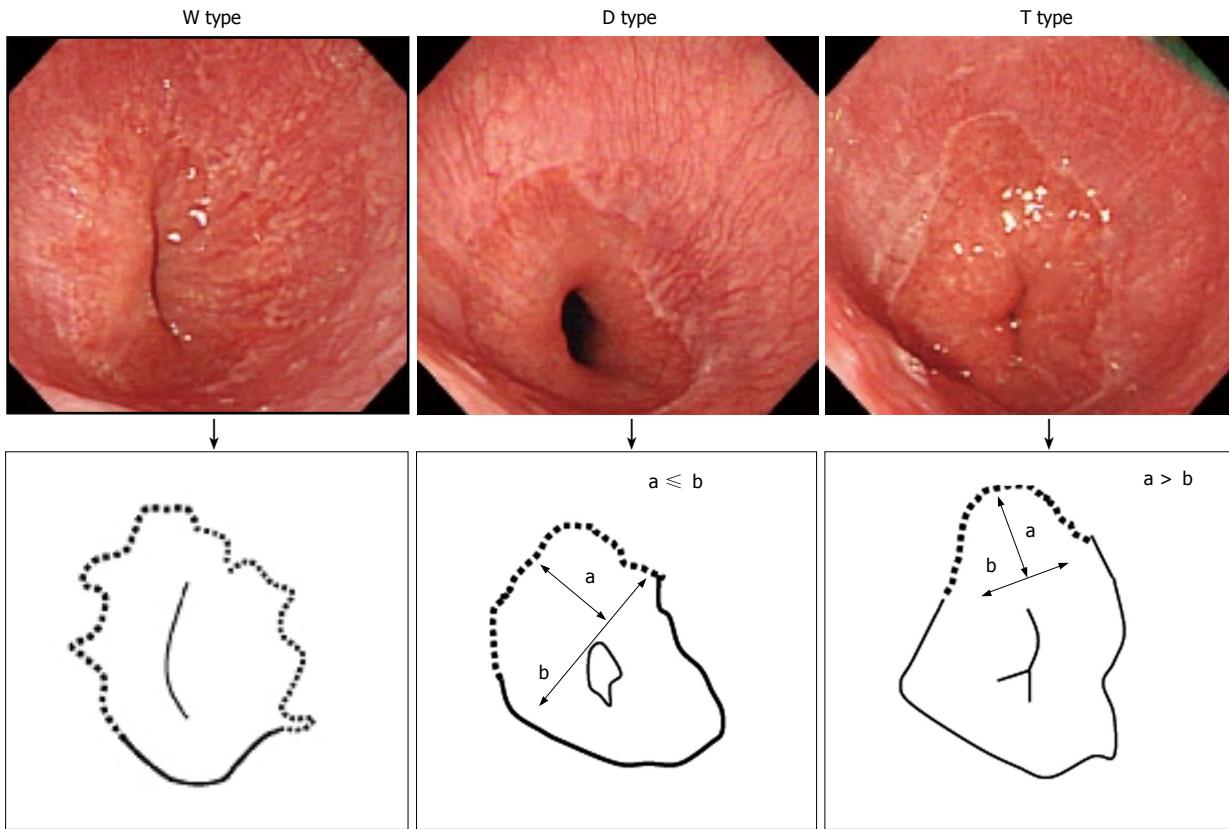
Although endoscopy with biopsy is the only validated technique to diagnose BE<sup>[6]</sup>, it has clear limitations as a screening tool due to cost, risk, and complexity. We therefore chose to assess ESEM in study patients. Diagnosed ESEM was defined as any length of columnar epithelium that extended continuously from the gastric lumen to the esophagus, and the GEJ was defined as the end of the palisade vessel of the lower esophagus<sup>[12]</sup>. If palisade vessels were not visible, the proximal margin of the gastric fold was used for detection of the GEJ. Furthermore, we also defined ESEM based on length and the following grading system was used.

### **Length of ESEM**

ESEM < 1: Defined as segments of columnar epithelium from the GEJ measuring <1 cm in length; 1 ≤ ESEM < 3: Defined as segments of columnar epithelium from the GEJ measuring 1 ≤ ESEM < 3 cm in length; ESEM ≥ 3: Defined as segments of columnar epithelium from the GEJ (measuring at the uppermost extent of red columnar epithelium) ≥ 3 cm in length, whether the columnar lining was circumferential or not.

### **Shape of ESEM**

We originally classified ESEM into 3 types based on shape. As shown in Figure 1, we classified the shape of ESEM as the following: (1) W type, in which the ESEM was defined by a columnar epithelium that extended continuously from the gastric lumen to the esophagus, in which the uppermost extent of the visible red columnar epithelium could be observed as a wave-like formation. The broken line in Figure 1 shows this type of ESEM; (2) D type, in which the length of basal part of the ESEM (b) was longer than the length of the major part of the ESEM (a) (i.e. a < b). The columnar epithelium of the ESEM is observed as a dome-like shape. The broken line in Figure 1 shows this type of ESEM; (3) T type,



**Figure 1** The shape of ESEM. We originally divided ESEM into 3 types based on its shape. The W type, in which the ESEM was defined by a columnar epithelium which extended continuously from the gastric lumen to the esophagus, in which the uppermost extent of the visible red columnar epithelium could be observed as a wave-like formation (W). The broken line in the illustration shows the ESEM. The D type, in which the length of basal part of the ESEM (b) was longer than the length of the major part of the ESEM (a; i.e.  $a < b$ ). The columnar epithelium of the ESEM is observed as a dome-like shape (D). The broken line in the illustration shows the ESEM. The T type in which the length of the major axis of the ESEM (a) is longer than the basal part of ESEM (b; i.e.  $a \geq b$ ). The columnar epithelium of the ESEM is observed as a tongue-like shape (T).

in which the length of the major axis of the ESEM (a) is longer than the basal part of ESEM (b) (i.e.  $a \geq b$ ). The columnar epithelium of the ESEM is observed as a tongue-like shape.

### Reflux esophagitis

RE is divided endoscopically into four grades, A to D, according to the severity of the mucosal breaks as defined by the Los Angeles (LA) classification<sup>[13]</sup>. A mucosal break was defined as "an area of slough or an area of erythema, with a discrete lined demarcation from the adjacent or normal looking mucosa"<sup>[13]</sup>.

### Localization of RE and ESEM

We also investigated the location in which mucosal breaks or ESEM occurred in the lower esophageal wall. The circumferential localization of mucosal breaks and the ESEM (according to shape) in the lower esophageal wall were determined according to the numbers on a clock face, with 12 o'clock always situated at the top of the photograph. When multiple mucosal breaks or ESEM were present, for example, grade A and B esophagitis or T type ESEM, the circumferential positions of all the mucosal breaks or ESEM were recorded. In cases of grade C esophagitis or W type ESEM, the transverse extent of the mucosal breaks or

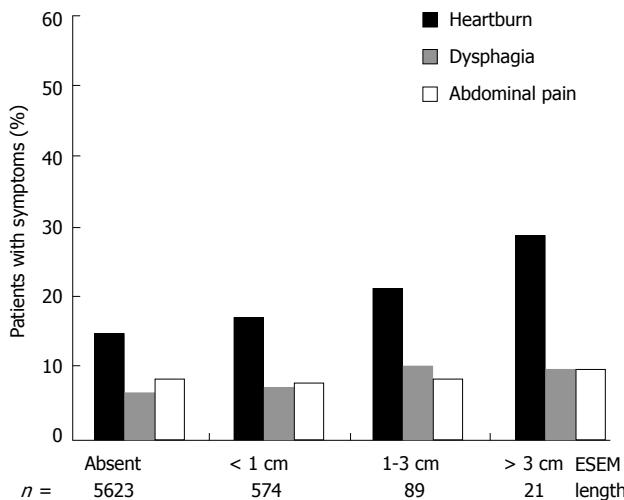
**Table 1** Questionnaire used in this study

Please circle the appropriate responses for each of the following symptoms during a 1-mo period	
Do you suffer from the symptom of dysphagia?	
1: yes	2: no
(a: usually; b: sometimes)	
(c: throat; d: chest; e: stomach)	
Do you suffer from the symptom of heartburn?	
1: yes	2: no
(a: usually; b: sometimes)	
Do you suffer from the symptom of abdominal pain?	
1: yes	2: no
(a: usually; b: sometimes)	
(f: before eating; g: after eating; h: no relation)	

the ESEM was assessed, and all the directions in which lesions existed were counted.

### Questionnaire

Table 1 showed the questionnaire used in this study. The subjects in each group were prospectively asked to complete the questionnaire concerning symptoms of heartburn, dysphagia, and abdominal pain within a one month period. The subjects were requested to simply answer "yes" or "no" to the symptoms, and if they answered "yes", they were further questioned



**Figure 2** Correlation between ESEM length grading and each clinical symptom. The prevalence of heartburn significantly increased in direct proportion to endoscopic ESEM length grading ( $P < 0.05$ ). No significance was observed between the length of ESEM and symptoms of dysphagia and abdominal pain.

on the frequency of symptoms ("usually" or "sometimes"). In addition, concerning the symptom of dysphagia, subjects were asked where the symptom of dysphagia occurred (throat, chest, or stomach). Regarding abdominal pain, its relationship with meals, if any, was elicited. This questionnaire was designed as a self-reporting instrument to measure symptoms experienced over one month previous to returning the completed questionnaire.

### Statistical analyses

The Mann-Whitney  $U$  test for nonparametric data was used for statistical analysis to compare results among the groups with different ESEM lengths and clinical symptoms. The Kruskal-Wallis rank test was used for statistical analysis to compare the results among the groups with different ESEM shapes. Values were additionally expressed as frequencies. Categorical variables were compared using the  $\chi^2$  test. A  $P$  value of less than 0.05 was considered significant.

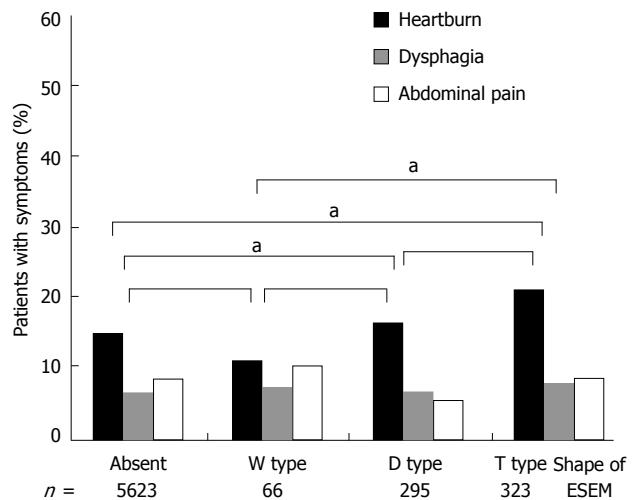
### The study protocol

The study protocol was approved by the ethical committee of the Tohoku University Graduate School of Medicine.

## RESULTS

### Study population

The mean age of the sample population was  $62.7 \pm 10.2$  years. All subjects responded to the questionnaire on heartburn, dysphagia and abdominal pain. Because the majority of subjects (99%) gave the frequency of the respective symptoms as "sometimes", the frequency of symptoms was omitted from our analysis. Among the 6504 subjects, 63 who had undergone some degree of stomach resection and 134 in whom judgment



**Figure 3** Correlation between the ESEM shape and each clinical symptom. The prevalence of heartburn significantly increased in the T type ESEM than the W type ESEM and ESEM-free patients ( $\chi^2$  test;  ${}^aP < 0.05$ ).

was difficult based on the digital photographic data were excluded from the final study analysis. Of the 6307 subjects in the final analysis, the mean age of the sample population was also  $62.7 \pm 10.2$  years. Females accounted for 50.8% of the interviewed subjects ( $n = 3206$ ).

### The prevalence of ESEM

ESEM was observed in 10.3% of subjects (ESEM  $< 1$  cm, 9.4%;  $1\text{ cm} \leqslant \text{ESEM} < 3$  cm, 1.7%; ESEM  $\geqslant 3$  cm, 0.5%). The frequency of ESEM was significantly higher in males compared with female subjects ( $\chi^2$  test).

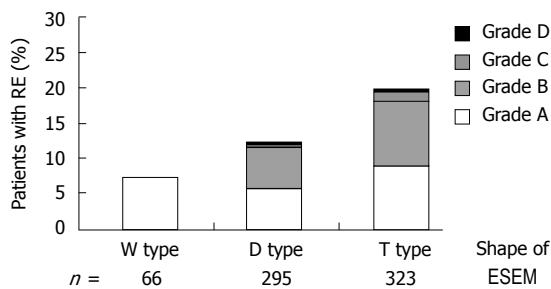
### Correlation between ESEM and clinical symptoms

Figure 2 shows the correlation between ESEM length grading and each clinical symptom. The prevalence of heartburn significantly increased concomitantly with the endoscopic ESEM length grading (Mann-Whitney  $U$  test). No significance was observed between the length of the ESEM and the prevalence of dysphagia and abdominal pain (Mann-Whitney  $U$  test).

Figure 3 shows the correlation between ESEM shape and each clinical symptom. The prevalence of heartburn was significantly higher in T type ESEM than W type ESEM and those subjects who did not have ESEM ( $\chi^2$  test). The subjects with T type ESEM tended to suffer from a high prevalence of heartburn compared to the subjects with D type ESEM, but without statistical significance ( $\chi^2$  test). No significance was observed between the shape of the ESEM and the prevalence of dysphagia and abdominal pain ( $\chi^2$  test).

### Correlation between ESEM and the prevalence of RE

Figure 4 shows the correlation between the shape of the ESEM and the prevalence of RE. The prevalence of RE was significantly higher in subjects with T type ESEM than those with the other shapes (Kruskal-Wallis rank test).



**Figure 4** Correlation between the shape of ESEM and the prevalence of RE. The prevalence of RE is significantly higher among those suffering from the T type ESEM (Kruskal-Wallis rank test,  $P < 0.05$ ).

### Localization of ESEM

Figure 5 shows the localization of ESEM. In subjects with T type ESEM, the ESEM was located mainly at the 12 o'clock and 1 o'clock wall (right anterior) of the lower esophagus, whereas in subjects with the D and W types, the ESEM was mainly located at the 9 o'clock to 12 o'clock wall (left anterior) of the lower esophagus. The circumferential location of the ESEM significantly differed among the different types of ESEM ( $\chi^2$  test).

### The prevalence of RE

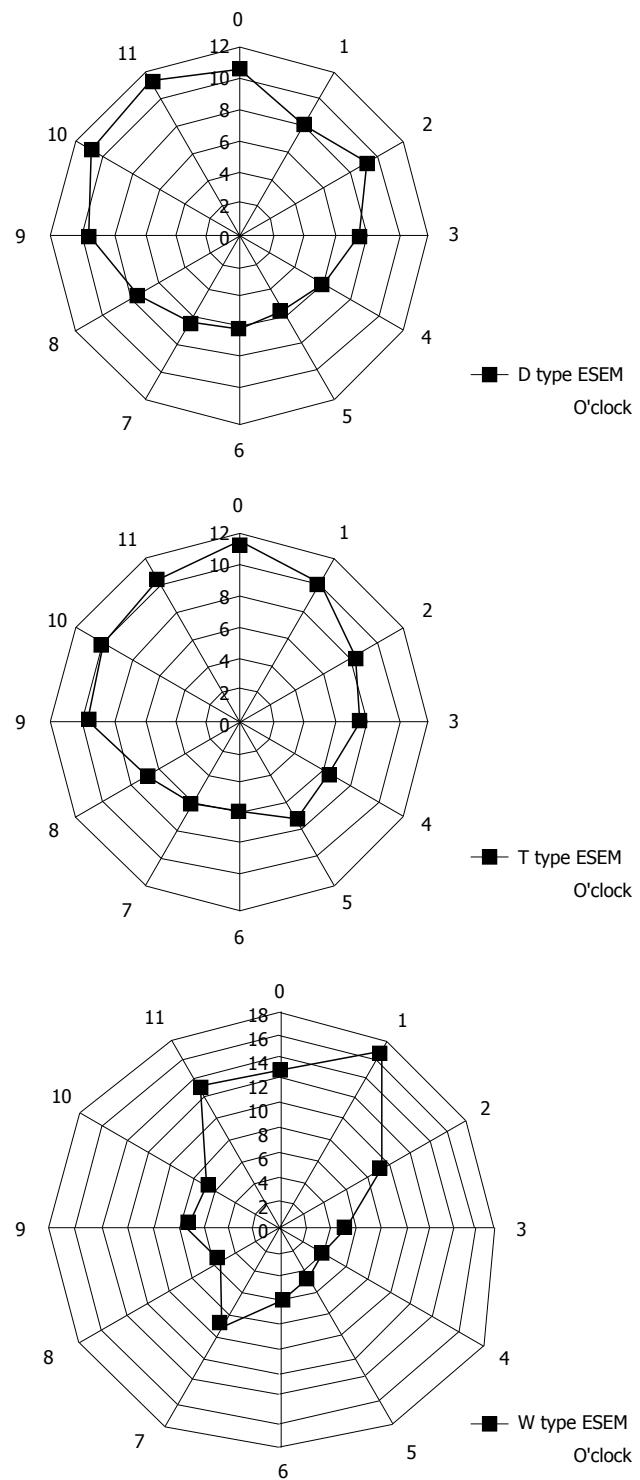
The endoscopic results revealed that 6.3% (grade A 3.7%, grade B 2.3%, grade C 0.2%, grade D 0.1%) of the subjects had RE according to the LA classification. The prevalence of RE was significantly higher in males (7.7%) than in females (6.3%,  $\chi^2$  test).

### Localization of RE

Figure 6 shows the localization of mucosal breaks in the lower esophageal wall. Subjects with grade A and B esophagitis had longitudinal mucosal breaks mainly at the 12 o'clock and 1 o'clock wall of the lower esophagus, whereas subjects with grade C and D esophagitis had transverse mucosal breaks mainly at the 4 o'clock to 6 o'clock wall of the lower esophagus (Figure 6). The circumferential locations of esophageal mucosal breaks significantly differed among different grades of esophagitis.

## DISCUSSION

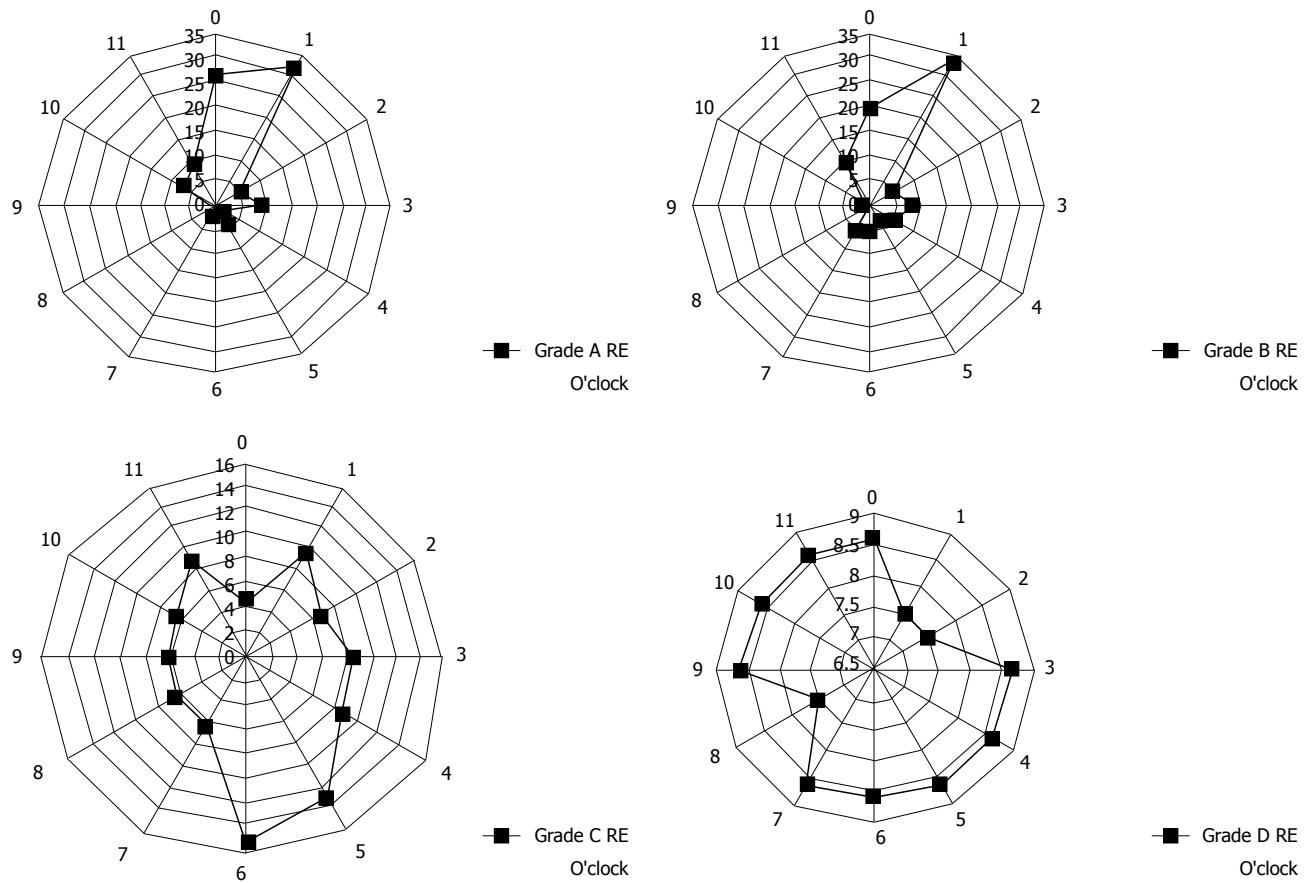
BE has been generally accepted as a complication of chronic and severe GERD<sup>[2-4]</sup>. It is currently accepted that BE places individuals at risk for the development of esophageal adenocarcinoma, which is the most rapidly increasing cancer in the United States<sup>[14]</sup>. Because the prevalence of RE in Japan is increasing to become near that of Western countries<sup>[15,16]</sup>, there might be a tendency for an increased number of esophageal adenocarcinomas in the Japanese population in the future. The fact that clinical risk factors with BE were also identified for esophageal adenocarcinoma attracted our attention<sup>[17]</sup>. The more frequent, more severe, and longer lasting the symptoms of acid reflux are, the greater the risk is for the development of



**Figure 5** The circumferential location of ESEM in subjects with the W, D, and T types of ESEM. Data are shown in terms of clock face orientation. The numbers represent the percentage of lesions on each side of the esophageal wall relative to the total number of lesions. The T-type ESEM is located mainly at the 12 and 1 o'clock wall (right anterior) of the lower esophagus, whereas the D and W types of ESEM are mainly located at the 9 to 12 o'clock wall (left anterior) of the lower esophagus. The circumferential distribution of ESEM differed significantly among subjects with different types of ESEM ( $\chi^2$  test:  $P < 0.05$ , T vs D, D vs W and W vs T).

adenocarcinoma of the esophagus<sup>[5]</sup>.

Although no one can doubt the importance of strong and chronic acid reflux in the development of BE, there is, however, doubt that all patients with BE



**Figure 6** The circumferential location of esophageal mucosal breaks in subjects with grade A-D RE. Data are shown in terms of clock face orientation. The numbers represent the percentage of lesions on each side of the esophageal wall relative to the total number of lesions. Patients with grade A and B esophagitis had longitudinal mucosal breaks mainly at the 12 o'clock and 1 o'clock wall of the lower esophagus, whereas patients with grade C and D esophagitis had transverse mucosal breaks mainly at the 4 o'clock to 6 o'clock wall of the lower esophagus. The circumferential distribution of esophageal mucosal breaks differed significantly among subjects with different grades of RE ( $\chi^2$  test:  $P < 0.05$ , A vs B, A vs C, A vs D, B vs C, B vs D and C vs D).

will show characteristic GERD symptoms. Ronkainen *et al* reported that BE may not develop as a late consequence of RE<sup>[9]</sup>. It is reported that obesity and smoking and drinking habits are also risk factors for developing BE<sup>[9,18-21]</sup>. A familial aggregation study of BE has been reported<sup>[22]</sup>. Several studies on BE have been carried out in Japan<sup>[7,8]</sup>. Kawano *et al* reported that there is a weak association between short segment BE and RE.

In summary, controversy exists regarding the potential of BE to develop as a late consequence of RE<sup>[10,11]</sup>. Paradoxical reports lead us to the idea that there are at least two types of short segment BE: one type that is closely associated with GERD and one that is not associated with GERD. We decided to investigate the relationship between ESEM endoscopic findings and clinical symptoms. We hypothesized that ESEM could be distinguished into two morphologically different types, one of which is closely associated with GERD and the other not associated with GERD.

We elucidated a significant correlation between the ESEM shape and each clinical symptom. The prevalence of heartburn was significantly higher in T type ESEM than in W type ESEM and ESEM-free subjects. Furthermore, subjects with T type ESEM tended to be

more symptomatic than the subjects with D type ESEM, and the prevalence of RE was highest in T type ESEM subjects. We found that T type ESEM was closely associated with GERD, whereas W type ESEM had a weak or no association with GERD.

We also hypothesized that T type ESEM might originate as a direct result of columnar replacement of areas damaged by RE. If this was correct, the localization of RE and localization of the T type ESEM should be in accord. Thus, we investigated the relationship between the localization of RE and ESEM and demonstrated that the localization of T type ESEM was also mainly at the 12 o'clock and 1 o'clock wall of the lower esophagus, which was similar in the case with grade A and B RE. This fact may support the proposition that RE plays an important part in the development of T type ESEM.

The reason why the circumferential location of esophageal mucosal breaks differs among different grades of esophagitis has not yet been fully investigated, but several speculative proposals have been put forward by Katsume *et al* and Winans<sup>[23,24]</sup>. Winans investigated the circumferential pressure of the lower esophageal sphincter, and confirmed that a significantly higher localized pressure existed in the orifices directed towards

the left posterior quadrant of the esophagus in normal subjects without hiatal hernia. Differing degrees of pressure due to the circumferential asymmetry of the lower esophageal sphincter could be a major cause of the differences in the localization of RE. With these dynamic causes in grade A and B RE, the mucosal breaks mainly exist at the 12 o'clock and 1 o'clock wall of the lower esophagus mucosal breaks. We may surmise that the mild type of RE, which is the most prevalent form of RE in Japan, may lead to the development of T type ESEM.

Two studies<sup>[25,26]</sup> in particular have attracted our attention. It has been reported that the localization of dysplasia in BE and cardiac cancer is dominant at the 12 o'clock and 3 o'clock wall of the lower esophagus<sup>[25,26]</sup>. However, we did not detect any corroborative findings in histological examinations in this study, thus more epidemiological studies will be required to examine this issue further.

The present study has a potential limitation. We collected data from subjects who had undergone further endoscopic testing among subjects who were suspected of having gastric cancer (cancer s/o) based on an X ray examination. Subjects who were suspected of having gastric cancer might have had a high degree of atrophic gastritis that resulted in low acid output, whereby the prevalence of GERD might be relatively low compared to the general Japanese population.

In conclusion, the prevalence of heartburn and RE was significantly higher in the T type of ESEM. The localization of ESEM was similar to the localization of mild RE, which is the most prevalent form of RE in Japan. We therefore propose that T type ESEM may arise as a result of RE.

## COMMENTS

### Background

Although Barrett esophagus (BE) is generally in place as a complication of Gastroesophageal reflux disease (GERD), some cases of BE, especially the short segment type, have an obscure connection with GERD.

### Research frontiers

We hypothesized that endoscopically suspected esophageal metaplasia (ESEM) could be distinguished into two morphologically different types, one of which is closely associated with GERD, and the other not associated with GERD.

### Innovations and breakthroughs

The controversy still exists regarding the potential of BE to develop as a late consequence of RE. These paradoxical reports lead us to the hypothesis that there are at least two type of short segment BE, namely one type that is closely associated with GERD, and one that is not associated with GERD.

### Applications

The prevalence of heartburn was significantly higher in the Tongue like (T-type) ESEM than in the Wave-like (W type) ESEM, and the prevalence of RE was highest in T type ESEM subjects. We found that the T type ESEM was closely associated with GERD, whereas the W type ESE had a weak or no association with GERD.

### Peer review

This is a good study that explores the possibility that Barrett's esophagus is not a homogeneous entity, but its shape and location may suggest a correlation with reflux symptoms and GERD. The authors have done a systematic job and have based their conclusions on sound reasoning.

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RAPID COMMUNICATION

# Prevalence and clinical significance of SEN virus infection in patients with non A-E hepatitis and volunteer blood donors in Shanghai

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## Abstract

**AIM:** To explore the prevalence of SEN virus (SENV) in patients with non A-E hepatitis and volunteer blood donors in Shanghai.

**METHODS:** According to the published gene sequences, primers from the conserved region were designed. Then, the prevalence of SEN virus in 30 samples from healthy voluntary blood donors and 30 samples from patients with non A-E hepatitis were detected by nested-PCR of SENV-D/H. Some PCR products were cloned and sequenced.

**RESULTS:** The specificity of genotype-specific PCR was confirmed by sequencing, the SENV DNA was detected in 53.3% of the patients with non A-E hepatitis and 10% of the blood donors. The prevalence of SENV-D/H viremia was significantly higher in patients with non A-E hepatitis than in blood donors ( $P = 0.0002$ ). SENV-H subtype and SENV-D subtype were found in 2 and 1 samples, respectively from blood donors. SENV-H subtype, SENV D subtype, mixed SENV-D and SENV-H subtype were found in 8, 6 and 2 samples, respectively, from patients with non A-E hepatitis.

**CONCLUSION:** The gene type of SENV in patients with non A-E hepatitis and blood donors in Shanghai is D or H subtype, and transfusion is not the only transmitting form of SENV.

## INTRODUCTION

A DNA virus-designated SEN virus (SENV), which was discovered in the serum of a human immunodeficiency virus type 1 (HIV-1)-infected patient, has been described recently<sup>[1]</sup>. It is assumed to be transmitted parenterally and to cause posttransfusion hepatitis in humans. SENV is described as a small single-stranded, non-enveloped circular DNA virus containing a genome of approximately 3800 nucleotides, possibly belonging to the circoviridae family. To date, eight distinct strains of SENV (A-H) have been identified<sup>[2]</sup>. There are 15%-50% sequence diversities among them. Phylogenetically, SENV is distantly related to TTV with which it shares a similar structural organization but only about 40%-60% sequence homologies<sup>[3]</sup>. According to previous studies, the prevalence of these eight different strains of SENV (A-H) is different in each infected group. The prevalence of SENV-D or SENV-H strains was 2.25% and 92.31% in different groups including healthy blood donors, patients with acute or chronic non A-E hepatitis, respectively, suggesting that SENV-D or SENV-H is significantly associated with the pathogenesis of non A-E hepatitis<sup>[3]</sup>.

To date, the prevalence of SENV in patients with various forms of liver disease has been reported in many countries and several districts of China, but the results are not consistent to a certain extent, and the role

of SENV infection in patients with non A-E hepatitis or other viral hepatitis, and the transmitting form of SENV are not very clear<sup>[4-7]</sup>. The purpose of this study was to investigate the prevalence and molecular biology characteristic of SENV in patients with non A-E hepatitis and volunteer blood donors in Shanghai, and the possible clinical significance of SENV infection in patients with non A-E hepatitis.

## MATERIALS AND METHODS

### **Patients**

A total of 30 serum samples from volunteer blood donors and 30 serum samples from patients with non A-E hepatitis from January 2005 to June 2007 in sixth people's Hospital affiliated to Shanghai Jiaotong University were studied. These cases included 42 men and 18 women, with a mean age of  $37.2 \pm 7.1$  years (range, 19-65 years). All the serum samples were negative for both HBsAg and anti-HCV. The patients with non A-E hepatitis were defined as negative control for known serologic markers, including IgM anti-HAV, IgM antibody to hepatitis B core antigen (anti-HBc), hepatitis B surface antigen (HBsAg), HBV DNA, and antibodies to HCV, HDV and HEV. The patients had no history of HAV, HDV, HEV, EBV, CMV infection, or of adiposis hepatica and hepatic lesions induced by drugs, alcohol, cholestatic and autoimmunity, or of transfusion.

### **Detection of SENV DNA**

The primers were designed according to the sequences submitted to GenBank with computer analysis to determine the outer primer by inner primer. All primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. The sequences of primers are as follows (W = A or T, Y = C or T, M = A or C). SENV common primers: P1, 5'-TW CYCMAACGACCAGCTAGACCT-3'; P2, 5'-GTTTG TGGTAGCAGAACCGGA-3'. SENV-D primers: P3, 5'-CTAAGCAGCCCTAACACTCATCCAG-3'; P4, 5'-GCAGTTGACCGCAAAGTTACAAGAG-3'. SENV-H primers: P5, 5'-TTTGGCTGCACCTCTGGTT-3'; P6, 5'-AGAAATGATGGGTGAGTGTAGGG-3'.

All blood samples were separated by centrifugation. The sera were stored at -80°C until SENV DNA analysis. Viral DNA was extracted from 200 μL serum with the QIAamp DNA blood mini kit (Qiagen) and resuspended in 100 μL elution buffer according to the manufacturer's instructions.

PCR mixture of 50 μL contained 0.5 μL (50 pmol/L) sense primer SENV-P2, 0.5 μL (50 pmol/L) antisense primer P1, 250 μmol/L of each dNTP, 6 μL DNA sample, and 2.5 U Taq DNA polymerase (TaKaRa). The reactions consisted of preheating at 94°C for 4 min, 35 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 50 s, extension at 72°C for 50 s, and a final at 72°C for 10 min.

The second PCR step was carried out with 50 μL PCR reaction mixture containing 10 μL of the first-

step amplification product. The same PCR buffer was used for the first PCR step, 0.5 μL (50 pmol/L) sense primer P4 and antisense primer P3 for SENV-D, 0.5 μL (50 pmol/L) sense primer P6 and antisense primer P5 for SENV-H, 250 μmol/L of each dNTP, and 2.5 U Taq DNA polymerase. PCR consisted of preheating at 94°C for 30 s, annealing at 55°C for 50 s, extension at 72°C for 50 s, and a final incubation at 72°C for 10 min.

### **Determination of SENV genotypes**

PCR amplified products were separated in 10 μL reaction mixture by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, and visualized using an ultraviolet transilluminator. Amplicons containing poly A tails and producing visible bands on agarose gel were excised from the gel and ligated to the pMD18-T vector (TaKaRa). DNA extracted from transformed *Escherichia Coli* was sequenced in Shanghai Sangon Biological Engineering Technology & Services Co, Ltd. The sequences excluding primer sequences were aligned with Cluster W to A-H SENV genotypes. The genotypes of SENV were determined by the phylogenetic trees.

### **Statistical analysis**

Descriptive statistical data such as means and proportions were calculated. Frequency was compared between groups using the chi-square test or Fisher's exact test, and group means were compared using Student's *t* test. Stepwise logistic regression method was used to analyze the data. *P* < 0.05 was considered statistically significant.

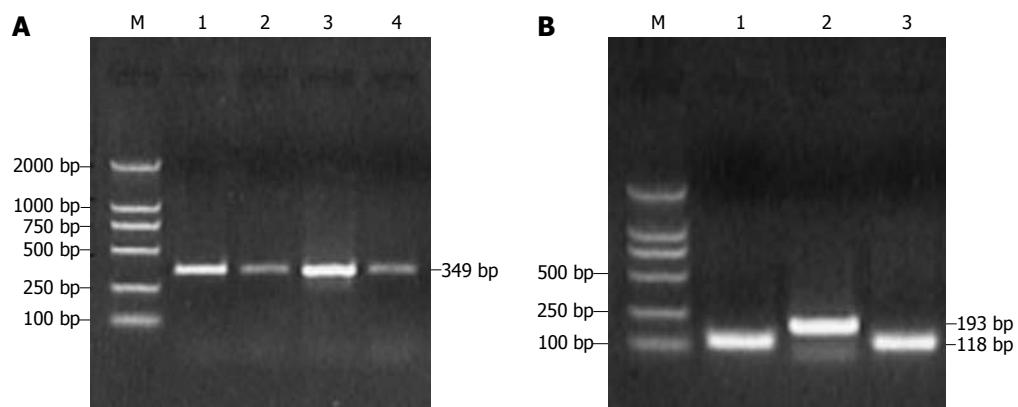
## RESULTS

### **Prevalence of SENV-D/H DNA in patients with non A-E hepatitis and blood donors**

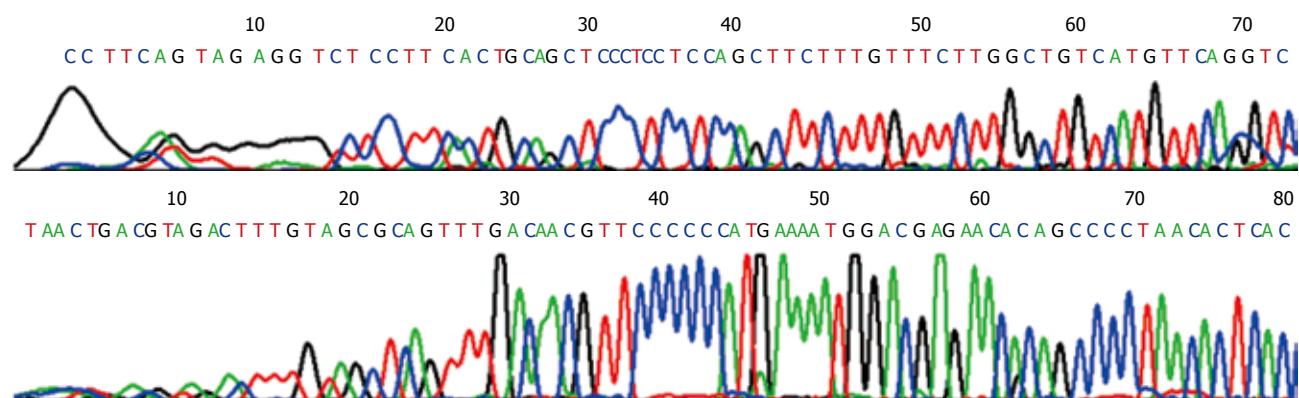
The nPCR results showed that 3 (10%) of the 30 volunteer blood donors and 16 (53.3%) of 30 patients with non A-E hepatitis were positive for SENV. Of 3 volunteer blood donors, 1 was positive for SENV-D and 2 were positive for SENV-H. Of the 16 patients with non A-E hepatitis, 6 were positive for SENV-D, 8 were positive for SENV-H, 2 were positive for both SENV-D and SENV-H DNA (SENV-D/H co-infection). The overall prevalence of SENV-D/H was 30%. After electrophoresis on 1.5% agarose gel stained with ethidium bromide on DNA product, the expected 349 bp, 118 bp and 193 bp bands were visualized on an UV transilluminator for SENV and SENV-D/-H, respectively (Figure 1). The positive rate of SENV infection was significantly higher in patients with non A-E hepatitis than in volunteer blood donors (*P* = 0.0002).

### **Sequencing PCR products and homology analysis**

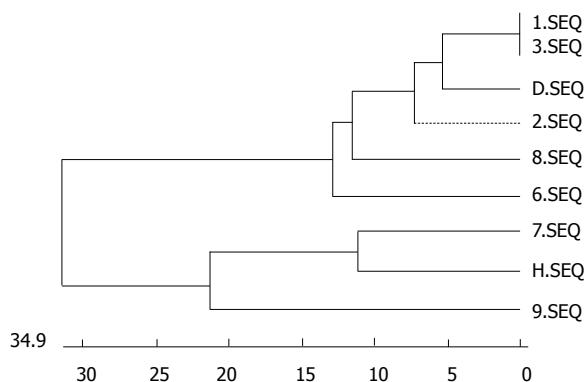
Randomly selected PCR products of P3 and P4 (118 bp) or P5 and P6 (193 bp) were sequenced. When the sequences of PCR positive products were compared with those of SENV by homology analysis, the



**Figure 1** Detection of SENV DNA (A) and SENV-D/H DNA (B). M: Marker DL2000; lane1-4: Primers and PCR products.



**Figure 2** Sequences of P3-P6 products.



**Figure 3** Phylogenetic tree of certain sequences of PCR products from patients with non A-E hepatitis and SENV-D and SENV-H subtype genes from GenBank.

homology was 98% between P3 and P4 amplification sequences of SENV-D, likewise, the homology was 98% between P5 and P6 amplification sequences of SENV-H. Some sequences of PCR positive products are shown in Figure 2. The phylogenetic tree for certain sequences of PCR products from patients with non A-E hepatitis and SENV-D/ H subtype genes from the GenBank (<http://www.ncbi.nlm.nih.gov/BLAST/>) are shown in Figure 3.

#### Distribution of SENV in blood donors and patients with non A-E hepatitis

SENV infection in different regions of China has been reported elsewhere<sup>[8-13]</sup>. The detected population was

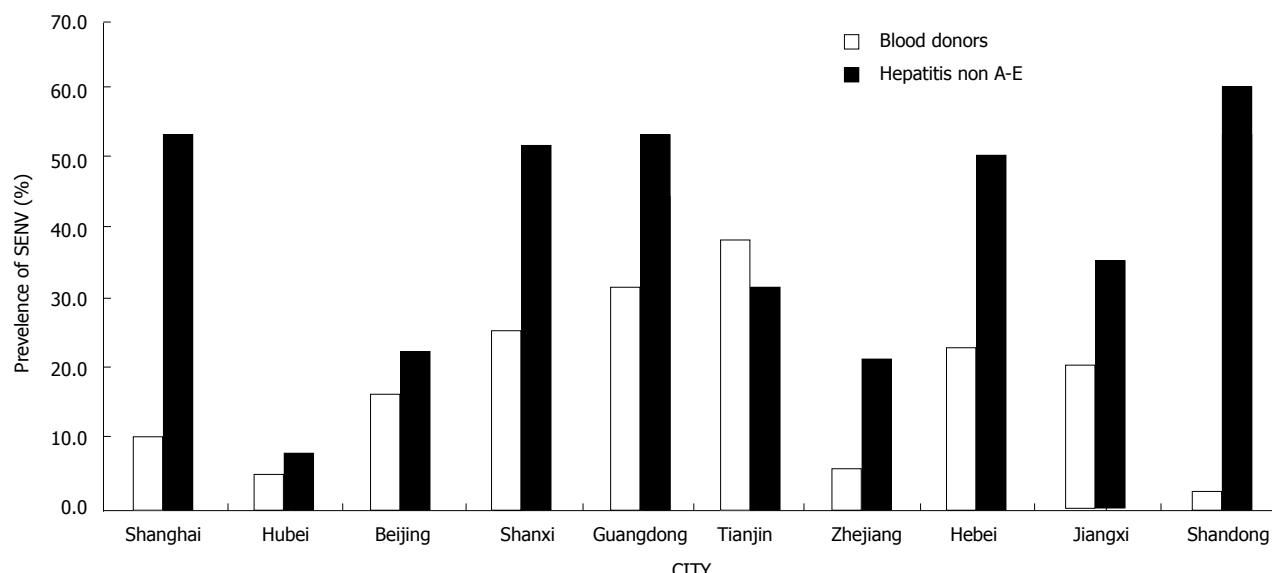
concentrated in areas of high CLD infection and the prevalence of SENV was different in these regions of China (Figure 4).

#### DISCUSSION

Hepatitis viruses can be divided into five types: A-E and cause 80%-90% hepatitis cases. Some viruses such as TTV are correlated with non A-E hepatitis<sup>[14-16]</sup>. However, no pathogenicity of TTV has been found in different populations<sup>[17-20]</sup>.

SENV has been recently identified as a candidate agent of non A-E hepatitis virus<sup>[21]</sup>. However, the exact role of this virus in the pathogenesis of chronic liver diseases, including chronic hepatitis and cirrhosis, and the development of hepatocellular carcinoma (HCC) remains to be verified<sup>[22]</sup>. The prevalence of transfusion-associated non A-E hepatitis is high and the SENV-D and SENV-H strains are significantly associated with transfusion-associated non A-E hepatitis.

Pirovano *et al*<sup>[23]</sup> found that aminotransferase level is transitly elevated in infants with positive SENV. Umemura *et al*<sup>[24]</sup> showed that SENV-D/H infection is related with posttransfusion hepatitis and special RNA molecule of SENV in which non A-E hepatic tissue probably is an intermedium of SENV replication. Recent data indicate that SENV DNA can be found in hepatic tissue from acute non A-E hepatitis patients and SENV perhaps causes hepatic lesions induced by *in situ* PCR. In addition, the detectable SENV DNA rate



**Figure 4** Prevalence of SENV in different provinces of China.

is 21% in patients with chronic hepatitis C<sup>[25-27]</sup>. In this study, the detectable SENV DNA rate was 10% (3/30) in 30 volunteer blood donors and 53.3% (16/30) in 30 patients with non A-E hepatitis.

In the present study, SENV infection was found in blood donors and patients with non A-E hepatitis in Shanghai. We found that approximately 3.2%-38.9% of blood donors and 9.3%-59.6% of non A-E hepatitis had positive SENV (Figure 4) and the positive SENV DNA rate was significantly different in different areas, suggesting that we must continue the detection of SENV DNA prevalence in other provinces. Interestingly, the prevalence rate of SENV was higher in blood donors than in patients with non A-E hepatitis (38.9% vs 32.3%) in Tianjin.

Furthermore, SENV-H was found in 2 volunteer blood donors and SENV-D was found in 1 volunteer blood donor infected with SENV. SENV-H, SENV-D and mixed SENV-D/H were found in 8, 6, and 2 patients with hepatitis, indicating that the prevalence of SENV in Shanghai is relatively low. However, SENV infection was not associated with blood transfusion in 16 SENV positive non A-E hepatitis patients with no blood-transfusion history, indicating that blood transfusion transmission is not the only way for people to infect with SENV. Because of the limited number of serum samples in our study, we could not explain why SENV infection is related with blood transfusion or non A-E hepatitis.

In conclusion, SENV infection is higher in patients with non A-E hepatitis than in blood donors and the homology is 98%. Further study with a large number of samples is required to analyze the relationship between SENV infection and non A-E hepatitis.

## COMMENTS

### Background

SEN virus (SENV) is described as a small single-stranded, non-enveloped circular DNA virus, possibly belonging to the circoviridae family. To date, eight

distinct strains of SENV (A-H) have been identified. The prevalence of these eight different strains SENV (A-H) is different in each infected group. In the present study, the prevalence of SENV-D or SENV-H strain was 2.25% and 92.31% in blood donors and patients with acute or chronic non A-E hepatitis, suggesting that SENV-D or SENV-H is significantly associated with the pathogenesis of non A-E hepatitis.

### Research frontiers

The prevalence of SENV in patients with various forms of liver disease has been reported in many countries and several regions of China, but the results are not consistent. The role of SENV infection in patients with non A-E hepatitis or other viral hepatitis and the transmitting form of SENV are not very clear. The clinical significance of SENV infection in patients with non A-E hepatitis is also not very clear.

### Innovations and breakthroughs

SENV infection was studied in blood donors and patients with non A-E hepatitis in Shanghai. The results indicate that the prevalence of SENV in blood donors of Shanghai was relatively lower than that in other regions of China. SENV infection was not associated with blood transfusion in SENV positive patients with non A-E hepatitis with no blood-transfusion history, suggesting that blood transfusion transmission is not the only way to spread SENV.

### Applications

The SENV infection rate is higher in non A-E hepatitis than in blood donors in Shanghai. Because of the limited number of serum samples in our study, we could not explain why SENV infection is associated with blood transfusion or non A-E hepatitis. Further study with a large number of samples is required to analyze the relationship between SENV infection and non A-E hepatitis.

### Peer review

This paper describes the prevalence of SEN virus in blood donors and hepatitis patients. The study is well designed and provides important information about the prevalence of SENV in hepatitis patients.

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RAPID COMMUNICATION

# Gene therapy for type 1 diabetes mellitus in rats by gastrointestinal administration of chitosan nanoparticles containing human insulin gene

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## Abstract

**AIM:** To study the expression of human insulin gene in gastrointestinal tracts of diabetic rats.

**METHODS:** pCMV.Ins, an expression plasmid of the human insulin gene, wrapped with chitosan nanoparticles, was transfected to the diabetic rats through lavage and colocephaly, respectively. Fasting blood glucose and plasma insulin levels were measured for 7 d. Reverse transcription polymerase chain reaction (RT-PCR) analysis and Western blot analysis were performed to confirm the expression of human insulin gene.

**RESULTS:** Compared with the control group, the fasting blood glucose levels in the lavage and colocephaly groups were decreased significantly in 4 d ( $5.63 \pm 0.48$  mmol/L and  $5.07 \pm 0.37$  mmol/L vs  $22.12 \pm 1.31$  mmol/L, respectively,  $P < 0.01$ ), while the plasma insulin levels were much higher ( $32.26 \pm 1.81$   $\mu$ IU/mL and  $32.79 \pm 1.84$   $\mu$ IU/mL vs  $14.23 \pm 1.38$   $\mu$ IU/mL, respectively,  $P < 0.01$ ). The human insulin gene mRNA and human insulin were only detected in the lavage and colocephaly groups.

**CONCLUSION:** Human insulin gene wrapped with chitosan nanoparticles can be successfully transfected to rats through gastrointestinal tract, indicating that chitosan is a promising non-viral vector.

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## INTRODUCTION

Type 1 diabetes mellitus is the result of insulin deficiency caused by the autoimmune destruction of insulin-producing pancreatic  $\beta$  cells. Hyperglycemia would cause a lot of long-term clinical problems, including renal failure, retinopathy, neuropathy and heart disease<sup>[1]</sup>. Although intensive exogenous insulin therapy can delay or prevent the onset of chronic complications, it is rather cumbersome and sometimes would cause hypoglycemia, which could be life-threatening. However, the development of gene therapy has also generated a greater hope and excitement for a possible “cure” of diabetes since insulin gene was first cloned and expressed in cultured cells in the late 1970s<sup>[2]</sup>. Many attempts have been made, including islet transplantation<sup>[3-5]</sup>, whole pancreas transplantation<sup>[6,7]</sup>, regeneration of  $\beta$  cells<sup>[8-10]</sup> and insulin gene therapy<sup>[11-13]</sup>.

In general, whole organ transplants have more sustained and durable function. Advances in islet transplantation procedures now mean that patients with the disease can be cured by transplantation of primary human islets of Langerhans. The major drawbacks of these strategies are the insufficient availability of donor islets, invasive procedure and high cost. Due to the limited available number of donor islet cells, researchers are looking for different

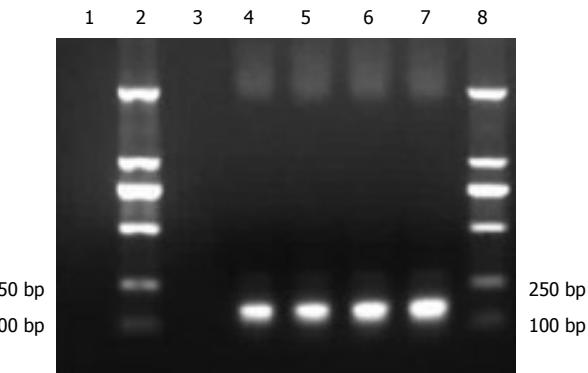
sources of pancreatic islet progenitor or stem cells. Stem cells with an extensive proliferative ability may provide a valuable source of islet progenitor cells. Several studies have demonstrated that progenitor/stem cells can be expanded *in vitro* to generate a large number of islet progenitor cells<sup>[14-16]</sup>. However, efficient and direct differentiation of these cells to an endocrine pancreatic lineage is difficult to achieve. Insulin gene therapy including any approach involving the introduction of a foreign gene into any cell type in the body can produce insulin<sup>[17]</sup>. The gene(s) introduced could be the insulin gene itself, perhaps under control of a tissue specific promoter, allowing for expression in a selected non-β-cell type, or in a gene encoding for a factor that activates the insulin gene, thereby allowing for ectopic insulin production. In insulin gene therapy, one of the key issues is the selection of carriers. Conventional viral vectors can introduce exogenous genes into cells precisely and effectively, but they can easily cause immune reactions because of the existence of antiviral immune system. More and more researchers are interested in non-viral vectors.

In this study, we constructed an expression plasmid pCMV.Ins expressing human insulin gene. Then, we wrapped the pCMV.Ins with chitosan nanoparticles, a non-viral vector, and transfected to diabetic rats through gastrointestinal tract to explore the gene therapy for type 1 diabetes mellitus. At present, there is no report on transfection of human insulin gene to gastrointestinal tract and application of chitosan as a vector in gene therapy for type 1 diabetes mellitus.

## MATERIALS AND METHODS

### Materials

The cDNA sequences of human insulin gene were cut from the PBAT16, hInsG1.M2 (presented by Doctor Michael German, Department of Hormone Research Institute, University of San Francisco, USA.) using *Bgl*II / *Not*I enzyme inserted into the expression vector of pCMV.eGFP in the cohesive end. Then the pCMV.Ins was transformed into *E.coli*. The top10F strains (Invitrogen) were screened. Plasmids isolated with a large-scale alkaline lysis procedure were purified, 0.1 g chitosan was dissolved in 100 mL acetic acid, and the pH was adjusted to 5.5 with sodium hydrate. The solution was stored at 4°C after determined with a micropore film, the aperture of which was 0.22 μm. Before it was used, the chitosan solution was diluted with ddH<sub>2</sub>O until the concentration reached 0.02%. One hundred μL DNA solution at a concentration of 200 μg/mL was added into 100 μL sodium sulfate solution at a concentration of 25 mmol/L. After the chitosan and DNA solutions were heated to 55°C in an aqueous bath for 15 min, respectively, they were mixed immediately and put into convolution for 1 min. The volume of reaction system should not excess 500 μL<sup>[18]</sup>. Experiments were carried out in 45



**Figure 1** PCR of recombinant plasmid pCMV.Ins. Lane 1: PCMV.eGFP, lanes 2 and 8: Molecular marker, lane 3: Negative control, lane 4: Recombinant plasmid pCMV.Ins, lanes 5-7: Bacterial suspension.

male Wistar rats (weighing 180-190 g) at the age of 8 wk. After an overnight fasting, the rats were given a single intraperitoneal injection of 60 mg/kg streptozotocin (STZ, Upjohn, Kalamazoo, MI, USA) in a 0.01 mol/L citrate buffer. Forty rats with their fasting blood glucose level > 16.7 mmol/L were used in the study. The rats were randomly divided into 4 groups. Chitosan-DNA nanoparticles were transfected to the diabetic rats through lavage and colocephaly, respectively. The two control groups were treated with naked chitosan and normal saline respectively. The rats had free access to diets and water.

### Identification of plasmid

pCMV.Ins was identified by PCR. The sequences of primers used for human insulin specifically are 5'-ATCACTGTCCTCTGCCA-3' (forward) and 5'-GGGTGTGTAGAAGAAAGCC-3' (reverse). The PCR products were analyzed by a 1.5% agarose gel electrophoresis, and the expected amplification length was 173 bp. The DNA sequencing of pCMV.Ins plasmid was performed in the Shanghai Sangon Sequencing Center.

### Measurement of fasting blood glucose and plasma insulin levels

The fasting blood glucose and plasma insulin levels were measured in all groups. The glucose levels in blood samples obtained from tail veins were measured with the one touch meter and test strips (Lifescan Inc, USA) every morning. The plasma human insulin was detected with a commercial human insulin radioimmunoassay (RIA) kit (Institute of Atomic Energy, China).

### Reverse transcription polymerase chain reaction (RT-PCR) analysis

RT-PCR was performed to verify the expression of human insulin gene in gastrointestinal tract. Half of the rats in each group were killed on the 4th d after they transfected with chitosan-DNA nanoparticles. Immediately upon death, stomachs, intestines and rectums of the treated and control animals were flash-frozen in liquid nitrogen, and stored at -70°C. Total RNA

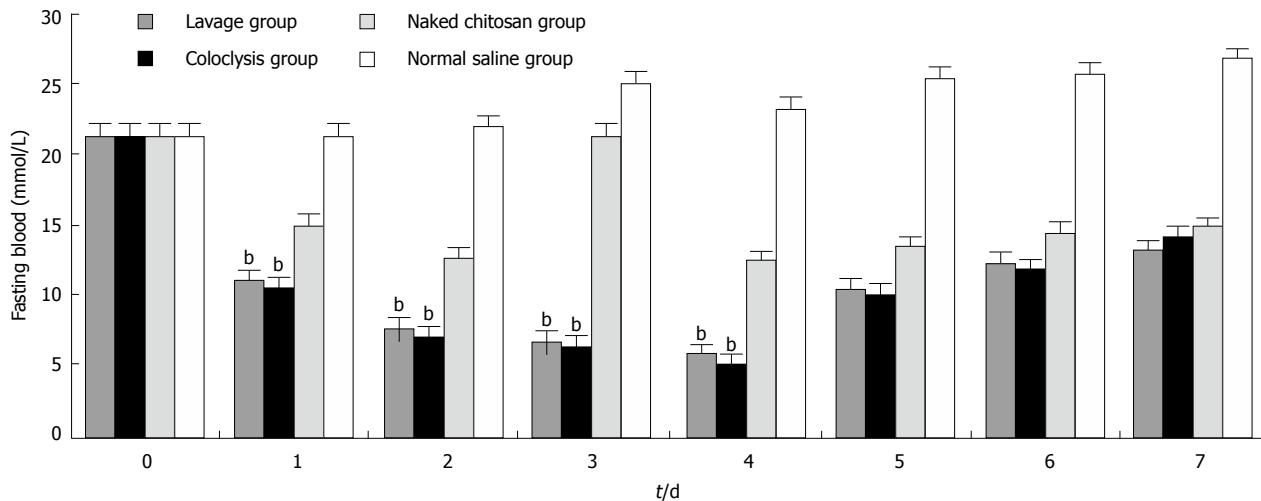


Figure 2 Fasting blood glucose levels in each group after the normal saline treatment. <sup>b</sup> $P < 0.01$  vs naked chitosan group and normal saline group ( $n = 10$ ).

(5 µg), obtained using Trizol (Gibco BRL, Gaithersburg, MD, USA) according to the manufacturer's instructions, was subjected to reverse transcription by using an oligo-dT21 primer, recombinant RNasin, and AMV reverse transcriptase (all Promega, Madison, WI, USA). AMV RT was inactivated at 95°C, and PCR was performed by using a gene amp PCR system 9600 thermal cycler (Perkin Elmer, Norwalk, CT, USA), and Taq DNA polymerase (Perkin Elmer). The cDNA-mixture was allowed to react for 19 (*GAPDH*), or 22 (insulin) cycles. The sequences of primers used for human insulin are 5'-ACCATGGCCCTGTGGATGCGC-3' (forward), and 5'-CTAGTTGCA GTAGTTCTC-CAG-3' (reverse). The sequences of primers for *GAPDH* are 5'-ACCACAGTCCATGCCATCAC-3' (forward) and 5'-TCCACCACCTGTTG CTGTA-3' (reverse). Insulin primers were designed to amplify human insulin specifically. Primers for *GAPDH* were used in control reactions. The RT-PCR products were analyzed by a 1.5% agarose gel electrophoresis, and the expected amplification lengths were 336 bp and 451 bp, respectively.

#### Western blot analysis

Four days after the transfection, stomachs and intestines were harvested and resuspended in a lysis buffer containing 1% nonidet P-40, 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 200 U/mL aprotinin, 1 mmol/L phenylmethanesulfonyl fluoride. The tissue lysates (50 mg of protein) were separated by 12% polyacrylamide gel electrophoresis and blotted onto poly-vinylidene difluoride membranes. Immunoblotting was performed with the antibody against human insulin (Sigma-Aldrich Corp, St Louis, MO, USA), and the molecular weight of human insulin was known as 56 kDa.

#### Statistical analysis

Data were expressed as mean  $\pm$  SD. The concentrations of blood glucose and plasma insulin were evaluated by

one-way ANOVA (SPSS for Windows 11.5).  $P < 0.05$  was considered statistically significant.

## RESULTS

#### Identification of recombinant plasmid pCMV.Ins

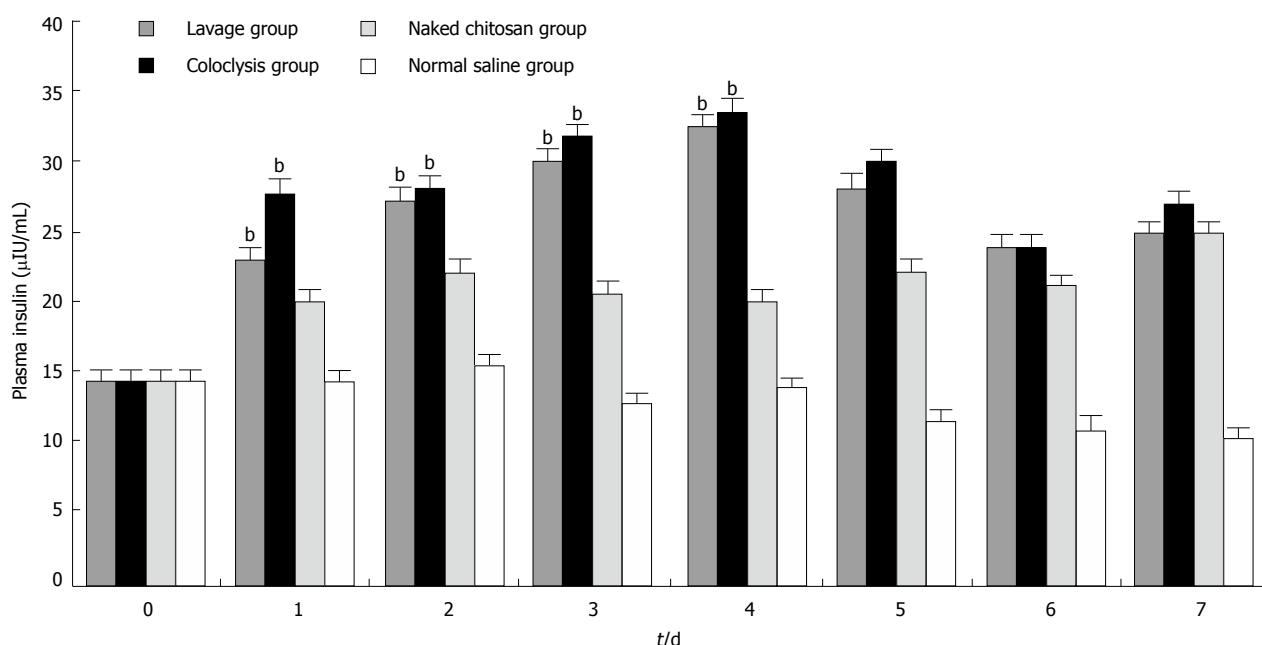
The PCR products of recombinant plasmid pCMV.Ins and bacterial suspension were amplified. One specific band was obtained in lanes 4-7, respectively, corresponding to the expected size of 173 bp. No fragments in lanes 1 and 3 were amplified from pCMV.eGFP and negative control (Figure 1), suggesting that the human insulin gene was successfully inserted into recombinant plasmid pCMV.Ins. The sequencing results also revealed that the recombinant pCMV.Ins plasmid was successfully constructed. The number of sequencing reports was LE142.

#### Change in fasting blood glucose

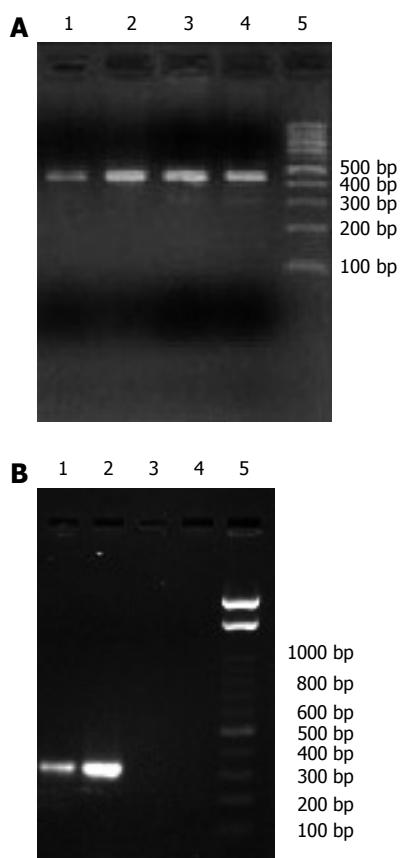
The fasting blood glucose level was decreased in lavage and coloclysis groups from  $22.12 \pm 1.31$  mmol/L to  $5.63 \pm 0.48$  mmol/L and  $5.07 \pm 0.37$  mmol/L, respectively, after transfected ( $n = 10$  each group). The levels of fasting blood glucose were significantly lower in the lavage group and coloclysis group transfected with chitosan-DNA nanoparticles ( $P < 0.01$ ) after the normal saline treatment from the 1st to 4th d (Figure 2). The blood glucose levels in the naked chitosan group also decreased significantly, because chitosan had the effect of decreasing blood glucose level. The blood glucose levels in the lavage and coloclysis groups were much lower than those in the naked chitosan group, and the differences were significant ( $P < 0.01$ ). There was no difference in blood glucose levels between the lavage and coloclysis groups.

#### Change in plasma insulin

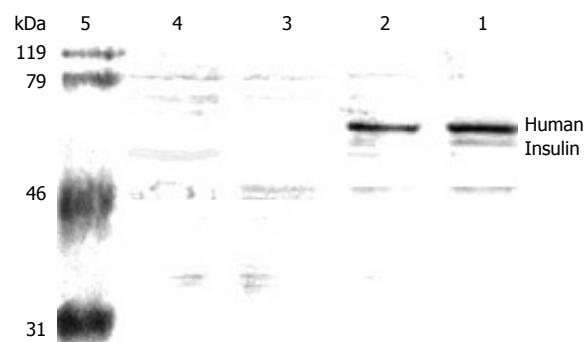
The plasma insulin in the lavage and coloclysis groups increased from  $14.23 \pm 1.38$  µIU/mL to  $32.26 \pm 1.81$  µIU/mL and  $32.7 \pm 1.84$  µIU/mL after transfection ( $n = 10$  each group). The plasma insulin levels in the



**Figure 3** Plasma insulin levels in each group after transfection. <sup>b</sup> $P < 0.01$  vs naked chitosan group and normal saline group ( $n = 10$ ).



**Figure 4** Amplification results of GAPDH (A) and human insulin gene mRNA (B) in each group. Lane 1: Lavage group, lane 2: Coloclysis group, lane 3: Naked chitosan group, lane 4: Normal saline group, lane 5: Molecular marker.



**Figure 5** Expression of human insulin in each group. Lane 1: Lavage group, lane 2: Coloclysis group, lane 3: Naked chitosan group, lane 4: Normal saline group, lane 5: Molecular marker.

#### Verification of human insulin gene expression

To analyze the expression of the human insulin gene, RT-PCR and Western blot analysis were carried out. Total RNA from the gastrointestinal tract was amplified using primers specific for GAPDH, revealing a 451 bp fragment in all groups (Figure 4A). However, human insulin specific primers produced a fragment, corresponding to the expected size of 336-bp in lanes 1 and 2, only in reactions containing RNA from the lavage and coloclysis groups, respectively (Figure 4B). No products were amplified from cDNA in the naked chitosan group (lane 3) and normal saline group (lane 4).

The molecular weight of human insulin was 56 kDa (Figure 5). One specific band was only obtained in the lavage and coloclysis groups in lanes 1 and 2, respectively. No products were obtained in the naked chitosan group (lane 3) and normal saline group (lane 4). These results suggest that the exogenous transferred pCMV.Ins plasmid but not the endogenous insulin gene expressed the mRNA and human insulin.

lavage, coloclysis and naked chitosan groups (Figure 3) were higher than those in the normal saline group from the Day 1 to the Day 4 ( $P < 0.01$ ). There were remarkable differences among the lavage, coloclysis and naked chitosan groups ( $P < 0.01$ ). The differences between the lavage and coloclysis groups did not show any statistical significance. These results were consistent with reduction in the fasting blood glucose levels.

## DISCUSSION

One factor critical to successful gene therapy is the development of efficient delivery systems. Despite the advances in gene transfer technology including viral and non-viral vectors, no ideal vector system is available at present<sup>[19]</sup>. Although viral vectors can introduce exogenous genes into cells precisely and effectively, they can easily cause immune reactions because of the existence of antiviral immune system. Due to the growing concerns over the toxicity and immunogenicity of viral DNA delivery systems, DNA delivery *via* improving viral routes has become more desirable and advantageous<sup>[20]</sup>.

A perfect vector should also be biocompatible, efficient, and modular so that it can be applied both in research and in clinical settings<sup>[21]</sup>. Taking into account this point, we selected chitosan nanoparticle, a kind of non-viral vector, in the study. We found that human insulin gene wrapped with chitosan nanoparticles could decrease the fasting blood glucose level and increase the insulin level in STZ diabetic rats. The mRNA in human insulin gene and human insulin was detectable in the gastrointestinal tract. These results demonstrate that chitosan nanoparticles can mediate the transfection of human insulin gene and that chitosan nanoparticles can be used as a good vector in gene therapy of type 1 diabetes mellitus. Köping-Höggård *et al*<sup>[22]</sup> reported that aerosol delivery formulated with chitosan oligomers can improve the distribution of pDNA polyplexes in the lungs and increase 6-fold of the efficiency of gene delivery *in vivo* over the commonly used intratracheal instillation method.

Chitosan nanoparticles are a kind of non-viral vector. Non-viral vector includes liposome<sup>[23-25]</sup>, composite<sup>[26]</sup>, microsphere, and nanoparticles, etc<sup>[27,28]</sup>, but the cytotoxicity of the bangosome limits its application *in vivo*. Owing to its loose constitution, the constancy of composites is poor. The diameter of microsphere is bigger than that of nanoparticles. Chitosan nanoparticles are a comparatively promising non-viral vector. Chitosan nanoparticles<sup>[29]</sup> have a good biocompatibility and no toxicity, and are economically available. The transfection efficiency of chitosan can be regulated by changing its molecular weight, plasmid concentration, and the chitosan/plasmid ratio. After the plasmid is embedded in chitosan, it can resist the degradation of nucleases. It also exhibits an antibacterial activity by inhibiting the bacterial metabolism.

In our study, the fasting blood glucose level was decreased during the first 4 d, due to the regeneration of gastrointestinal tract epithelial cells, which is consistent with the reported data<sup>[30]</sup>.

Further study should be performed to detect the cells intaking the plasmid. We speculate that gut K-cells may intake the expression plasmid. The gut K-cells in the epithelium mucosa of gastrointestinal tract secrete glucose-dependent insulinotropic polypeptide (GIP)<sup>[31]</sup>, an incretin hormone secreted by endocrine K-cells in response to nutrient absorption. GIP can

stimulate β cells to release insulin, and promote the regeneration of β cells<sup>[32]</sup>. GLP accounts for about 60% of the stimulation of insulin by oral glucose, but the determinants of their secretion from the small intestine are poorly understood<sup>[33]</sup>. There are many similarities between the release of GIP and the secretion of insulin. The concentration of GIP will obviously increase several minutes after glucose ingestion, and return to its basal level after 2 h. It was reported that the antagonist of GIP can remarkably degrade the secretion of insulin<sup>[34]</sup>. Furthermore, the promoter of GIP can only be activated in K-cells.

One of the key points in gene therapy for diabetes is the modification (processing) of proinsulin to insulin. Most non-β cells functioning as target cells in gene therapy for diabetes are lack of typical prohormone convertases which are essential to the processing of proinsulin, whereas K-cells can produce PC2 and PC3, which can help process the proinsulin correctly<sup>[35]</sup>. Palizban *et al* have transfected rat small intestine K-cells with pGIP/Ins plasmid by DOTAP liposome<sup>[36]</sup>. As mentioned above, K-cells might be the best target cells in gene therapy for type 1 diabetes mellitus due to their response to glucose and resistance to destruction mediated by cytokines and free radicals.

In conclusion, the human insulin gene can be transfected successfully by chitosan-DNA nanoparticles and expressed efficiently in the gastrointestinal tract of diabetic rats, and chitosan is a promising non-viral vector. If it is applied in clinic practice, it would be accepted by patients. Although much work remains to be done, the rapid progress in insulin gene therapy provides an optimistic outlook for its clinical applications in the treatment of type 1 diabetes mellitus.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Gastrointestinal K-cells might be the best target cells in gene therapy for type 1 diabetes mellitus due to their response to glucose and the resistance to the destruction mediated by cytokines and free radicals. A perfect vector should also be biocompatible, efficient, and modular so that it can be applied both in research and in clinical settings. Chitosan is an ideal non-viral vector and has drawn wide attention.

### Research frontiers

There are considerable endocrine cells in the gastrointestinal tract. Gastrointestinal K-cells are the potential and ideal target cells in gene therapy for diabetes. Progress in gene therapy has produced promising results that translate experimental research into clinical treatment. The main barrier in gene transfer is a safe and effective gene delivery system.

### Innovations and breakthroughs

The exogenous insulin genes can be transfected by chitosan nanoparticles and expressed efficiently in the gastrointestinal tract of diabetic rats, indicating that chitosan is a promising non-viral vector. At present, there is no report on

the transfection of human insulin gene to the gastrointestinal tract and on the application of chitosan as a vector in gene therapy for type 1 diabetes mellitus.

### Applications

The superiority of human insulin gene expression in gastrointestinal tract by chitosan nanoparticles is its safety without any wound. If it can be applied in clinic practice, it would be accepted by patients. Chitosan is an ideal non-viral vector and can be widely used in gene transfer.

### Terminology

Gastrointestinal K-cells exist in the epithelium mucosa of gastrointestinal tract and can secrete glucose-dependent insulinotropic polypeptide (GIP), which can stimulate  $\beta$  cells to release insulin and promote the regeneration of  $\beta$  cells. Chitosan nanoparticles are a kind of non-viral vector and have a good biocompatibility without any toxicity, and are economically available. The transfection efficiency of chitosan can be regulated easily. After the plasmid is embedded in chitosan, it can resist the degradation of nucleases.

### Peer review

This paper reports the expression of human insulin gene in the gastrointestinal tract by chitosan nanoparticles, thus providing new technologies of gene transfer to endocrine cells in the gastrointestinal tract. This study is well designed and interesting.

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RAPID COMMUNICATION

## Effect of lymphadenectomy extent on advanced gastric cancer located in the cardia and fundus

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the cardia and fundus, removing at least 20 LNs for stage II, 25 LNs for stage III, and 30 LNs for stage IV patients during D2 radical dissection is recommended.

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### Abstract

**AIM:** To analyze the prognostic impact of lymphadenectomy extent in advanced gastric cancer located in the cardia and fundus.

**METHODS:** Two hundred and thirty-six patients with advanced gastric cancer located in the cardia and fundus who underwent D2 curative resection were analyzed retrospectively. Relationships between the numbers of lymph nodes (LNs) dissected and survival was analyzed among different clinical stage subgroups.

**RESULTS:** The 5-year overall survival rate of the entire cohort was 37.5%. Multivariate prognostic variables were total LNs dissected ( $P < 0.0001$ ; or number of negative LNs examined,  $P < 0.0001$ ), number of positive LNs ( $P < 0.0001$ ), T category ( $P < 0.0001$ ) and tumor size ( $P = 0.015$ ). The greatest survival differences were observed at cutoff values of 20 LNs resected for stage II ( $P = 0.0136$ ), 25 for stage III ( $P < 0.0001$ ), 30 for stage IV ( $P = 0.0002$ ), and 15 for all patients ( $P = 0.0024$ ). Based on the statistically assumed linearity as best fit, linear regression showed a significant survival enhancement based on increasing negative LNs for patients of stages III ( $P = 0.013$ ) and IV ( $P = 0.035$ ).

**CONCLUSION:** To improve the long-term survival of patients with advanced gastric cancer located in

### INTRODUCTION

At present, surgery is the most effective treatment for gastric cancer. As the standard procedure for advanced gastric cancer, D2 radical resection has been widely accepted and practiced<sup>[1-3]</sup>. The final results of a randomized Dutch trial<sup>[4]</sup> showed that patients with N2 disease might benefit from a D2 dissection, which required removing all the lymph nodes (LNs) of Group 1 and Group 2. According to another randomized Italian trial<sup>[5]</sup>, a D2 lymphadenectomy was also advised. Lymph node metastasis is considered one of the most important prognostic factors<sup>[6,7]</sup>, and adequate lymphadenectomy is advocated for gastric cancer. However, the number of LNs that should be removed and examined when performing a D2 lymphadenectomy has not been determined for advanced gastric cancer located in the cardia and fundus<sup>[8]</sup>. Therefore, the aim of this retrospective study was to evaluate the relative contributions of both the number of total resected LNs and the number of negative LNs to the outcome of patients with advanced gastric cancer located in the cardia and fundus, and provide further evidence for rational lymphadenectomy.

## MATERIALS AND METHODS

### Materials

Between January, 1996 and June, 2002, 236 patients diagnosed with primary gastric cancer located in the cardia and fundus was treated with curative resection at the Department of Oncology, Affiliated Union Hospital of Fujian Medical University, Fuzhou, China. The surgical procedure was defined as curative when no grossly visible tumor tissue (metastasis or LN involvement) remained after the resection and the resection margins were histologically normal. There were 197 male and 39 female patients whose ages ranged from 30 to 79 years ( $58.8 \pm 9.8$  years). All patients received a D2 or more extended dissection according to the Japanese Classification of Gastric Carcinoma (JCGC). Lymph nodes were meticulously dissected from the en bloc specimens, and the classification of the dissected LNs was determined by specialist surgeons who reviewed the excised specimens after surgery based on the Japanese Classification of Gastric Carcinoma<sup>[9]</sup>. The clinical and histopathologic data of each patient were collected and recorded in a specifically designed form. The tumors were histologically classified according to the WHO classification criteria and staged according to the 5th Edition of the TNM system<sup>[10]</sup>, as listed in Table 1. The follow-up was carried out by trained investigators through mailings, telephone calls, visiting patients or recording the patients' consultations at the outpatient service. The survival time was the time from diagnosis until the last contact, the date of death, or the date that the survival information was collected. All surviving patients were followed for more than five years. The median follow up for the entire cohort was 44 mo (range, 1-136 mo). The follow-up rate was 94.0%, with 222 cases involved.

### Methods

Patients were stratified into five groups based on the number of total LNs removed as follows: < 15 LNs (36 cases), 15-19 LNs (43 cases), 20-24 LNs (62 cases), 25-29 LNs (40 cases) and  $\geq 30$  LNs (55 cases). Meanwhile, four groups were established based on the number of negative LNs as follows: 0-9 LNs (61 cases), 10-19 LNs (93 cases), 20-29 LNs (63 cases) and  $\geq 30$  LNs (19 cases). All calculations were performed using the SPSS 11.5 statistical package. Actuarial survival was determined via the Kaplan-Meier method, with univariate comparisons between groups through the log-rank test. Cox regression was used for multivariate analysis, with a backward elimination model for all covariates. A regression model to correlate negative LN counts with survival was obtained based on Kaplan-Meier 5-year survival estimates for each LN count interval, using the LN count interval midpoints to construct the independent variable. Significance of differences was assumed at  $P$  values less than 0.05 for all analyses.

**Table 1** Clinical characteristics of the 236 patients

Characteristics	n	Percentage
Gender		
Male	197	83.5
Female	39	16.5
Age (yr)		
< 60	103	43.6
$\geq 60$	133	56.4
Tumor size (cm)		
< 3	88	37.3
3-6	97	41.1
> 6	51	21.6
Borrmann's type		
Type I, II	172	72.9
Type III, IV	64	27.1
Histological classification		
Papillary adenocarcinomas	47	20
Tubular adenocarcinomas	101	42.8
Mucinous adenocarcinomas	29	12.3
Poorly differentiated adenocarcinomas	36	15.3
Undifferentiated carcinomas	8	3.4
Others	15	6.4
Depth of invasion (T category)		
Muscularis (pT2)	25	10.6
Serosa (pT3)	118	50
Invasion to adjacent organs (pT4)	93	39.4
Lymph node involvement (N category)		
pN0	42	17.8
pN1	97	41.1
pN2	68	28.8
pN3	29	12.3
Stage		
II	48	20.3
III	128	54.2
IV	60	25.4
Type of gastrectomy		
TG	190	80.5
PSG	46	19.5

## RESULTS

### Analysis based on total and negative LNs examined

Overall, 82.2% of patients (194/236) had LN metastasis. The incidences of LN metastasis were 56% for T2, 78.8% for T3 and 93.5% for T4. A total of 5615 LNs were picked up for histological examination, with 4005 being negative. The median of total LN number was 23 (range, 7-74; mean  $23.8 \pm 8.8$ ) per patient, and the median of negative LNs was 16 (range, 0-48; mean  $16.9 \pm 9.3$ ) per patient. There were no differences between T subcategories regarding total LNs resected [median (range): T2, 22 (9-31); T3, 26 (7-47); T4, 23 (7-74); Kruskal-Wallis,  $P = 0.062$ ] or negative LN counts [median (range): T2, 12 (9-28); T3, 21 (1-45); T4, 17 (1-48); Kruskal-Wallis,  $P = 0.502$ ].

### Multivariate survival analysis

The five-year overall survival rate of the entire cohort was 37.5%. The backwards elimination model yielded the following independent prognostic variables: total LN count (or number of negative LNs examined;  $P < 0.0001$ ), number of positive LNs ( $P < 0.0001$ ), T category ( $P < 0.0001$ ) and tumor size ( $P = 0.015$ ). The covariates gender ( $P = 0.052$ ), age ( $P = 0.329$ ),

**Table 2** Multiple stepwise regression analysis with the Cox proportional hazards model ( $n = 236$ )

Characteristics	$\beta$	Wald	P	RR	95% CI
Result 1					
Tumor size		8.40	0.015		
3-6 cm vs < 3 cm	0.303	3.77	0.052	1.354	0.997-1.840
> 6 cm vs < 3 cm	0.530	7.88	0.005	1.699	1.174-2.460
pT category		37.34	0.000		
T3 vs T2	0.899	13.51	0.000	2.457	1.522-3.969
T4 vs T2	1.498	33.55	0.000	4.471	2.693-7.420
Number of total LNs	-0.042	18.56	0.000	0.959	0.941-0.977
Number of positive LNs	0.094	53.05	0.000	1.098	1.071-1.126
Result 2 <sup>1</sup>					
Tumor size		8.83	0.012		
3-6 cm vs < 3 cm	0.299	3.67	0.055	1.349	0.993-1.833
> 6 cm vs < 3 cm	0.547	8.41	0.004	1.727	1.194-2.499
pT category		37.97	0.000		
T3 vs T2	0.899	13.50	0.000	2.456	1.521-3.967
T4 vs T2	1.507	33.96	0.000	4.511	2.718-7.488
Number of positive LNs	0.052	20.20	0.000	1.053	1.030-1.077
Number of negative LNs	-0.045	19.69	0.000	0.956	0.938-0.975

<sup>1</sup>Obtained by using number of negative LNs as the variable instead of number of total LNs.

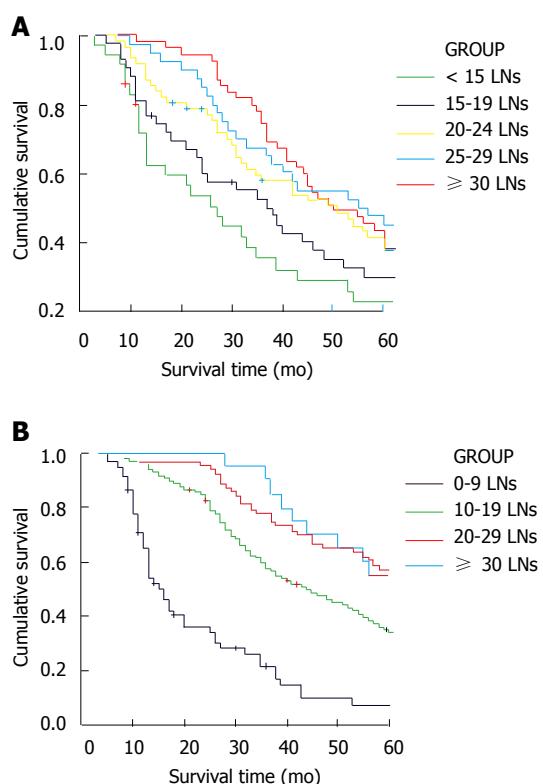
Borrmann type ( $P = 0.373$ ), histological grade ( $P = 0.132$ ), and type of gastrectomy ( $P = 0.093$ ) all failed to retain significance levels in this model. The risk ratios and 95% confidence intervals are listed in Table 2. The number of negative LNs and the total number of LNs examined behaved interchangeably and maintained a similar significance level when substituted for each other. This suggests that the number of negative LN can reflect the extent of lymphadenectomy, as can the total number of LNs.

### Impact of total LN counts by univariate survival analysis

When five-year overall survival was compared by increasing total LN count, higher LN counts were generally associated with better survival (Figure 1A). Of the entire cohort, for the T4, N2 and N3 categories, the best survival outcomes were observed with total LN counts no less than 30. For the N1 category, the best survival outcomes were observed with total LN counts between 25 and 29, as shown in Table 3. When adjusted for the T category, the survival rate of the 25-29 LNs group was higher than that of the < 15 ( $\chi^2 = 13.41$ ,  $P = 0.0002$ ), 15-19 ( $\chi^2 = 7.06$ ,  $P = 0.008$ ) and 20-24 LNs groups ( $\chi^2 = 5.69$ ,  $P = 0.017$ ). The survival rate of the ≥ 30 LNs group was higher than that of the < 15 ( $\chi^2 = 7.03$ ,  $P = 0.008$ ), and the 15-19 LNs groups ( $\chi^2 = 4.91$ ,  $P = 0.03$ ). When adjusted for the N category, the survival rate of the 25-29 LNs group was higher than that of 15-19 ( $\chi^2 = 9.67$ ,  $P = 0.002$ ) and 20-24 LNs groups ( $\chi^2 = 5.68$ ,  $P = 0.02$ ); the survival rate of the ≥ 30 LNs group was higher than that of the 15-19 ( $\chi^2 = 6.56$ ,  $P = 0.01$ ) and 20-24 LNs groups ( $\chi^2 = 4.56$ ,  $P = 0.03$ ).

### Cut point analysis of survival differences relating to total LNs dissected

In an attempt to identify the optimal total LN count cutoff, survival comparisons were made for all stage



**Figure 1** A: Five-year survival curve for all patients according to five total LN strata; B: Five-year survival curve for all patients according to four different negative LN strata.

groups at increasing total LN counts between 7 and 35. The greatest discrepancies, as measured by the chi-square test, were stage dependent, and varied from the cutoff levels of 20 (stage II), 25 (stage III), and 30 (stage IV) to the cutoff level of 15 (entire cohort), as listed in Table 4.

### Impact of negative LN counts by univariate survival analysis

The five-year survival, based on categories, showed considerable variations with increasing counts of negative LNs. An obvious trend toward better survival results for higher negative LN counts was observed (Table 5). Figure 1B shows the overall survival curve for all patients according to different negative LN strata. When adjusted for T category, the survival rate of the 20-29 LNs group was higher than that of the 10-19 LNs group ( $\chi^2 = 10.51$ ,  $P = 0.0012$ ); when adjusted for N category, the survival rate of the 20-29 LNs group was higher than that of either the 0-9 ( $\chi^2 = 14.99$ ,  $P = 0.0001$ ) or the 10-19 LNs group ( $\chi^2 = 5.23$ ,  $P = 0.02$ ); the survival rate of the 10-19 LNs group was higher than that of the 0-9 LNs group ( $\chi^2 = 19.05$ ,  $P < 0.0001$ ).

### Projected impact of negative LN counts on overall survival

Based on the statistical linearity regression, the impact of negative LN counts on overall survival was analyzed. For the stage II subgroup, the hypothetical baseline five-year survival (based on the y-intercept, i.e. no negative

**Table 3** Five-year overall survival by stage subgroups and total number of LNs dissected

	< 15		15-19		20-24		25-29		≥ 30		P
	n	OS (%)	n	OS (%)	n	OS (%)	n	OS (%)	n	OS (%)	
<b>pTcategory</b>											
T2	4	25.0	8	50.0	9	55.6	3	33.3	1	0	0.2612
T3	20	13.8	23	22.6	25	45.1	24	37.0	26	48.5	0.6093
T4	12	4.6	12	7.5	28	13.5	13	23.1	28	37.5	0.0015
<b>pNcategory</b>											
N0	8	15.0	10	41.7	11	60.0	8	62.5	5	40.0	0.2487
N1	16	8.7	15	13.3	24	37.8	15	52.9	27	40.7	0.0005
N2	12	7.7	17	8.3	19	12.5	10	25.0	10	30.0	0.0034
N3	NA		1	0	8	0	7	0	13	7.6	0.0016
Total	36	19.4	43	25.0	62	37.4	40	45.0	55	38.2	0.0009

NA: Not applicable; OS: Overall survival rate.

**Table 4** Overall survival by total LN count, each stage subgroup cut point analysis

Total LN count	Stage II		Stage III		Stage IV		Total	
	$\chi^2$	P	$\chi^2$	P	$\chi^2$	P	$\chi^2$	P
7-14 vs ≥ 15	0.17	0.6842	20.04	< 0.0001	4.36	0.0369	9.23	0.0024
≤ 19 vs ≥ 20	5.22	0.0136	18.46	< 0.0001	8.70	0.0032	2.15	0.0425
≤ 24 vs ≥ 25	0.27	0.6051	22.05	< 0.0001	9.07	0.0026	6.49	0.0109
≤ 29 vs ≥ 30	1.20	0.2726	8.70	0.0032	13.70	0.0002	5.89	0.0153

LN) was 39.6%. Similarly, for the stage III subgroup, the baseline five-year survival (with an assumed zero negative LNs) was 15.4%. For the stage IV subgroup, the baseline five-year survival (also with an assumed zero negative LNs) was 2.5%. For all patients, the calculated five-year survival rate at baseline was 11.2%. For every ten extra LNs added to the negative LN count, the calculated overall survival increased by 5.77% (stage II), 6.09% (stage III), 7.65% (stage IV) or 6.24% (the entire cohort). In this setting, the regression showed a statistically significant survival improvement based on increasing negative LN number only for patients with stages III ( $P = 0.013$ ) and stages IV ( $P = 0.035$ ). The results for stage II had no statistical significance ( $P = 0.195$ ).

## DISCUSSION

Over the last few decades, an increase in the incidence of upper third gastric cancer has been reported by many investigators around the world<sup>[11-13]</sup>. It is generally accepted that the prognosis of patients with this type of carcinoma is worse than for tumors in other parts of the stomach<sup>[14,15]</sup>. The only potentially curative treatment for this disease is complete surgical resection, with an en bloc LN dissection. The D2 radical resection has been regarded as the standard surgical procedure for advanced gastric cancer<sup>[16]</sup>. Lymph node dissection during the D2 radical resection is not confined to the anatomical extent, but also to the number of LNs dissected. The current edition of the UICC staging manual recommends examining at least 15 LNs for adequate gastric cancer staging. However, is that adequate for advanced gastric cancer located in the

**Table 5** Overall five-year survival by stage subgroup and number of negative LNs

	0-9		10-19		20-29		≥ 30		Log-rank P value
	n	OS (%)	n	OS (%)	n	OS (%)	n	OS (%)	
<b>pTcategory</b>									
T2	4	0	15	47.1	6	83.3	NA		0.4907
T3	23	6.7	46	38.2	37	51.4	12	58.3	< 0.0001
T4	35	3.6	32	12.0	20	40.0	6	16.7	< 0.0001
<b>pNcategory</b>									
N0	1	0	19	65.8	18	66.7	4	40.0	0.0317
N1	12	15.2	37	24.5	34	47.1	14	42.9	0.0328
N2	30	5.1	28	22.4	9	33.3	1	0	0.0055
N3	18	0	9	11.1	2	50.0	NA		0.0114
Total	61	4.8	93	33.8	63	53.9	19	45.0	< 0.0001

NA: Not applicable; OS: Overall survival rate.

cardia and fundus? It was reported that patients with advanced gastric cancer located in the cardia and fundus often showed a higher frequency of perigastric LNs and higher proportion of overall LN metastasis<sup>[17]</sup>. Koufuji *et al*<sup>[18]</sup> investigated 49 cases with an upper gastric cancer invading the esophagus who underwent surgical treatment. The incidences of LN metastasis were 0% for T1, 67% for T2, 81% for T3 and 80% for T4. Ichikura *et al*<sup>[19]</sup> retrospectively analyzed 65 cases with cardiac carcinoma and found that the incidences of LN metastasis were 68% for Siewert's type II tumors, and 94% for Siewert's type III tumors. In the present study, 82.2% of patients had LN metastasis. For these patients, it is better to determine the extent of lymphadenectomy according to the extent of LN metastases preoperatively or intraoperatively. However, this is difficult to achieve as there is lack of reliable measures for clinical diagnosis preoperatively and for comprehensive LN biopsy intraoperatively<sup>[20,21]</sup>. If the LNs were not completely removed, the probability of residual tumor cells would increase, leading to poor prognosis. Therefore, some investigators advocated removing adequate LNs intraoperatively to avoid this situation<sup>[22-24]</sup>. However, it is not certain how many LNs need to be dissected in a D2 lymphadenectomy. Liu *et al*<sup>[25]</sup> showed, in 147 patients with adenocarcinoma of the stomach who had undergone gastrectomy with curative intent, that for stage III disease, removal of >15 LNs

appeared to provide a considerable survival advantage, compared with removal of < 15 LNs. Another study favoring this opinion was from Barbour *et al*<sup>[26]</sup>. They suggested that patients with Siewert type II and III adenocarcinoma of the gastroesophageal junction (GEJ) should undergo adequate lymphadenectomy to permit examination of ≥ 15 LNs, allowing accurate identification of prognostic variables. Removal of ≥ 15 LNs is associated with more accurate survival estimates for patients with advanced disease. For adenocarcinoma of the GEJ, a minimum removal of 25 LNs was recommended by Gee *et al*<sup>[27]</sup>. Our present observations showed that better long-term survival outcomes were obtained with higher numbers of LNs resected during D2 lymphadenectomy. This is consistent with the research results of Schwarz *et al*<sup>[28]</sup>. We suggest that for adequate LN resection, including total LNs and the number of negative LNs, the removal of 20 LNs for stage II, 25 LNs for stage III, 30 LNs for stage IV and 15 LNs for the entire cohort be recommended during D2 radical dissection.

Total LNs and number of negative LNs can reflect the extent of LN dissection and influence survival<sup>[29,30]</sup>. In the present study, total LN count or negative LN counts turned out to be independent protective factors, according to the symbols of regression coefficient. These two factors behaved interchangeably, and maintained a similar significance level when substituted for each other. Furthermore, an impact of total LNs or negative LNs on survival was observed. In the univariate survival analysis, higher total LN counts may translate into better outcomes. The effect was much more significant with T4 and N2, N3 disease. We thus postulate that even in locally advanced disease with adjacent organ invasion or advanced nodal involvement that is still resectable, adequate LN dissection influences survival.

The contribution of negative LN counts to the prognosis of patients is partly due to LN micrometastases. In patients without lymph metastases identified by HE staining, about 20% had LN micrometastases<sup>[31]</sup>. del Casar *et al*<sup>[32]</sup> reviewed 144 patients with primary gastric adenocarcinoma who underwent surgery and found that lymphatic and/or blood vessel tumoral invasion (LBVI) was present in 46 patients (31.9%), which was significantly associated with a poorer overall patients' survival. Therefore, curability was reported as one of the most reliable predictors of long-term survival for LN-negative gastric carcinoma patients<sup>[33]</sup>. A similar study<sup>[34]</sup> examined the impact of the number of negative LNs on survival. The study was conducted in patients with stage III colon cancer, and demonstrated that a higher number of negative nodes was associated with better survival. In our study, the impact of negative LN on survival in these stage patients also showed an obvious trend toward better survival for higher negative LN counts. For every ten extra LNs added to the negative LN count, the calculated overall survival increased by 6.09% for stage III, and 7.65% for stage IV patients, based on the linear regression. Generally, better long term survival was observed with higher total LNs

or negative LNs, showing the contribution of sufficient lymphadenectomy toward reducing residual tumor cells. For the curative-intent gastrectomy of locally advanced disease, retrieval and examination of adequate numbers of LNs is suggested for gastric cancer located in the cardia and fundus.

## COMMENTS

### Background

The incidence rates of upper third gastric cancer, mostly located in the cardia and fundus, has increased in recent years. Few studies have investigated the relative contributions of both the number of total resected lymph nodes (LNs) and the number of negative LNs to the outcome of patients with advanced gastric cancer located in the cardia and fundus.

### Research frontiers

Some researches have considered D2 radical dissection to be standard procedure for advanced gastric cancer located the cardia and fundus, which requires the removal of Group 1 and Group 2 LNs. The dissected LN count is found to have strong association with patient survival.

### Innovations and breakthroughs

The authors retrospectively reviewed 236 patients with gastric cancer in the cardia and fundus who were treated with D2 radical resection in a hospital in Fujian between 1996 and 2002 to investigate how the numbers of dissected LNs affect patients' survival outcomes.

### Applications

The authors suggest that importance should be attached to removing enough LNs so as to reduce residual tumor cells in patients with advanced gastric cancer located in the cardia and fundus.

### Peer review

This article demonstrated that in advanced gastric cancer located in the cardia and fundus, the number of lymphadenectomy with/without metastasis of gastric cancer cells influenced the prognosis of the patients. This finding has important information for gastrointestinal surgeons.

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RAPID COMMUNICATION

# Predictive factors for lymph node metastasis in poorly differentiated early gastric cancer and their impact on the surgical strategy

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have metastasis, 29 (65.9%) and 15 (34.1%) of the lymph nodes involved were within N1 and beyond N1, respectively, in 12 patients with LNM.

**CONCLUSION:** Endoscopic mucosal resection alone may be sufficient to treat poorly differentiated intramucosal EGC ( $\leq 2.0$  cm in diameter) with no histologically-confirmed lymphatic vessel involvement. When lymphatic vessels are involved, lymph node dissection beyond limited (D1) dissection or D1+ lymph node dissection should be performed depending on the tumor location.

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**Key words:** Poorly differentiated early gastric cancer; Lymph node metastasis; Clinicopathological characteristics; Endoscopic mucosal resection

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## Abstract

**AIM:** To identify the predictive clinicopathological factors for lymph node metastasis (LNM) in poorly differentiated early gastric cancer (EGC) and to further expand the possibility of using endoscopic mucosal resection (EMR) for the treatment of poorly differentiated EGC.

**METHODS:** Data were collected from 85 poorly-differentiated EGC patients who were surgically treated. Association between the clinicopathological factors and the presence of LNM was retrospectively analyzed by univariate and multivariate logistic regression analyses.

**RESULTS:** Univariate analysis showed that tumor size (OR = 5.814, 95% CI = 1.050 - 32.172,  $P = 0.044$ ), depth of invasion (OR = 10.763, 95% CI = 1.259 - 92.026,  $P = 0.030$ ) and lymphatic vessel involvement (OR = 61.697, 95% CI = 2.144 - 175.485,  $P = 0.007$ ) were the significant and independent risk factors for LNM. The LNM rate was 5.4%, 42.9% and 50%, respectively, in poorly differentiated EGC patients with one, two and three of the risk factors, respectively. No LNM was found in 25 patients without the three risk factors. Forty-four lymph nodes were found to

## INTRODUCTION

Endoscopic mucosal resection (EMR) is widely accepted as an alternative treatment for early gastric cancer (EGC)<sup>[1-3]</sup>. The application of EMR has been limited to differentiated EGC because of the higher risk of lymph node metastasis (LNM) in undifferentiated EGC, compared to differentiated EGC<sup>[4-6]</sup>. Therefore, gastrectomy with lymphadenectomy is considered an essential treatment for patients with undifferentiated EGC. Undifferentiated gastric cancers include poorly differentiated adenocarcinoma, signet ring cell carcinoma, and mucinous adenocarcinoma<sup>[7]</sup>. However, almost all (96.6%) surgical cases of poorly differentiated EGC confined to the mucosa

have no LNM<sup>[8]</sup>, suggesting that gastrectomy with lymphadenectomy may be an overtreatment for these cases.

We carried out this retrospectively study to determine the predictive clinicopathological factors for LNM in poorly differentiated EGC. Furthermore, we established a simple criterion for the use of EMR in the treatment of poorly differentiated EGC.

## MATERIALS AND METHODS

### **Patients**

Patients who underwent a radical operation due to EGC in Department of Oncology, First Affiliated Hospital of China Medical University (Shenyang, China) between January 1980 and December 2002, were included in this retrospective study.

The inclusion criteria were (1) possible lymph node dissection beyond limited (D1) dissection (i.e. D1 dissection + dissection of lymph nodes along the left gastric artery, D1 dissection + dissection of lymph nodes along the common hepatic artery, D1 dissection + dissection of lymph nodes along the celiac artery) or extended (D2) dissection, (2) pathological analysis of the resected specimens and lymph nodes and poorly differentiated EGC diagnosed according to the Japanese classification of gastric carcinoma (JCGC)<sup>[7]</sup>, and (3) availability of medical record of patients from the database.

During the past 23 years, operation was performed in 243 EGC patients. Of the 115 patients who were histologically diagnosed as undifferentiated EGC, 87 had poorly differentiated EGC and 28 had undifferentiated EGC. Among the 87 patients with poorly differentiated EGC, complete medical record of 2 patients was not available. Thus, 85 poorly differentiated EGC patients (60 males, 25 females, mean age of 54 years, range 19-78 years) met the inclusion criteria for further analysis.

The study protocol was approved by the Ethics Committee of China Medical University.

### **Dissection and classification of lymph nodes**

Lymph nodes were meticulously dissected from the *en bloc* specimens, and the dissected lymph nodes by a surgeon after he/she carefully reviewed the excised specimens based on the JCGC<sup>[7]</sup>. Briefly, lymph nodes were classified into two groups: group 1 as the perigastric lymph nodes and group 2 as the lymph nodes along the left gastric artery, the common hepatic artery, and the splenic artery or around the celiac axis<sup>[7]</sup>.

### **Classification of lymphadenectomy**

Accordingly, lymphadenectomy was classified as D1 (i.e. dissection of all the group 1 lymph nodes, D1 + (i.e. dissection of all the lymph nodes along the left gastric artery, lymph nodes along the common hepatic artery, or lymph nodes along the celiac artery), and

**Table 1 Distribution of metastatic lymph nodes**

Lymph node station	Tumor location in the stomach			
	Upper	Middle	Lower	Overall
Group 1 (N1)	1	3	25	29
No. 1, right paracardial nodes	1	1	0	2
No. 2, left paracardial nodes	0	0	0	0
No. 3, lesser curvature nodes	0	1	5	6
No. 4, great curvature nodes	0	1	5	6
No. 5, suprapyloric nodes	0	0	0	0
No. 6, infrapyloric nodes	0	0	15	15
Group 2 (N2)	0	3	12	15
No. 7, left gastric artery nodes	0	3	8	11
No. 8, common hepatic nodes	0	0	3	3
No. 9, celiac artery nodes	0	0	1	1

D2 (i.e. dissection of all the lymph nodes in groups 1 and 2).

### **Assessment and classification of LNM**

The resected lymph nodes were cut into sections which were stained with hematoxylin and eosin, and examined by pathologists for metastasis and lymphatic vessel involvement (LVI).

For classification of LNM, the symbol “No.” was used to indicate the lymph node station number while “N” was used to indicate the lymph node group. For example, No. 1 indicates the right paracardial lymph nodes (Table 1). No. indicates that there was no evidence of LNM. N1 indicates that metastasis was limited to lymph nodes in group 1. N2 indicates that metastasis extended to group 2 lymph nodes (Table 1).

### **Association between clinicopathological parameters and LNM**

The following clinicopathological parameters covered in the JCGC<sup>[7]</sup> were included in this study, namely the gender (male and female), age (< 60 years, ≥ 60 years), family medical history of gastric cancer, number of tumors (single or multitude), tumor location (upper, middle, or lower of the stomach), tumor size (maximum diameter ≤ 2 cm, or > 2 cm), macroscopic types including protruded (type I), superficially elevated (type II a), flat (type II b), superficially depressed (type II c), or excavated (type III), depth of invasion (mucosa, submucosa), lymphatic vessel involvement.

The associations between various clinicopathological factors and LNM was examined as described below.

### **Statistical analysis**

All data were analyzed using SPSS13.0 statistical software (Chicago, IL, United States). Differences in the clinicopathological parameters between patients with and without LNM were determined with chi-square test. Multivariate stepwise logistic regression analysis was performed subsequently in order to identify the independent risk factors for LNM. Hazard ratio and 95% confidence interval (CI) were calculated.  $P < 0.05$  was considered statistically significant.

**Table 2 Univariate analysis of potential risk characteristics for lymph node metastasis**

Factor	Lymph node metastasis n (%)	P
Sex		
Male (n = 60)	7 (11.7)	0.315
Female (n = 25)	5 (20)	
Age (yr)		
< 60 (n = 53)	9 (17.0)	0.329
≥ 60 (n = 32)	3 (9.4)	
Family medical history		
Positive (n = 6)	1 (16.7)	0.852
Negative (n = 79)	11 (13.9)	
Number of tumors		
Single (n = 82)	12 (14.6)	0.475
Multitude (n = 3)	0 (0)	
Location		
Upper (n = 5)	1 (20.0)	0.925
Middle (n = 14)	2 (14.3)	
Lower (n = 66)	9 (13.6)	
Tumor size in diameter		
≤ 2 cm (n = 45)	2 (5.1)	0.007
> 2 cm (n = 40)	10 (21.7)	
Macroscopic type		
I (n = 2)	1 (50.0)	0.183
II (n = 70)	8 (11.4)	
III (n = 13)	3 (23.1)	
Depth of invasion		
Mucosa (n = 44)	2 (4.5)	0.009
Submucosa (n = 41)	10 (24.4)	
Lymphatic vessel involvement		
Positive (n = 4)	3 (75.0)	< 0.001
Negative (n = 81)	9 (11.1)	

## RESULTS

### Distribution of metastatic lymph nodes

LNM was histologically confirmed in 12 (14.1%) of the 85 patients. Forty-four metastatic lymph nodes were found in these 12 patients, of which 29 (65.9%) were classified as N1 and 15 (34.1%) as N2. LNM was located in the upper stomach of 1 patient and the observed metastatic lymph nodes belonged to N1. LNM was located in the middle stomach of 2 patients. Of the six observed metastatic lymph nodes, three (50%) belonged to N1 and three (50%) belonged to N2. Of the 37 metastatic lymph nodes observed in the lower stomach of 9 LNM patients, 25 (67.6%) belonged to N1 and 12 (32.4%) belonged to N2 (Table 1).

### Association between clinicopathological factors and LNM

The association between various clinicopathological factors and LNM was first analyzed with chi-square test (Table 2). Tumor larger than 2.0 cm in diameter ( $P = 0.007$ ), submucosal invasion ( $P = 0.009$ ), and the presence of LVI ( $P < 0.001$ ) were significantly associated with a higher incidence rate of LNM.

However, gender, age, family medical history of gastric cancer, number, location, and macroscopic type were not associated with LNM.

### Multivariate analysis of potential independent risk factors for LNM

Univariate analysis showed that tumor size (OR= 5.814,

95% CI = 1.050 - 32.172,  $P = 0.044$ ), depth of invasion (OR = 10.763, 95% CI = 1.259 - 92.026,  $P = 0.030$ ) and LVI (OR = 61.697, 95% CI = 2.144 - 175.485,  $P = 0.007$ ) were significantly associated with LNM, while multivariate analysis revealed that they were the significant and independent risk factors for LNM.

### Lymph node metastasis of poorly differentiated EGC

The LNM rate was 5.4%, 42.9% and 50%, respectively, in poorly differentiated EGC patients with one, two and three of the risk factors, respectively. No LNM was detected in 25 patients without the three risk factors.

## DISCUSSION

One of the critical factors in choosing EMR for EGC would be the precise prediction of whether the patient has LNM. To achieve this goal, several studies have attempted to identify the predictive risk factors for LNM of EGC<sup>[9-11]</sup>. Few reports, however, have focused on the applicability of endoscopic treatment for poorly differentiated EGC<sup>[12,13]</sup>.

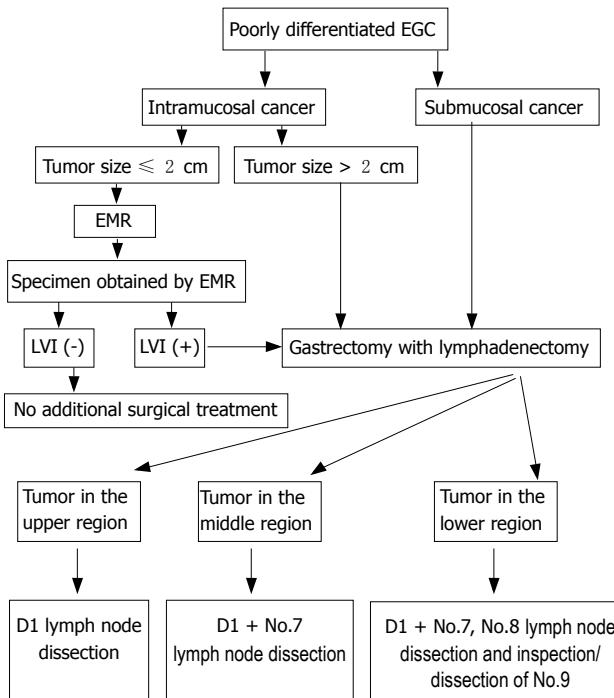
In the present study, multivariate analysis revealed that tumor larger than 2.0 cm in diameter, submucosal invasion, and the presence of LVI were the significant predictive factors for LNM in patients with poorly differentiated EGC, which is in consistent the reported findings<sup>[9-13]</sup>.

We then attempted to identify a subgroup of poorly differentiated EGC patients in whom the risk of LNM could be ruled out. As a result, no LNM was found in patients with intramucosal cancer if the tumor is less than or equal to 2.0 cm in diameter without LVI, indicating that EMR is sufficient to treat these patients.

We further examined the relationship between the three predictive factors and the LNM rate in order to establish a simple criterion for the treatment of poorly differentiated EGC. In the present study, the LNM rate was 5.4%, 42.9% and 50%, respectively, in poorly differentiated EGC patients with one, two and three of the risk factors, suggesting that gastrectomy with lymphadenectomy can be performed for these patients with the risk factors.

All the information on these predictive factors, particularly LVI, became evident after histological assessment of the entire specimen. Thus, an accurate histological examination of the endoscopically resected specimen was required to determine whether EMR alone can achieve curative effect. The new EMR technique, complete removal of a large lesion in a single fragment using an insulation-tipped diathermic knife<sup>[14-16]</sup>, is a promising procedure for this purpose.

According to the results of this study, a treatment strategy was proposed for the poorly differentiated EGC patients (Figure 1). EMR alone may be sufficient to treat poorly differentiated intramucosal EGC if it is less than or equal to 2.0 cm in diameter with no LVI. When LVI is found in specimens, an additional gastrectomy with lymphadenectomy should be



**Figure 1** Flow chart of the therapeutic strategy for patients with poorly differentiated EGC.

recommended, with the tumor location taken into consideration. For example, for patients with EGC in the upper stomach, D1 should be performed. However, for patients with EGC in the middle stomach, No.7 lymph nodes should be dissected. For patients with EGC in the lower stomach, No.7 and No.8 lymph nodes should be dissected and No.9 lymph node should be inspected carefully and dissected if LNM is suspected.

In conclusion, EMR alone may be sufficient to treat poorly differentiated intramucosal EGC if it is less than or equal to 2.0 cm in diameter with no LVI. When LVI is found in specimens, D1 or D1+ lymph node dissection should be performed depending on the tumor location.

## COMMENTS

### Background

Gastrectomy with lymphadenectomy is the standard therapy for poorly differentiated early gastric cancer (EGC) with lymph node metastasis (LNM). However, because approximately 96.6% of poorly differentiated intramucosal EGC have no LNM, gastrectomy with lymphadenectomy may be an overtreatment for such patients. We attempted to identify a subgroup of poorly differentiated EGC patients in whom the risk of LNM can be ruled out and treated with endoscopic mucosal resection, which may serve as a breakthrough management of poorly differentiated EGC.

### Research frontiers

Many clinicians and researchers are undertaking studies of the predictive factors for LNM in EGC. It is widely accepted that lymph node involvement and depth of invasion are closely correlated with LNM. However, the relationship between metastasis and diameter of tumor, histological type and macroscopic type is still controversial. Since LNM remains one of the most important predictors for survival, reduction in lymphadenectomy

will probably result in residue of metastatic lymph nodes. Unnecessarily extended resection will induce complications, thus resulting in a poor quality of life.

### Innovations and breakthroughs

Tumor size, depth of invasion, and lymphatic vessel involvement are the independent risk factors for LNM. Lymph node dissection is the treatment of choice for poorly differentiated EGC.

### Applications

Based on the predictive factors for LNM, Lymph node dissection is the treatment of choice for poorly differentiated EGC.

### Peer review

This manuscript presents a rational surgical therapy for poorly differentiated EGC with lymph node dissection and is of interest to clinicians and researchers.

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RAPID COMMUNICATION

# Mechanisms underlying aspirin-mediated growth inhibition and apoptosis induction of cyclooxygenase-2 negative colon cancer cell line SW480

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**Author contributions:** Lai MY and Huang JA designed the research; Lai MY, Liang ZH and Jiang HX performed the research; Huang JA contributed to the new reagents/analytic tools; Liang ZH and Jiang HX analyzed the data; Lai MY and Tang GD wrote the paper.

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expression in cells, but the expression of *Bax* was down regulated.

**CONCLUSION:** Aspirin inhibits proliferation and induces apoptosis of SW480 cells. Its anti-tumor mechanism may arrest cell cycle and shift *Bax/Bcl-2* balance in cells.

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**Key words:** Aspirin; Colorectal cancer; Proliferation; Apoptosis

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Lai MY, Huang JA, Liang ZH, Jiang HX, Tang GD. Mechanisms underlying aspirin-mediated growth inhibition and apoptosis induction of cyclooxygenase-2 negative colon cancer cell line SW480. *World J Gastroenterol* 2008; 14(26): 4227-4233 Available from: URL: <http://www.wjnet.com/1007-9327/14/4227.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4227>

## Abstract

**AIM:** To investigate the effects of aspirin (acetylsalicylic acid) on proliferation and apoptosis of colorectal cancer cell line SW480 and its mechanism.

**METHODS:** Cyclooxygenase (COX)-2 negative colorectal cancer cell line SW480 was treated with aspirin at concentrations of 2.5 mmol/L, 5.0 mmol/L, 10.0 mmol/L for different periods *in vitro*. Anti-proliferation effect of aspirin on SW480 was detected by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell cycle and apoptosis were observed by flow cytometry (FCM). Transmission electron microscope (TEM) was used for morphological study. Apoptosis-associated genes were detected by immunohistochemical staining and Western blotting.

**RESULTS:** Aspirin inhibited SW480 proliferation and induced apoptosis in a dose- and time-dependent manner. Treatment with different concentrations of aspirin significantly increased the proportions of cells at the G<sub>0</sub>/G<sub>1</sub> phase and decreased the proportions of cells at the S- and G<sub>2</sub>/M phases in a concentration-dependent manner. Aspirin not only induced apoptosis but also caused cell necrosis at a high concentration as well. After treatment with aspirin, SW480 cells displayed typically morphological features of apoptosis and necrosis under TEM, and increased the *Bcl-2*

## INTRODUCTION

Colorectal cancer is a common disease that remains the major cause of cancer-related mortality in developed countries. With improvement in economic status, its incidence increases in developing countries including China and severely threatens the health of human beings. Increasing evidence from human epidemiological studies, animal models, and experiments *in vitro* revealed that administration of nonsteroidal anti-inflammatory drugs (NSAIDs) including aspirin (acetylsalicylic acid) represents a treatment of choice for colon cancer<sup>[1-3]</sup>. Data indicate that use of NSAIDs, including aspirin, is inversely associated with the risk of colorectal cancer, and clinical trials in patients with familial adenomatous polyposis showed that use of NSAIDs can lead to the regression of colorectal adenomas by exerting their protective effects on the stomach and esophagus<sup>[4]</sup>. However, the molecular mechanism by which aspirin exhibits its anticancer effects is not completely clear.

The best-defined molecular target for aspirin and

other NSAIDs is cyclooxygenase (COX). COX-2, the regulated isoform of COX, plays an important role in the carcinogenesis<sup>[5]</sup> and angiogenesis<sup>[6]</sup>. A possible mechanism underlying the antitumor properties of aspirin has been ascribed to its direct inhibition of COX-2 in colorectal cancer tissue<sup>[7]</sup>. However, several lines of evidence suggest that the wide range of antiproliferative potencies of aspirin does not correlate exclusively with COX-2 inhibitory activity, because NSAIDs can induce apoptosis in colon cancer cell lines that lack detectable expression of COX-2 protein<sup>[8,9]</sup>. Until now, most researchers recruit COX-2 positive colon cancer cell lines to study the effect of aspirin on their apoptosis and proliferation. However, few studies of the effect of aspirin on COX-2 negative colon cancer cells and few systematic analyses of aspirin-induced morphologic changes are available<sup>[10-12]</sup>. Furthermore, the results of certain studies are controversial<sup>[12,13]</sup>. The purpose of this study was to investigate the effect of aspirin on proliferation, and apoptosis of SW480 cells, a COX-2 negative human colon cancer cell line.

## MATERIALS AND METHODS

### Materials

Aspirin, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), penicillin/streptomycin and RNase A were from Sigma (St. Louis, MO, USA). Mouse monoclonal antibodies against Bcl-2 and Bax as well as biotin-labeled anti-mouse IgG, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). RPMI1640 medium was purchased from Life Technologies, Inc (Grand Island, NY) and fetal bovine serum was from Gibco (GIBCO BRL). Annexin V-Cy5 apoptosis detection kit was purchased from PharMingen (San Diego, CA).

### Cell culture and culture conditions

The human colon cancer cell line SW480 was obtained from the American Type Culture Collection and cultured in RPMI 1640 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin solution at 37°C in a humidified atmosphere containing 50 mL/L CO<sub>2</sub>.

### Aspirin treatment

We dissolved aspirin in 1 mol/L Tris-HCl (pH 7.5) to a stock concentration of 1 mol/L and adjusted the pH to 7.2 with 4 mol/L HCl. Twenty-four hours before aspirin treatment, exponentially growing human colon cancer cells were seeded at a density of 10<sup>5</sup> cells/100 mm culture dish (Becton Dickinson, Franklin Lakes, NJ). The cells were washed once with phosphate buffered saline (PBS) and treated for an indicated time by adding various volumes of stock to obtain the final aspirin concentrations of 1 mmol/L, 2.5 mmol/L, 5 mmol/L, or 10 mmol/L, respectively. Control cells were treated with an equivalent volume of Tris-HCl (pH 7.2).

### Cell proliferation analysis

SW480 colon cancer cells were seeded at a density of

3 × 10<sup>4</sup> cells/well onto a 96-well plate. Twenty-four hours after seeding, the cells were exposed to different concentrations of aspirin (0 mmol/L, 2.5 mmol/L, 5 mmol/L, 10 mmol/L) and maintained in culture for 1-7 d at 37°C in a humidified atmosphere containing 50 mL/L CO<sub>2</sub>. Individual cells were taken from suspension after exposure to trypsin/EDTA in PBS. Aliquots were counted with a haemocytometer and tested for their viability by trypan blue exclusion. Each assay was performed in triplicate.

### MTT assay

The effect of aspirin on cellular viability was also evaluated by MTT assay. SW480 cells were plated in 96-well microtiter plates at a density of 10<sup>4</sup> cells/well in a final volume of 100 μL of RPMI 1640 medium. Twenty-four hours after the initial seeding, the cells were treated with various concentrations of aspirin for 1-7 d. The untreated cells (appropriate volume of buffer solution was added) served as controls. After treatment, the cells were incubated for 3 h at 37°C with a solution of MTT at a concentration of 50 μg/100 μL, lysed for 12 h at room temperature in a buffer containing 10% SDS and 0.01 mol/L HCl as previously described<sup>[14]</sup>. For each sample, the absorbance of the reduced intracellular formazan product was read at 570 nm on a microtiter plate reader (Bio-Rad Laboratories, Hercules, CA). Each assay was performed in triplicate.

### Apoptosis determination

SW480 cells were plated at a density of 10<sup>6</sup> cells/10 cm dishes and treated with aspirin at the concentration of 0, 2.5, 5 and 10 mmol/L, respectively. After treatment, the cells were harvested by trypsinization and washed once with 10 mL of PBS. We used the Annexin V-Cy5 apoptosis detection kit according to the manufacturer's instructions to stain the apoptosis marker and cell surface phosphatidylserine. Briefly, the cells were resuspended in 200 μL of 1 × annexin binding buffer containing 10 mmol/L HEPES, 140 mmol/L NaCl, 2.5 mmol/L CaCl<sub>2</sub> (pH 7.4) and stained with 5 μL of annexin V-Cy5 (1 μmol/mL). After incubation for 15 min at room temperature, the cells were incubated with 10 μL of 7-AAD for another 15 min at 25°C in the dark. The cell suspension was gently centrifuged, into which 400 mL of 1 × annexin binding buffer was added. The cells were analyzed by flow cytometry (FCM) within 1 h of 7-AAD staining. Appropriate controls were used to subtract background counts. We used the FACScalibur flow cytometer (Becton Dickinson, San Jose, CA) for two-color analysis of apoptosis. Fluorescence compensation was adjusted to minimize overlap of the Cy5 and 7-AAD signals.

### Cell cycle analysis

The effect of aspirin treatment on cell proliferation was evaluated by measuring the distribution of cells in different phases of the cell cycle by FCM based on the measurement of the DNA content of nuclei

labeled with propidium iodide (PI). Cell suspension from either aspirin-treated or untreated cell cultures was prepared by trypsinizing the cells and washing them twice with cold PBS. The cells were resuspended at a density of  $10^6$  cells/mL in cold PBS and fixed with 75% ethanol overnight at -20°C. Ethanol-fixed cells were washed with cold PBS, resuspended in 300  $\mu$ L of PBS containing 0.15% boiled and renatured RNase A at 37°C for 30 min, and stained with 80  $\mu$ g/mL of PI for 30 min. The cells were analyzed for DNA content with the FACScalibur flow cytometer at an excitation of 488 nm with detection at 620 nm for red fluorescence. The cell cycle data were analyzed using the Multicycle Software Autofit Version 2.50 (Phoenix Flow Systems, San Diego, CA).

#### Transmission electron microscopy

For electron microscopic analysis of apoptosis, pretreated SW480 cells were fixed in 1% glutaraldehyde and 4% paraformaldehyde in PBS, postfixed in 1% osmium tetroxide in PBS, dehydrated and subsequently embedded in epoxy resin. Ultrathin sections (80 nm) were stained with uranyl and lead acetates and examined under a Hitachi H-600 electron microscope at 80 kV (Hitachi, Tokyo, Japan).

#### Immunocytochemical staining

SW480 cells were seeded at a density of  $4 \times 10^5$  cells/well onto 24-well plates and cultured for 24 h. After treatment for 48 h with ASPIRIN at final concentration of 2.5, 5.0 and 10.0 mmol/L, respectively, the untreated cells (appropriate volumes of buffer solution added) served as controls. The cells were fixed for 10 min in 100% methanol at room temperature, washed with PBS, treated with 3% hydrogen peroxide to block the endogenous peroxidase activity and incubated with 2% nonfat dry milk. After incubated for 2 h at room temperature with primary antibody, the cells were washed three times with PBS (10 min each time) and incubated with biotin-labeled anti-mouse IgG for 20 min at room temperature. After washed three times with PBS (10 min each time), the cells were stained using a streptavidin-peroxidase detection system (Novo Castra, Newcastle, UK) and viewed under a Hoffman modulation contrast microscope.

#### Measurement of Bax and Bcl-2 protein levels in cells

Bax, Bcl-2 and  $\beta$ -actin protein levels in cells were measured by Western blotting. Twenty micrograms of protein from a 20% (wt/vol) cell homogenate was separated on a 15% (wt/vol) polyacrylamide denaturing gel, and then electroblotted onto Hybond-enhanced chemiluminescence (ECL) nitrocellulose (Amersham Pharmacia Biotech Ltd). The membranes were blocked by addition of 3% (wt/vol) bovine serum albumin (BSA) into 0.1% (vol/vol) Tween-20 tris-buffered saline (TBS) at 4°C overnight. Western blots were probed with a polyclonal rabbit anti-rat Bax diluted at 1:2000 (P-19) or a monoclonal mouse anti-rat Bcl-2 diluted at 1:2000 at room temperature for 2 h. Primary Bax or Bcl-2

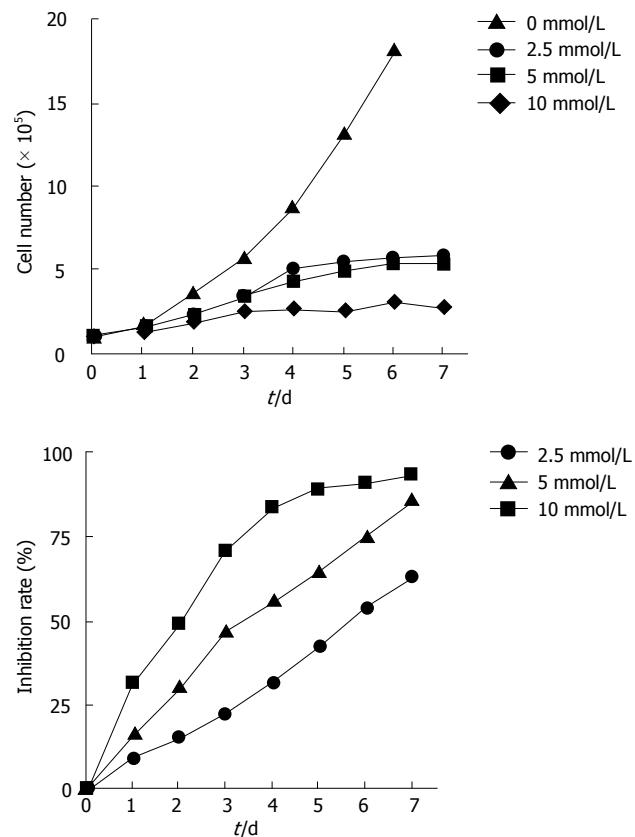


Figure 1 Effect of aspirin on proliferation of SW480 cells.

antibody binding was revealed using an anti-rabbit-HRP or an anti-mouse-HRP antibody diluted at 1:2000 or at 1:1000 for 1 h and the ECL detection system. Developed films were quantitatively analyzed using a volume densitometer and Molecular Analyst version 4 software (Bio-Rad Laboratories Ltd). The Bax to Bcl-2 ratio was determined for each sample individually by reprobing the same membrane and dividing the volume density for Bax by that for Bcl-2.

#### Statistical analyses

Data were expressed as mean  $\pm$  SE. Statistical difference was assessed by a single factor variance (ANOVA) or the Student *t* test. For each test, *P* < 0.05 was considered statistically significant.

## RESULTS

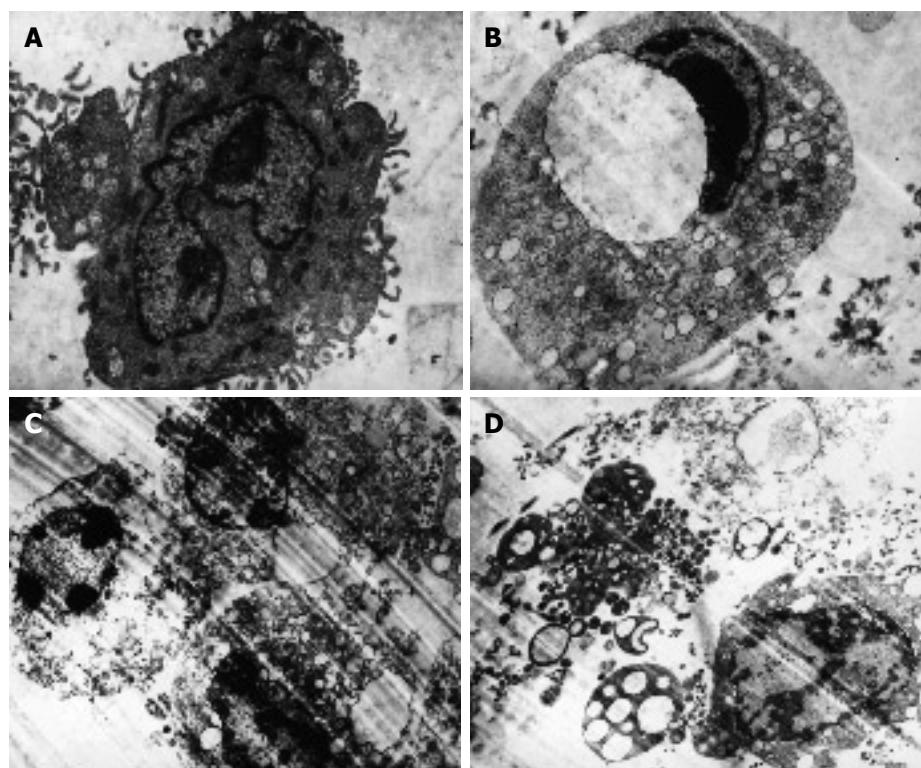
#### Aspirin inhibited the growth of SW480 cells

In the present study, SW480 cell proliferation was assessed by counting the cells after stimulation of aspirin at different concentrations for 7 d. As shown in Figure 1, the untreated cells grew in a linear manner. After aspirin treatment, the growth of SW480 cells was inhibited in a concentration-dependent manner. Moreover, aspirin modulated the growth of SW480 cells in a time-dependent manner. Being consistent with the results of cell counting, aspirin showed an anti-proliferation effect on the growth of SW480 cells in a concentration-and time-dependent manner.

**Table 1** Effect of aspirin on cell cycle distribution in SW480 cells (mean  $\pm$  SE)

Aspirin Conc (mmol/L)	Cell cycle			Proliferation index (%)	Apoptosis rate (%)
	G <sub>0</sub> /G <sub>1</sub> (%)	S (%)	G <sub>2</sub> /M (%)		
0	42.3 $\pm$ 2.49	33.0 $\pm$ 2.16	24.7 $\pm$ 0.47	57.7 $\pm$ 2.49	3.17 $\pm$ 0.37
2.5	61.0 $\pm$ 1.63 <sup>b</sup>	24.3 $\pm$ 1.25 <sup>b</sup>	14.7 $\pm$ 1.70 <sup>b</sup>	39.0 $\pm$ 1.63 <sup>b</sup>	8.92 $\pm$ 0.49 <sup>b</sup>
5	73.3 $\pm$ 3.36 <sup>b</sup>	13.7 $\pm$ 1.24 <sup>b</sup>	13.0 $\pm$ 2.42 <sup>b</sup>	26.6 $\pm$ 3.63 <sup>b</sup>	13.14 $\pm$ 1.29 <sup>b</sup>
10	80.3 $\pm$ 2.49 <sup>b</sup>	8.7 $\pm$ 0.47 <sup>b</sup>	11.0 $\pm$ 2.94 <sup>b</sup>	19.7 $\pm$ 2.49 <sup>b</sup>	37.92 $\pm$ 1.37 <sup>b</sup>

<sup>b</sup>P < 0.01 vs control group.



**Figure 2** Effect of aspirin on morphological change in normal SW480 cells (A), after treatment with aspirin at the concentration of 2.5 mmol/L (B), 5.0 mmol/L (C), and 10.0 mmol/L (D) for 72 h, respectively.

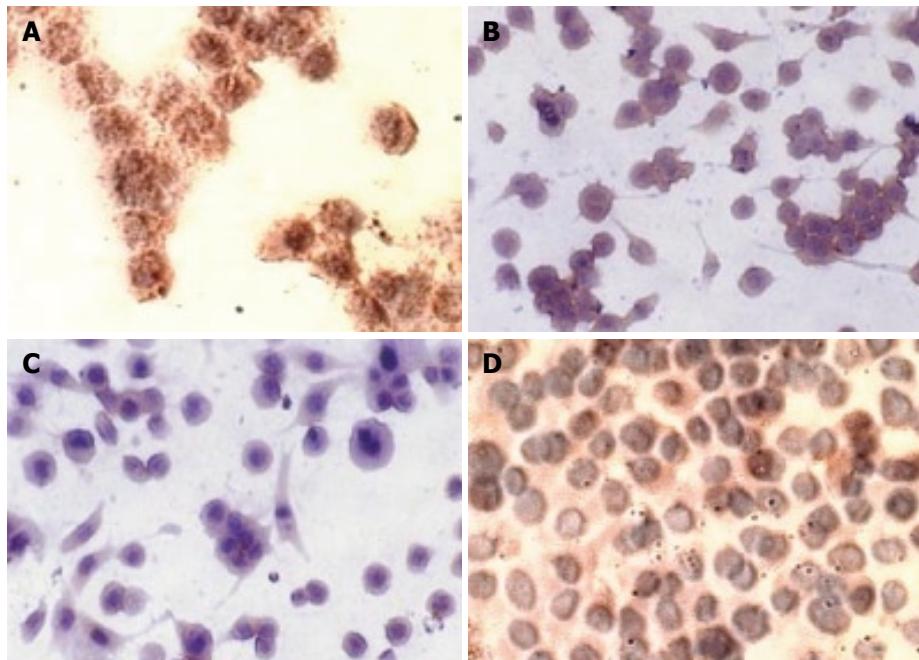
#### Aspirin arrested human colon cancer cell at the G<sub>0</sub>/G<sub>1</sub> phase and increased apoptosis of SW480 cells

To study the effect of aspirin treatment on cell proliferation at different phases of the cell cycle, we treated exponentially growing cells with aspirin at different concentrations for 72 h. Conventional DNA FCM showed that 39%-46% of the untreated SW480 cells were found at the G<sub>0</sub>/G<sub>1</sub> phase, 31%-35% at the S phase, and the remaining cells at the G<sub>2</sub>/M phase, depending on the dose of aspirin used and the percentage of cells decreased (Table 1). However, the presence of aspirin at different concentrations resulted in an accumulation of cells at the G<sub>0</sub>/G<sub>1</sub> phase. The fraction of sub-G<sub>1</sub> DNA content accounted for 3.17%, indicating that apoptosis was decayed in the untreated control cells. After treatment with aspirin at the concentrations of 2.5, 5.0 and 10.0 mmol/L, the fraction of sub-G<sub>1</sub> DNA content increased to 8.9%, 13.1% and 39.9%, respectively, indicating that aspirin could not only arrest cell cycle at the G<sub>0</sub>/G<sub>1</sub> phase, but also induce apoptosis in a concentration-dependent manner (Table 1).

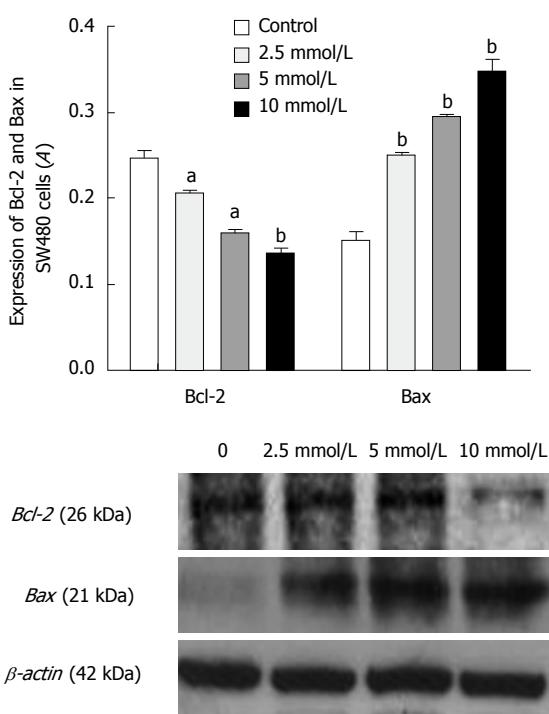
#### Effect of aspirin on morphology of SW480 cells

Electron microscopy of the aspirin-treated SW480

cells showed typical apoptosis characterized by volume reduction, chromatin condensation, nuclear fragmentation, and presence of apoptotic bodies (Figure 2) when compared to the untreated cells (Figure 2A). The severity of morphological changes increased in a dose-dependent manner. After 72 h of treatment with 2.5 mmol/L aspirin, the SW480 cells showed early changes in apoptosis with loss of microvilli, nuclear chromatin condensation that formed a crescent-like clump (Figure 2B). Intermediate and late stages of apoptosis, characterized by more extensive nuclear chromatin condensation, cytoplasmic blebbing, nuclear membrane splitting, fragmentation of nuclei, and formation of apoptotic bodies, were induced by treatment with 5 mmol/L aspirin for 72 h (Figure 2C). Aspirin induced secondary postapoptotic necrosis, defined as degeneration of subcellular organelles and rupture of the plasma membrane despite the presence of apoptotic condensation of chromatin when its concentration was increased to 10 mmol/L. Certain SW480 cells showed the features of oncotic necrosis, such as oncotic nuclear change, disrupted plasma membrane, degeneration of cytoplasmic organelles, swollen mitochondria with amorphous dense bodies (Figure 2D).



**Figure 3** Effect of aspirin on the expression of Bcl-2 in normal SW480 cells (**A, C**) and Bax after treatment with aspirin at the concentration of 5 mmol/L (**B, D**).



**Figure 4** Effect of aspirin on the expression of Bcl-2 and Bax in SW480 cells. The expression of Bcl-2 protein was down-regulated while that of Bax protein was up-regulated in a dose-depend manner. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 vs control group.

#### Aspirin inhibited Bcl-2 and increased Bax expression in SW480 cells

In the untreated SW480 cells, there was a strong cytoplasmic staining for Bcl-2 as detected by immunocytochemistry (Figure 3). After incubation with 5 mmol/L aspirin for 72 h, cytoplasmic Bcl-2 staining was substantially diminished (Figure 3) while immunocytochemistry staining for Bax was significantly increased after treatment with aspirin. Being consistent

with immunocytochemistry staining, the expression of Bcl-2 protein measured by Western blotting, was reduced in aspirin-treated SW480 cells while that of Bax protein was increased, suggesting that aspirin could down-regulate Bcl-2 protein expression and up-regulate Bax protein expression (Figure 4).

#### DISCUSSION

The molecular mechanism underlying the chemopreventive effects of NSAIDs is not well understood. One most widely accepted mechanism underlying the anticancer effect of NSAIDs is the reduced PG synthesis by inhibiting the COX activity. In the present study, we used a COX-2 negative colon cancer cell line SW480 as a model to exclude the pathway of COX-2. Being consistent with previous findings<sup>[10,11,15]</sup>, treatment with aspirin at different concentrations inhibited the proliferation of SW480 cells in a dose- and time-dependent, arrested the cell cycle at the G<sub>0</sub>/G<sub>1</sub> phase, decreased the cell proportions at the S- and G<sub>2</sub>/M- phases in a concentration-dependent manner. SW480 cell apoptosis induced by aspirin was confirmed by flow cytometric analyses of cellular DNA content and transmission electron microscopy (TEM). After treatment with aspirin at the concentration of 2.5–10 mmol/L for 72 h, typical morphological alterations of apoptosis were observed<sup>[16]</sup>, including cytoplasmic blebbing, nuclear membrane splitting, fragmentation of nuclei, and formation of apoptotic bodies. The mechanism underlying aspirin-induced cell cycle arrest is not known. It was reported that NSAIDs can arrest HT-29 cell cycle at the G<sub>0</sub>/G<sub>1</sub> phase by inhibiting the expression of cdc2, cdc4 and cyclin, decreasing the phosphorylation of Rb, and increasing the p21<sup>WAF-1/CIP1</sup> expression in HT-29 cells<sup>[17]</sup>. Moreover, Perugini<sup>[18]</sup> found that aspirin induces cell

cycle arrest at the  $G_1$  phase in human pancreatic cancer cells by inhibiting cyclinD1. Whether aspirin shares the same mechanism against SW480 cells still needs to be explored.

Several protein families modulating apoptosis have been described, among which the *Bcl-2* family of proteins is best characterized<sup>[19,20]</sup>. *Bcl-2* appears to function as an inhibitor of apoptosis whereas *Bax* promotes this process. It has been shown that *Bax* can form heterodimers with *Bcl-2* to inhibit the anti-apoptotic function of *Bcl-2*<sup>[21]</sup>. Our results show that aspirin could inhibit the expression of *Bcl-2* in colon cancer cells but increase *Bax* expression. The *Bcl-2/Bax* ratio is important for apoptosis and the decline of this ratio contributes to apoptosis induction. In addition, *Bax* dimers or oligomers directly form channels in mitochondrial outer membrane in apoptosis<sup>[20,21]</sup>. It is reasonable to conclude that, at least in part, aspirin promotes apoptosis of SW480 cells by down-regulating *Bcl-2* and up-regulating *Bax* expression.

In summary, aspirin inhibits the proliferation and induces apoptosis of COX-2 negative colon cancer SW480 cells by down-regulating *Bcl-2* and up-regulating *Bax* expression, which might be a potential mechanism by which aspirin plays a positive role in apoptosis of SW480 cells.

## COMMENTS

### Background

Increasing evidence from human epidemiological studies, animal models, and experiments *in vitro* reveals that administration of nonsteroidal anti-inflammatory drugs (NSAIDs) including aspirin (acetylsalicylic acid) represents a treatment of choice for colon cancer. Data indicate that use of NSAIDs, including aspirin, is inversely associated with the risk of colorectal cancer, and clinical trials in patients with familial adenomatous polyposis showed that use of NSAIDs can lead to the regression of colorectal adenomas. In addition, data derived from various animal models of chemical carcinogenesis also suggest that NSAIDs can protect the stomach and esophagus against development of cancer. However, the molecular mechanism by which aspirin exhibits its anticancer effects is not completely clear.

### Research frontiers

A possible mechanism under lying the antitumour properties of aspirin against cancer has been ascribed to its direct inhibition of cyclooxygenase (COX)-2 in colorectal cancer tissue. However, several lines of evidence suggest that the wide range of antiproliferative potencies of aspirin do not correlate exclusively with the COX-2 inhibitory activity, because some reports showed that NSAIDs can induce apoptosis in colon cancer cells that lack detectable expression of COX-2 protein. Until now, most of researchers recruited COX-2 positive colon cancer cells to study the effect of aspirin on apoptosis and proliferation of colon cancer cells. However, few studies on the effect of aspirin on COX-2 negative colon cancer cells and lack systematic analyses of aspirin-induced morphologic changes are available. Meanwhile, whether aspirin induces apoptosis of COX-2 negative colon cancer cells is controversial. The purpose of this study was to investigate the effect of aspirin on proliferation and apoptosis of SW480 cells, a COX-2 negative human colon cancer cell line.

### Innovations and breakthroughs

Aspirin could not only induce apoptosis but also necrosis of cells at high concentrations. After treatment with aspirin, SW480 cells displayed a typically morphological feature of apoptosis and necrosis under transmission electron microscope (TEM), and increased the *Bcl-2* expression, but decreased the expression of *Bax*.

### Applications

The results of this study confirmed that aspirin could inhibit the proliferation and induce apoptosis of COX-2 negative colon cancer cell line SW480 by

down-regulating the *Bcl-2* expression, and up-regulating the *Bax* expression, which might be a potential mechanism by which aspirin plays a positive role in apoptosis of this cell line. This may benefit to the exploration of new anticancer drugs in the future.

### Peer review

This paper describes the antiproliferative effect of aspirin on Cox-2 negative colon cancer cell via the activation of apoptosis, cell cycle arrest and change in the *Bax/Bcl-2* balance. The manuscript contains interesting data, and benefits to the elucidation of the pathogenesis of colon cancer.

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CASE REPORT

## Successful photodynamic therapy for biliary papillomatosis: A case report

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loma without the need for intervention. In early 2006, the patient died of multi organ failure without signs of extrahepatic cholestasis or cholangitis at the age of 75, 10 years after the diagnosis of biliary papillomatosis was established. The patient exceeded the average life expectancy of patients with biliary papillomatosis by far. Thus, PDT might be a sufficient therapeutic option for recurrent papillomatosis patients with no significant side effects.

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**Key words:** Biliary papillomatosis; Cholangioscopy; Photodynamic therapy

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### Abstract

Papillomatosis of the bile duct is a rare disease with a high risk of malignant transformation. Therapeutic options include partial hepatectomy and liver transplantation. A previously healthy 65-years old male developed jaundice and right upper abdominal quadrant pain in 1996. A villous adenoma of the distal bile duct was diagnosed. A Whipple procedure was performed. In 2002 the patient turned symptomatic again. Another adenoma was found in the right hepatic duct resulting in a right hepatectomy. Two years later the patient again developed cholestasis. After drainage of the left hepatic duct with a percutaneous transhepatic cholangial drainage (PTCD) catheter, a recurrent biliary adenomatosis was diagnosed by cholangioscopy. As there was no surgical option left, the patient received photodynamic therapy (PDT) for the recurrent biliary papillomatosis. Three mo after he received further photodynamic therapies, the bile duct epithelium appeared normal and the patient had no signs of adenomatosis, both macroscopically and histologically. The follow-up cholangioscopy in late 2005 revealed only a small papil-

### INTRODUCTION

Biliary papillomatosis is a rare disease with less than 100 cases reported in the literature<sup>[1,2]</sup>. Biliary adenomatosis is a disease of advanced age and more common in males than in females. Similar to the adenoma-carcinoma sequence found in colon cancer, a progression to cholangiocarcinoma is presumed for biliary adenoma<sup>[3]</sup>. Therefore, patients with bile duct adenomatosis are at an increased risk of developing malignancies<sup>[1]</sup>. So far, resection of the involved liver segments and liver transplantation are the only established therapeutic options for patients with biliary papillomatosis<sup>[4,5]</sup>. Because of the advanced age and concomitant cardiovascular risk factors, many patients are neither eligible for extended liver resection nor transplantation, limiting the therapeutic options for the majority of patients with bile duct papillomatosis.

Photodynamic therapy (PDT) is a relatively new endoscopic procedure established in the local therapy for

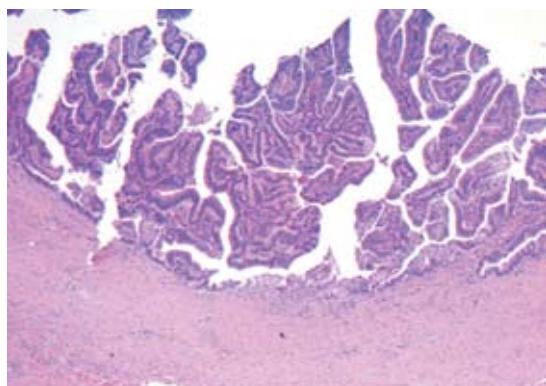
Barrett's esophagus<sup>[6]</sup>, non small-cell lung cancer<sup>[7]</sup> and newly established for the palliative treatment of malignant neoplasms of the bile duct<sup>[8-13]</sup>. After local or systemic application of a photosensitizer, the agent accumulates in neoplastic tissue while it is cleared from most other tissues within 40-60 h. Local radiation of the neoplasm with non-thermal laser activates the compound resulting in destruction of the neoplasm by a photochemical process generating oxygen radicals<sup>[11]</sup>. Effects occur with a penetration depth of 5-6 mm, depending on the physical attributes of the surrounding tissue (particle scatter, light absorption, etc.). This procedure was first reported in 1991 by Mc Coughan in the treatment of bile duct cancer<sup>[14]</sup>. In this palliative setting, PDT shows good results in terms of quality of life, relief from cholestasis and extension of lifespan<sup>[8,10]</sup>. Side effects include fever, abdominal pain, nausea, vomiting and insomnia though rare. Increased photosensitivity of the skin and eyes is observed and precautions should include avoidance of direct sunlight and bright indoor light. The skin should be covered and sunglasses should be worn when being outdoors.

## CASE REPORT

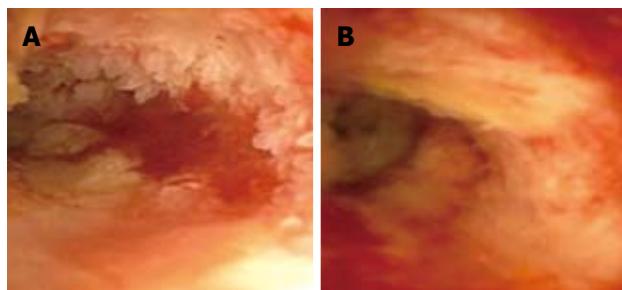
A previously healthy 65-years old male developed jaundice and right upper abdominal quadrant pain in 1996. An ERCP revealed a tumor in the common bile duct. Thus a Whipple procedure was performed. Histologically, a villous adenoma of the distal bile duct was diagnosed. The patient recovered well until he became symptomatic again in 2002. Another large adenoma was seen in a previously performed cholangioscopy of the right hepatectomy and hepatico-jejunostomy. The histological examination again revealed a villous adenoma with mild inflammation of the adjacent proximal parts of the bile duct and the surrounding liver tissue (Figure 1).

Two years later, the patient developed cholestasis again. After drainage of the left hepatic duct with a percutaneous transhepatic cholangial drainage (PTCD) catheter, a recurrent biliary adenomatosis was diagnosed via cholangioscopy. Following the Whipple-procedure, hemihepatectomy and hepatico-jejunostomy in the past, another surgical procedure was found to be of little benefit for the 72-years old patient. Thus we decided to perform a therapeutic trial of photodynamic therapy (PDT). Forty-eight hours after injection of 2 mg/kg of photosensitizer Photofrin II®, we performed laser irradiation (633 nm) of the bile duct via videocholangioscopy (Olympus) with 400 W/cm. A Yamakawa catheter was placed for drainage of the biliary tract. The patient was instructed to protect against sunlight for the following 6 wk and discharged. No side effects of the PDT procedure were seen. He recovered well and was regularly readmitted 6 mo later for a follow-up cholangioscopy.

This time, the biliary papillomatosis appeared less extensive and the Yamakawa-drain was exchanged. Another PDT procedure was performed using the same protocol as in the first attempt. In June 2005, the patient was regularly readmitted for another follow-up. This



**Figure 1** HE staining for samples collected at the right-sided hemihepatectomy and hepatico-jejunostomy of the left hepatic duct ( $\times 40$ ) showing a villous adenoma with mild inflammation of the adjacent proximal parts of the bile duct and the surrounding liver tissue but no invasive growth.



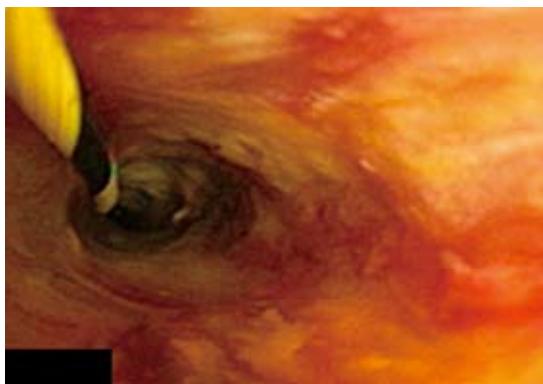
**Figure 2** Recurrent papillomatosis before the third course of photodynamic therapy without signs of a functional stenosis of the bile duct (A) and one month after the second course of PDT (B).

time a mild recurrent papillomatosis was diagnosed and another PDT procedure was performed (Figure 2). Three months later, we repeated photodynamic therapy with only remnants of the papillomatosis seen cholangioscopically. By that time histology revealed a papillary adenoma with focal high-grade dysplasia. The patient remained asymptomatic for months. Three months after the third treatment, the bile duct epithelium appeared normal and the patient had no signs of adenomata or dysplasia, both macroscopically and histologically. Another follow-up cholangioscopy in late 2005 revealed only a small adenoma without the need for intervention (Figure 3).

In early 2006, the patient presented with signs of end-stage liver disease accompanying portal hypertension, ascites, spontaneous bacterial peritonitis and caput medusae. The underlying liver disease was most likely secondary biliary cirrhosis, caused by chronic cholestasis in the past. In March 2006, he was admitted to hospital with severe pneumonia and decompensated liver cirrhosis. A few weeks later, the patient died of multi organ failure. By that time, he had no clinical or sonographical signs of extrahepatic cholestasis or cholangitis.

## DISCUSSION

We report the first successful photodynamic therapy for biliary adenomatosis without any serious side effects.



**Figure 3** Cholangioscopy showing only minor adenomatosis 3 mo after the third course of PDT.

Photodynamic therapy is an established intervention in the treatment of Barret's esophagus<sup>[6]</sup>, lung cancer<sup>[7]</sup> and bile duct cancer<sup>[8]</sup>. It was reported that photodynamic therapy can improve the quality of life and the survival of such patients<sup>[8,10,15,16]</sup>.

Our patient survived ten years after the diagnosis of biliary papillomatosis was established, exceeding the average survival time of biliary adenomatosis patients ranging from 28<sup>[1]</sup> to 32 mo<sup>[2]</sup>. Yeung *et al*<sup>[2]</sup> reviewed 78 cases of biliary papillomatosis and postulated a median survival of 28 mo after radical resection irrespective of the histological signs of dysplasia, while the median survival time was only 11 mo when no radical resection was performed. Only few cases having a survival time of more than 4 years after the diagnosis of biliary papillomatosis have been reported. In the patient presented here, the first biliary adenoma was diagnosed in 1996 and recurred in 2002, resulting in hemihepatectomy. After initiation of the PDT, the patient survived another four years with a good quality of life until admission for decompensated liver cirrhosis, a few weeks before he died. From the first manifestation of the disease he even survived 10 years.

Depending on the location and extension of the disease so far, the Whipple procedure and hemihepatectomy are the therapies of choice for biliary papillomatosis resulting in an average survival time of approximately 17 mo<sup>[2,17]</sup>. Extensive preoperative diagnosis is required to determine the resectability and the extent of the disease by ERCP, CT, cholangioscopy and intraoperative ultrasound studies<sup>[18]</sup>. In cases of diffuse hepatic manifestations, liver transplantation should be considered<sup>[4]</sup>. Bile duct papillomatosis is a disease of the elderly (mean age at time of diagnosis: 63 years), thus many patients would not be eligible for transplantation limiting the therapeutic options.

Apart from other local palliative procedures (i.e. stenting, drainage), few cases of local ablation have been reported<sup>[19,20]</sup>, most of them in the palliative setting with advanced disease plus cholangiocarcinoma were treated with a conventional laser. Data on the long term survival of these patients are lacking so far. However, PDT ablation seems to be more specific targeting dysplastic tissue

with less harm to the surrounding tissue<sup>[11]</sup>. Thus, using PDT in this particular setting might improve the quality of life and prolong life expectation of this group of patients without exposing them to risky procedures. The use of PDT in this setting has not been reported in the literature so far.

Side effects of PDT include fever, abdominal pain, nausea, vomiting and insomnia though rare. Increased photosensitivity of the skin and eyes is observed and precautions should include avoidance of direct sunlight and bright indoor light<sup>[11]</sup>. In this case only minor adverse effects are reported including mild inflammatory reaction two days after the PDT and mild skin reaction. The quality of life was significantly improved with relief from cholestasis and cholangitis for months and the patient exceeded the survival rate of most other cases reported in literature<sup>[2]</sup>.

In conclusion, PDT might be a therapeutic option for patients with recurrent biliary papillomatosis after resection as well as for those who are not eligible for surgery. Further studies are needed to prove the role of PDT in the treatment of biliary papillomatosis.

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CASE REPORT

## Chronic hepatitis C infection in a patient with bone marrow hypoplasia

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**Author contributions:** Bethlen S collected the data about the patient; Chandrikakumari K wrote the article; Giot JB, Mukeba D, Leonard P and Meuris C followed up the patient; Delwaide J performed fibroscan and fibrotest; Moutschen M and Frippiat F reviewed the article; de Leval L performed PCR of the bone marrow biopsy and genetic analysis.

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### Abstract

Chronic hepatitis C virus (HCV) infection is associated with multifarious extra-hepatic manifestations; the most described and discussed being mixed cryoglobulinemia which is strongly related to B-cell lymphoproliferative disorders (LPDs). We present a case of chronic HCV infection and mixed cryoglobulinemia, with minimal liver involvement. The case is a 53-year-old patient who was diagnosed as having bone marrow hypoplasia at the age of three. She received several blood transfusions to normalize her haemoglobin. At the age of 31, she was diagnosed with rheumatoid arthritis on account of her diffuse joint pain and inflammation, elevated rheumatoid factor (RF) and Raynaud's phenomenon. Twenty years later, monoclonal gammopathy of IgG Lambda (one year later, changed to IgM Kappa) was detected during a routine examination. A bone marrow biopsy showed hypoplasia, Kappa positive B-lymphocytes and low-grade malignant lymphoma cells. PCR of the bone marrow aspirate was not contributory. No treatment was initiated owing to her

poor bone marrow function and she is under regular follow-up.

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**Key words:** Chronic hepatitis C infection; Mixed cryoglobulinemia; Prognosis

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Bethlen S, Chandrikakumari K, de Leval L, Giot JB, Mukeba D, Leonard P, Frippiat F, Meuris C, Delwaide J, Moutschen M. Chronic hepatitis C infection in a patient with bone marrow hypoplasia. *World J Gastroenterol* 2008; 14(26): 4238-4240 Available from: URL: <http://www.wjgnet.com/1007-9327/14/4238.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4238>

### INTRODUCTION

Natural history of chronic hepatitis C virus (HCV) infection is still evolving. Several factors contribute to the long term consequences. Although the exact mechanism is not known, the ability of virus to modulate the immune system plays a significant role in the long term consequences. It has been observed that the extra-hepatic manifestations which appear during the chronic course do not alter HCV infection clinically. In this context we present a case of chronic HCV infection in a patient with bone marrow failure.

### CASE REPORT

The patient was a 53-year-old woman. In 2004, she was found positive by a screening in a public program to track HCV infected individuals. She presented to our institution immediately and HCV genotype 1b was detected on further evaluation. The liver function test, fibrotest and ultrasound transient elastography (fibroscan) indicated liver in non-fibrosis stage (F0-F1). Retrospection into her medical history revealed a slow evolution of chronic HCV infection, paradoxically mixed cryoglobulinemia was the first diagnosis. At 3 years of age, the patient was diagnosed as having cytopenia involving leukocyte

and erythrocyte lineages secondary to Chloramphenicol toxicity. To normalize her very low hemoglobin, she received several blood transfusions at unspecified intervals. However, an early or late transfusion reaction was never reported. At age 19, she developed severe bone marrow hypoplasia and anaemia and was treated with Prednisone for 5 years until her bone marrow recovered. Twelve years later, she presented with pain, redness and swelling of inter-phalangeal joints of hand. The symptoms were characteristically bilateral and progressively involved other joints, such as wrists, shoulders, knees and hips. Simultaneously she developed Raynaud's phenomenon and blood examination showed an elevated rheumatoid factor (RF). Although the symptoms were not very typical, a diagnosis of rheumatoid arthritis was established based on her clinical spectrum. Radiography of the joints taken during the course of the disease showed neither destructive and erosive features nor obvious deformities. In 2001, a blood test showed monoclonal gammopathy of IgG Lambda isotype. In the following year, aggravating cytopenia and change in isotype of monoclonal immunoglobulin to IgM Kappa were noted. A bone marrow biopsy showed hypoplasia and infiltration with Kappa positive small B lymphocytes, with mature chromatin and a high cytoplasmic-nuclear ratio. Dispersed between the B lymphocytes were low-grade malignant lymphoma cells, however, in view of her hypoplastic bone marrow, no treatment was initiated. Persistent joint symptoms, Raynaud's phenomenon and steadily increasing values of RF (from 146 IU/mL in 1998 to 7400 IU/mL in 2004) led to the detection of mixed cryoglobulinemias (MC) in 2003. Anti-cyclic citrullinated peptide (CCP) and anti-keratin antibodies were negative, suggesting the arthritis is a manifestation of cryoglobulinemia. With time she developed paraesthesia of upper and lower limbs. Nevertheless, neither sensory nor motor neuropathy was detected objectively. The common manifestation of MC, i.e. vasculitis and skin involvement was never seen in our patient. Her persisting anaemia was treated with blood transfusion and a subsequently elevated serum ferritin with Desferrioxamine.

## DISCUSSION

Chronic HCV infection is associated with a variety of extra-hepatic manifestations, the prototype being MC with a prevalence rate of 19%-50%<sup>[1]</sup>. MC is associated with lymphoproliferative disorder (LPD) of small B lymphocytes in the bone marrow or the liver<sup>[2]</sup>. Two distinct mechanisms of LPD development have been suggested, the first being specific binding of HCV E2 protein with CD81 on the B-cell which promotes consistent polyclonal response to the viral antigens and favor the development of LPD, and the second being HCV induced mutations in Ig genes and oncogenes such as *Bcl-2* rearrangement (t14; 18 translocation). However, Zignego *et al* suggested a multi-step process of pathogenesis involving both mechanisms. Persistent stimulation of B-cells followed by mistakes in Ig gene rearrangement and t (14; 18) translocation which favors the survival of abnormal B-cells<sup>[2,3]</sup>. It is estimated

that 8-10% of type II MC evolves into lymphoma (Bcell non-Hodgkins lymphoma)<sup>[2]</sup>.

Based on the type of Ig, MC is classified into three types, of which, type II MC (polyclonal IgG and Monoclonal IgM) and type III (polyclonal IgG and IgM) are commonly seen in chronic HCV infection. The Ig participates in the formation of circulating immune complexes and exerts RF like activity, usually by IgM component which frequently displays WA cross-reactive idiotype. RF is positive in 50%-80% of the patients with MC and clinically present as polyarthritis involving large joints in contrast to rheumatoid arthritis involving smaller joints<sup>[1,3]</sup>.

MC is a systemic vasculitis and circulating immune complexes are deposited in small and medium sized vessels<sup>[3]</sup>. Nevertheless, low levels of cryoglobulins can remain undetected for a long duration due to the absence of specific symptoms<sup>[2,3]</sup>. Clinical manifestations are observed in 10%-30% of the patients and the most common symptoms are weakness, arthralgias and purpura (Meltzer and Franklin triad)<sup>[2]</sup>. Apart from the triad, bilaterally symmetrical and distal neuropathy (mostly sensory) is the most frequent clinical feature of MC. Patients have variable degrees of paraesthesia and multiple mononeuritis and mononeuropathies are diagnosed infrequently<sup>[2,3]</sup>. Renal involvement is recognized in 20% of the patients with MC and is considered as one of the worst prognostic indices. Patients present with haematuria, proteinuria, edema and renal failure of variable grade. Precipitation of cryoglobulins in the capillary loops gives a histological picture similar to idiopathic membranoproliferative glomerulonephritis<sup>[2]</sup>. However, progression of renal pathology is slow and less than 15% of the affected patients develop renal failure requiring dialysis<sup>[3]</sup>. Additionally, the large spectrum of extra-hepatic manifestations also include thyroid disorders, anti-thyroid antibodies, porphyria cutanea tarda, lichen planus, diabetes mellitus, sicca syndrome, cardiomyopathy, amyloidosis, alveolitis, lung fibrosis, etc<sup>[1-3]</sup>.

There are no definite criteria for the diagnosis of MC, therefore clinical features and laboratory results such as elevated RF, reduced C4 values and presence of cryoglobulins might provide the clue<sup>[1,2]</sup>. Even in the absence of symptoms it is prudent to monitor the patients with positive HCV RNA at regular intervals for the development of MC. The mainstay treatment for HCV infection is Interferon-alpha. Several studies have proven the linear relation between clinico-immunological and virological response to anti-HCV treatment with pegylated interferon alpha (IFN- $\alpha$ ) and Ribavirin. Virological relapse on discontinuing treatment is also associated with expansion of B-cell clones bearing t (14; 18) translocation. IFN- $\alpha$  exhibits antiproliferative and immunomodulatory effects. It effectively inhibits viral replication and B-cell clonal expansion which is considered as the pathogenetic basis of MC. Interestingly, long-term analysis of treated patients with sustained virological response have developed expansion of B-cell clone with translocation, suggesting persistent lymphatic infection<sup>[2]</sup>.

The ultimate prognosis in chronic HCV infection is

determined by the development of liver cirrhosis and MC is considered as a negative prognostic indicator in this context by many authors<sup>[2-4]</sup>. In a recent publication on the natural history of chronic HCV by Vigano *et al*, 343 patients were followed up for 10 years<sup>[5]</sup>. They found that cryoglobulins did not affect the clinical course of HCV infection and had little impact on the development of extrahepatic complications. Additionally, they also cited that MC had no influence on the development of liver decompensation or hepatocellular carcinoma in chronic HCV infection. However, they could not prospectively assess any association between MC and development of cirrhosis<sup>[5]</sup>. Several factors such as male gender, older age at infection, excessive alcohol consumption and secondary hemochromatosis (due to blood transfusion) enhance the fibrotic process. Furthermore, progression to cirrhosis is more rapid in patients with compromised immunity, hepatic steatosis, obesity and diabetes. Interestingly, viral factors such as viral load and genotype determine the response to treatment; however, they do not influence the fibrosis progression<sup>[6]</sup>.

The time gap between the diagnosis of rheumatoid arthritis and MC in our patient was more than twenty years. During this period, she developed progressive paraesthesia, worsening anaemia, MC and small B-cell lymphoma confined to the bone marrow. No other classical extra-hepatic manifestations were noted. Low C4 values were observed after the diagnosis of rheumatoid arthritis. It is suggested that elevated serum alanine aminotransferase (ALT) level indicates the progression of fibrosis<sup>[4]</sup> and in our patient elevated ALT values had been observed since August 2004, but the average values were always two times lower than the normal values (recent values, SGOT/AST = 41 IU/L; normal values 14-40 IU/L, and SGPT/ALT = 66 IU/L; normal values 6-40 IU/L). Patients with chronic HCV infection might demonstrate anti-LKM1 antibody as in autoimmune hepatitis type 2<sup>[4]</sup> and it was negative in our patient. The first in 2004 and recent fibroscan showed the liver in non-fibrosis stage (F0-F1). Renal manifestations and amyloidosis were excluded by a normal renal function test and normal β2 microglobulin. Her persistent anaemia was treated with regular blood transfusion after 2003 and accordingly elevated serum ferritin with Desferrioxamine. On account of her bone marrow hypoplasia anti-HCV treatment was

deferred.

We believe that our patient might have acquired the virus by blood transfusion in childhood. However, acute hepatitis C is rarely recognized in childhood and children with chronic infection are typically asymptomatic<sup>[6]</sup>. Considering this fact, it was hypothesized that children under immunosuppression (induced by chemotherapeutic agents for the treatment of leukaemia) acquiring HCV through blood transfusion might not develop an immune response that could cause chronic injury<sup>[6]</sup>. Although, this postulate was questioned due to several conflicting reports, we support it in view of the normal liver function and absent fibrosis in our patient with bone marrow hypoplasia. This could also explain the absence of other organ involvement. However, we agree that the patient should be kept under surveillance for the development of other complications.

## CONCLUSION

Chronic HCV infection might present with more serious extra-hepatic manifestations than hepatic disease itself. Patients' immunological status might influence the extent of liver injury and extra-hepatic manifestations.

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CASE REPORT

## Hemobilia as the initial manifestation of cholangiocarcinoma in a hemophilia B patient

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### INTRODUCTION

The presence of blood into the biliary tree, as a result of hemorrhage through the biliary tract, has first been reported by Francis Glisson<sup>[1-3]</sup>. The term hemobilia, however, which is used to describe this phenomenon, was coined by Sandblom<sup>[1-3]</sup>. Hemobilia usually presents with one or more constituents of the following triad of symptoms and signs: upper quadrant pain, upper gastrointestinal bleeding and jaundice<sup>[3]</sup>. Hemobilia can occur due to trauma (both accidental and iatrogenic), gallstone disease, acalculous cholecystitis, cholangitis, ascariasis or hydatid, hepatic abscess, malignancies of the liver and pancreas and biliary tract, polyarteritis nodosa, vascular malformations of hepatic artery aneurysm or coagulopathy<sup>[3]</sup>. As far as coagulopathies are concerned, Bernard-Soulier syndrome<sup>[4]</sup>, idiopathic thrombocytopenic purpura<sup>[5]</sup>, treatment with anticoagulants<sup>[6]</sup> and hemophilia<sup>[7,8]</sup> have been implicated in the induction of hemobilia. To our knowledge, seven cases of hemobilia in patients with hemophilia have been described<sup>[7-13]</sup>, five of them following liver biopsy<sup>[9-13]</sup> and only two cases of spontaneous hemobilia<sup>[7,8]</sup>, while several cases of malignancy-associated hemobilia<sup>[14-16]</sup> have been reported. A case, however, which combines all the three conditions, has never been published. We describe a case of hemobilia as the initial manifestation of cholangiocarcinoma in a patient with hemophilia B.

### Abstract

Hemobilia is a rare manifestation of hemophilia and is usually iatrogenic following liver biopsy. There are only few reports of spontaneous hemobilia in hemophilia patients. Cholangiocarcinoma is a well-established cause of hemobilia. We describe a case of a 70-year-old male, with known haemophilia B and a past history of papillotomy, who presented with classical symptoms of hemobilia. The initial diagnostic work-up failed to demonstrate a potential cause of bleeding other than the coagulopathy. Three months later, he was readmitted to our hospital with a second episode of hemobilia. During the second work-up, a cholangiocarcinoma was diagnosed both by imaging studies and by a significant elevation of cancer antigen 19-9. Although hemobilia could be attributed to hemophilia, especially in a patient with previous papillotomy, an underlying malignancy of the biliary tree should be suspected.

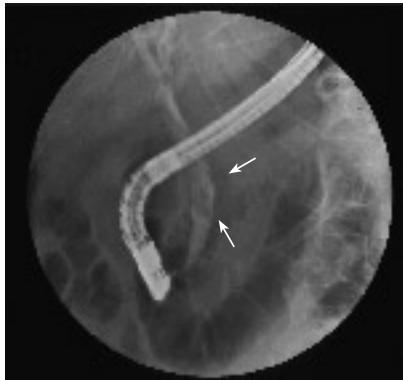
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**Key words:** Hemobilia; Hemophilia; Cholangiocarcinoma; Cancer antigen 19-9; Cholangiopancreatography

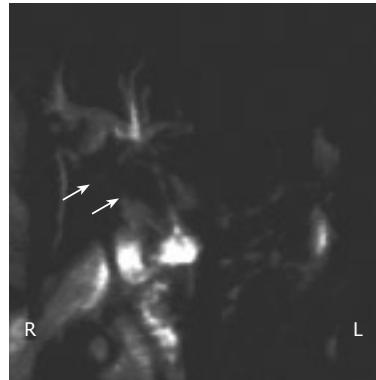
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### CASE REPORT

A 70-year-old male was admitted to our department during his second episode of hematemesis, melena, fever, and right upper quadrant pain. He was a known hemophilia B patient with liver cirrhosis, who was



**Figure 1** Endoscopic retrograde cholangiopancreatography showing string-like filling defects (arrows) suggestive of intraductal blood clots.



**Figure 2** MRCP image showing stricture (arrows) of the proximal segment of the common bile duct.

seropositive for hepatitis C virus and referred to our department three months ago due to a similar episode. He had a history of cholecystitis and underwent a papillotomy for choledocholithiasis and common bile duct stones two years ago. The work-up performed during his first admission revealed higher than normal serum levels of cancer antigen (CA) 19-9 (112.5 U/mL), along with ejection of a blood clot through the sphincterotomized papilla of Vater-hemobilia by side-viewing endoscopy and the presence of string-like filling defects in the biliary tree *via* endoscopic retrograde cholangiopancreatography (ERCP, Figure 1). Magnetic resonance angiography (MRA) showed no evidence of hepatic artery pseudoaneurysm or any other vascular malformation. No potential cause for hemobilia other than the underlying coagulopathy was identified since ultrasonography, magnetic resonance imaging (MRI), magnetic resonance cholangiopancreatography (MRCP) and ERCP were not suggestive of another etiology. The patient was treated at that time with factor IX concentrate, fresh frozen plasma (FFP) and blood transfusions until the prolonged activated partial thromboplastin time (APTT) and the decreased hematocrit reached their normal levels. Moreover, cholestasis resulting from intrabiliary clots was successfully managed by removing these clots with a balloon catheter during ERCP, which led to lysis of the remaining smaller clots and a significant decrease in levels of bilirubin, gamma-glutamyltransferase (gamma-GT), alanine aminotransferase (ALT), and aspartate aminotransferase (AST). As soon as the above-mentioned markers returned to normal and the patient's clinical condition improved, he was discharged and remained free of any bleeding or cholestasis-related manifestations for almost 3 mo. Then, he was hospitalized for a week at a peripheral hospital with low-grade fever and right upper quadrant pain. During hospitalization, he had episodes of hematemesis and melena, but the site of bleeding was not identified. The patient was then referred to our hospital.

Physical examination at admission revealed that his temperature was 37.4°C, blood pressure was 160/70 mmHg and heart rate was 55 beats per minute.

Right upper quadrant tenderness, jaundice and melena were also present. Laboratory findings included 7.2 g/dL hemoglobin,  $2.65 \times 10^6/\mu\text{L}$  erythrocytes,  $227 \times 10^3/\mu\text{L}$  platelets,  $6.1 \times 10^3/\mu\text{L}$  leucocytes with 55.6% polymorphs, 6.9 mg/dL total serum bilirubin, 4.03 mg/dL direct bilirubin, 199 IU/L AST, 53 IU/L ALT, 633 IU/L gamma-GT, 1466 IU/L alkaline phosphatase (ALP), 36.2 s APTT. Moreover, the determination of serum tumor markers revealed significantly elevated levels of CA 19-9 (3456 U/mL). Abdominal ultrasonography showed echogenic non-shadowing debris in the common bile duct and dilation of the intrahepatic bile ducts in the right liver lobe. Side-viewing endoscopy revealed blood in the lumen of the second part of the duodenum and a prominent blood clot at the papilla of Vater. Finally, MRCP revealed dilation of the intrahepatic bile ducts and identified the cause of hemobilia: a cholangiocarcinoma involving a 3 cm-long proximal segment of the common bile duct (Figure 2). These findings were confirmed by ERCP. The intrabiliary blood clots were removed using a balloon catheter and a 10 cm-long (10 Fr) stent was placed to stop the bleeding and decrease the level of bilirubin, gamma-GT and ALP. Factor IX concentrates, erythrocyte concentrates and FFP were also transfused until APTT, hemoglobin and erythrocyte count levels returned to normal. In addition, he was also treated with antibiotics. The patient was doing well, no recurrence of bleeding or cholangitis was recorded and he was discharged. Two weeks later, almost 6 wk before a scheduled stent was replaced, he presented with high-grade fever, jaundice and upper quadrant pain. The patient was septic at admittance. Despite full antibiotic coverage, he died of septic shock soon.

## DISCUSSION

Hemophilia is a rare cause of non-iatrogenic hemobilia. To our knowledge, only two cases of spontaneous hemobilia in hemophiliacs have been described so far<sup>[7,8]</sup>. Malignancies, on the other hand, are a more common cause of hemobilia, compared to hemophilia. Malignant tumors, however, do not account for more than 10% of total hemobilia incidents<sup>[3]</sup>. Hemophilia and malignancy, two potential causes for hemobilia, were both present in our patient. Hemorrhagic diathesis induced by

hemophilia B, increased the susceptibility to bleeding originating from a tumor-infiltrated ductal system. What seems to be of some importance is the fact that hemobilia was proved to be an early manifestation of the underlying cholangiocarcinoma, and that ultrasonography, ERCP and MR techniques (MRI, MRCP, MRA) failed to identify it. A mild elevation of CA 19-9 was also recorded during the patient's first admission. It was reported that use of this tumor marker in the diagnosis of cholangiocarcinoma shows a sensitivity of 53%, when a CA19-9 value  $> 100$  U/mL is used<sup>[17]</sup>, and is considered to be misleading when such patients have gallstone disease and/or cholangitis<sup>[18]</sup>. In our case, the elevation of CA19-9 was initially attributed to chronic ascending cholangitis following papillotomy which in turn could affect the arterial wall, thus leading to the formation of hepatic artery pseudoaneurysm, a known cause of hemobilia<sup>[19]</sup>.

Although cholangiocarcinoma was not detected during the initial work-up, hemobilia was successfully recorded during both work-ups. The combination of upper quadrant pain, upper gastrointestinal bleeding (hematemesis, melena) and jaundice as well as the evidence provided by the abdominal ultrasonography were all indicative of hemobilia. It was not, however, until side-viewing endoscopy and ERCP were performed, hemobilia was diagnosed.

In view of the classical triad of hemobilia, all the three constituents are present in up to 22% of patients<sup>[3]</sup>. Side-viewing endoscopy performed independently or as a part of ERCP can reveal active bleeding<sup>[6,20]</sup> or blood clots at the papilla of Vater (as in this case). Moreover, cholangiography may show string-like, as reported here, or spherical filling defects<sup>[3]</sup>. Angiography also plays a significant role in the confirmation and management of hemobilia and in the identification of vascular malformations as a potential cause of bleeding<sup>[3]</sup>. Ultrasonography, on the other hand, offers circumstantial evidence of bile duct dilation along with the presence of echogenic, non-shadowing, non-mobile formations of blood clots that evolve less reflective masses<sup>[3]</sup>. Computed tomography<sup>[21]</sup> and radioisotope studies<sup>[3]</sup> can also be used.

As far as the management of hemobilia is concerned, different approaches have been applied, depending on the etiology and the underlying diseases. It was reported that decompression of the biliary tree encourages resolution of jaundice and contributes to the arrest of hemobilia<sup>[3,8]</sup>. As shown in a study performed by Sandblom, bile enzymes can perform lysis of fibrin, but a free flow of bile is needed so that this lytic activity can occur<sup>[22]</sup>. Embolization techniques, such as transarterial embolization<sup>[23]</sup> or even surgery<sup>[3]</sup>, have also been applied in the management of hemobilia. In our case, decompression of the biliary tree (by means of a balloon catheter and stenting during ERCP) along with correction of the patient's bleeding diathesis was adequate for both resolution of jaundice and control of bleeding.

In summary, underlying malignancy should be

suspected in cases of non-traumatic, non-iatrogenic hemobilia irrespective of any coincident coagulopathies, such as hemophilia. Hemobilia should not be easily attributed to hemophilia, even when the first work-up fails to identify a potential cause other than coagulopathy. ERCP may serve as a diagnostic tool for both hemobilia and underlying malignancy as well as a therapeutic technique for the management of obstructive jaundice and, in part, of bleeding. The correction of bleeding diathesis by treatment with factor IX, FFP and erythrocyte concentrates is mandatory for the arrest of hemobilia.

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CASE REPORT

# Successful laparoscopic splenectomy after living-donor liver transplantation for thrombocytopenia caused by antiviral therapy

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conclusion, LS can be performed safely even after LDLT, and LS after LDLT is a feasible and less invasive modality for thrombocytopenia caused by antiviral therapy.

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**Key words:** Concomitant splenectomy; Portal vein thrombosis; Ribavirin

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Kato H, Usui M, Azumi Y, Ohsawa I, Kishiwada M, Sakurai H, Tabata M, Isaji S. Successful laparoscopic splenectomy after living-donor liver transplantation for thrombocytopenia caused by antiviral therapy. *World J Gastroenterol* 2008; 14(26): 4245-4248 Available from: URL: <http://www.wjgnet.com/1007-9327/14/4245.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4245>

## Abstract

Although interferon (IFN) based therapy for recurrent hepatitis C virus (HCV) infection after liver transplantation has been widely accepted, it induces various adverse effects such as thrombocytopenia, resulting in its interruption. Recently, concomitant splenectomy at the time of living donor liver transplantation (LDLT) has been tried to overcome this problem, but this procedure leads to several complications such as excessive intraoperative bleeding and serious infection. A 60-year-old female received LDLT using a left lobe graft from her second son for liver failure caused by hepatitis C-related cirrhosis. Six months after LDLT, she was diagnosed as recurrent HCV infection by liver biopsy. IFN monotherapy was started from 7 mo after LDLT and her platelet count decreased to less than 50 000/ $\mu$ L, which thus made it necessary to discontinue the treatment. We decided to attempt laparoscopic splenectomy (LS) under general anesthesia. Since intra-abdominal findings did not show any adhesion formations around the spleen, LS could be successfully performed. After LS, since her platelet count immediately increased to 225 000/ $\mu$ L 14 d after operation, IFN therapy was restarted and we could convert the combination therapy of IFN and ribavirin, resulting in no detectable viral marker. In

## INTRODUCTION

Recurrence of hepatitis C virus (HCV) infection is a serious problem of HCV positive patients receiving liver transplantation, because it has been universally documented as the leading cause of allograft destruction<sup>[1,2]</sup>. Although interferon (IFN) based therapy in combination with ribavirin for recurrent HCV infection has been widely accepted after liver transplantation, it induces various adverse effects related to bone marrow suppression such as thrombocytopenia and leukopenia, resulting in its interruption. Until recently, no approved treatment for thrombocytopenia in patients with HCV infection is available except for splenectomy or partial splenic embolization (PSE). However, it was most recently reported that the efficacy of eltrombopag, an orally active thrombopoietin-receptor agonist, for

thrombocytopenia in patients with cirrhosis is associated with hepatitis C<sup>[3]</sup>. At present, this drug is not available in our country, and thus splenectomy is the treatment of choice to overcome this problem. Kishi *et al*<sup>[4]</sup> performed splenectomy concurrently with liver transplantation for all HCV-positive liver transplant patients. However, the indication for concomitant splenectomy still remains controversial.

As for splenectomy, laparoscopic splenectomy (LS) is employed as the minimal invasive surgery for various diseases including a normal-size spleen in idiopathic thrombocytopenic purpura (ITP) as well as a huge-size spleen in hypersplenism due to liver cirrhosis<sup>[5]</sup>. It is believed that LS is relatively contra-indicated for patients with previous abdominal surgery, especially with upper abdominal surgery<sup>[6]</sup>. However, it was reported that the incidence of adhesion after organ transplantation is very low<sup>[7,8]</sup>. Wasserberg *et al*<sup>[9]</sup> revealed that post-surgical adhesion formation is significantly reduced in rats receiving tacrolimus immunosuppression after intestinal transplantation.

We therefore performed LS in a female patient with recurrent HCV infection after living-donor liver transplantation (LDLT), which did not lead to any complications. She developed severe thrombocytopenia after antiviral therapy using IFN. This is the first case of LS after LDLT.

## CASE REPORT

A 60-year-old female received LDLT with an ABO identical left lobe graft from her second son for liver failure due to hepatitis C-related cirrhosis at our institution. She was discharged without any postoperative complications. Six months after LDLT, however, her liver function was elevated and she was diagnosed as recurrent HCV infection by liver biopsy. IFN (alpha 2b) monotherapy (3 mega units, 3 times a week) was started 7 mo after LDLT and the levels of liver enzyme and HCV-RNA were notably improved. After 11 mo, however, her platelet count decreased to 32 000/ $\mu$ L, and thus INF treatment was discontinued. After interruption, her liver enzyme and HCV-RNA levels were remarkably elevated again 13 mo after LDLT. IFN therapy was started again when her platelet count was 70 000/ $\mu$ L and discontinued when her platelet count decreased to 39 000/ $\mu$ L (Figure 1).

She was then admitted to our institution to receive splenectomy for thrombocytopenia 15 mo after LDLT. At admission, her laboratory test showed elevated levels of liver and biliary enzymes (157 IU/L AST, 184 IU/L ALT, 201 IU/L  $\gamma$ -GTP, 3.5 mg/L T-Bil, 2.7 mg/L D-Bil) and viral load of HCV-RNA was 5.4 LogIU/mL (genotype 1b). An abdominal enhanced CT demonstrated the enlarged spleen of 140 mm in maximum diameter and collateral vessels derived from the splenic vein (Figure 2A-B).

We decided to perform LS even after LDLT, because the trial of LS was acceptable since LS is potentially

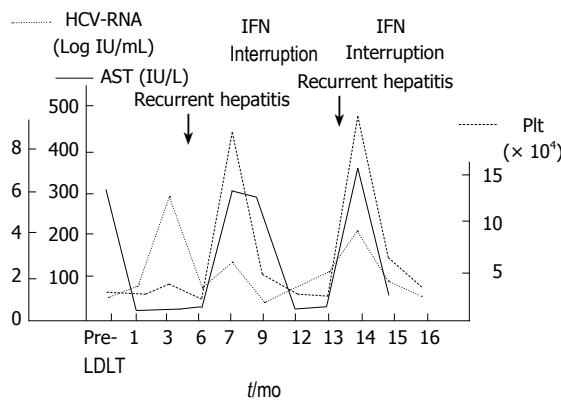


Figure 1 Changes in serum levels of AST, HCV-RNA and platelet after LDLT.

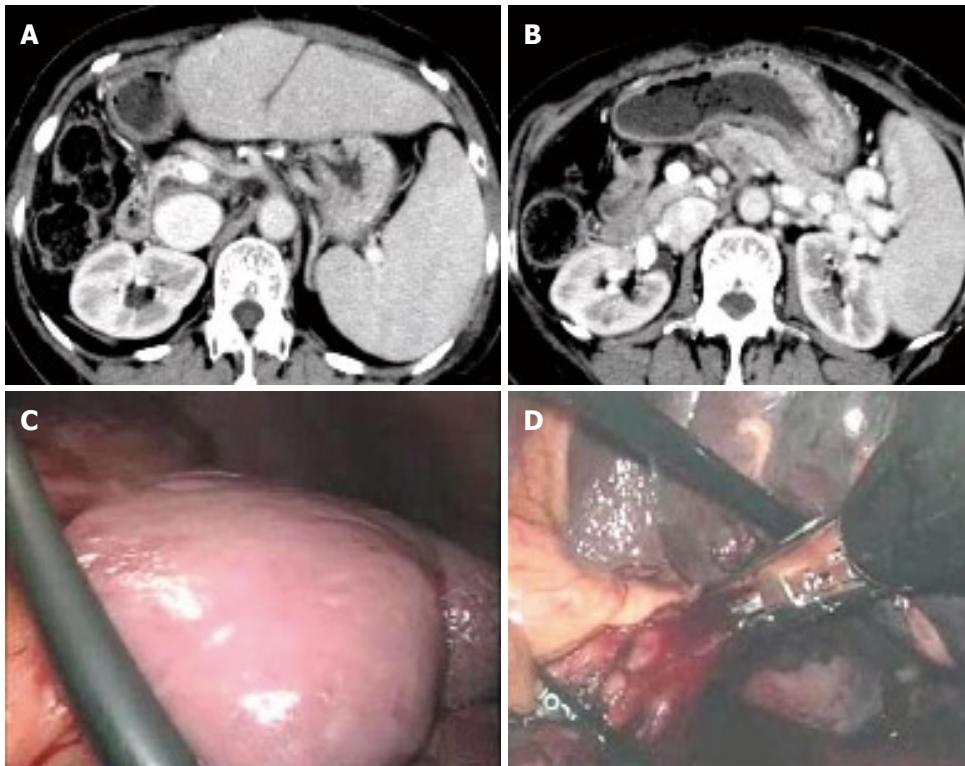
successful because the operation field of LDLT is mainly the right upper abdomen and the mobilization of spleen from the retroperitoneum under laparoscopic approach as much as possible makes a skin incision smaller even if LS is converted to open surgery. In addition, as LS after LDLT is not widely recognized and also has significant short term and long term considerable risks such as infectious complications, we gave the patient sufficient information about splenectomy.

The patient was placed at a right semidecubitus position under general anesthesia. The pneumoperitoneum was introduced with the open Hasson's technique at the left lateral to the umbilicus and intra-abdominal findings did not show any adhesion to abdominal wall and around the spleen (Figure 2C). Thus, we started dissection of the splenocolic ligament and parietal peritoneum using the vessel sealing system (Ligasure AtlasTM) from the lower pole to the upper pole through the lateral side of spleen. After enough mobilization of the spleen, we stapled the splenic hilum with linear staplers (Figure 2D) and delivered the crushed spleen from the extended wound. The operation time was 2 h and 32 min and the amount of bleeding was 72 g. The weight of spleen was 260 g.

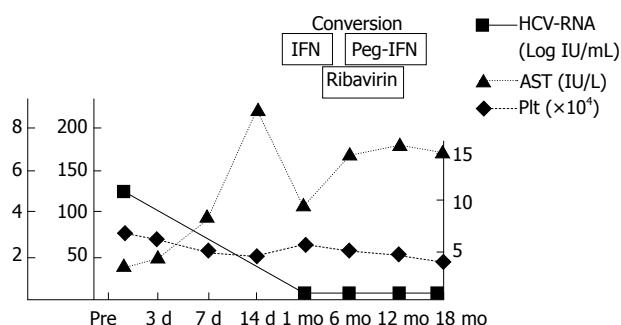
After splenectomy, her platelet count immediately increased and we could restart IFN monotherapy from postoperative day (POD) 7. Furthermore, since her platelet count increased to 225 000/ $\mu$ L on POD 14, we could convert IFN monotherapy to combination therapy of IFN (convert to peg-IFN 1 mo after operation) and ribavirin, resulting in no detectable viral marker 18 mo after LS and achievement of sustained viral response (Figure 3).

## DISCUSSION

Since Delaitre<sup>[10]</sup> performed the first laparoscopic splenectomy (LS) in the last decade, it has gained more and more respect because of its minimal invasion. Similarly to other laparoscopic procedures, it is characterized by shorter hospital stay, lower perioperative complication rate, better cosmetic result and earlier return to full working activity, which are the most important advantages over the open approach.



**Figure 2** Abdominal enhanced CT showing splenomegaly with 140 mm in maximum diameter (**A**) and development of collateral veins around the splenic vein (**B**), no peritoneal adhesion to abdominal wall and around the spleen (**C**), and splenic hilum stapled with linear staplers (**D**).



**Figure 3** Changes in serum levels of AST, HCV-RNA and platelet count after operation.

In recent years, extension of its indications has been noted and this operation has been employed for diseases with normal size spleen such as ITP and huge size spleen such as hypersplenism related to liver cirrhosis. Furthermore, patients with thrombocytopenia caused by IFN therapy for recurrent HCV infection are good candidates for LS in order to overcome this problem<sup>[5,11]</sup>. Kent *et al*<sup>[5]</sup> reported that patients undergoing LS for thrombocytopenia associated with IFN therapy can improve their thrombocytopenia and their platelet count remains above 100 000/ $\mu$ L during subsequent pegylated-IFN therapy. However, no reports are available on successful LS after LDLT for the improvement of thrombocytopenia, as a result of PubMed search using laparoscopic splenectomy and liver transplantation as the key words.

It was reported that concomitant splenectomy with LDLT is necessary to prevent the progression of thrombocytopenia caused by postoperative antiviral therapy<sup>[4,12]</sup>. On the other hand, it is well known that

concomitant splenectomy with LDLT induces several severe complications, such as postoperative portal vein thrombosis, re-bleeding, increasing intraoperative bleeding, and severe infection<sup>[13,14]</sup>. Therefore, indications for concomitant splenectomy should be decided carefully. Although we perform splenectomy concurrently with LDLT to modulate portal venous pressure, especially when the portal venous pressure is over 20 mmHg after reflowing<sup>[15]</sup>, we do not perform concomitant splenectomy for introduction of IFN therapy routinely at present.

Sohara *et al*<sup>[11]</sup> have reported the efficacy of PSE on overcoming IFN-related thrombocytopenia after liver transplantation. But in our hospital, LS is regarded as the first-line treatment for thrombocytopenia in patients with cirrhosis associated with hepatitis C, because PSE induces various severe complications, such as abscess of spleen, persistent fever, severe abdominal pain and the efficacy of PSE lasts for only one year and it is necessary to perform several times of PSE for good results<sup>[16-18]</sup>.

Although LS has been accepted as a minimum invasive method, abdominal surgery used to be a relative contraindication to LS because of some complications related to peritoneal adhesions including other organ injury<sup>[6]</sup>. DeRoover<sup>[19]</sup> reported the first case of LS after orthotopic liver transplantation and described mild peritoneal adhesion found at intra-operation. In addition, various laparoscopic surgeries including incisional hernia repair, proctocolectomy and Roux-en-Y gastric bypass after liver transplantation have been successfully performed in recent years<sup>[20-22]</sup> with a very low incidence of adhesion<sup>[7-9]</sup>. We successfully performed LS after LDLT.

In conclusion, LS can be performed safely even after

LDLT, and LS after LDLT is a feasible and less invasive modality for thrombocytopenia caused by antiviral therapy.

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CASE REPORT

# Ischemic colitis secondary to inferior mesenteric arteriovenous fistula and portal vein stenosis in a liver transplant recipient

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## INTRODUCTION

Arteriovenous fistula (AVF) in the portal circulation is rare and usually involves the hepatic, splenic, or superior mesenteric vessels<sup>[1]</sup>. Inferior mesenteric AVF is uncommon, and only isolated cases have been reported<sup>[2,3]</sup>. Though inferior mesenteric AVF may be congenital<sup>[4-6]</sup>, it results more often from penetrating traumas, such as a gunshot or knife wound, or may be a complication of arterial catheterization or abdominal surgery with bowel resection<sup>[2,3,7-9]</sup>. Inferior mesenteric AVF usually presents with abdominal pain, mass, or features of established portal hypertension<sup>[2,3]</sup>. Colonic ischemia is a less common and more serious manifestation of AVF<sup>[2]</sup>.

Portal vein stenosis is a relatively rare vascular complication affecting 1%-2% of patients who have undergone orthotopic liver transplantation<sup>[10,11]</sup>. Clinical symptoms of portal vein stenosis are usually less pronounced. Patients carrying this diagnosis may develop portal hypertension with ascites and esophageal varices, but portal vein stenosis is potentially devastating, leading to graft failure<sup>[10,11]</sup>.

We report a case of ischemic colitis secondary to an inferior mesenteric AVF in a patient who underwent a previous liver transplantation, subsequently developed portal vein stenosis, and then presented with acute lower gastrointestinal bleeding.

## CASE REPORT

A 46-year-old man was admitted to our hospital for abdominal pain and hematochezia. Two years previously, he underwent cadaveric, orthotopic, whole liver transplantation due to hepatitis B-associated decompensated hepatic cirrhosis. Forty-two days

## Abstract

Arteriovenous fistula (AVF) involving the inferior mesenteric vessels is rare, and the affected patients usually present with abdominal pain, mass, or features of established portal hypertension. Colonic ischemia is a less common and more serious manifestation of AVF. We report a case of ischemic colitis secondary to inferior mesenteric AVF in a patient who underwent a previous liver transplantation, subsequently developed portal vein stenosis, and then presented with acute lower gastrointestinal bleeding. He underwent percutaneous transhepatic placement of a portal vein stent and left colectomy.

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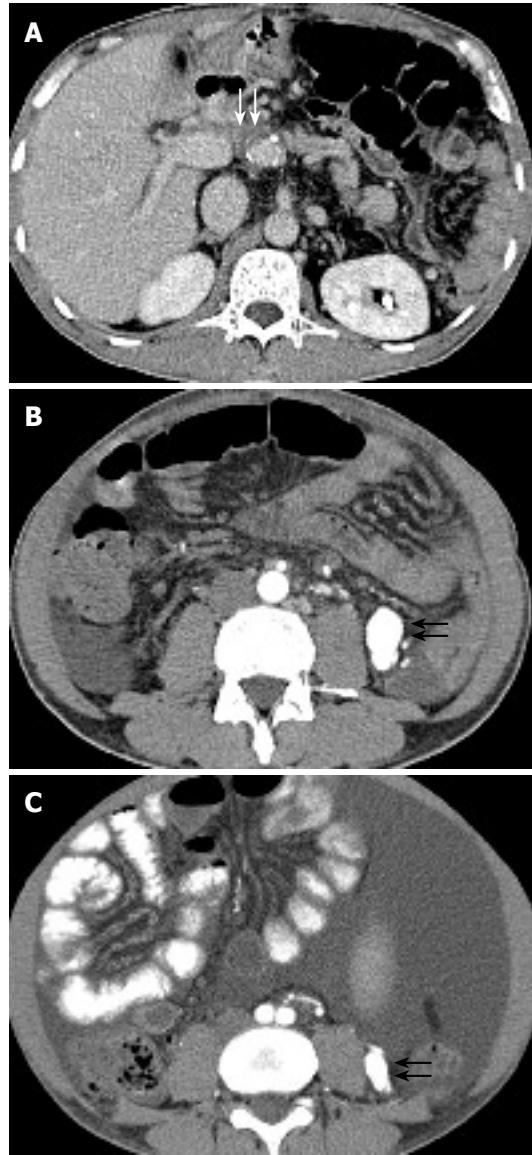
**Key words:** Ischemic colitis; Inferior mesenteric; Arteriovenous fistula; Portal vein stenosis



**Figure 1** Colonoscopy revealing diffuse colonic ulceration, exudate, and hemorrhage in the sigmoid and descending colon.

after liver transplantation, the patient developed cytomegalovirus colitis which was treated with intravenous ganciclovir, 5 mg/kg, twice per day for 4 wk. Follow-up colonoscopy showed healing of ulcers, and serology showed negative cytomegalovirus antigenemia. The patient had no history of abdominal trauma or abdominal surgery other than liver transplantation.

At the first clinical examination, the lower abdomen was distended and painful, but bowel sounds were decreased. Hematologic, biochemical and liver function tests showed  $10.2 \times 10^3 / \text{mm}^3$  white blood cells,  $29.5 \times 10^3 / \text{mm}^3$  red blood cells, 9.7 g/dL hemoglobin,  $167 \times 10^3 / \text{mm}^3$  platelet count, 75.3 mg/L C-reactive protein (range  $\leq 5$ ), 32 mg/dL blood urea nitrogen, 0.68 mg/dL serum creatinine, 45 U/L alanine aminotransferase, 15 U/L aspartate aminotransferase, 1.24 mg/dL total bilirubin, and 3.2 g/dL serum albumin. Colonoscopy showed diffuse ulceration, exudate, and hemorrhage in the sigmoid and descending colon, associated with narrowing of the lumen (Figure 1). Histopathologic evaluation of biopsy specimens from the colon revealed mucosal and submucosal hemorrhage and edema, with partial necrosis and ulceration of the mucosa. Blood and tissue analysis for cytomegalovirus and *Clostridium difficile* was negative. Stool cultures and samples were also negative for bacteria and parasites. A clinical diagnosis of ischemic colitis was made. Contrast-enhanced computed tomography (CT) showed severe stenosis at the anastomosis of the donor portal vein and the native portal vein (Figure 2A), and a 30 mm  $\times$  18 mm enhancing vascular structure in the left lower abdominal cavity connecting to the inferior mesenteric vein associated with venous stasis in the mesenteric vein (Figure 2B). Diffuse thickening of the wall of the sigmoid colon without mucosal enhancement was also seen. We reviewed a previous CT scan performed before liver transplantation and found a 10 mm  $\times$  8 mm enhancing vascular structure at the same location, indicating that the structure was increased in size over the two years following transplantation (Figure 2C). Angiography of the inferior mesenteric artery showed both the artery and its partner vein emerging from the lesion, along with very early



**Figure 2** Contrast-enhanced CT of the abdomen showing portal vein stenosis (white arrows) (A), an approximately 30 mm  $\times$  18 mm contrast-enhancing vascular mass (black arrows) (B), and an approximately 10 mm  $\times$  8 mm enhancing vascular structure before transplantation (black arrows) (C).

opacification of the markedly dilated inferior mesenteric vein (Figure 3). Based on this, the mass-like vascular lesion was diagnosed as an AVF. Angiography also showed an additional small inferior mesenteric AVF with venous dilatation and decreased perfusion of the sigmoid colon. We felt that portal hypertension secondary to portal vein stenosis developed after liver transplantation, which contributed to enlargement of the inferior mesenteric AVF and venous congestion, and then resulted in decreased perfusion of the sigmoid colon and ischemic colitis. Therefore, we decided to attempt a percutaneous angioplasty for the portal vein stenosis and surgical repair of the inferior mesenteric AVF associated with severe ischemic change in the sigmoid colon.

The patient underwent a percutaneous transhepatic portography for portal vein stenosis. Portogram revealed an almost complete obstruction at the extrahepatic



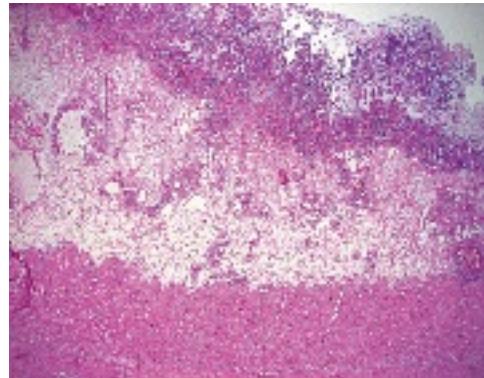
**Figure 3** Angiography of the inferior mesenteric artery showing an AVF (arrow) with early opacification of a dilated vein.



**Figure 4** Transhepatic portal venography showing portal vein stenosis (white arrow) with stasis in the mesenteric vein (A) and good patency of the portal vein following deployment of the metal stent (black arrows) (B).

anastomosis of the portal vein and a marked delay of venous drainage of the mesenteric vein (Figure 4A). A 14 mm × 4 cm self-expandable bare metallic stent was deployed in the stenotic portal vein area, and a 10 mm × 4 cm balloon was dilated for residual stenosis. Portogram performed after stent deployment revealed good patency of the portal vein and no continuous contrast filling in the mesenteric vein (Figure 4B).

Two days after percutaneous angioplasty, the patient underwent a laparotomy for colon ischemia. After the mesocolon was ligated and divided, the sigmoid and descending colon with fistulae were resected. Pathological examination of the resected specimens demonstrated extensive necrosis of mucosa, diffuse inflammatory infiltrations extending into submucosa, and focally hyalinized thick-wall vessels, which were compatible with the symptoms of ischemic colitis (Figure 5). The patient's



**Figure 5** Histopathologic examination of the resected sigmoid showing diffuse ischemic necrosis inflammatory infiltrations (HE  $\times 20$ ).

postoperative course was uneventful, and an abdominal CT scan 6-mo later showed a mildly dilated inferior mesenteric vein without aneurysmal dilatation. The portal vein stent patency was good, with homogeneous enhancement of the liver.

## DISCUSSION

Inferior mesenteric AVF is a rare splanchnic AVF. Only 11 cases have been reported in the literature<sup>[3]</sup>. Our patient had no history of abdominal trauma or abdominal surgery other than liver transplantation. An abdominal CT scan performed before liver transplantation demonstrated a small inferior mesenteric AVF, suggesting that the fistula is congenital in origin and increases in size after transplantation, resulting in the patient's presentation.

The common clinical symptoms and signs of inferior mesenteric AVF include abdominal pain, mass, or bruit, or a combination of these symptoms<sup>[2,3]</sup>. More serious manifestations of mesenteric AVF are associated with portal hypertension, which is present in about 50% of patients with splanchnic AVF<sup>[1]</sup>. Okada *et al*<sup>[3]</sup> reported that 7 of 11 patients with inferior mesenteric AVF had either signs or symptoms of portal hypertension (mainly ascites, esophageal varices, or splenomegaly) or elevated portal venous pressure. Portal hypertension in an inferior mesenteric AVF is called "forward" or "hyperkinetic" portal hypertension and may result from both increased blood flow into the portal system and from compensatory increase in hepatic vascular resistance<sup>[2,3,12]</sup>. Inferior mesenteric AVF may also be a factor predisposing to non-occlusive ischemic colitis<sup>[2]</sup>. An AVF is usually associated with decreased arterial blood flow to the tissue beyond it and increased venous pressure distal to it<sup>[2,12]</sup>.

Portal venous stenosis in patients who have undergone orthotopic liver transplantation may be caused by disparity in the diameters of recipient and donor portal veins (particularly in pediatric transplants). Cryopreserved grafts, excessively long vessel stumps or thrombotic occlusion of the veno-venous bypass, or portosystemic shunt surgery can be used prior

to transplantation<sup>[10,11,13]</sup>. The majority of those patients who are found to have portal vein stenosis are otherwise asymptomatic and detected on routine screening ultrasound<sup>[10]</sup>. Contrast-enhanced CT scan and magnetic resonance imaging can clearly show portal vein stenosis. When patients are symptomatic, they also present with typical signs of portal hypertension, including ascites, esophageal varices, and splenomegaly with or without thrombocytopenia.

The present patient reported no usual symptoms of ischemic colitis, such as hematochezia, melena, or abdominal pain before liver transplantation. However, he suffered from esophageal variceal hemorrhages and ascites, which were thought to be caused by advanced hepatic cirrhosis and portal hypertension. Two years after transplantation, he presented with abdominal pain and hematochezia. Colonoscopy and histopathologic evaluation of biopsy specimens revealed ischemic colitis in the sigmoid and descending colon, and radiologic evaluation showed an inferior mesenteric AVF and portal vein stenosis, suggesting that the portal vein stenosis induced portal hypertension is associated with hepatofugal flow. High arterial flow due to inferior mesenteric AVF and venous stasis in the fistula due to hepatofugal flow, may have contributed to the pseudoaneurysmal dilatation of the inferior mesenteric vein in our patient, leading to augmentation of the preexisting inferior mesenteric AVF and induced the development of ischemic colitis due to steal phenomenon.

Percutaneous transhepatic balloon angioplasty or placement of metal stent has been widely accepted as a safe and effective procedure for portal vein stenosis following liver transplantation<sup>[13]</sup>. Our patient underwent percutaneous transhepatic deployment of a metal stent with balloon angioplasty. The stent patency was good, the stenosis was disappeared after treatment.

The choice of therapy for inferior mesenteric AVF is surgical correction with or without associated bowel resection<sup>[3]</sup>. Some studies reported that percutaneous endovascular embolization of the feeding artery may be useful in selected cases, particularly in critically ill patients<sup>[2,4,7]</sup>. In our case, the combination of clinical state, CT and endoscopic images and the presence of the large AVF all prompted a surgical repair.

Inferior mesenteric AVF is rare, but may be associated with portal hypertension and non-occlusive ischemic colitis. To our knowledge, this is the first report of inferior mesenteric AVF-induced ischemic colitis

complicated by portal vein stenosis developed after liver transplantation.

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## Coexistence of esophageal blue nevus, hair follicles and basaloid squamous carcinoma: A case report

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### Abstract

We present the case of a 57-year-old man who underwent esophagectomy for esophageal carcinoma found at barium meal and gastroscopic examination. He was diagnosed as esophageal basaloid squamous carcinoma (BSC) and gastric stromal tumor, which were associated with focal proliferation of melanocytes/pigmentophages and hair follicles in esophageal mucosa. Melanocytic hyperplasia (melanocytosis) has previously been recognized as an occasional reactive lesion, which can accompany esophageal inflammation and invasive squamous carcinoma. The present case is unusual because of its hyperplasia of not only melanocytes but also hair follicles. To our knowledge, this is the first report of esophageal blue nevus and hair follicle coexisting with BSC.

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**Key words:** Esophagus; Basaloid squamous carcinoma; Blue nevus; Hair follicle; Gastric stromal tumor

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### INTRODUCTION

Scattered melanocytes at the epithelio-stromal junction of esophageal mucosa are an incidental finding. This phenomenon was first described in 1963 by De la Pava, who found melanocytes in 4% of his autopsy material from the esophagus<sup>[1]</sup>. Subsequent studies showed that the incidence of esophageal melanocytes ranges 2.5%-8%<sup>[2,3]</sup>.

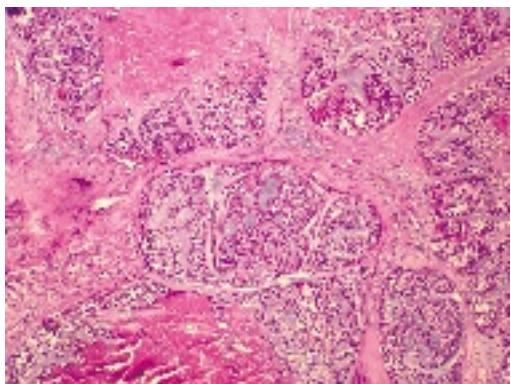
The increased number of esophageal melanocytes along the epithelio-stromal junction is considered benign and has been described in association with chronic esophagitis, squamous epithelial hyperplasia and infiltrating squamous carcinoma of the esophageal mucosa<sup>[2]</sup>.

In the present report, we describe a very unusual proliferation form of melanocytes and hair follicles in esophageal mucosa, which is associated with basaloid squamous carcinoma (BSC) of esophagus and gastric stromal tumor. To our knowledge, esophageal blue nevus coexisting with hair follicles is first described.

### CASE REPORT

#### Clinical history

A 57-year-old man complained of feeling an aggravating obstruction when taking food for 3-mo, which was more severe and affected his normal diet when taking solid food. He had no vomiting, abdominal pain or melena. Physical examination revealed no obvious anemia. The lung and heart sounds were normal, the abdomen was soft and flat with no hepatomegaly or splenomegaly or palpable abdominal mass. Rectal examination found no abnormalities and no enlargement of the superficial lymph nodes. He had an over 30-year history of gastroesophageal reflux disease, and an over 10-year history of chronic gastritis with occasional duodenal ulcer. To cure these diseases, he took soda tablets/powder and other drugs in the past 6-7 years. The patient smoked more than twenty cigarettes and drank 100-150 mL alcohol per day. His family medical history and physical examination were negative. At the lower third esophagus, barium meal examination indicated an esophageal carcinoma, gastroscopic examination showed a 1 cm × 1 cm smooth protrusion and a 0.5 cm × 0.5 cm mucosa erosion on its top, identified by subsequent biopsy as a low-differentiation carcinoma. The



**Figure 1** Arrangement of basaloid cells in the form of anastomosing trabeculae and microcystic structures. The microcystic spaces contained basophilic mucoid matrix, mimicking an adenoid cystic carcinoma. The cells at the edges of basaloid islands tended to show peripheral nuclear palisading. Comedo necrosis was observed in the center of basaloid lobules (HE,  $\times 40$ ).

patient underwent a lower third esophagectomy with gastric tube reconstructed.

### Materials and methods

The surgical specimen including the dissected lymph nodes was fixed in a 4% buffered formalin solution. All the samples taken from the esophagus and stomach were embedded in paraffin for routine histology. All sections were stained with hematoxylin and eosin (HE).

Immunohistochemistry was performed on selected paraffin sections using a standard avidin-biotin complex method with diaminobenzidine as chromogen. Monoclonal antibodies against CD117, CD34, SMA, S100 protein (S-100), were used.

### Pathologic findings

The surgical specimen consisted of the lower-third esophageal tissue and the proximal tissue of the stomach including two tumid lymph nodes around the esophagus and one lymph node from the lesser gastric curvature. Another specimen was resected from the subserosa of gastric anterior wall.

Gross examination revealed that the esophageal mucosa was regular and smooth. A 2.5 cm  $\times$  1.5 cm tumor was found 1 cm away from the lower margin and 4 cm near the upper margin. The tumor was found to be gray-white with weak mucus shine, and easy to be separated from muscularis. No visible pigmentation was found in esophageal mucosa. The specimen from the gastric part including the squamo-columnar junction appeared normal.

Microscopy revealed that the tumor occupied the area from lamina propria to submucosa and crossed the muscularis mucosa in the lower third of esophagus. Histologically, the basaloid cells were arranged mainly in the form of solid, smooth-contoured lobules, or in the form of solid sheets, anastomosing trabeculae, or microcystic structures. Eosinophilic hyaline materials were found in the intertrabecular spaces and stroma of the tumor. The microcystic spaces contained basophilic mucoid matrix. The basaloid cells were round or oval in

shape with scant amphophilic cytoplasm, but sometimes they were abundant and clear. The nuclei showed either dark hyperchromatin or vacuolated nucleoplasm with 1 to 3 small distinct nucleoli. The number of mitoses was 15 under 10 high-power fields. The cells at the edges of basaloid islands tended to show peripheral nuclear palisading. Comedo necrosis was found within the basaloid lobules (Figure 1), mimicking adenoid cystic carcinoma and basaloid carcinoma without association with dysplasia, carcinoma *in situ*, squamous cell carcinoma, or focal squamous cell differentiation. It did not invade the muscularis. No malignant cells were observed in the two resection margins. All lymph nodes were free of malignant cells.

The mucosa of the upper resection margin exhibited a population of melanocytes/pigmentophages. The melanocytes were focally distributed in keratinocytes of the basal esophageal mucosa layer. Heavily pigmented, spindled or dendritic melanocytes/pigmentophages were located predominately in the superficial lamina propria, which were parallel to the covering epithelium and vertical to the long esophagus axis. The spindle and dendritic cells displayed no cytologic atypia and mitoses, in which the small nuclei were covered by the pigment. The pigmented cells occupied about 1 mm, and the thickness was no more than 1 mm. We did not find pigmented cells any more when we recut the specimen embedded in paraffin (Figure 2A). Moreover, there was single or cluster of hair follicles and horn cysts in the mucosa with melanocytes (Figure 2A-E). Similarly, below the esophageal carcinoma, melanocytes were focally distributed in keratinocytes of the basal esophageal mucosa layer, but not in hair follicles or horn cysts (Figure 2F).

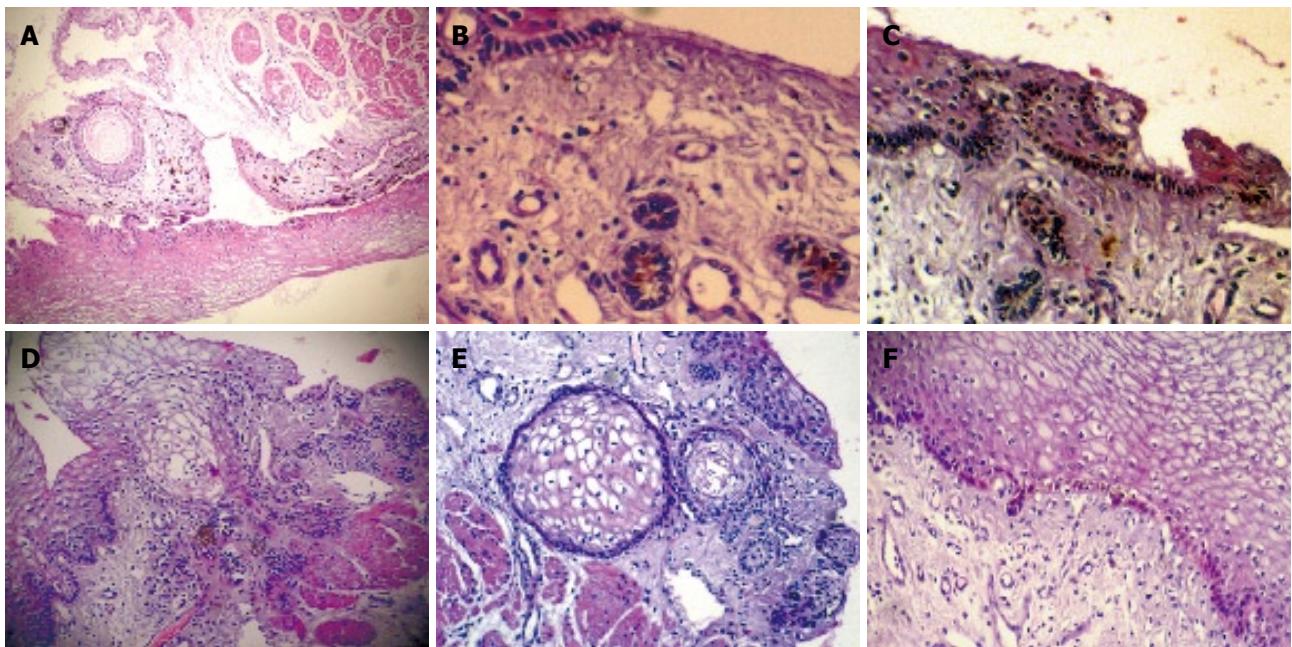
The gastric tumor consisted of basophilic spindle-cells, which were arranged in short fascicles but could be aligned in a strikingly Schwannian pattern with prominent nuclear palisading but no mitotic activity and necrosis (Figure 3A). Immunohistochemistry showed that these cells exhibited a strong immunoreactivity on CD117 (Figure 3B) and CD34 (Figure 3C). No specific reaction to the antibodies SMA (Figure 3D) and S-100 (Figure 3E) was observed.

Based on morphology and marker pattern, a diagnosis of esophageal BSC and low malignant potential gastric stromal tumor was made with blue nevus and hair follicle proliferation.

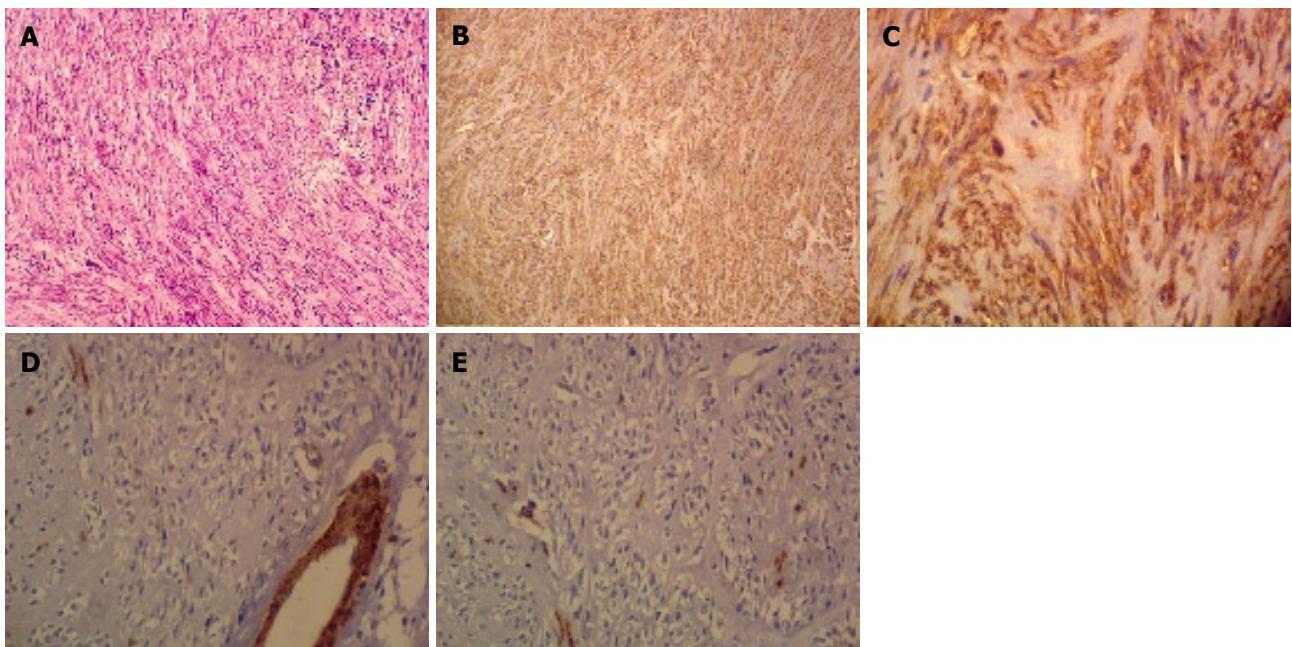
## DISCUSSION

During early embryogenesis, melanocytes migrate from the neural crest to the epidermis, hair follicles, oral cavity, nasopharynx, uvea, leptomeninges and inner ear. In the normal human esophagus, scattered melanocytes may occur at the epithelial-stromal junction. In 1963, Dela Pava first described pre-existing esophageal melanocytes found in 4 out of 100 autopsies and found that they are not related to esophageal disorders<sup>[1]</sup>.

In the normal esophagus, the number of intramucosal melanocytes is low and their presence can



**Figure 2** Focal distribution of melanocytes in keratinocytes of the basal esophageal mucosa layer, heavily pigmented, spindled or dendritic melanocytes/pigmentophages locate predominately in the superficial lamina propria. Moreover, there are several hair follicles and a horn cyst in the mucosa, with melanocytes in the hair follicle (HE,  $\times 40$ ) (A), and distribution of melanocytes in keratinocytes of the basal esophageal mucosa layer and hair follicles in the mucosal lamina propria with melanocytes (B,C) (B: HE,  $\times 200$ ; C: HE,  $\times 40$ ), cluster of hair follicles and horn cysts in the mucosa (D,E) (HE,  $\times 40$ ), and focal distribution of melanocytes in keratinocytes of the basal esophageal mucosa layer below the BSC (HE,  $\times 40$ ) (F).



**Figure 3** Gastric tumor consisting of basophilic spindle-cells arranged in short fascicles and aligned in a strikingly Schwannian pattern with prominent nuclear palisading but no mitotic activity and necrosis (HE,  $\times 40$ ) (A), and immunohistochemistry showing strong immunoreactivity of basophilic spindle-cells on CD117 ( $\times 40$ ) and CD34 (C) ( $\times 400$ ) but no specific reaction to the antibodies SMA (D) ( $\times 200$ ) and S-100 (E) ( $\times 200$ ).

easily be overlooked, but many pathologic conditions can increase the number of melanocytes at the epithelial-stromal junction<sup>[4]</sup>, suggesting that proliferation of melanocytes is based on pre-existing melanocytes in the interface between epithelium and underlying stroma. However, the proliferation of melanocytes might also arise from the scattered stromal neuroectodermal cells or from pluripotent epithelial stem cells<sup>[2]</sup>. The

proliferation of melanocytes is rarely observed with an estimated incidence of about 0.07%-0.15% in patients undergoing endoscopy<sup>[3]</sup>. Results of a recent study<sup>[2]</sup> and our case, however, indicate that melanocytes are much more frequently seen in association with reactive changes in the squamous epithelium, such as chronic esophagitis. Moreover, melanocytes seem to be more frequently found in carcinoma specimens than in

unselected autopsy material, which supports the notion that melanocytes may develop due to the long-standing inflammatory irritation of the mucosa. Our patient had a 30-year history of reflux, soda, smoking and alcohol which might indicate that esophageal BSC and proliferation of melanocytes are a general tissue reaction pattern in response to acid, basic and noxious stimuli. Hair follicles in the esophagus have not been reported up to now and may be due to the tissue reaction to the stimuli too, suggesting that melanocytes and hair follicles might develop from pluripotent epithelial stem cells. Up to now, no report is available on the association between esophageal BSC and hair follicles. Melanocytic nevi are uncommonly seen in esophageal mucosa. To our knowledge, only two cases of blue nevus in the esophagus are reported<sup>[5]</sup>. The blue nevus may be derived from the aboriginal melanocytes or pluripotent epithelial stem cells, and may be transformed into melanoma. What physiological and/or pathologic potential the hair follicles and blue nevus have in the esophageal mucosa needs to be further investigated.

The present case is very unusual because not only melanocytes and hair follicles proliferate in esophagus but also are associated with esophageal BSC and gastric stromal tumor. The epidemiology and etiology remain uncertain, although esophageal melanocytes are mostly located in the middle and lower thirds of the esophagus<sup>[6-9]</sup>. The intrinsic pathogenesis underlying

these pathological changes in the gastrointestinal tract remains largely a mystery.

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LETTERS TO THE EDITOR

## Laparoscopic fenestration of multiple giant biliary mucinous cystadenomas of the liver

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### TO THE EDITOR

We have previously described a case of huge biliary cystadenoma in a middle aged woman and presented an extensive review of this uncommon entity<sup>[1]</sup>. We report, from the same institution, a case of synchronous, multiple, huge biliary mucinous cystadenomas with unique features.

A 70-year old woman presented in our institution with a 1-year history of low back pain. Abdominal ultrasonography revealed the presence of multiple hepatic cysts (the largest one measuring 16 cm in diameter) and cholelithiasis. Subsequent abdominal computer tomography (CT) scan showed multiple hepatic cysts in the right lobe of the liver and a smaller cyst in the left lobe (Figure 1). The cysts were unilocular, smooth, without septa but with homogeneous content. The cysts were well defined, had no irregular thickness, mural nodules or papillary projections. The radiological findings were consistent with simple liver cysts. Laboratory examination showed an elevation of CA 125 to 94.50 U/mL (reference value: ≤ 35 U/mL) and CA 15.3 to 45.10 U/mL (reference value: ≤ 35 U/mL), while carcinoembryonic antigen (CEA), α-fetoprotein (AFP) and CA19.9 were within the normal values.

The liver function tests and serum bilirubin levels were also within the normal range. The patient underwent a wide laparoscopic fenestration of the cysts and laparoscopic cholecystectomy, under general anaesthesia. Intraoperatively, three cysts were identified (Figure 2). Laparoscopic Lin fenestration<sup>[2]</sup> was performed following the decompression of the cysts with aspiration of their mucinous content. The cysts were unroofed and haemostasis was obtained with UltraCision harmonic scalpel. The frozen section examination was unrevealing. Finally, a typical laparoscopic cholecystectomy was performed. The postoperative period was uneventful. The patient was discharged on the second postoperative day.

Pathologic evaluation of the paraffin-fixed material revealed that the cyst wall was comprised of a single layer of cuboidal-to-columnar epithelium, a moderately

### Abstract

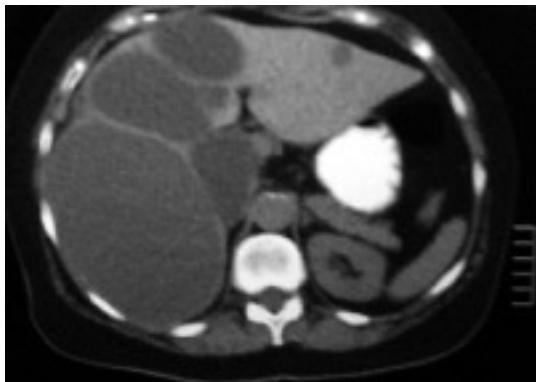
Biliary cystadenomas of the liver are rare, cystic neoplasms of the biliary ductal system usually occur in middle aged women. We report a case of synchronous multiple huge biliary mucinous cystadenomas with unique features. This is, according to our knowledge, the first report in the literature about three synchronously occurring hepatobiliary cystadenomas. Cystadenomas have a strong tendency to recur, particularly following incomplete excision, and a potential of malignant transformation. A therapeutic re-evaluation may be necessary when the diagnosis of hepatobiliary cystadenoma is made after the operation and an open liver resection should be considered.

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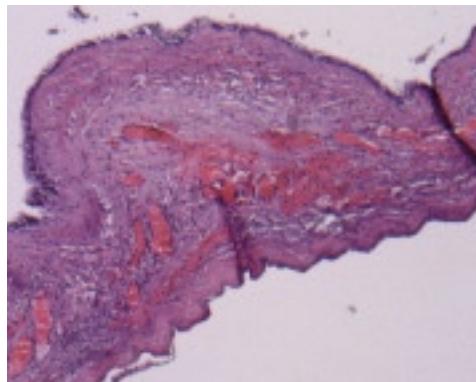
**Key words:** Biliary cystadenoma; Liver cysts; Laparoscopic fenestration

**Peer reviewer:** Simon D Taylor-Robinson, MD, Department of Medicine A, Imperial College London, Hammersmith Hospital, Du Cane Road, London W12 0HS, United Kingdom

Manouras A, Lagoudianakis E, Alevizos L, Markogiannakis H, Kafiri G, Bramis C, Filis K, Toutouzas K. Laparoscopic fenestration of multiple giant biliary mucinous cystadenomas of the liver. *World J Gastroenterol* 2008; 14(26):



**Figure 1** CT scan demonstrating multiple unilocular liver cysts. The cysts are well delineated, smooth with uniform content, consistent with simple liver cysts.



**Figure 3** The cysts are lined with cuboidal to columnar non ciliated mucin secreting cells. A moderate cellular stroma and an outer collagenous layer comprise the rest of the cyst wall (HE,  $\times 40$ ).



**Figure 2** Laparoscopic view of the cysts and gallbladder.

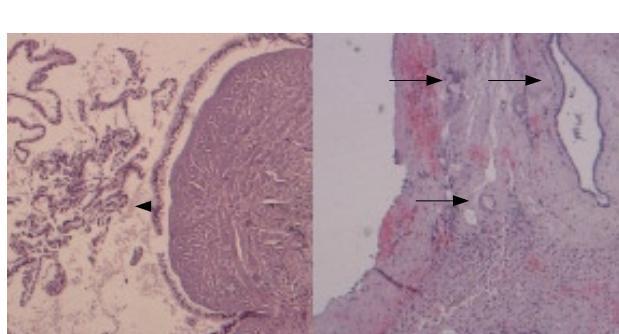
cellular stroma and an outer dense layer of collagenous connective tissue (Figure 3). These findings were consistent with cystadenoma without evidence of malignancy.

The pathologic examination of the specimen from the largest cyst, measuring 17 cm in diameter, showed papillary projections, internal smaller cysts, calcifications and hyaline degeneration of the stroma (Figure 4). These findings were not found in the smaller cysts and might represent the natural course of the cystadenoma. The absence of cellular atypia and intestinal metaplasia indicated the benign nature of this tumour.

The patient was informed about the possibility of malignant degeneration of the lesions and declined a further surgical intervention. No evidence of recurrence was found after a 6-mo follow-up period.

This is, according to our knowledge, the first report in the literature about three synchronously occurring hepatobiliary cystadenomas.

Hepatobiliary cystadenoma is an unusual cystic lesion, occurring more commonly in middle age women in the fifth decade of life and its size varies from 2 to 25 cm<sup>[3,4]</sup>. The exact etiology of biliary cystadenoma is unknown. The tumor is thought to result from the development of ectopic rests of primitive foregut sequestered within the liver or from the obstruction of the congenitally aberrant bile duct<sup>[5]</sup>. Malignant transformation is known to occur from hepatobiliary cystadenoma to cystadenocarcinoma<sup>[3]</sup>.



**Figure 4** The wall of the largest cyst showing papillary projections of the lining epithelium (arrowhead), presence of microscopic cysts with the same epithelial lining (arrows), calcifications and hyaline degeneration of the stroma (HE,  $\times 40$ ).

The diagnosis of liver cyst can be easily made by ultrasonography, CT and magnetic resonance imaging (MRI). Hepatobiliary cystadenoma can be differentiated from simple cysts due to internal echogenic content or other ultrasonographic features<sup>[6]</sup>. However, in our patient, the differentiation of the cystadenomas from simple cysts was not possible by either ultrasound or even CT scan.

Radical excision of the tumor is the first choice of treatment. Operative removal is essentially important considering the high rate of recurrence (90%) if excision is not complete, and the possibility of a malignant transformation. Frozen sections are used to direct the surgical treatment but they can miss the diagnosis of hepatobiliary cystadenoma during a fenestration as was the case in our patient<sup>[7]</sup>. Thomas *et al*<sup>[8]</sup> reported that tumor recurrence occurs in two thirds of the patients, who have only local or pericystic excision. In contrast, only 10% of the patients, who have hepatic lobectomy, hemihepatectomy or radical excision of cystadenoma with a rim of normal tissue with a diameter of 2 cm, require further surgery for recurrence. Therefore, the detection of hepatobiliary cystadenoma after a laparoscopic surgical approach implies many challenging therapeutic dilemmas<sup>[8,9]</sup>.

In conclusion, differential diagnosis of cystic lesions of the liver should always include hepatobiliary cystadenoma. There are no major reports addressing the diagnosis, treatment and follow-up outcome of laparoscopic surgery

for hepatobiliary cystadenoma. A therapeutic evaluation may be necessary when the diagnosis of hepatobiliary cystadenoma is made after the operation and an open liver resection should be considered.

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## Meetings

### Events Calendar 2008-2009

FALK SYMPOSIA 2008  
January 24-25, Frankfurt, Germany  
Falk Workshop: Perspectives in Liver Transplantation

International Gastroenterological Congresses 2008  
February 14-16, Paris, France  
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies  
[www.easl.ch/hepatitis-conference](http://www.easl.ch/hepatitis-conference)

February 14-17, Berlin, Germany  
8<sup>th</sup> International Conference on New Trends in Immunosuppression and Immunotherapy  
[www.kenes.com/immuno](http://www.kenes.com/immuno)

February 28, Lyon, France  
3<sup>rd</sup> Congress of ECCO - the European Crohn's and Colitis Organisation Inflammatory Bowel Diseases 2008  
[www.ecco-ibd.eu](http://www.ecco-ibd.eu)

February 29, Québec, Canada  
Canadian Association of Gastroenterology  
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK  
British Society of Gastroenterology Annual Meeting  
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China  
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea  
Asian Pacific Association for the Study of the Liver  
18<sup>th</sup> Conference of APASL: New Horizons in Hepatology  
[www.apaslseoul2008.org](http://www.apaslseoul2008.org)

March 29-April 1, Shanghai, China  
Shanghai-Hong Kong International Liver Congress  
[www.livercongress.org](http://www.livercongress.org)

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco  
OESO 9<sup>th</sup> World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas  
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA  
SAGES 2008 Annual Meeting - part of Surgical Spring Week  
[www.sages.org/08program/html/](http://www.sages.org/08program/html/)

April 18-22, Buenos Aires, Argentina  
9<sup>th</sup> World Congress of the International Hepato-Pancreato Biliary Association  
Association for the Study of the Liver  
[www.ca-ihpba.com.ar](http://www.ca-ihpba.com.ar)

April 23-27, Milan, Italy  
43<sup>rd</sup> Annual Meeting of the European Association for the Study of the Liver  
[www.easl.ch](http://www.easl.ch)

May 2-3, Budapest, Hungary  
Falk Symposium 164: Intestinal

### Disorders

May 18-21, San Diego, California, USA  
Digestive Disease Week 2008

May 21-22, California, USA  
ASGE Annual Postgraduate Course Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion  
E-mail: education@sgo.org

June 4-7, Helsinki, Finland  
The 39<sup>th</sup> Nordic Meeting of Gastroenterology  
[www.congrex.com/ngc2008](http://www.congrex.com/ngc2008)

June 5-8, Sitges (Barcelona), Spain  
Semana de las Enfermedades Digestivas  
E-mail: sepd@sepdi.es

June 6-8, Prague, Czech Republic  
3<sup>rd</sup> Annual European Meeting: Perspectives in Inflammatory Bowel Diseases  
E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey  
ESGAR 2008 19<sup>th</sup> Annual Meeting and Postgraduate Course  
E-mail: fca@netvisao.pt

June 11-13, Stockholm, Sweden  
16<sup>th</sup> International Congress of the European Association for Endoscopic Surgery  
E-mail: info@aes-eur.org

June 13-14, Amsterdam, Netherlands  
Falk Symposium 165: XX International Bile Acid Meeting, Bile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic  
Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management  
E-mail: idca2008@guantar.cz

June 25-28, Barcelona, Spain  
10<sup>th</sup> World Congress on Gastrointestinal Cancer  
Imedex and ESMO  
E-mail: meetings@imedex.com

June 25-28, Lodz, Poland  
Joint Meeting of the European Pancreatic Club (EPC) and the International Association of Pancreatology (IAP)  
E-mail: office@epc-iap2008.org  
[www.e-p-c.org](http://www.e-p-c.org)  
[www.pancreatology.org](http://www.pancreatology.org)

June 26-28, Bratislava, Slovakia  
5<sup>th</sup> Central European Gastroenterology Meeting  
[www.ceurgem2008.cz](http://www.ceurgem2008.cz)

July 9-12, Paris, France  
ILTS 14<sup>th</sup> Annual International Congress  
[www.ilts.org](http://www.ilts.org)

September 10-13, Budapest, Hungary  
11<sup>th</sup> World Congress of the International Society for Diseases of the Esophagus  
E-mail: isde@isde.net

September 13-16, New Delhi, India  
Asia Pacific Digestive Week  
E-mail: apdw@apdw2008.net

APDW 2008  
September 13-16, New Delhi, India  
Organized: Indian Society of Gastroenterology

III FALK GASTRO-CONFERENCE

September 17, Mainz, Germany  
Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany  
Falk Symposium 166: GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic  
Prague Hepatology Meeting 2008  
[www.czech-hepatology.cz/phm2008](http://www.czech-hepatology.cz/phm2008)

September 20-21, Mainz, Germany  
Falk Symposium 167: Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France  
Third Annual Meeting  
European Society of Coloproctology  
[www.escp.eu.com](http://www.escp.eu.com)



October 8-11, Istanbul, Turkey  
18<sup>th</sup> World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists  
E-mail: orkun.sahin@serenas.com.tr

October 18-22, Vienna, Austria  
16<sup>th</sup> United European Gastroenterology Week  
[www.negf.org](http://www.negf.org)  
[www.acv.at](http://www.acv.at)

October 22-25, Minnesota, USA  
Anstralian Gastroenterology Week 2008  
E-mail: gesa@gesa.org.au

October 22-25, Brisbane, Australia  
71<sup>st</sup> Annual Colon and Rectal Surgery Conference  
E-mail: info@colonrectalcourse.org

October 31-November 4, Moscone West Convention Center, San Francisco, CA  
59<sup>th</sup> AASLD Annual Meeting and Postgraduate Course  
The Liver Meeting Information: [www.aasld.org](http://www.aasld.org)

November 6-9, Lucerne, Switzerland  
Neurogastroenterology & Motility Joint International Meeting 2008  
E-mail: ngm2008@mci-group.com  
[www.ngm2008.com](http://www.ngm2008.com)

November 12, Santiago de Chile, Chile  
Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

November 28-29, Cairo, Egypt  
1<sup>st</sup> Hepatology and Gastroenterology Post Graduate Course  
[www.egyptgastrohep.com](http://www.egyptgastrohep.com)

December 7-9, Seoul, Korea  
6<sup>th</sup> International Meeting Hepatocellular Carcinoma: Eastern and Western Experiences  
E-mail: sglee@amc.seoul.kr

INFORMATION FOR ALL  
FALK FOUNDATION e.V.  
E-mail: [symposia@falkfoundation.de](mailto:symposia@falkfoundation.de)  
[www.falkfoundation.de](http://www.falkfoundation.de)

Advanced Courses - European

Institute of Telesurgery EITS - 2008 Strasbourg, France  
January 18-19, March 28-29, June 6-7, October 3-4

N.O.T.E.S  
April 3-5, November 27-29  
Laparoscopic Digestive Surgery

June 27-28, November 7-8  
Laparoscopic Colorectal Surgery

July 3-5  
Interventional GI Endoscopy Techniques  
Contact address for all courses:  
E-mail: [info@eits.fr](mailto:info@eits.fr)

International Gastroenterological Congresses 2009  
March 23-26, Glasgow, Scotland  
Meeting of the British Society of Gastroenterology (BSG)  
E-mail: [bsg@mailbox.ulcc.ac.uk](mailto:bsg@mailbox.ulcc.ac.uk)

May 17-20, Denver, Colorado, USA  
Digestive Disease Week 2009

November 21-25, London, UK  
Gastro 2009 UEGW/World Congress of Gastroenterology  
[www.gastro2009.org](http://www.gastro2009.org)



### Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London, The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.



## Instructions to authors

### GENERAL INFORMATION

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- 2 Lin GZ, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixudiarrhoea. *Shijie Huaren Xiaohua Zaishi* 1999; **7**: 285-287

In press

- 3 Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

- 5 Vallancien G, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

Volume with supplement

- 7 Geraud G, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment

of migraine and in comparison with sumatriptan. *Headache* 2002; 42 Suppl 2: S93-99 [PMID: 12028325]

*Issue with no volume*

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (401): 230-238 [PMID: 12151900]

*No volume or issue*

- 9 Outreach: Bringing HIV-positive individuals into care. *HRS A Careaction* 2002; 1-6 [PMID: 12154804]

## Books

*Personal author(s)*

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

*Chapter in a book (list all authors)*

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

*Author(s) and editor(s)*

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

*Conference proceedings*

- 13 **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

*Conference paper*

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

**Electronic journal** (list all authors)

- 15 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/eid.htm>

**Patent** (list all authors)

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[1]Passed away on October 20, 2007

[2]Passed away on June 11, 2007



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## Roles of *Helicobacter pylori* BabA in gastroduodenal pathogenesis

Yoshio Yamaoka

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### Abstract

Interactions between BabA and Lewis b ( $Le^b$ ) related antigens are the best characterized adhesin-receptor interactions in *Helicobacter pylori* (*H pylori*). Several mechanisms for the regulation of BabA expression are predicted, including at both transcriptional and translational levels. The formation of chimeric proteins (*babA/B* or *babB/A* chimeras) seems to play an especially important role in translational regulation. Chimeric BabB/A protein had the potential to bind  $Le^b$ ; however, protein production was subject to phase variation through slipped strand mispairing. The *babA* gene was cloned initially from strain CCUG17875, which contains a silent *babA1* gene and an expressed *babA2* gene. The sequence of these two genes differs only by the presence of a 10 bp deletion in the signal peptide sequence of *babA1* that eliminates its translational initiation codon. However, the *babA1* type deletion was found only in strain CCUG17875. A few studies evaluated BabA status by immunoblot and confirmed that BabA-positive status in Western strains was closely associated with severe clinical outcomes. BabA-positive status also was associated with the presence of other virulence factors (e.g. *cagA*-positive status and *vacA* s1 genotype). A small class of strains produced low levels of the BabA protein and lacked  $Le^b$  binding activity. These were more likely to be associated with increased mucosal inflammation and severe clinical outcomes than BabA-positive strains that exhibited  $Le^b$  binding activity. The underlying mechanism is unclear, and further studies will be necessary to investigate how the complex BabA-receptor network is functionally

coordinated during the interaction of *H pylori* with the gastric mucosa.

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**Key words:** *Helicobacter pylori*; BabA; Pathogenesis; Lewis antigens

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### INTRODUCTION

The adherence of *Helicobacter pylori* (*H pylori*) to the gastric mucosa is widely assumed to play an important role in the initial colonization and long-term persistence in the human gastric mucosa. Analysis of the three completed *H pylori* genomes (strains 26695, J99, and HPAG1) has confirmed the presence of five major outer membrane protein (OMPs) families, which comprise approximately 4% of the *H pylori* genome. Among the families, members of the large Hop (*Helicobacter* outer membrane protein) family were the first characterized OMPs in *H pylori*. Several OMPs in the Hop family have been reported to act as adhesion molecules including the blood group antigen binding adhesin (BabA), sialic acid binding adhesin (SabA), adherence-associated lipoprotein (AlpA and AlpB), outer membrane inflammatory protein (OipA), and HopZ. Lewis b antigen ( $Le^b$ ) and related fucosylated ABO blood group antigens are recognized by BabA<sup>[1]</sup>, whereas sialyl-Lewis x and sialyl-Lewis a antigens ( $sLe^x$  and  $sLe^a$ ) are recognized by SabA<sup>[2]</sup>. The corresponding receptors for AlpAB, OipA, and HopZ remain unknown. To date, BabA- $Le^b$  is the best-characterized adhesin-receptor interaction in *H pylori*. In this review, I summarize recent data giving new insight into BabA and its role in pathogenesis.

### IDENTIFICATION OF BABA

It is well known that  $Le^b$  is the dominant antigen in the

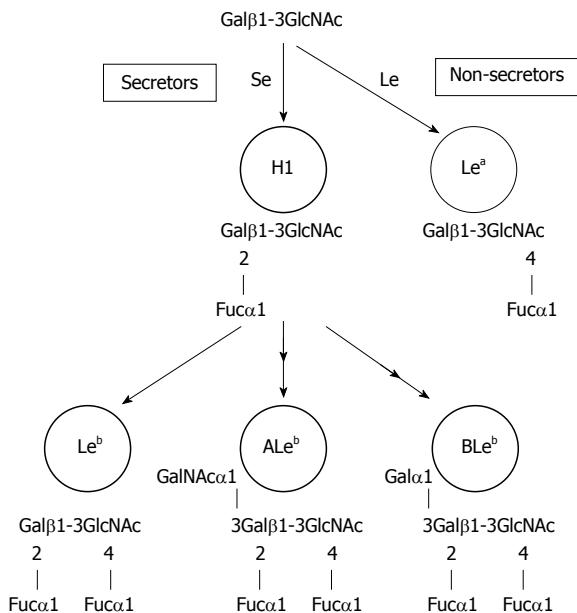
gastric mucosa of secretor-positive individuals<sup>[3]</sup>, and the negative secretor status is associated with a Lewis a ( $\text{Le}^a$ )-dominant phenotype in the gastric mucosa (Figure 1). In 1993, two studies showed that *H pylori* can bind to fucosylated glycoconjugates containing  $\text{Le}^b$  structures on the surface of gastric epithelial cells within human biopsy specimens<sup>[4,5]</sup>. Studies using transgenic mice expressing the human  $\text{Le}^b$  epitope in gastric epithelial cells indicated that  $\text{Le}^b$  functions as a receptor for an *H pylori*-specific adhesin and mediates its attachment to the gastric pit and surface mucous cells<sup>[6]</sup>. Further studies using the same transgenic mice showed that *H pylori* was adherent to the surface of gastric epithelial cells, resulting in severe chronic gastric inflammation and atrophy; whereas *H pylori* was localized in the mucous layer in non-transgenic control mice<sup>[7]</sup>.

In 1998, Ilver *et al* analyzed the blood group antigen-binding activity by measuring binding of *H pylori* to  $^{125}\text{I}$ -labeled fucosylated blood group antigens<sup>[1]</sup>. Among 100 *H pylori* isolates examined, 66% bound the  $\text{Le}^b$  antigen; whereas 95% of the isolates did not bind the related  $\text{Le}^a$ , H-2,  $\text{Le}^x$ , or  $\text{Le}^y$  antigens. The 78 K adhesin recognizing the  $\text{Le}^b$  antigen was detected on the bacterial cell outer membrane and was isolated by a combined ligand identification and purification technique and designated as blood group antigen-binding adhesin (BabA)<sup>[1]</sup>. Additional analyses revealed two sets of clones that encode two proteins with almost identical NH<sub>2</sub>-terminal domains and completely identical COOH-terminal domains, but with divergent central domains. The corresponding genes were designated *babA* and *babB*; BabA but not BabB had  $\text{Le}^b$  antigen-binding activity. Therefore, the central domain in *babA* is believed to determine the specificity of receptor binding<sup>[1, 8-12]</sup>; however, the motifs of the *babA* gene that are involved in binding are still unknown.

## FUNCTION OF BABA

BabA originally was defined as an adhesin binding to the  $\text{Le}^b$  antigen. The H-1 antigen is the carbohydrate structure that defines the blood group O phenotype in the ABO blood group system.  $\text{Le}^b$ , which is difucosylated, is formed by the addition of a branched fucose (Fuc) residue to H-1. The antigens that define blood group A and B phenotypes and corresponding antigens in the Lewis blood group system are formed by terminal N-acetylgalactosamine (GalNAc) or galactose (Gal) substitutions of H-1 and  $\text{Le}^b$  [A-1 and A-Lewis b ( $\text{ALe}^b$ ), and B-1 and B-Lewis b ( $\text{BLLe}^b$ ) antigens, respectively; Figure 1].

Recently, Aspholm-Hurtig *et al* investigated the ability of BabA to bind  $\text{Le}^b$ ,  $\text{ALe}^b$  and  $\text{BLLe}^b$ <sup>[8]</sup>. Among 265  $\text{Le}^b$ -binding *H pylori* strains from various geographic regions, more than 95% of *H pylori* strains are “generalists” (able to bind  $\text{ALe}^b$  and  $\text{BLLe}^b$  in addition to  $\text{Le}^b$ ); whereas a small subset of strains bind exclusively to  $\text{ALe}^b$ , and are called “specialist” strains. The authors proposed that the middle region of BabA was responsible for determining the different binding patterns; however, the



**Figure 1** Biosynthetic pathways of Lewis antigens starting from type 1 lacto series core chains. Starting from type 1 core chains, an  $\alpha 1,2$ -fucosyltransferase (Se) transfers fucose (Fuc) to the terminal galactose (Gal), resulting in the H-1 antigen (H1). H-1 antigen is a target for GalNAc- or Gal-transferases (in blood group A or B individuals) or remains unmodified (in blood group O individuals). These intermediates are modified for the fucosylation step by an  $\alpha 1,3/4$ -fucosyltransferase (Le), resulting in the difucosylated histo-blood group antigens  $\text{ALe}^b$ ,  $\text{ALe}^b$  and  $\text{Le}^b$ . Non-secretors are unable to produce an active Se product, and are only targets for the Le gene product  $\text{Le}^a$ .

specific motifs could not be identified<sup>[8]</sup>. Interestingly, “specialist” strains originated predominantly from South American individuals (where 60% of strains were classified as “specialist”), who are known to express almost entirely the blood group O phenotype. South American isolates in their study were from Peruvian and Venezuelan Amazon Amerindian populations and also from a Colombian mestizo (mixed Amerindian-European ancestry) population; probably most of these strains came mainly from European strains<sup>[13,14]</sup>. These data suggest that most specialist *babA* alleles may have arisen by mutation and/or recombination within the last 500 years. Therefore, the authors propose that such rapid evolution of BabA in response to host mucosal glycosylation patterns would enable the pathogen to adapt to their individual hosts while avoiding host immune responses, and contributes importantly to the extraordinary chronicity of human *H pylori* infection worldwide.

The mucins secreted by gastric mucous cells form a mucous gel layer covering the gastric mucosa. This gel layer is considered the first line of gastric mucosal defense against luminal noxious agents<sup>[15-17]</sup>, and damage to the mucous gel is thought to precede gastric mucosal injury. The gastric surface mucous cells and gland mucous cells express the secretory mucins MUC5AC and MUC6/MUC5B, respectively<sup>[18,19]</sup>. The majority of *H pylori* reside in the gastric mucus overlying the epithelium. It is reported that *H pylori* could be co-localized with MUC5AC gastric mucin, but not with MUC6-producing cells in the glandular areas, suggesting

that adhesion is predominantly toward MUC5AC-specific ligands in gastric mucosa<sup>[20]</sup>. Subsequently, this binding phenotype could be correlated with the expression of an active BabA protein in *H pylori* and the presentation of the Le<sup>b</sup> antigen in the gastric mucin MUC5AC<sup>[21,22]</sup>. However, since BabA-positive strains also attached to Le<sup>b</sup>-negative MUC5AC of non-secretors, the involvement of additional epitopes and/or adhesins also must be involved<sup>[21]</sup>. In addition, binding of *H pylori* to MUC5B had been described<sup>[23]</sup> and a recent study confirmed that the binding was predominantly mediated by BabA and to a lesser degree by SabA adhesin<sup>[24]</sup>.

## LOCATION OF THE *BABA* GENE IN THE *H PYLORI* GENOME

*H pylori* 26695, J99 and HPG1 each possess one *babA* allele (HP1243/JHP0833/HPG1\_0876) and one *babB* allele (HP0896/JHP1164/HPG1\_0320)<sup>[25-27]</sup>. Interestingly, the genomic locations of *babA* and *babB* genes are completely different among three strains (Figure 2). In strain J99, *babA* (JHP0833) is downstream of *hypD* (JHP0835) with a J99-specific gene (JHP0834) intervening, and *babB* (JHP1164) is downstream of *s18* (JHP1165). In strain 26695, the locations of *babA* (HP1243) and *babB* (HP0317) are reversed. The chromosomal locations downstream of *hypD* and *s18* are referred to as locus A and locus B, respectively. In strain 26695, one gene encoding OMPs homologous to *babA* and *babB* (HP0317: denoted *babC*) with unknown function have been identified<sup>[27-29]</sup>. The location is referred to as locus C; interestingly, in strain HPG1, the *babB* gene is located at locus C and *babC* gene at locus B. The *bab* genes initially were cloned from the strain CCUG17875, and this strain has two *babA* genes and one *babB* gene<sup>[1]</sup>. Gene inactivation experiments identified that only one gene (denoted *babA2*) had Le<sup>b</sup> antigen-binding activity; whereas another gene (*babA1*) did not; *babA1* was located at locus B; however the locus of *babA2* was not determined<sup>[1]</sup>.

The location of *babA* and *babB* in various clinical isolates of *H pylori* recently has been reported<sup>[29,30]</sup>. Hennig *et al*<sup>[30]</sup> analyzed a panel of 35 *H pylori* isolates and found that 24 (69%) contained *babA* sequences. In contrast, the *babB* gene was identified in 34 strains (97%). The *babA* gene was located at locus A for 19 strains (54%), at locus B for four strains (11%), and at locus C for three strains (9%). Four strains contain two copies of the *babA* gene, and the *babA* sequences found at two loci were identical in three strains and almost identical in one strain (i.e. three substitutions near the 5' ends of the genes in one strain), indicating that the multiple copies of *babA* presumably resulted from gene conversion (intragenomic nonreciprocal recombination) events. Importantly, two strains possessed the *babA* gene; however, the locus could not be identified, suggesting that there are additional unidentified chromosomal loci for *babA*, although *babA* may be found in one of three chromosomal loci in most cases.

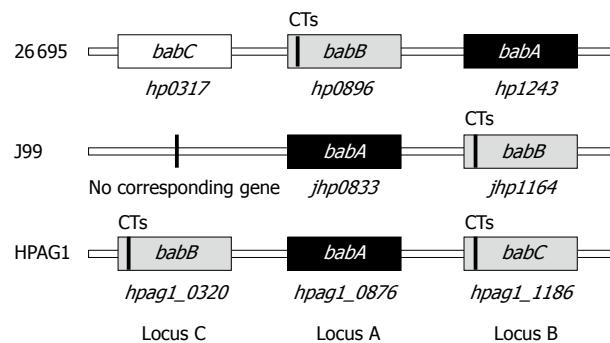


Figure 2 Genomic location of the *babA*, *babB*, and *babC* genes in strains J99, 26695, and HPG1. CT: CT dinucleotide repeats.

Colbeck *et al*<sup>[29]</sup> analyzed a panel of 44 *H pylori* strains and found that 32 (73%) contained *babA* sequences. In contrast, the *babB* gene was identified in 41 (95%) isolates. The *babA* gene was located at locus A for 25 strains (57%) and at locus B for 18 strains (41%); locus C was not evaluated. Interestingly, although chromosomal DNA from low-passage-number, single-colony isolates was used, there was a mixed genotype in 30% (13/44) of the isolates, where the population of cells contained both *babA* and *babB* at the same locus. As a result, 11 strains were found to contain two copies of the *babA* gene including eight with mixed *babA* and *babB* at locus B.

Overall, from two detailed studies I conclude that the *babA* gene prefers to be located at locus A, some strains do not possess the *babA* gene, some strains possess multiple copies of the *babA* gene, and most strains possess the *babB* gene. The presence of *babB* might confer a stronger selective advantage than the presence of *babA*.

## REGULATION OF BABA

### Chimera formation between *babA* and *babB*

Several different mechanisms for regulation of BabA expression are predicted, including at the transcriptional and translational levels. As for translational regulation, the formation of chimeric proteins seems to play an important role. Chimera formation between *babA* and *babB* initially was reported by Pride and Blaser<sup>[11]</sup> who found that in two of 42 (5%) clinical isolates studied, the 5' regions of *babB* were replaced with the first 56 bp of *babA* (*babA/B* chimera). In addition, these authors showed that gene conversions frequently ( $10^{-3}$ ) occur in *H pylori*, and the events are *recA*-dependent and DNase-resistant, indicating that they likely result from intragenomic recombination. *babA/B* chimeras also have been reported experimentally during *H pylori* infection in Rhesus monkeys<sup>[10]</sup>.

In addition to *babA/B* chimeras, *babB/A* chimeras have been observed<sup>[9]</sup>. A *babA2* mutant from strain CCUG17875, defective in Le<sup>b</sup>-binding, regained its activity by homologous recombination of a silent *babA1* gene into the *babB* locus, resulting in a chimeric *babB/A* gene. A silent wild-type *babA1* gene still was present.

The frequency of the *babA* mutant with Le<sup>b</sup>-binding was approximately 10<sup>-5</sup>. Detailed analyses of the chimeric *babB/babA* gene showed that the first 47 bp were *babB*-specific, the following 66 bp were shared between both *babA* and *babB*, and the remaining sequence was *babA*-specific. The second crossover event likely occurred within a region where the sequences of the *babA1* and the *babB* locus were identical. The chimeric BabB/A protein has the potential to bind Le<sup>b</sup>; however, protein production was subject to phase variation through slipped-strand mispairing based on the number of Cysteine-Threonine (CT) dinucleotide repeats in the 5' region of the *babB* gene (switch "on" = functional and switch "off" = non-functional).

Initially, only five genes encoding the OMPs in *H pylori* (*oipA*, *sabA*, *sabB*, *babB* and *hopZ*) were reported to undergo phase variations in the 5' region such that not all strains produce functional proteins<sup>[25,27]</sup>. However, recent studies confirmed that phase variation is a method of regulating BabA production in some strains<sup>[10,29,30]</sup>. CT repeats were observed in 13 of 43 (30%) strains<sup>[29]</sup> and 4 of 22 (18%) strains<sup>[30]</sup>. Importantly, detailed analyses of the *babA* gene with CT repeats showed that the signal peptides are closely related to signal peptides of paralogous BabB proteins, whereas sequences further downstream were typical BabA sequences<sup>[30]</sup>. Taken together, these data suggest that the *babA* gene with the CT repeat might be the result of the translocation of *babA* into *babB* thereby generating a chimeric *babB/babA* gene. Interestingly, the *babC* gene in strain HPAG1 possessed CT repeats in the 5' coding region, whereas the *babC* gene in strain 26695 did not. These data suggest that *babB/C* chimera also might have occurred in some strains.

As described above, Colbeck *et al* found that there were cases with mixed *babA* and *babB* genes, especially at locus B<sup>[29]</sup>. The frequency of *babA* translocated at the *babB* locus was between 10<sup>-3</sup> and 10<sup>-4</sup>, which is in agreement with the frequency in strain CCUG17875<sup>[9]</sup>. Detailed investigation of 10 strains showed that the recombination event was identified from approximately 50 to 200 bp downstream of the ATG in five strains (all recombination occurred at locus B) and upstream of the ATG in the other five strains<sup>[29]</sup>. In the former case, the resulting gene forms the *babB/A* chimera, whereas complete recombination occurred in the latter case.

Overall, frequent translocation between *babA* and *babB* genes appears to be the main mechanism of regulating BabA expression. Therefore, *H pylori* uses both antigenic variation and phase variation to regulate *babA* expression.

#### **Genomic mutations in the coding region of the *babA* gene**

The *babA* gene initially was cloned from strain CCUG17875, which contains a silent *babA1* gene and an expressed *babA2* gene<sup>[1]</sup>. The sequence of these two genes differed only by the presence of a 10 bp deletion in the signal peptide sequence of *babA1* that eliminates its translational initiation codon. However, my group re-

cently found that all 80 strains from a panel of Western and East Asian isolates contained an intact ATG start codon in the *babA* gene<sup>[31]</sup>, and another group also reported the absence of the *babA1* type deletion<sup>[11,12,29,30,32]</sup>. Overall, the absence of a translation initiation codon, as described for *babA1* from CCUG17875, should be rare. Point mutations leading to stop codon, deletion and insertion in other parts of the *babA* gene also are not common; Hennig *et al* found one of 24 *babA*-positive strains (4%) contained a frameshift mutation that prevented expression of a full-length BabA protein (amino acid position at 55)<sup>[30]</sup>.

#### **Transcriptional regulation of BabA**

Transcriptional regulation of BabA also has been reported. Backstrom *et al* found that only *babA2*, but not *babA1* was transcribed in strain CCUG17875<sup>[9]</sup>. Their analyses showed that *babA* transcription seemed to be regulated by the number of adenine [poly(A)] nucleotides within the -10 to -35 region of the *babA* promoter. The -10 and -35 region of the *babA2* sequences are highly homologous to the consensus for *E.coli*  $\sigma^{70}$  promoter sequences. This region was stable when the number of adenines was 10 (*babA2*) but would become non-functional when the number was 14 (*babA1*). The authors hypothesized that the poly(A) sequences between the -10 and the -35 sites could be prone to slippage mutations that allow changes in the level of transcription of downstream genes. However, other studies could not confirm that the -10 to -35 spacing played an important role in regulating *babA* expression<sup>[30,31]</sup>. Further studies will be necessary to fully interrogate the roles of transcriptional regulation of BabA.

Overall, there are several predicted mechanisms that may control BabA expression in some strains; however, there are many cases that remain unexplained. *H pylori* strains that do not produce BabA can be divided into five types, as shown in Table 1.

#### **RELATIONSHIP BETWEEN BABA AND LE<sup>B</sup> BINDING ACTIVITY**

My group recently examined BabA protein and Le<sup>b</sup> binding activity for 80 strains (40 from Japan and 40 from Colombia)<sup>[31]</sup>. BabA protein was measured by immunoblot analyses using anti-BabA antiserum (AK277), and Le<sup>b</sup> binding activity was measured by binding of *H pylori* to <sup>125</sup>I-labeled fucosylated blood group antigens. *H pylori* strains were divided into two major groups: BabA-positive (76 strains) or BabA-negative (four strains). Semi-quantitative analyses of the BabA-positive strains allowed the BabA-positive strains to be classified into two distinct groups: those with high levels of BabA expression (68 strains) or those with low levels of BabA expression (eight strains). All of the 68 strains that exhibited Le<sup>b</sup> binding activity produced high levels of BabA. The low and non-producer strains did not exhibit Le<sup>b</sup> binding activity. Based on this finding, my group classified the strains into three distinct groups

**Table 1** Five major types of *H pylori* strains that do not produce BabA

<i>babA</i> gene	Status
Negative	Include <i>babA/B</i> chimeras
Present	Regulated by slipped strand repairing and the status is "off" (probably equal to <i>babB/A</i> chimeras)
Present	Lack a translation initiation codon (single case of <i>babA1</i> in strain CCUG17875)
Present	Have a frameshift mutation(s) causing non-productive translation
Present	Without apparent mutations and without a hypothesis for the lack of expression

based on their expression levels of BabA: (1) BabA-high producers (BabA-H), which produce BabA protein at high enough levels to mediate Le<sup>b</sup> binding, (2) BabA-low producers (BabA-L), which produce a small amount of BabA but not enough to mediate Le<sup>b</sup> binding, and (3) BabA-negative strains, which do not produce any BabA protein.

## BABA, LE<sup>b</sup> BINDING ACTIVITY AND CLINICAL OUTCOMES

There currently are only a few studies that correlate the importance of BabA with clinical outcomes using immunoblot analyses<sup>[31,33,34]</sup>. My group recently performed large scale studies of 520 geographically diverse patients presenting with different clinical symptoms to evaluate BabA status by immunoblot analysis<sup>[31]</sup>. A total of 250 isolates from Western countries (150 strains from Colombia, 100 from the U.S.) and 270 isolates from East Asia (150 from Korea and 120 from Japan) were studied. All strains from East Asia expressed BabA protein. Twenty-four (9.8%) of Western strains were BabA-negative and were associated with milder gastric injury and lower *H pylori* density than BabA-positive status. BabA-negative status was inversely correlated with *cagA* status or *vacA* s1 genotype (i.e. only one (4.2%) and none (0%) of these BabA-negative strains were *cagA*- or *vacA* s1-positive, respectively). This is in agreement with previous studies that the *cagA* status was related to the presence of Le<sup>b</sup> binding activity<sup>[11]</sup> and the presence of the *babA* gene<sup>[30]</sup>.

Importantly, a small class of strains were BabA-positive but produced low levels of the BabA protein and lacked Le<sup>b</sup> binding activity (BabA-L)<sup>[31]</sup>. Although these strains were functionally BabA-negative and were typically CagA-positive, they were more likely to be associated with duodenal ulcer, gastric cancer, and increased mucosal inflammation and atrophy than BabA-positive strains that exhibited *in vitro* Le<sup>b</sup> binding activity (BabA-H strains) and BabA-negative strains. This finding suggests that either *in vitro* Le<sup>b</sup> binding activity does not accurately reflect the severity of mucosal damage or that the clinical outcome or *in vitro* binding activity does not accurately reflect *in vivo* conditions. The underlying reason why strains with BabA-L status were more highly correlated with severe diseases than strains with BabA-H status is unknown, and it remains unclear whether expressing low levels of BabA have a direct role in the pathogenesis of gastroduodenal diseases. It is possible that BabA expression is influenced by the

intragastric environment and that the phenotype of the BabA-L strains is an epiphenomenon rather than a cause of disease. It is possible that strong Le<sup>b</sup> binding activity is associated with an inappropriate immune response resulting in severely inflamed mucosa. If so, the ability to change the BabA status from a high producer to low producer (i.e. Le<sup>b</sup> binding to Le<sup>b</sup> non-binding) would be advantageous for the organism, and a low producer might reflect an adaptation of *H pylori* that enhances survival in inflamed gastric mucosa. It also is possible that BabA expression down-regulates the proinflammatory effects of other putative virulence factors, such as the *cag* PAI and OipA.

## DETECTION OF FUNCTIONAL BABA GENE

Most previous studies evaluating BabA (*babA*) status have used PCR techniques based on detection of the 10 bp deletion to distinguish between the *babA2* and *babA1* genes (Table 2)<sup>[35-53]</sup>. However, as described above, strains carrying the prototypical silent *babA1* gene are very rare, and in addition, the BabA protein levels often do not match the presence of the *babA* (*babA2*) gene<sup>[31]</sup>. Current terminology for *babA1* and *babA2* in the literature is confusing, and many researchers mistakenly understand that *H pylori* strains that do not produce BabA are either *babA* gene-negative or *babA1*-positive (= *babA* gene lacking a translation initiation codon). However, only one case with *babA1* has been reported, and BabA non-producing strains also usually possess non-functional silent *babA* gene sequences (i.e. 2, 4, and 5 in Table 2). Unfortunately, current PCR methods regard non-functional *babA* status as *babA2*-positive. In addition, a recent study confirmed that the PCR method used to detect *babA2* with only one primer pair previously designed yielded many false-negative results, probably due to sequence variation among strains<sup>[31]</sup>.

Only a few studies have used a forward primer that is within the promoter region of the *babA* gene, a region that is identical to the sequence of *babA2* but different from that of *babA1* in strain CCUG17875<sup>[32,54,55]</sup>; however, recent analyses showed that the primers could also detect *babB* gene<sup>[31]</sup>. Overall, the information gained from currently used PCR-based methods must be interpreted with caution. In addition, I propose that researchers should not use current PCR-based methods in future studies.

Nonetheless, approximately half of the studies have suggested a correlation between *babA2*-positive *H pylori* in Western countries and increased risk of

**Table 2** PCR-based genotyping for the *babA2* gene in *H pylori* positive cases n (%)

Study	Year	Population	Number studied	Prevalence of <i>babA2</i> gene							
				Total	Gastritis	PUD	Cancer	MALT	Duodenitis	Related to diseases	
Western countries											
Gerhard <i>et al</i>	1999	Germany	114	82 (72)	18 (51)	23 (100)	21 (78)	20 (69)		Yes	
Prinz <i>et al</i> <sup>1,2</sup>	2001	Germany	145	57 (39)	57 (39)					-	
Rad <i>et al</i> <sup>1</sup>	2002	Germany	141	54 (38)	54 (38)					-	
Zambon <i>et al</i>	2003	Italy	167	60 (36)	26 (28)	20 (49)		14 (42)	Yes		
Oleastro <i>et al</i>	2003	Portugal	140	45 (32)	24 (23)	21 (58)				Yes	
Podzorski <i>et al</i>	2003	USA	61	33 (36)	22 (36)					-	
Oliveira <i>et al</i>	2003	Brazil	208	96 (46)	24 (32)	43 (54)	29 (56)			Yes	
Rad <i>et al</i> <sup>3</sup>	2004	Germany	207	73 (35)	73 (35)					-	
Lehours <i>et al</i>	2004	France	82	40 (49)	21 (54)		19 (44)			No	
Gatti <i>et al</i>	2005	Brazil	89	42 (47)	37 (53)	3 (20)	1 (100)	1 (33)		No	
Olfat <i>et al</i>	2005	Germany	92	41 (45)	19 (28)	22 (88)				Yes	
		Sweden	74	33 (45)	21 (48)	12 (40)				No	
		Portugal	91	31 (34)	12 (20)	19 (63)				Yes	
		Finland	57	34 (60)	12 (46)	22 (71)				Borderline ( <i>P</i> = 0.06)	
Gatti <i>et al</i>	2006	Brazil	94	38 (40)	18 (41)	20 (40)				No	
Asian countries											
Mizushima <i>et al</i>	2001	Japan	179	152 (85)	34 (81)	73 (85)	36 (90)	9 (82)		No	
Yu <i>et al</i>	2002	China	104	83 (80)	83 (80)					-	
Lai <i>et al</i>	2002	Taiwan	101	101 (100)	41 (100)	46 (100)	14 (100)			No	
Han <i>et al</i>	2004	China	141	90 (64)	28 (65)	50 (65)	12 (57)			Yes (DU vs GU)	
Zheng <i>et al</i>	2006	China	72	28 (39)	11 (39)	17 (40)				No	
Lee <i>et al</i>	2006	Korea	135	83 (61)	64 (57)		19 (86)			Yes	
Erzin <i>et al</i>	2006	Turkey	91	49 (54)	7 (23)	12 (43)	24 (73)			Yes	

PUD: Peptic ulcer disease; MALT: Mucosal-associated lymphoid tissue; DU: Duodenal ulcer; GU: Gastric ulcer. <sup>1</sup>88% had German nationality and 12% were from other European countries; <sup>2</sup>Samples were examined from the antrum and the corpus, and the corpus data are presented (in the antrum, 55 were *babA2*-positive); <sup>3</sup>89% had German nationality and 11% were from other southern European countries.

developing significant clinical outcomes<sup>[38,44-46,52]</sup> and are in agreement with protein data as described above<sup>[31,33,34]</sup>. The prevalence of clinical isolates with a non-functional *babA2* gene without production of BabA protein may be low and negligible in some studies.

## CONCLUSION

Several different mechanisms for regulation of BabA expression are predicted, including at both the transcriptional and translational levels. The formation of chimeric proteins seems to play an especially important role in translational regulation. The chimeric BabB/A protein has the potential to bind Le<sup>b</sup>; however, the production was subject to phase variation through slipped-strand mispairing. Currently used PCR-based methods to evaluate BabA status do not take this mechanism of regulation into account, and information gained from currently used PCR-based methods must be interpreted with caution. I strongly recommend that researchers should not use PCR-based methods in their future studies. Recent studies evaluating BabA status by immunoblot confirmed that BabA-positive status in Western strains was closely associated with severe gastric injury, high *H pylori* density, and severe clinical outcomes. A small class of strains produced low levels of the BabA protein and lacked Le<sup>b</sup> binding activity. Surprisingly, they were more likely to be associated with increased mucosal inflammation, atrophy, and severe clinical outcomes than BabA-positive strains that exhibit Le<sup>b</sup> binding activity.

The underlying reason is unclear, and further studies will be necessary to investigate how the complex BabA-receptor network is functionally coordinated during the interaction of *H pylori* with the gastric mucosa.

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EDITORIAL

## Stem cells, a two-edged sword: Risks and potentials of regenerative medicine

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### Abstract

The recent advancements in stem cell (SC) biology have led to the concept of regenerative medicine, which is based on the potential of SC for therapies aimed to facilitate the repair of degenerating or injured tissues. Nonetheless, prior to large scale clinical applications, critical aspects need to be further addressed, including the long-term safety, tolerability, and efficacy of SC-based treatments. Most problematic among the risks of SC-based therapies, in addition to the possible rejection or loss of function of the infused cells, is their potential neoplastic transformation. Indeed, SCs may be used to cure devastating diseases, but their specific properties of self-renewal and clonogenicity may render them prone to generate cancers. In this respect, 'Stemness' might be seen as a two-edged sword, its bright side being represented by normal SCs, its dark side by cancer SCs. A better understanding of SC biology will help fulfill the promise of regenerative medicine aimed at curing human pathologies and fighting cancer from its roots.

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**Key words:** Stem cells; Regenerative medicine; Gastrointestinal diseases; Chronic liver diseases; Cell-based therapy

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### A LESSON FROM THE PAST: STEM CELLS AND TUMORS

The recorded history of human cancer begins with an Egyptian papyrus, dating between 3000 and 2000 B.C., which describes breast tumors in humans. The nomenclature 'cancer' was first proposed by Hippocrates. The term derives from the Greek words carcinos and carcinoma, both literally meaning crab. Galen offered a possible explanation for this name based on similitude with either the morphology of cancer or with its tenacious and parasitic behavior. According to legend, Celsus attempted to classify pathologic masses into three categories: secundum naturam, associated with pregnancy; propter naturam, the tumefaction which develops following traumas and leads to tissue repair; and contra natura, synonymous with cancer<sup>[1,2]</sup>. After two millennia, this classification has found renewed perspective based on recent advancements in cell biology, with particular emphasis on the concepts of stemness and SCs.

In multicellular organisms, tissues are organized in a hierarchical manner, with SCs residing at the apex of the developmental pathway. SCs are defined as undifferentiated cells capable of self-renewal and differentiation into diverse mature progenies<sup>[3,4]</sup>. Therefore, SCs play a central role in tissue genesis, regeneration and homeostasis by providing new elements to increase tissue mass during pre- and post-natal growth, and replacing cell loss due to senescence or damage<sup>[5,6]</sup>. SCs are thought to alternate symmetric and asymmetric divisions, hence maintaining the property of self-renewal<sup>[7]</sup>. SCs possess a hierarchy of potentialities: from the totipotency of the zygote and its immediate progeny, to the pluripotency of embryonic SCs (ESCs), and the multi/unipotency of adult SCs (ASCs)<sup>[5,8,9]</sup>. ESCs are pluripotent cells derived from the inner cell mass of the blastocyst. ESCs can generate

any differentiated phenotype of the three primary germ layers by a process called determination<sup>[9,10]</sup>. At the end of embryogenesis, each tissue contains a heterogeneous population of cells at different stages of maturation, including relatively undifferentiated, self-renewing cells, termed adult SCs (ASCs). ASCs have a limited differentiation potential and are responsible for turnover and repair within the tissue of origin. ASCs have been identified in several organs, such as bone marrow (BM), gastrointestinal epithelium, skin, brain, muscle, and liver<sup>[8,11,12]</sup>. SCs colocalize with supporting cells in a physiologically limited and specialized niche, that varies in nature and location depending upon the tissue type<sup>[13,14]</sup>. The reciprocal interactions between SCs and their niche influence SC behavior: a complex network of developing signals regulates the balance between quiescence and the dividing state, leading ASCs toward self-renewal or differentiation<sup>[15,16]</sup>. According to the hierarchical model, long-term SCs (true SCs, extremely rare, with high differentiation potential and proliferative capacity) can give rise to short-term SCs (transit-amplifying or committed progenitors), which in turn are able to differentiate into mature elements providing tissue-specific functions<sup>[5,17]</sup>. Despite the paradigm of unidirectional cell determination, recent studies have shown that ASCs are endowed with an unexpected plasticity, as circulating adult progenitor cells can differentiate into mature cells of other tissue types<sup>[10,14,18]</sup>. A particularly high degree of plasticity is shown by bone marrow SCs (BM-SCs), which *in vivo* and *in vitro* studies have proved to be able to differentiate into a wide range of non-hematopoietic phenotypes<sup>[19-22]</sup>. It has also been demonstrated that BM-SCs normally circulate in the peripheral blood, and that the number of circulating SCs committed toward neuronal and hepatic differentiation increases following treatment with mobilizing agents<sup>[23]</sup>. This phenomenon has led to speculation about the existence of BM-derived pluripotent SCs, which could migrate from the peripheral blood into various tissues and contribute to normal turnover and repair following injury<sup>[5,15,24]</sup>.

## REGENERATIVE MEDICINE BASED ON STEM CELLS

The recent advancements in SC biology have led to the concept of regenerative medicine, which is based on SC potential for therapies aimed to facilitate the repair of degenerating or injured tissues<sup>[6]</sup>. SC-based therapies could be used to cure degenerative disorders associated with the loss of ASC functions, such as hematologic, cardiovascular, muscular and neurological diseases, gastrointestinal pathologies and chronic hepatopathies. SCs can be obtained from various sources, including embryos, fetal tissues, umbilical cord blood and adult organs. Once isolated, these cells may be forced to expand and differentiate into functional progenies suitable for cell replacement and tissue engineering<sup>[24]</sup>. ESCs, which have been isolated from humans and mice, can be maintained in an undifferentiated state indefinitely, though

they seem to develop genetic abnormalities over long periods in culture<sup>[15,25]</sup>. ESCs and their derivatives might constitute an easily available source to obtain a large number of transplantable cells for regenerative treatments. Nevertheless, the possibility of immune rejection and teratoma/teratocarcinoma formation in the recipients represent major obstacles to the success and safety of ESC clinical applications<sup>[26]</sup>. A promising alternative source for SC-based treatments may be represented by cells established from fetal organs and placental tissues, which do not seem to form teratomas/teratocarcinomas in humans. In particular, several studies have indicated that umbilical cord blood SCs (CBSCs) are an easily accessible source of multipotent SCs, which may be readily available for transplantation, or for further expansion and manipulation prior to cellular therapies<sup>[8]</sup>. The plasticity and accessibility of CBSCs has provided the rationale for creation of CBSC unit banks, where these cells can be collected and stored for future use<sup>[24]</sup>. Finally, the manipulation and/or stimulation of ASCs seems to be the most promising tool for SC-based treatments, as it could improve the endogenous regenerative potential without risk of rejection and overcome the ethical and political issues related to embryonic and fetal SCs<sup>[6,8,24]</sup>.

Focusing on ASC-based therapies in Gastroenterology, first attempts to translate regenerative medicine from theory to clinical practice have been made for various diseases, including celiac disease and inflammatory bowel disorders (IBD). In particular, following autologous BM transplantation, in a selected group of refractory celiac patients, significant histological improvement associated with impressive clinical progresses has been recorded<sup>[27,28]</sup>. Crohn's disease (CD) and ulcerative colitis (UC) are characterized by a status of chronic inflammation, mainly as a result of local immunological imbalance<sup>[29]</sup>. Several studies have suggested that either allogeneic or autologous BM-SC transplants may be effective in inducing CD and UC remission<sup>[30]</sup>. Various authors report their experience with IBD patients who underwent BM-SC transplantation for haematological malignancies and maintained a complete remission of their intestinal disease following transplantation<sup>[30]</sup>. The specific pathways and molecular mechanisms underlying the beneficial effects of HSC transplantation in IBD are still largely undefined. The immune system ablation followed by allogeneic transplantation of BM-SCs might provide a reset of the host immune system imbalance. Moreover, BM cells might contribute to tissue repair by facilitating neoangiogenesis and might also differentiate into epithelial cells and myofibroblasts<sup>[30,31]</sup>. The potential of BM-derived SCs in the treatment of IBD is currently being analyzed in clinical trials<sup>[30]</sup>. SCs might be used to cure other gastrointestinal pathologies, such as gastric ulcers, gastrointestinal motility disorders, and diabetes mellitus (DM)<sup>[32,33]</sup>. Regarding the latter, a major challenge in the treatment of DM is to provide patients with an insulin source that regulates glucose levels on a mandatory minute-to-minute basis<sup>[34,35]</sup>. In recent decades, new therapeutic strategies for the treatment of DM type I have been proposed, such as growth factor

administration, islet cell transplantation and also SC infusion to replace the dysfunctional beta-cells<sup>[35]</sup>. Different adult sources of extra-pancreatic SCs have been investigated, including CBSCs, whose efficacy for the treatment of DM has been shown in diabetic mice<sup>[36-39]</sup>. Another candidate for DM regenerative therapy is represented by BM-SCs. Numerous reports have showed that the infusion of BM-SCs can restore chemically-induced DM in mice<sup>[40,41]</sup>. Along with extra-pancreatic SC-based therapies, other researchers have focused their interest on endogenous pancreatic SCs (PSCs). The quest for an organ-bound PSC has received growing attention by the scientific community, because PSCs hold several advantages over extra-pancreatic sources, combining the ability for prolonged proliferation with an already established pancreatic commitment<sup>[35,42]</sup>. Finally, recent reports have demonstrated that extra-pancreatic, organ-bound SCs, such as liver SCs<sup>[43-46]</sup>, human adipose tissue-derived mesenchymal SCs<sup>[47]</sup> and gastrointestinal SCs<sup>[48]</sup> can differentiate into islet cells. Unfortunately, there are still no functional studies that show biphasic insulin release upon glucose challenge by these cells.

In Hepatology, the most appealing application for SC-based therapies consists in the treatment of end-stage hepatic diseases. Chronic liver pathologies affect almost a fifth of the general population, often requiring an orthotopic liver transplantation (OLT)<sup>[49]</sup>. Given the donor organ shortage, various alternatives to OLT have been evaluated, including cell-based therapies which are currently under investigation all over the world. Cell-therapies in hepatology have numerous advantages when compared to OLT: the cells can be expanded *in vitro*, genetically manipulated, cryopreserved, obtained from the same patient and infused without major surgery. Possible cell-based treatments consist of hepatocyte transplantation and the development of bio-artificial liver systems (BALs). BALs have been mainly applied as supportive devices in patients excluded from or waiting for OLT and hepatocyte transplantation has limited overall success, related to the large amount of cells required to achieve acceptable function<sup>[50,51]</sup>. Therefore, SC-based therapies are emerging as new alternatives to OLT for end-stage liver pathologies. The most promising source for SC-based therapies is currently represented by BM-SCs and/or by mobilizing/proliferating agents, such as granulocyte-colony stimulating factor (G-CSF), which is able to both enhance the BM-SC mobilization into the peripheral blood and facilitate the endogenous liver SC activation<sup>[52,53]</sup>. BM-SCs seem to be physiologically involved in the processes of liver repair in humans<sup>[54,55]</sup>. The possible therapeutic potential of these cells has been investigated by intraportal autologous transplantation of BM-SC, which achieved some clinical improvement<sup>[56,57]</sup>. However, some authors reported negative results regarding BM-SC-therapies for end-stage liver disorders<sup>[58]</sup>. Other clinical approaches have been based upon the administration of G-CSF alone or in combination with the reinfusion of the mobilized BM-SCs. The feasibility, safety, and pattern of BM-SC mobilization following G-CSF treatment in patients affected by cir-

rhosis has been evaluated in a few clinical trials<sup>[59-64]</sup>.

Overall, the use of ASCs for the treatment of gastrointestinal and hepatic disorders holds several advantages, such as easy accessibility, unlimited supply (given the possibility to expand the collected cells *in vitro*) and no risks of rejection or need for immunosuppressive therapies when autologous cells are employed. Nonetheless, some conceptual issues still limit the diffusion of such treatments into clinical practice. Firstly, on the basis of preclinical data, BM cells seem to facilitate gastrointestinal and hepatic regeneration mainly by a microenvironment modulation, which is likely to be transitory. In such a case, multiple treatments would presumably be required to achieve significant and lasting clinical results. Moreover, it has been observed that in some models of apparent transdifferentiation, SCs may actually be fusing with cells in the host tissue. Fusion phenomena between BM-SCs and other cells (Purkinje cells, cardiomyocytes and hepatocytes) have been shown both *in vitro* and *in vivo*<sup>[15,24]</sup>. The implications of this discovery are notable: fusion and transdifferentiation are not synonymous, since transdifferentiation requires that a specific SC program be activated on the basis of extracellular signals, whereas in the case of fusion, the plasticity is triggered by endogenous factors upon mixing of the cytoplasm and joining of the nuclei. It must also be noted that the fused cells are aneuploid and potentially unstable<sup>[15]</sup>. Consequently, the possibility of cell fusion and the risk of malignant transformation of the transplanted cells, especially those pre-expanded *in vitro* before reinfusion, cannot be excluded and impose a need for careful evaluation and longer follow-up periods for assessing the safety and efficacy of these SC-based treatments<sup>[24]</sup>.

## STEM CELL ORIGIN OF CANCER AND CANCER STEM CELLS

In the nineteenth century, Virchow and Cohnheim proposed that some tumors, such as teratocarcinomas, exhibiting features of a whole range of different organs and therefore mimicking fetal development, could originate from embryonic rests<sup>[10,65-67]</sup>. Over 150 years later, the hypothesis of a SC origin of cancer lends itself to a modern-day interpretation of this theory: in a given tissue, somatic tumors could originate from the malignant transformation of a SC or its progeny during the determination process, a phenomenon called maturation arrest<sup>[10]</sup>. It is well accepted that carcinogenesis is a multi-step process, involving accumulation of genetic mutations leading to the transformation of normal cells into tumorigenic cells. Every proliferating cell within a tissue may be targeted by carcinogenic stimuli and undergo the process of transformation. Because of the specific characteristics of SCs, mutations within the SC compartment may result in cancer transformation<sup>[15]</sup>. Similarly, tumors might also arise from mutated progenitor cells which have regained the property of self-renewal, thereby dedifferentiating towards a SC phenotype<sup>[68-72]</sup>. Presumably, fewer mutagenic changes are required to transform a SC, in which the machinery to specify and

regulate self-renewal is already active, as compared to more committed progenitor cells, in which self-renewal must be activated ectopically<sup>[70]</sup>. Another potential source of tumorigenic cells may be represented by circulating pluripotent cells, originating from the BM and able to migrate into non-hematopoietic sites. The existence of such a population of SCs, whose properties are reminiscent of ESCs, has been suggested in humans and experimental animal models<sup>[73]</sup>. Once recruited, these cells may behave as normal SCs, and, therefore may accumulate mutations over time and initiate malignancies. Indeed, a recent report described a mouse model of gastric cancer induced by *H Pylori* infection, in which BM-derived cells were able to contribute to cancer development<sup>[74]</sup>. The hypothesis of a SC origin of tumors imposes caution when proposing SC-based therapies to treat human diseases. It is well known that ESCs may give rise to tumors, while cancers derived from ASC-therapies have never been reported. Nonetheless, the long-term safety of ASC infusion has not been adequately tested: preclinical studies and clinical trials with longer follow-up periods should be recommended prior to large-scale clinical applications of such cell-based therapies.

Along with the possible role of SCs in the cellular origin of tumors, mounting evidence suggests that cancer might be considered as a SC disease. Over the past 30 years, several studies have demonstrated that most cancers possess a hierarchic organization: the great majority of cancer cells cannot sustain the tumor mass, nor establish secondary lesions elsewhere in the body. Only a minority of cancer cells appear to be tumor-initiating and possess the metastatic phenotype. These cells have the property of self-renewal, can differentiate into any cell within the tumor population, and can migrate, establishing metastases. Given the similarities between normal SCs and tumor-initiating cells, the latter have been termed cancer SCs (CSCs)<sup>[75]</sup>. Studies on acute myelogenous leukemia (AML) firstly showed that only a small subset of cancer cells was capable of extensive proliferation both *in vitro* and *in vivo*. Two models have been proposed to explain this phenomenon: the stochastic theory and the cancer SC theory<sup>[76]</sup>. In the first model, processes of self-renewal versus differentiation occur randomly, so that every cancer cell has an equal probability of retaining self-renewal capacity. Conversely, the cancer SC theory postulates a hierarchical organization of functionally distinct cell subpopulations, at the apex of which resides a small population of tumor-initiating cells, responsible for cancer growth and progression. Such a hierarchical organization was first documented in hematological malignancies by Dick *et al*, who showed that only AML-initiating cells could induce AML when transplanted into SCID mice<sup>[77,78]</sup>. These results represented both the first direct demonstration of the existence of CSCs, and a proof of principle extendible to solid tumors. Currently, distinct populations of CSCs have been identified within the hematopoietic system<sup>[77,79]</sup>, breast<sup>[80]</sup>, brain<sup>[81]</sup>, prostate<sup>[82,83]</sup>, lung<sup>[84]</sup>, skin, bone, kidney, ovary, head and neck cancers, and also gastrointestinal and liver tumors<sup>[85-90]</sup>.

Tumor-initiating cells mimic SC properties to sustain

the growth and spread of the tumor, while eluding the intrinsic and extrinsic controls that regulate homeostasis within SC populations. The unique properties of CSCs explain the failure of traditional chemotherapeutic strategies aimed at reduction of tumor mass by targeting proliferating cells: CSCs are usually quiescent and thus refractory to these treatments. The cancer SC hypothesis offers new insights for the development of therapeutic strategies in oncology, which will require a deep understanding of CSC molecular profile and biological behaviour<sup>[15,65]</sup>. Potential targets for CSC-based therapies in oncology might be found by comparing SC and CSC properties. i.e. it is well known that CSCs share molecular pathways involved in the maintenance of stemness (such as Wnt, Sonic Hedgehog, and Notch signalling) with SCs and that they are responsive to similar motomorphogens involved in both SC migration and cancer metastasis. The development of drugs antagonizing these signals may be helpful in inhibiting CSC proliferation and mobilization, therefore blocking cancer growth and metastasis<sup>[15,65]</sup>. Moreover, SCs and CSCs are able to secrete cytokines and angiopoietic factors which are critical for sustaining tumors, and that can be specifically targeted by anti-angiogenetic therapies<sup>[24]</sup>. However, an ideal CSC-based therapy would require targeting of CSCs, while sparing normal SCs. Indeed, despite similarities in terms of immunophenotype with their normal counterparts, some cell-surface markers and metabolic pathways must differ in CSCs compared with SCs, implying a biological uniqueness of CSCs. As a consequence, the identification of specific CSC-markers and pathways appears to be fundamental in order to develop novel therapeutic strategies in oncology. The quest for a surface marker which will enable isolation and further characterization of tumor-initiating cells within human cancers has already begun. Several studies have suggested that the CSC fraction within various tumors might be identified by the expression of CD133, a trans-membrane glycoprotein<sup>[91]</sup>. CD133 is expressed by progenitor cells belonging to neuronal, hematopoietic, epithelial and endothelial lineages and its expression has been reported in several tumor tissues, including melanomas, kidney, ovarian, colon and liver cancers<sup>[85-91]</sup>. In our opinion, CD133 might be useful to enrich the CSC fraction within some tumors, but it cannot be considered as a specific cancer SC-antigen. Indeed, CD133 is expressed by various normal SCs and also progenitor cells; moreover, upon a careful examination of the published studies, it seems that only a minority of CD133+ cancer cells is tumor-initiating<sup>[85-91]</sup>.

## STEMNESS AS A TWO-EDGED SWORD

A regenerative medicine based on SCs is no longer a future perspective, since SC research is already supporting an escalating industry, engaged in testing treatments for every sort of disease. Nonetheless, critical aspects need to be further addressed, including the long-term safety, tolerability, and efficacy of SC-based treatments, as well as their carcinogenic potential. Indeed, SCs represent the key to tissue genesis, regeneration and homeosta-

sis. However, for their specific characteristics, SCs may also represent a unique target for tumorigenic stimuli<sup>[16]</sup>. Stemness might be seen as a two-edged sword, its bright side being represented by normal SCs, its dark side by CSCs. This scenario leads to a reinterpretation of the previously mentioned Celsus' tumor classification, where ESCs represent the source of tumors secundum naturam; normal ASCs restore homeostasis following injuries, being responsible for tumors propter naturam; CSCs mimic normal ASCs in respect to self-renewal potential, but elude homeostatic regulation, resulting in tumors contra natura.

The CSC hypothesis imposes caution when proposing SC-based therapies, because infused SCs may degenerate into CSCs and give rise to neoplasms. This possibility should impose further preclinical studies prior to large-scale clinical applications of SC-based therapies. However, the CSC hypothesis also offers new insights for anti-cancer treatments, based upon the similarities and differences between SCs and CSCs. As a consequence, normal SC and CSC research must proceed side-by-side, because the identification of unique CSC targets requires a deep understanding of normal SC molecular profile and properties. The promise of regenerative medicine based on SCs imposes a better knowledge of SC and CSC biology, to help prevent and cure human pathologies and fight cancers from their roots.

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EDITORIAL

## Role of cytokines in inflammatory bowel disease

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## INTRODUCTION

Inflammatory bowel disease (IBD) comprises two forms, Ulcerative Colitis (UC) and Crohn's disease (CD). Currently, the pathogenesis of UC and CD is not completely understood, although the chronic relapsing inflammation is thought to be result from a dysregulated, aberrant immune response to intestinal flora in a context of genetic predisposition. In IBD, this loss of immune tolerance toward the enteric flora it is mediated by different molecules.

Cytokines are key signals in the intestinal immune system, and are known to participate in the disruption of the so-called normal state of controlled inflammation (physiological inflammation of the gut)<sup>[1]</sup>. Cytokines are small peptide proteins produced mainly by immune cells that facilitate communication between cells, stimulate the proliferation of antigen specific effector cells, and mediate the local and systemic inflammation in an autocrine, paracrine, and endocrine pathways<sup>[2]</sup>. In IBD, the innate immune response plays a critical role. Activated dendritic cells (DC) and macrophages secrete several cytokines that actively regulate the inflammatory response in UC and CD. Once secreted by these antigen presenting cells (APC), these cytokines trigger and differentiate many T cells activating the adaptive immune response. IBD has also a T cell dysregulation where clearance of overreactive and autoreactive cells is disturbed, in addition to an imbalance of Treg/Th1, Th2 and newly described Th17 cells populations in the activated state. The lack of appropriate regulation from T cells, or an over-production of effector T cells, participates in the development and exacerbation of IBD<sup>[3]</sup>.

Altogether, APCs, Th1, Th2, T regulatory cells and

## Abstract

Inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC), represents a group of chronic disorders characterized by inflammation of the gastrointestinal tract, typically with a relapsing and remitting clinical course. Mucosal macrophages play an important role in the mucosal immune system, and an increase in the number of newly recruited monocytes and activated macrophages has been noted in the inflamed gut of patients with IBD. Activated macrophages are thought to be major contributors to the production of inflammatory cytokines in the gut, and imbalance of cytokines is contributing to the pathogenesis of IBD. The intestinal inflammation in IBD is controlled by a complex interplay of innate and adaptive immune mechanisms. Cytokines play a key role in IBD that determine T cell differentiation of Th1, Th2, T regulatory and newly described Th17 cells. Cytokines levels in time and space orchestrate the development, recurrence and exacerbation of the inflammatory process in IBD. Therefore, several cytokine therapies have been developed and tested for the treatment of IBD patients.

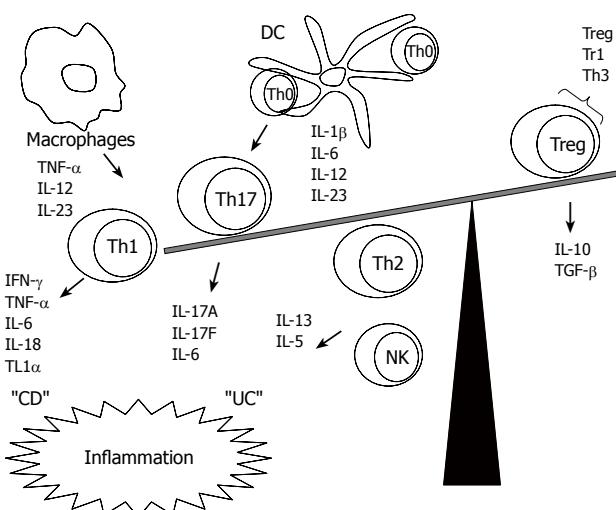
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**Key words:** Cytokines; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Inflammation

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**Table 1** Role of cytokines and cell lines involved in their production in patients with IBD

Cytokine	UC	CD	Cells involved in the production
TNF- $\alpha$	Up-regulated	Up-regulated	Macrophages
TL1 $\alpha$	Unknown	Up-regulated	Th1
IL-1 $\beta$	IL-1ra/IL-1 ratio	IL-1ra/IL-1 ratio	Macrophages
IL-6	Up-regulated	Up-regulated	Macrophages, DC, Th17 and others
IL-18	Not	Yes, not in all patients	Macrophages
TGF- $\beta$	Not clear, maybe defective signalling	Not clear, maybe defective signalling	Th0, Th3, Treg
IL-10	Not clear	Yes, up-regulated	Tr1 and Breg
IL-4	Not clear	Not clear	Th2, NK
IL-12	Up-regulated	Up-regulated	Macrophages, DC
IL-23	Yes	Yes	Macrophages, DC
IL-27	Not clear	Up-regulated	APCs
IL-17	Up-regulated	Up-regulated	Th17
IL-13	Up-regulated	Not	Th1, NK
IL-5	Up-regulated	Not	Th2, NK

**Figure 1** Cytokines imbalance between effector and T regulatory cells in IBD.

most recently characterized Th17 and their cytokine products play a complex role in IBD<sup>[4]</sup>. These cellular interactions are modulated by both traditionally studied cytokines (such as TNF- $\alpha$ , INF- $\gamma$ , IL-1, IL-6, IL-4, IL-5, IL10, TGF- $\beta$ ) and others recently characterized (like IL-13, IL-12, IL-18, IL-23), considered to be either pro or anti-inflammatory, as shown in Table 1<sup>[5]</sup>. Although many common responses in IBD are mediated by cytokines, such as the regulation of the production of inflammatory mediators, reactive oxygen metabolites, nitric oxide, leukotriens, platelet-activating factor, and prostaglandins, activation of the nuclear factor  $\kappa$ B (NF- $\kappa$ B) and inhibition of apoptosis, how cytokines determine the nature of the immune response in IBD may be quite different among IBD forms<sup>[6]</sup>. CD is associated with a Th1 T cell mediated response, characterized by enhanced production of IFN- $\gamma$  and TNF- $\alpha$ . IL-12 and IL-23 govern the Th1 differentiation which in combination with IL-15, IL-18 and IL-21 will induce the stabilization of polarized Th1. On the other hand, in UC, the local immune response is less polarized, but it is characterized by CD1 reactive natural killer T cell production of IL-13 and Th2 cytokine production, as shown in Figure 1<sup>[7]</sup>.

## CLASSICAL PRO-INFLAMMATORY CYTOKINES

Lymphocytes and APCs orchestrate a lot of the inflammation in IBD, mainly the production of TNF- $\alpha$ , a 17-kD pleiotropic cytokine produced by innate immune cells as macrophages, monocytes, and also by differentiated T cells. TNF- $\alpha$  exerts its pro-inflammatory effects through increased production of IL-1 $\beta$  and IL-6, expression of adhesion molecules, proliferation of fibroblasts and procoagulant factors, as well as initiation of cytotoxic, apoptotic, acute-phase responses, and inhibition of apoptosis<sup>[8,9]</sup>. TNF- $\alpha$  expression in human macrophages was discovered in the colonic tissue and macrophages in both patients with CD and UC<sup>[10]</sup> and serum levels of TNF- $\alpha$  correlate with clinical and laboratory indices of intestinal disease activity<sup>[11]</sup>. Clinical studies have reported a dramatic improvement in CD patients treated with anti-TNF- $\alpha$  therapy such as infliximab, adalimumab and certolizumab pegol<sup>[12]</sup>. Reductions in the number of IFN- $\gamma$  producing, lamina propria mononuclear cells (LPMC) in colonic biopsies results from anti-TNF- $\alpha$  treated patients<sup>[13]</sup>.

The signalling of TNF- $\alpha$  starts with serum soluble TNF receptor I and II (sTNF-R I, II) levels correlate with disease activity in IBD patients. More specifically, sTNF-RI is up-regulated in the serum of IBD patients compared to healthy controls and could be used as a marker for disease activity<sup>[14]</sup>. sTNF-RII levels are significantly more elevated in serum from active CD patients as compared to UC and could be used as an additional parameter to discriminate both diseases<sup>[12]</sup>. Recently TNF receptor type 1-dependent activation of innate responses was shown to reduce intestinal damage-associated mortality<sup>[15]</sup>.

Related to the TNF- $\alpha$ , the TNF-like factor (TL1A) seems to stimulate IFN- $\gamma$  secretion by binding to the death receptor 3 (DR3). A higher percentage of cells express the TL1A receptor DR3 in mucosal biopsies taken in CD and UC, and increased synthesis of IFN- $\gamma$  has been observed to correlate with severity of disease in IBD patients<sup>[16]</sup>. This molecule links TNF related apoptosis in inflammatory intestinal epithelial lesions,

tumour-necrosis-factor related apoptosis inducing ligand (TRAIL) messenger RNA and protein were markedly up-regulated in IEC and lamina propria lymphocytes in animal model. Interferon-gamma and TNF-alpha potently induced TRAIL in IEC and TRAIL is highly up-regulated in IEC in inflammatory ileum and colon<sup>[19]</sup>.

In addition to TNF- $\alpha$ , IL-1 seems to be important in the pathogenesis of IBD because of its immunological up-regulatory and pro-inflammatory activities. The IL-1 system consists of IL-1 $\alpha$  and IL-1 $\beta$ , both of which are produced by various cell types through the initiation of cyclooxygenase type 2, phospholipase A, and inducible nitric oxide synthase (iNOS)<sup>[17]</sup>. The IL-1 system can be also highly regulated by IL-1 receptor antagonist (IL-1Ra), as supported by the findings of high plasma and tissue levels of IL-1Ra in patients with IBD, indicating that IL-1Ra may be part of the host mechanism for downregulation of inflammation<sup>[18]</sup>. The IL-1Ra/IL-1 ratio decreases with increasing IBD activity, while remaining constant in uninvolving CD and inflammatory control specimens: this may contribute to the pathogenesis of chronic gut inflammation<sup>[19]</sup>. Increased levels of IL-1 in IBD may be result of stimulation of colonic macrophages that can activate interleukin (IL)-1 converting enzyme (ICE) and hence release mature IL-1 $\beta$  into the colonic mucosa<sup>[20]</sup>.

In contrast to other cytokines, IL-6 is a pleiotropic cytokine that exerts its proinflammatory effects largely by means of its soluble IL-6 receptor (sIL-6R). The combination of soluble IL-6 receptor (sIL-6R) and IL-6 stimulates cells that only express gp130 and not IL-6R, a process known as trans-signalling. IL-6 signalling through signal transducer and activator of transcription-3 (STAT3) has been extensively studied<sup>[21]</sup>. This system plays a central role in several immunologic reactions during the development of IBD, and circulating levels of IL-6 and sIL-6R correlate with many clinical features of CD and UC<sup>[22-24]</sup>. Blockade of IL-6 trans-signalling causes T-cell apoptosis, indicating that the IL-6-sIL-6R system mediates the resistance of T cells to apoptosis in CD<sup>[24]</sup>. Yamamoto *et al* (Kallen KJ<sup>[25]</sup>) introduced the anti-IL-6 receptor monoclonal antibody to a murine colitis model and found that the treatment with this antibody reduced IFN- $\gamma$ , TNF- $\alpha$ , and IL-1 $\beta$  mRNA, and suppressed expression of several intracellular adhesion molecules in the colonic vascular endothelium. The anti-IL-6 receptor monoclonal antibody also abrogates murine colitis by effectively blocking the recruitment of leukocytes and increasing T-cell apoptosis<sup>[26]</sup>. Peripheral immune cells as well as colon epithelial and lamina propria cells with an active form of IL-6/STAT3 system may be responsible of the high correlation with the degree of mucosal inflammation<sup>[27]</sup>. The signaling of IL-6/STAT3 in the activation of mucosal T cells has been suggested as a major therapeutic target for the future<sup>[28]</sup>. STAT-3 itself induces the anti-apoptotic factors Bcl-2 and Bcl-xL, thus resulting in T-cell resistance against apoptosis. This circle of T-cell accumulation, mediated by apoptosis resistance, finally leading to chronic

inflammation, can be blocked by anti-IL-6 receptor antibodies<sup>[29]</sup>.

## IL-18

IL-18 is produced by intestinal epithelial cells and was originally identified as an IFN- $\gamma$  inducing factor, shares similarities with the IL-1 family in terms of its structure, processing, receptor, signal transduction pathway, and pro-inflammatory properties<sup>[30]</sup>. Recent studies have shown that the balance between this pleiotropic pro-inflammatory cytokine and its natural inhibitor, IL-18-binding protein (IL-18BP), may contribute to the pathogenesis of IBD<sup>[31]</sup>. A local increase of IL-18 expression has been demonstrated in chronic lesions of CD compared with uninvolving areas or normal controls<sup>[32]</sup>, and an increase in IL-18 was also shown to be accompanied by marked increases in IL-18 receptor-positive immune cells as well as intense transcription of IL-18 induced by cytokines, such as IFN- $\gamma$ , IL-1 $\beta$ , and TNF- $\alpha$ <sup>[33]</sup>. In a recent study has been reported that IL-18 up-regulation may be found only in a minority of patients with CD<sup>[34]</sup>. Furthermore, in the presence of IL-18, T cells from the inflamed CD tissue have been shown to produce less IL-10 than control tissue<sup>[35]</sup>. Although recombinant IL-18 alone induces significant proliferative responses in freshly isolated mucosal lymphocytes from CD patients<sup>[36,37]</sup>, a synergy between IL-12 and IL-18 in activated macrophages may be a regulatory mechanism driving lamina propria lymphocytes toward a Th1 response in IBD<sup>[38]</sup>. It has been reported that cytokine IL-12 may act in synergy with IL-18 to promote the induction of IFN- $\gamma$ , leading to severe gut inflammation in mice<sup>[39]</sup>. The development of Th1 CD4+ T cells in the intestinal mucosa is driven by IL-12, produced from activated macrophages, and IL-18, produced from activated macrophages and colonic epithelial cells. The synergistic effect is mainly caused by mechanisms involving the up-regulation of the IL-18 receptor by IL-12<sup>[40]</sup>.

## ANTI-INFLAMMATORY AND IMMUNOMODULATORY CYTOKINES

### IL-10

IL-10 is an anti-inflammatory cytokine that inhibits both antigen presentation and subsequent release of pro-inflammatory cytokines, thereby attenuating mucosal inflammation. The pivotal role played by IL-10 within the mucosal immune system has been extensively studied in the chronic ileo-colitis that develops in gene-targeted IL-10 knockout mice and by its therapeutic efficacy in several animal models of colitis<sup>[41]</sup>. An inactivation of IL-10 in mice results in an increased production of IL-12 and IFN- $\gamma$ <sup>[42,43]</sup>. Inflamed tissues and granulomas of CD show low IL-10<sup>[44]</sup>. Melgar *et al*<sup>[45]</sup> reported a highly significant increase in IL-10 mRNA levels in T lymphocytes and in IL-10-positive cells in the colons of UC patients. Recently produc-

tion of IL-10 by regulatory T cells has been implicated as important issue in IBD<sup>[46]</sup>. Other regulatory cells that may participate in UC through the production of IL-10 are a regulatory B cells subtype called Bregs<sup>[47]</sup>. The importance of IL-10 production by B cells has been evidenced in IBD models and in humans<sup>[48,49]</sup>, Mizoguchi *et al* showed that Bregs can be responsible for the suppression and/or recovery from acquired immune mediated inflammations by mechanisms that include IL-10 and TGF-β1 in IBD<sup>[47]</sup>.

### **IL-4 and TGF-β**

Overall, anti-inflammatory cytokines whose roles are less well characterized in IBD include IL-4 and TGF-β. IL-4 is a stimulatory molecule for B and T cells, and has known immunosuppressive effects in the intestine<sup>[50]</sup>. T-cell receptor alpha chain-deficient mice (TCR -/-) treated with anti-IL-4 monoclonal antibody showed a decrease in Th2-type mRNA cytokine production and an increase in expression of IFN-γ, suggesting that IL-4 plays a major role in inducing Th2-type CD4+ cells in the gut to shift towards a Th1 response<sup>[51]</sup>. Also another study showed that development of colitis in the TCR -/- mice depends on IL-4 rather than IFN-γ<sup>[52]</sup>. A study reported that the administration of IL-4 led to a significant reduction of the vascular endothelial growth factor (VEGF) production by peripheral blood mononuclear cells in active CD and UC patients<sup>[53]</sup>.

Similarly, TGF-β is an inhibitory cytokine recognized as a key regulator of immunological homeostasis and inflammatory responses. Reduced TGF-β activity is considered to be responsible for the development of autoimmune disorders in several pathologic conditions including IBD<sup>[54]</sup>. Defective transforming growth factor TGF-β1 signaling due to high levels of Smad7 is a feature of IBD<sup>[55]</sup>. UC patients have exhibited increased production of TGF-β1 by LPMC as compared with both CD patients and controls, highlighting that although TGF-β acts on the systemic immune system to promote a potent immunosuppressive effect, locally TGF-β may demonstrate pro-inflammatory properties<sup>[56]</sup>. Evidence suggests that TGF-β can act in concert with epidermal, insulin-like, fibroblast growth factors, as well as VEGF to protect host tissue from luminal challenges and facilitate repair of mucosal injury in IBD<sup>[57,58]</sup>. As future therapy, the inhibition of Smad7 may reestablish TGF-β1 function and the suppression of colitis as proven in experimental models of colitis<sup>[59]</sup>.

### **IL-12 and related cytokines**

IL-12 and IL-23 belong to the IL-12 family of pro-inflammatory heterodimeric cytokines and comprises IL-12p40/IL-12p35 and IL-12p40/IL-23p19 subunits<sup>[60]</sup>. They are mainly produced by activated APCs and accessory cells such as DC and phagocytes<sup>[61]</sup>. The receptors for these cytokines are also heterodimeric IL-12 binds an IL-12Rβ-IL-12Rβ2 heterodimer, whereas IL-23 binds an IL-12Rβ1-IL-23R heterodimer<sup>[60]</sup>. The receptors for both IL-12 and IL-23 are mainly expressed on T cells,

NK cells, and NKT cells. However, low levels of the receptor for IL-23 are also expressed on monocytes, macrophages, and DCs<sup>[61]</sup>. Both cytokines activate TYK2 and JAK2 as well as STAT1, STAT3, STAT4, and STAT5<sup>[60]</sup>. Although IL-12 activates STAT4 most efficiently, IL-23 preferentially activates STAT3<sup>[60]</sup>. Despite the similarities in receptor subunit and signaling, recent studies have shown that IL-12 and IL-23 drive divergent immunological pathways.

The expression of IL-12 is up-regulated in both active UC and CD biopsies and it correlates with activity index score<sup>[62]</sup>. Levels of IL12p40 and IL12Rβ2 are higher in early rather than in late CD suggesting that IL12-mediated modulation is strongly dependent on the stage of disease<sup>[63]</sup>. In particular, to drive adaptive immune responses, DCs (that sense the nature of the micro-organisms in the intestine) are key producers of IL-12 in IBD<sup>[64]</sup>.

In animal models, IL-23 showed to be essential for manifestation of chronic intestinal inflammation, whereas IL-12 is not. A critical target of IL-23 is a unique subset of tissue-homing memory T cells, which are specifically activated by IL-23 to produce the pro-inflammatory mediators IL-17 and IL-6<sup>[65]</sup>.

Recently, another IL-12-related cytokine, IL-27, was described. IL-27 consists of EBI3, an IL-12p40-related protein, and p28, a newly discovered IL-12p35-related polypeptide. Mucosal expression of IL-23p19 and IL-27p28 transcripts correlate with the inflammatory activity in IBD both CD and UC. Particularly, IL-27p28 transcripts and EBI3 transcripts were significantly elevated only in active CD<sup>[66]</sup>.

### **IL-17 and Th17 cells**

Recently, a new T cell subset named “Th17”, characterized by the production of IL-17, was identified as an important player in inflammatory responses<sup>[67]</sup>. Sequencing the human genome resulted in the discovery of an additional five members of the IL-17 family that were consecutively named IL-17B to IL-17F. IL-17A is exclusively produced by Th17 cells<sup>[68]</sup>. The production of IL-17 relies on STAT3 activation triggered by IL-23<sup>[69]</sup>. IL-17 in general induces the recruitment of immune cells to peripheral tissues, a response that requires NF-κB activation after IL-17 receptor engagement<sup>[70,71]</sup>. IL-17 also leads to the induction of many pro-inflammatory factors, including TNF-α, IL-6, and IL-1β, suggesting an important role for IL-17 in localizing and amplifying inflammation<sup>[72-74]</sup>. Furthermore, TNF-α and IL-6, which are both produced by Th17 cells, not only support Th17 cell development but also synergize with IL-17 to enhance the production of pro-inflammatory mediators<sup>[74]</sup>. Regulatory T cells CD4+CD25-Foxp3- could be a source of Th17 cells<sup>[75]</sup>. In human cells, IL-1, IL-6, and IL-23 promote human CD4+ to Th17 differentiation, but TGF-β1 is not needed like in mouse<sup>[76]</sup>. IL-17 as well as Th17 cells have both been found to be elevated in serum and intestinal tissue of IBD patients. IL-17 was not detected in inactive patients tissue as well as other colitis<sup>[77]</sup>.

### **IL-13 and T cell response in UC**

UC is characterized by a Th2 immune response in which IL-13, which is produced by specialized cells such as NK T-cells, was identified as an important effector cytokine<sup>[78]</sup>. In UC, IL-13 may impair epithelial barrier function by affecting epithelial apoptosis, tight junctions, and restitution velocity<sup>[79]</sup>. Both discoveries were made by determining the cytokine profile of LPMC isolated from tissue recovered from colonic resection from UC and CD patients. It was found that LPMC from UC patients secreted high amounts of Th2 cytokines IL-13 and IL-5<sup>[78,79]</sup>. This research group found that the IL-13 and IL-5 LPMC cells bear NK specific markers CD161 and recognize CD1d, indicating that they are NK T-cells<sup>[78]</sup>. These NK T-cells are considered "non-classical". The NK T-cells isolated from UC patients exhibited cytotoxicity towards an epithelial cell line (HT-29)<sup>[78]</sup>. This cell population possibly could be the cells causing epithelial cell cytotoxicity in UC described in the 1980s<sup>[80]</sup>. IL-13 signalling through the IL-13 $\alpha$ 2 receptor (IL-13R $\alpha$ ) in general is involved in induction of TGF- $\beta$ 1 production and fibrosis<sup>[81]</sup>. The signalling through IL-13R $\alpha$  was important in the fibrosis caused by TGF- $\beta$ 1 in an animal model<sup>[82]</sup>. However, the extent to which this leads to the ultimate cascade of inflammation in UC remains to be determined.

### **NOVEL CYTOKINES INVOLVED IN IBD**

Other cytokines like IL-21 and IL-22, which have been implicated in the pathophysiology of inflammatory and autoimmune diseases such as asthma, arthritis and lupus, play also an important role in IBD. IL-21 is a T cell derived cytokine member of the common gamma-chain-dependent cytokine family, which in general acts on intestinal epithelium helping to maintain the ongoing Th1 inflammation by inducing the production of IFN- $\gamma$ <sup>[83,84]</sup>. IL-21 also has been shown to enhance the expansion of NK cells<sup>[85]</sup>. IL-21 is expressed by immune T and B cells and non-immune cells like fibroblasts, where it activates the metalloproteinase 1 production, and signalling through its receptor IL-21R it activates STAT-3 in T cells<sup>[86]</sup>. IL-21, like IL-6 and IL-23 is also involved in Th17 cell differentiation<sup>[87]</sup> and it is over-expressed in both CD and UC, with higher levels being found in CD<sup>[88]</sup>.

IL-22 was originally described as an IL-9-induced gene and was named as IL-10-related T cell-derived inducible factor (IL-TIF)<sup>[89]</sup>. This cytokine shows 22% amino acid identity with IL-10 and belongs to a family of cytokines with limited homology to IL-10. IL-22 binds at the cell surface to a receptor complex composed of two chains belonging to the class II cytokine receptor family (CRF2): IL-22R1 and IL-10R2<sup>[90,91]</sup>. In the intestinal cells, particularly innate immune cells, the binding of IL-22 to its respective R1 chain induces a conformational change that enables IL-10R2 to interact with the newly formed ligand-receptor complexes. This in turn, activates a signal transduction cascade that re-

sults in rapid activation of several transcription factors, including STAT1/3 proteins<sup>[91]</sup>. The principal sources of IL-22 are natural killer and activated T and B cells. Th17 has proven a very important role in this matter<sup>[92]</sup>. IL-22 has proinflammatory functions in IEC and is upregulated in CD both in tissue and in serum<sup>[93,94]</sup>. Surprisingly, in a murine model of UC, Sugimoto *et al* demonstrated a novel protective role for IL-22, in which IL-22 attenuates in the intestine inflammation by inducing mucin membrane bound production by goblet cells<sup>[93,94]</sup>. Another recent paper showed that IL23R genotypes affect IL-22 serum concentrations, linking for the first time genetic CD susceptibility to Th17 cell function<sup>[94]</sup>.

### **POTENTIAL BIOLOGICAL THERAPIES DIRECTED TO CYTOKINES**

Controlling the expression, production and activity of IL-23 as well as IL-17 is an approach that would allow the development of a novel treatment strategy with more anti-inflammatory efficacy and potentially with less suppressive effects on host defenses<sup>[95]</sup>. There are different biologic therapies directed to several cytokines tested in patients with IBD: fontolizumab (anti-interferon  $\gamma$ ) is a humanized monoclonal antibody to interferon gamma. A small phase 2 study of fontolizumab at subcutaneous doses of 10 mg/kg in patients with moderate to severe CD demonstrated efficacy and safety<sup>[96]</sup>. A randomized clinical trial of 79 patients with CD receiving 1 mg or 3 mg of anti-IL-12 monoclonal antibody *versus* placebo demonstrated a response in 75% of CD patients compared with 25% in the placebo group<sup>[97]</sup>. Other antibodies have been generated against to T-cell subsets blockade including CD3+ cells (visilizumab) and CD25+ cells (daclizumab and basiliximab) for UC. Pilot studies have shown promising results in steroid-resistant UC patients<sup>[98]</sup>. IL-6 participates in a variety of critical functions, including T cell growth and differentiation, as well as B-cell proliferation. In a pilot study, where patients with active CD were treated with an antibody directed against the IL-6 receptor (Atlixumab), 80% responded at the full dose compared with 31% in the placebo group<sup>[99]</sup>. IL-11 is produced by cells of mesenchymal origin. A placebo controlled trial of subcutaneous IL-11 in patients with active CD did not demonstrate clear efficacy<sup>[100]</sup>.

### **CONCLUSION**

Cytokines are important in the pathogenesis of IBD and their manipulation has successfully reduced disease severity and maintained remission. Following the discovery of novel cytokines and the role they may play in gut mucosal immunity, as well as the emergence of new concepts and changing paradigms in IBD pathogenesis, the roles of several cytokines have been elucidated and tested in both preclinical animal models and clinical trials of patients with IBD. Complementary to this, proof of

concept for new cytokine targets is rapidly developing, with the possibility of future cytokine-based therapies that may offer greater specificity and decreased toxicity for the treatment of IBD. In addition, further applications of cytokine-based therapies in human clinical trials and preclinical animal studies are ongoing.

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CLINICAL PRACTICE GUIDELINE

# Perspective on the practical indications of endoscopic submucosal dissection of gastrointestinal neoplasms

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## GENERAL CONCEPT TO APPLY ENDOSCOPIC SUBMUCOSAL DISSECTION FOR GASTROINTESTINAL NEOPLASMS

Endoscopic submucosal dissection (ESD) is a new endoluminal therapeutic technique involving the use of cutting devices to permit a larger resection of the tissue over the muscularis propria in three steps: injecting fluid into the submucosa to elevate the lesion from the muscularis propria, precutting the surrounding mucosa of the lesion, and dissecting the connective tissue of the submucosa beneath the lesion. The major advantages of the technique in comparison with polypectomy and endoscopic mucosal resection (EMR) are these: the resected size and shape can be controlled; *en bloc* resection is possible even for a large lesion; and the lesions with ulcerative findings are also resectable<sup>[1,2]</sup>. Retrospective analyses of the comparison between ESD and EMR for the stomach epithelial neoplasms showed that ESD increased *en bloc* and histologically complete resection rates compared with EMR but was associated with longer average operation times and a higher incidence of intraoperative bleeding and perforation<sup>[3,4]</sup>.

Two aspects are considered to determine the application of ESD for each lesion by each operator (Figure 1). The first is a little likelihood of lymph node metastasis and the second is the technical resectability. The former has been determined by the large numbers of surgically resected cases in each organ before establishment of ESD and the latter may be determined by the applied technique, the expertise of the operators, the location of the lesions or their characteristics. In terms of technical resectability, *en bloc* resection is more desirable than piecemeal resection for accurate assessment of the appropriateness of the therapy, because the depth of invasion and lymphovascular infiltration of cancer cells (that are considerable risk factors for nodal metastasis) are not accurately assessed by piecemeal resection. Almost all possible node-negative epithelial neoplasms can be resected *en bloc* by

## Abstract

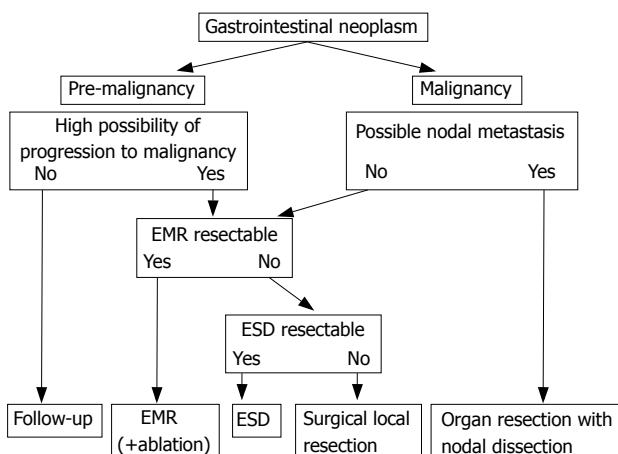
Endoscopic submucosal dissection (ESD) is a new endoluminal therapeutic technique involving the use of cutting devices to permit a larger resection of the tissue over the muscularis propria. The major advantages of the technique in comparison with polypectomy and endoscopic mucosal resection are controllable resection size and shape and *en bloc* resection of a large lesion or a lesion with ulcerative findings. This technique is applied for the endoscopic treatment of epithelial neoplasms in the gastrointestinal tract from the pharynx to the rectum. Furthermore, some carcinoids and submucosal tumors in the gastrointestinal tract are treated by ESD. To determine the indication, two aspects should be considered. The first is a little likelihood of lymph node metastasis and the second is the technical resectability. In this review, practical guidelines of ESD for the gastrointestinal neoplasms are discussed based on the evidence found in the literature.

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**Key words:** Endoscopic submucosal dissection; Endoscopic mucosal resection; Gastrointestinal neoplasm; Treatment guideline; Lymph node metastasis

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**Figure 1** Algorism for endoscopic submucosal dissection of gastrointestinal neoplasms.

ESD, when they are treated by very experienced hands. This does not mean that all endoscopic resection should be performed as ESD. Polypectomy or endoscopic mucosal resection (EMR) are beneficial for patients with pedunculated neoplasms or small neoplasms because of the little invasiveness<sup>[5]</sup>. If the lesions are apparently pre-malignant neoplasms, piecemeal resection by using EMR may be permissible with the best balance of risks and benefits. Surgical organ resection with lymphadenectomy should be applied to those neoplasms with high probability of positive lymph nodes or failure in complete removal by ESD. Recurrent lesions can be also indicated for ESD, if they fulfill the criteria of no nodal metastasis, but indication should be carefully determined considering the risks of accompanying complications.

## STOMACH EPITHELIAL NEOPLASMS

### Aspects of nodal metastasis

Pre-malignant stomach epithelial neoplasms, gastric adenomas, have no nodal metastases. It is still controversial whether we should treat gastric adenomas endoscopically or follow them. A series with a small number of cases with a preoperative diagnosis of pre-malignant lesion revealed that 37% (16/43) of them were finally diagnosed as adenocarcinoma and a lesion > 1 cm was considered to pose a risk of malignancy<sup>[6]</sup>. Another study revealed that 6.8% (8/118) of cases were finally diagnosed as adenocarcinoma and high-grade dysplasia by endoscopic biopsy was considered to be an independent risk factor for malignancy<sup>[7]</sup>. Furthermore, preoperative diagnosis of depressed adenoma is considered to represent a higher risk of malignancy than protruding adenoma<sup>[8]</sup>. So, when the lesions have these characteristics, endoscopic treatments are recommended, similarly to intramucosal carcinomas. Although local recurrence should be taken into account, piecemeal resection by using EMR techniques to remove the apparent gastric adenomas is allowed.

In terms of malignant stomach epithelial neoplasms, the following types of early gastric cancers without lymphovascular infiltration of cancer cells may have

little likelihood of nodal metastases: (1) intramucosal, differentiated adenocarcinoma without ulcer findings of any size; (2) intramucosal, differentiated adenocarcinoma with ulcer findings when the lesion is ≤ 3 cm; (3) intramucosal, undifferentiated adenocarcinoma without ulceration when the lesion is ≤ 2 cm; and (4) differentiated adenocarcinoma with minute submucosal penetration (500 micrometers below the muscularis mucosa; sm1) when the lesion is ≤ 3 cm<sup>[9]</sup>.

### Technical aspects

When the endoscopists are well trained for ESD, the technical aspects may not restrict indications to perform ESD, based on the above criteria of no nodal metastasis. However, in our opinion, cases of ulcer findings with fusion of the muscle layer and the mucosal layer and cases of undifferentiated adenocarcinoma may be excluded from the indication or be carefully resected, at least until now. The former cases occur in cancers that previously had a deep ulcer extending into the proper muscle layer, where it is difficult to identify the gastric wall plane during submucosal dissection, which increases the possibility of perforation or incomplete resection by ESD<sup>[10]</sup>. In the latter cases, first, the margin is very unclear and the possibility of incomplete resection is fairly high, second, the clinical course after recurrence may be more miserable than that of differentiated-type, and third, the differentiation between ulcerative finding or biopsy-inducing fibrosis is sometimes difficult, even though small intramucosal undifferentiated adenocarcinoma with ulcer findings may be associated with nodal metastases<sup>[9]</sup>.

## ESOPHAGEAL SQUAMOUS EPITHELIAL NEOPLASMS

### Aspects of nodal metastases

Low- and high-grade squamous intraepithelial neoplasms, including carcinoma *in situ* (m1), have no nodal metastases. It is still controversial whether one should treat these intraepithelial neoplasms endoscopically or just follow them. However, when the lesions are diagnosed as high-grade intraepithelial neoplasms, endoscopic treatment is recommended, to avoid future development of invasive carcinoma or to contain foci of invasive carcinoma<sup>[11,12]</sup>. Although local recurrence should be taken into account, piecemeal resection by using EMR techniques to remove the apparent intraepithelial neoplasms is allowed<sup>[13-15]</sup>.

Esophageal squamous cell carcinomas invading the lamina propria (m2) pose little risk of nodal metastases. For those invading the muscularis mucosa (m3) and those with minute submucosal invasion (< 200 micrometers below the muscularis mucosa; sm1), the nodal metastases rate is 9.3% and 19.6%, respectively. The nodal metastases rate of m3 or sm1 cancers with 0-II type, < 5 cm, well or moderately differentiated type, and no lymphovascular infiltration of cancer cells is 4.2%<sup>[16]</sup>. It has been reported that no nodal metastasis was found in patients with sm1, low

histologic grades, and no lymphovascular infiltration of cancer cells<sup>[17]</sup>. Therefore, for patients unwilling to undergo esophagectomy or chemoradiation and patients with comorbid diseases, ESD may be applied taking into consideration the risks of nodal metastases and treatment-related morbidity.

#### **Technical aspects**

When the endoscopists are well trained for ESD, the technical aspects by themselves may not restrict indications to perform ESD, except in special circumstances, such as lesions located in the diverticulum. When lesions spreading > 3/4 of circumference are resected as circular or semi-circular resection, post-operative stricture occurs to a high rate<sup>[18]</sup>. So, it is controversial to treat these lesions endoscopically. However, intensive balloon dilatations or tentative stent insertion may rescue from the stricture.

## **ESOPHAGEAL BARRETT NEOPLASMS**

#### **Aspects of nodal metastases**

Columnar intraepithelial neoplasms have no nodal metastases. Although local recurrence should be taken into account, piecemeal resection by using EMR techniques and additional ablation therapy to remove the apparent intraepithelial neoplasms is allowed<sup>[19-23]</sup>.

There are no data about nodal metastases from the large numbers of surgically resected cases due to limited number of cases of esophageal columnar epithelial carcinomas at an early stage, although a small number of cases revealed no nodal metastasis for the intramucosal and sm1 cancer, where sm1 was determined by upper third of the submucosa<sup>[24]</sup>. There is no consensus whether one should apply to this kind of malignancy the same criteria that are applied to stomach epithelial neoplasms or esophageal squamous epithelial neoplasms as far as the depth of sm1 to be measured. International workshops of esophagogastric neoplasms adopted the cut-off line of 500 micrometers below the deeper muscularis mucosae, similarly to the stomach<sup>[25,26]</sup>.

#### **Technical aspects**

Similarly to esophageal squamous epithelial neoplasms, the technical aspects by themselves may not restrict indications to carry out ESD, when the endoscopists are well trained for ESD. When lesions spreading > 3/4 of the circumference of the esophagus (a situation which commonly occurs in long segment Barrett epithelium) are resected (with circular or semi-circular resection), post-operative strictures occur at a high rate<sup>[19-23]</sup>.

## **RECTAL EPITHELIAL NEOPLASMS**

#### **Aspects of nodal metastases**

Pre-malignant rectal epithelial neoplasms, rectal adenomas, have no nodal metastases. From the standpoint of adenoma-carcinoma sequence, all adenomas, including diminutive polyps, are targets for

endoscopic resection<sup>[27,28]</sup>, although some investigators agree with endoscopic removal only if the size is > 5 mm<sup>[29]</sup>. *En bloc* resection is not always necessary for rectal adenoma or intramucosal carcinoma. However, higher rate of local recurrence was reported when multiple resections were performed<sup>[30-32]</sup>. Intramucosal carcinomas and those with slight submucosal invasion (< 1000 micrometers below the muscularis mucosa; sm1) without lymphovascular infiltration have little risk of nodal metastasis<sup>[33]</sup>.

Tumor morphology and surface pit pattern are good endoscopic indicators for submucosal invasion. From this aspect, depressed lesions, laterally spreading tumors of non-granular type (LST-NG) and large protruding tumors are considered as good candidates for ESD because these lesions have a high risk of submucosal invasion, which may be difficult to diagnose preoperatively, and a thorough histopathological assessment of the resected specimen is essential. It is controversial whether one should perform ESD or piecemeal EMR for laterally spreading tumors of granular type (LST-G), because most lesions are intramucosal and the endoscopic prediction of invasiveness is highly feasible<sup>[34]</sup>.

#### **Technical aspects**

Even for lesions that meet the criteria above, laparoscopic or open surgery may be selected in some institutions considering the location and size of the lesion. The lesions with submucosal fibrosis due to previous endoscopic treatment or biopsy are also resectable by ESD, even though the indication should be carefully weighed considering risks and benefits of ESD *vs* surgery<sup>[35,36]</sup>. The rectum is fixed to the retroperitoneum, therefore the endoscope is more easily manoeuvred than in other organs of the gastrointestinal tract. Furthermore, panperitonitis may be less likely than in the rest of the colorectum, even if the muscularis propria is teared, although penetration leads to air accumulation in the retroperitoneal space, which may then spread to a wider area<sup>[37,38]</sup>.

## **COLONIC EPITHELIAL NEOPLASMS**

#### **Aspects of nodal metastases**

The criteria for absence of nodal metastases are the same as those of rectal epithelial neoplasms (see above).

#### **Technical aspects**

There are several tortuous folds in the colon. Peristalsis and residual feces may sometimes disturb ESD procedure. So it is commonly believed that the technical difficulty of colon ESD exceeds those of the stomach, the esophagus, and the rectum, although there are many differences. In all cases, should one consider the substantial risks and expected benefits of ESD. However, promising results of ESD are reported from very experienced endoscopists at advanced institutions, similarly to those of the rectal epithelial neoplasms<sup>[39-43]</sup>.

## EPITHELIAL NEOPLASMS IN THE SMALL INTESTINE, INCLUDING DUODENUM

### Aspects of nodal metastasis

Pre-malignant epithelial neoplasms in the small intestine have no nodal metastases. Although local recurrence should be taken into account, piecemeal resection by using EMR techniques and ablation therapy to remove the apparent intraepithelial neoplasms is allowed<sup>[44]</sup>. There are no data about nodal metastases from the large numbers of surgically resected cases due to limited number of cases of epithelial carcinomas in the small intestine. There is no consensus whether one should apply the same criteria of stomach epithelial neoplasms or colorectal epithelial neoplasms to this malignancy.

### Technical aspects

The small intestine, including the duodenum, is considered to be the most difficult organ where to perform ESD. The endoscope does not easily reach the target lesion and the organ is not fixed tightly except at the level of the duodenum, which results in fairly bad maneuverability. Peristalsis is the most active and the wall is the thinnest among the other gastrointestinal organs. Even if the resection is completed successfully, pancreatic juice and bile cause chemical damage to the mucosal wound, which may lead to prolonged bleeding and perforation. In our opinion, closure of the mucosal wound is recommended after ESD. When considering these issues, indication to perform ESD in the small intestine should be carefully assessed and limited. Due to the structural specificity of the papilla, ESD for ampullary neoplasms is not performed.

## PHARYNGEAL EPITHELIAL NEOPLASMS

### Aspects of nodal metastasis

Pre-malignant epithelial pharyngeal neoplasms have no nodal metastases. Although local recurrence should be taken into account, piecemeal resection by using EMR techniques and ablation therapy to remove the apparent intraepithelial neoplasms is permissible<sup>[45]</sup>. There are no data about nodal metastasis from the large numbers of surgically resected cases due to limited number of cases of pharyngeal epithelial carcinomas at an early stage. So, indication for invasive carcinoma is still controversial due to the lack of data. Owing to the structural differences, it is impossible to apply the criteria of esophageal squamous epithelial carcinomas for this malignancy.

### Technical aspects

ESD is technically possible in this organ, and ESD may be the optimal endoscopic treatment not only because it enables an *en bloc* resection but also because it can prevent removal of excess mucosa of the pharynx, which is a very narrow and important organ related to swallowing and speech<sup>[46]</sup>.

## CARCINOID

### Aspects of nodal metastasis

Carcinoids are classified based on organ site and cell of origin and occur most frequently in the gastrointestinal tract (67%) where they are most common in small intestine (25%), appendix (12%), and rectum (14%)<sup>[47]</sup>. Primary size > 2 cm, serosal penetration, and primary site in the small intestine are considered to be risk factors for metastases in the case of gastrointestinal carcinoids<sup>[48]</sup>.

Nodal metastases are most commonly found with small intestine carcinoids (20%-45%), providing the rationale for an extended resection including the adjacent lymph node drainage area. Carcinoids of the appendix < 1 cm rarely metastasize, simply requiring appendectomy for treatment. Rectal carcinoids < 2 cm rarely metastasize, directing local excision, including endoscopic resection<sup>[49]</sup>. Another group revealed that colorectal carcinoids < 1 cm without lymphovascular infiltration could be curatively treated by local resection, but others would need radical nodal dissection<sup>[50]</sup>. Duodenal carcinoids < 2 cm may be excised locally because they rarely metastasize<sup>[51]</sup>.

Multiple gastric carcinoids, usually no more than 1 cm, can be followed up by endoscopy and biopsy<sup>[52,53]</sup>. Sporadic gastric carcinoids should be treated by gastrectomy with lymphadenectomy, because some of those have nodal metastases even when they have a small size<sup>[54-56]</sup>. However, differentiation of types of gastric carcinoids is not always easy, so endoscopic resection, as a first step to obtain histology, may be acceptable for small gastric carcinoids < 1 cm to predict nodal metastases.

### Technical aspects

Because almost all lesions for local resection are less than 1 cm in all the gastrointestinal organs, band ligation resection<sup>[57,58]</sup>, cap-technique<sup>[59]</sup> or strip biopsy<sup>[60-62]</sup> result in good outcome. So the application of ESD for carcinoids may be limited. When the lesions are in intermediate size, such as 1-2 cm, or invade massively the submucosal layer, which may result in tumor-positive margin resection, ESD should be applied<sup>[56,63]</sup>.

## SUBMUCOSAL TUMOR

### Aspects of metastases

Submucosal tumors (SMTs) are mesenchymal tumors, which may have very diverse origins. SMTs are classified and defined as benign or malignant based on a combination of size, histological, immunohistochemical, and ultrastructural criteria. The majority of them are classified into gastrointestinal stromal tumor (GIST), of muscular origin, of neurogenic origin, of vascular origin, and of adipose tissue origin. SMTs < 3 cm are generally considered benign tumors. SMTs > 3 cm with high mitotic counts are considered tumors at high-risk of malignancy. In case of GIST, the cutoff of the size between pre-malignancy and malignancy may be

2 cm. Sarcomas including malignant GIST generally do not metastasize to regional lymph nodes, but instead spread hematogenously to the liver or metastasize to the peritoneum<sup>[64]</sup>. Benign SMTs should generally only be treated if they are symptomatic. So the SMTs > 2 cm or 3 cm without evidence of metastasis may be candidates for local resection<sup>[65]</sup>.

### Technical aspects

From the rationale of ESD, the targets should originate from over the muscularis propria. The lesions originating from the inner layer of the muscularis propria may be resectable by careful resection over the outer layer of the muscularis propria, but the high probability of perforation and the artificial peritoneal dissemination by tear of the tumor capsule should be taken into consideration. When considering that the small size lesions located in the mucosal or submucosal layers are mostly benign, the indication of ESD for SMTs is quite limited, although some investigators reported promising results of ESD for SMTs<sup>[66,67]</sup>.

### FUTURE PERSPECTIVES

The perspectives on the current indication of ESD are described based on a review of data available in the literature until the end of 2007. Further investigations in both aspects, the assessment of nodal metastases and the technical innovations, may change widely the above perspectives in the future. Recently, a new application of ESD is being investigated in cooperation with laparoscopic surgeons for the treatment of possible node-positive gastric carcinoma and gastric GIST<sup>[68,69]</sup>. There is no doubt that these attempts will expand ESD into a new field, which will be added to the upcoming practical guidelines for ESD.

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# "Melanosis" in the small and large intestine

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## Abstract

Deposition of pigment in the intestinal mucosa is commonly observed by the endoscopist, especially within the colon, and particularly during investigations for constipation. Pigment may also be detected in the small intestine. Although labeled as melanosis, electron microscopy and X-ray analytical methods have provided evidence that this pigment is not melanin at all, but lipofuscin. Often, herbal remedies or anthracene containing laxatives are often historically implicated, and experimental studies in both humans and animal models have also confirmed the intimate relationship with these pharmacological or pseudo-pharmacological remedies. The appearance of melanosis coli during colonoscopy is largely due to pigment granule deposition in macrophages located in the colonic mucosa. The pigment intensity is not uniform, being more intense in the cecum and proximal colon compared to the distal colon. Possibly, this reflects higher luminal concentrations of an offending agent in the proximal compared to distal colon, differential absorption along the length of the colon, or finally, differences in macrophage distribution within the colon. Mucosal lymphoid aggregates normally display a distinct absence of pigment producing a "starry sky" appearance, especially in the rectosigmoid region. Interestingly, some focal, usually sessile, colonic mucosal neoplastic lesions, rather than submucosal lesions, may be better appreciated as pigment deposition may be absent or limited. If detected, removal and further histopathologic analysis of the polyp may be facilitated.

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**Key words:** Melanosis; Lipofuscin; Melanosis coli; Constipation; Hemosiderin; Melanosis ilei; Melanosis duodeni; Anthraquinones

## INTRODUCTION

Prior studies using electron microscopy and X-ray analysis refined information regarding the composition of pigment granules in the intestinal tract. In particular, the distinction between lipofuscin, melanin and hemosiderin was more readily accomplished since separation of these pigment materials appeared to be possible based solely on their morphological characteristics<sup>[1]</sup>. X-ray analysis also permitted *in situ* analysis of tiny cellular inclusions in sectioned material with a high degree of sensitivity and sensitivity. As a result, a better understanding of pigments found in the intestinal tract has emerged.

## PIGMENT GRANULES

Lipofuscin granules are residual bodies with undigested and/or oxidized lipids. These granules are thought to result from the processes of sequestration and enzymatic dissolution of cellular organelles within lysosomes. Electron microscopy is reported to show single membrane-bound bodies containing electron-dense lipid material along with electron-lucent or medium density neutral fat. Melanin is synthesized in the melanosome by oxidation of tyrosine to dopa and, eventually, melanin. Characteristic striated rod-like structures are observed under electron microscopy. Hemosiderin develops in residual bodies that result from macrophage phagocytosis of erythrocytes and/or their breakdown products. Lysosomal digestion results in electron-dense iron-containing particles. Each granule type is distinctive and has been well illustrated by others elsewhere<sup>[1]</sup>.

## MELANOSIS COLI

Melanosis coli is probably the most common pigmentation change seen in the intestinal tract mucosa



**Figure 1** Typical alligator or snake-skin appearance of melanosis coli. Despite routine colon preparation, residual fecal debris is common, likely reflecting reduced colonic propulsive activity.



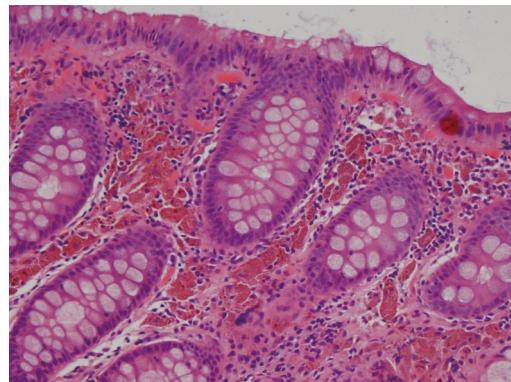
**Figure 2** Rectosigmoid mucosa illustrating focal areas of patchy intense pigmentation as well as focal areas of absent pigment, the latter reflecting the presence of normal mucosal aggregates of lymphoid cells (so-called "starry sky" appearance of melanosis coli).

during endoscopic evaluation and in biopsy materials submitted for histopathologic evaluation. It was, however, first described by Cruveilhier in 1829 before the emergence of endoscopic technologies. The abnormally brown or black pigmentation can even be visualized in the biopsy specimen in tissue forceps or on the filter paper prior to fixation immersion. Representative endoscopic appearances of melanosis coli are shown in Figures 1-3.

As shown, the pigment in melanosis coli is well localized within the colon as there is usually no pigment deposition in the more proximal small intestine, including the ileum. Occasionally, the pigment may extend through the entire colon, including the appendix and into the most distal ileal transition region with the cecum. It appears to be most intense and most readily detected in the cecum and ascending colon, but pigment changes are variable in their intensity, even within the same colon. In some areas of the colonic mucosa where lymphoid cell aggregates in the lamina propria are numerous, such as the rectum, the pigmented colonic mucosa shows a characteristic "starry sky" appearance, as these lymphoid cells do not accumulate intracellular pigment. As shown in Figure 4, a biopsy routinely treated with a standard hematoxylin-eosin stain shows



**Figure 3** Ileal and cecal mucosa in melanosis coli. Melanosis is generally confined to the cecal mucosa although there is a very limited area of transition into ileal mucosa that is not pigmented.



**Figure 4** Photomicrograph showing typical pigment granule laden macrophages in the lamina propria.

lamina propria macrophages filled with brown colored pigment granules.

A variation in the intensity of the pigmentation through the length of the colon appreciated in some patients with melanosis coli at endoscopy may reflect differences in the luminal concentrations of possible offending agents (i.e., higher in proximal colon), such as laxatives or their byproducts. Alternatively, there may be differential regional rates of mucosal absorption within the colon. Finally, there may be colonic regional differences in the topographic distribution of macrophages within the colonic lamina propria. Studies on granule composition have demonstrated primarily lipofuscin, rather than the melanin pigmentation historically suggested by histochemical staining reactions. Indeed, it has been suggested by others that this entity might be more precisely labeled "pseudomelanosis coli" or colonic "lipofuscinosis". Recently, however, a lectin method for application to formalin-based and paraffin-embedded colon<sup>[2]</sup> was used to explore changes in biopsies from patients with melanosis coli associated with laxative use<sup>[3]</sup>. These studies revealed increased apoptotic bodies in the colonic epithelium with pigment accumulation in macrophages. The results based on application of these lectins were typical of lipofuscin as well as ceroids. In addition, however, an intense argentaffin reaction abolished by bleaching was present,



**Figure 5** Easily visualized small sessile polypoid lesion in ascending colon with adjacent background pigmented mucosa typical of melanosis coli. Resected specimen confirmed absence of pigmented macrophages in the body of the resected adenoma.



**Figure 6** Small pigmented polypoid lesion similar to background pigmented colonic mucosa. Resected specimen revealed a submucosal leiomyoma along with pigmented macrophages in the overlying colonic mucosa due to melanosis coli.

most typical of a melanic substance. The apoptotic bodies in the colonic epithelium were thought to be due to laxative-induced cell death, not from natural programmed cell renewal. In addition, these apoptotic epithelial cells were believed to be the source of the pigment saccharides (as detected by lectins) while the melanic substance appeared to be derived directly from the anthraquinones<sup>[3]</sup>.

Melanosis coli is most often detected during investigation for long-standing constipation, often in conjunction with a history of the chronic use of anthracene cathartics (including cascara, senna, aloes and rhubarb). Experimental studies in different mammalian species along with humans previously documented the appearance, disappearance and re-appearance of the pigment in colorectal mucosa with repeated cycles of laxative administration. Interestingly, as shown in Figure 5, pigment deposition also may spare some neoplastic colonic lesions, including both adenomas and carcinomas. Thus, biopsy or removal of these non-pigmented foci in melanosis coli has been recommended elsewhere to exclude the presence of neoplastic epithelial cells. Other lesions, including submucosal leiomyomas shown in Figure 6, however, may still have pigment deposits within mucosal macrophages overlying these

focal submucosal lesions.

Anthraquinones appear to damage the colonic epithelial cells causing irreversible injury to some organelles. These cells are either shed into the colonic lumen, or the damaged organelles are sequestered in autolysosomes in macrophages where digestion to residual lipofuscin bodies results. In some, lymph node involvement has also been observed. It is possible that this relatively selective colonic mucosal involvement may reflect the qualitative or quantitative differences in colonic microbial flora (as opposed to the small intestine). Alternatively, some other structural difference in colonic cells or their response to anthraquinone cathartics may be responsible for the colonic mucosal regionalization of the lipofuscin pigment deposition.

Treatment of this condition has not been established. Often, a recommendation is made to manage symptomatic constipation with fiber-containing foods or substances with mucilage, including psyllium, along with avoidance of anthraquinone cathartics.

## MELANOSIS OF SMALL INTESTINE

Melanosis has been rarely recorded in the small intestine, at least, in the most readily visualized areas during routine endoscopic evaluation, including the duodenum or distal ileum. As noted earlier, pigment may extend for a very limited distance into the most distal ileum transitional mucosa in association with melanosis coli. Melanosis ilei alone and exclusive of pigmentation elsewhere, however, has also been noted, usually as an incidental observation during autopsy. In this setting, it has also been historically recorded to occur with carcinoma of the colon<sup>[4]</sup>. Pigment in the ileum is thought to arrive there through ingested materials found either in food or even inhaled dust that has been swallowed<sup>[5,6]</sup>. Although aluminum and silicon-containing compounds are common and may be detected as food additives or in medicines, hemosiderin pigment has also been detected in the ileum and hypothesized to result from intermittent bleeding within the upper gastrointestinal tract. Similarly, melanosis duodeni has also been related to occasional bleeding, usually from peptic ulceration<sup>[7-10]</sup>.

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## TOPIC HIGHLIGHT

Simon D Taylor-Robinson, MD, Series Editor

# Hepatocellular carcinoma: Epidemiology, risk factors and pathogenesis

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## EPIDEMIOLOGY

Hepatocellular carcinoma (HCC) is the commonest primary cancer of the liver. Incidence is increasing and HCC has risen to become the 5th commonest malignancy worldwide and the third leading cause of cancer-related death, exceeded only by cancers of the lung and stomach<sup>[1]</sup>. The estimated incidence of new cases is about 500 000-1 000 000 per year, causing 600 000 deaths globally per year<sup>[2-6]</sup>. However, important differences have been noted between countries. Most cases of HCC occur in Asia<sup>[1]</sup> where several countries, particularly in East Asia, have a very high incidence (over 20 cases/100 000 population). For example, the incidence is 99 per 100 000 persons in Mongolia, 49 per 100 000 in Korea, 29 per 100 000 in Japan, and 35 per 100 000 in China<sup>[3]</sup>. Hong Kong and Thailand also have similarly high rates. Another region of concern is sub-Saharan Africa, particularly the western region of Africa, including Gambia, Guinea, and Mali, and also the Republic of Mozambique in south-east Africa. Areas with moderately high risk (11 cases/100 000-20 cases/100 000) include Italy, Spain and Latin American countries, and those at intermediate risk (5 cases/100 000-10 cases/100 000) include France, the United Kingdom, and the Federal Republic of Germany. A relatively low incidence (less than 5 cases/10 000) is found in the United States, Canada, and in Scandinavia. However, there are large areas of the world where the incidence is still unknown<sup>[3,7,8]</sup>.

## Abstract

Hepatocellular carcinoma (HCC) is the commonest primary malignant cancer of the liver in the world. Given that the burden of chronic liver disease is expected to rise owing to increasing rates of alcoholism, hepatitis B and C prevalence and obesity-related fatty liver disease, it is expected that the incidence of HCC will also increase in the foreseeable future. This article summarizes the international epidemiology, the risk factors and the pathogenesis of HCC, including the roles of viral hepatitis, toxins, such as alcohol and aflatoxin, and insulin resistance.

**Table 1 Global frequency of new cases of hepatocellular carcinoma**

Year (reference)	Total number	Males	Females
1990	437408	316300	121100
2000	564300	398364	165972
2002 (The World health report, 2003)	714600	504600	210000

Adapted from Parkin *et al.*, 2001, 2005<sup>[2,5]</sup> and Bosch *et al.*<sup>[8]</sup>.

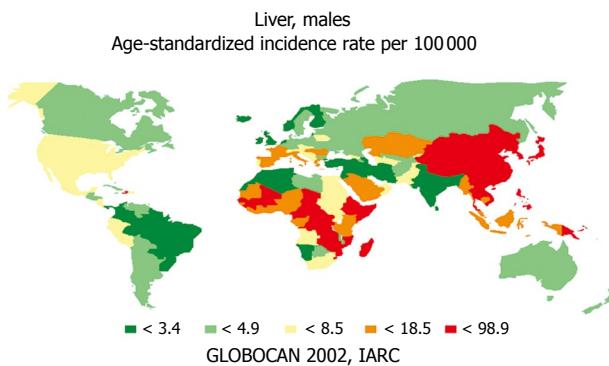
Although currently relatively low, the incidence of HCC is rising in developed western countries<sup>[9-12]</sup>. In the United States, there has been an increase of about 80% in the annual incidence of HCC during the past two decades. HCC rates increased from 1.4/100 000 per year from 1976-1980 to 2.4/100 000 per year from 1991-1995<sup>[3,10]</sup>. This increase has been most marked in men, with African-American men having higher incidence rates than US Caucasian men. This was explained by the emergence of hepatitis C during this same period, although the rise in immigration from HBV-endemic countries may also have played a role<sup>[9,10]</sup>.

Other developed western countries have noted similar increasing trends. An increase in incidence of HCC has been reported in Italy, the United Kingdom, Canada, Japan, and Australia. The increase was reported among immigrants from parts of the world with high prevalence, such as sub-Saharan Africa and parts of Asia, being associated with a parallel increase in hospitalization and mortality for HCC<sup>[3,10]</sup>. In Egypt, between 1993 and 2002, there was an almost twofold increase in HCC amongst chronic liver patients<sup>[13]</sup>.

However, it is not obvious when this rising trend, observed in many countries, will reach a peak. The Disease Control Center in Atlanta has estimated that deaths related to chronic hepatitis C in the United States will triple from the current rates of 8-10 000 per year during the next decade. While most of these deaths will be due to liver failure and its complications, a considerable proportion can be expected to be due to HCC as well<sup>[9]</sup>.

The most recent World Health report (World Health Organization<sup>[14]</sup>, Table 1) indicated a total of 714 600 new cases of HCC worldwide, with 71% among men (Figure 1). HCC is the 4th commonest cause of death due to cancer, after cancers of the respiratory system, stomach, and colon/rectum. Liver cancer ranked 3rd for male subjects and 5th for women. Geographically, there were 45 000 liver cancer deaths in Africa, 37 000 in the Americas, 15 000 in the eastern Mediterranean, 67 000 in Europe, 61 000 in South-East Asia, and 394 000 in the western Pacific region, including China and Japan. In the same year, 783 000 persons died from cirrhosis, of which 501 000 were men and 282 000 were women<sup>[15]</sup>.

The incidence of HCC increases with age, reaching its highest prevalence among those aged over 65 years<sup>[16,17]</sup>. Although HCC is rare before the age of 50 years in North America and Western Europe<sup>[18]</sup>, a shift in incidence towards younger persons has been noted in the last two decades. HCC tends to occur in the background of cirrhosis of the liver. In western countries, this holds



**Figure 1** Age-standardized incidence rates of liver cancer in males per 100 000 population (Adapted from GLOBOCAN 2002 with permission)<sup>[134]</sup>.

true in over 90% of cases, whereas in Asia and Africa the percentage of cases of HCC is higher in individuals with non-cirrhotic livers, compared to those with cirrhotic livers<sup>[3,19]</sup>.

## RISK FACTORS

The major risk factor for the development of HCC is cirrhosis of the liver. However, about one quarter of HCC cases diagnosed in the United States do not have any known predisposing risk factors. The major known risk factors for HCC are viral (chronic hepatitis B and hepatitis C), toxic (alcohol and aflatoxins), metabolic (diabetes and non-alcoholic fatty liver disease, hereditary haemochromatosis) and immune-related (primary biliary cirrhosis and autoimmune hepatitis)<sup>[17]</sup>. Recently, the geographical variability in the incidence of HCC has been attributed to the changing distribution and the natural history of Hepatitis B virus (HBV) and Hepatitis C virus (HCV) infection<sup>[20]</sup>.

## HCV

HCV is the most important risk factor for HCC in western European and North American countries, since epidemiological studies have shown up to 70% of patients with HCC have anti-HCV antibody in the serum<sup>[3,21-23]</sup>. Liver cancer has a higher prevalence in patients with HCV-associated cirrhosis than in non-viral aetiologies of chronic liver disease, while only a few cases of HCV-associated HCC have been reported in the non-cirrhotic liver, indicating that the virus possibly has a mutagenic effect<sup>[3,24,25]</sup>.

The prevalence of HCV infection varies considerably by geographical region. African and Asian countries reported high HCV infection prevalence rates, while rates in North America, Europe and Australia have usually reported lower rates<sup>[26,27]</sup>. Egypt has the highest prevalence of HCV in the world<sup>[28-34]</sup> (predominantly genotype 4), which has been attributed to previous public health eradication schemes for schistosomiasis<sup>[28,34]</sup>. Even higher HCV infection rates, up to 60%, have been reported in older individuals, in rural areas such as the Nile delta, and in lower social classes<sup>[28,30,32,34]</sup>.

The natural history of HCV infection has been investigated in several studies<sup>[3]</sup>. A Japanese study<sup>[35]</sup> reported a time lag of 13 years from infection by transfusion of

HCV infected blood to the development of chronic hepatitis. This time period was reported to be approximately 10 years in an American study and it took about 20 years for the same patients to develop cirrhosis of the liver<sup>[56]</sup>. Development of HCC took 28 years in the American subjects and 29 years in the Japanese cohort<sup>[35,56]</sup>. The annual risk of developing HCC in HCV-infected patients depends on the presence and severity of the underlying liver disease<sup>[3]</sup>.

Up to 80% of HCV-infected individuals fail to eliminate the virus acutely and progress to chronic HCV infection<sup>[27,37-40]</sup>. Continuous inflammation and hepatocyte regeneration in the setting of chronic hepatitis and subsequent progression to cirrhosis is thought to lead to chromosomal damage and possibly to initiate hepatic carcinogenesis<sup>[27,41]</sup>.

The rate of fibrotic progression following HCV infection is markedly variable, since the natural history of the disease typically extends over several decades<sup>[40,41]</sup>. The rate of fibrotic progression in HCV-infected patients is influenced by age at the time of infection, male sex, HCV genotype and alcohol consumption<sup>[42-50]</sup>.

It is not clear whether any of these factors affect the onset of liver-related complications by mechanisms other than their effects on the rate of fibrotic progression. To determine which interactive variables were independent determinants of adverse clinical outcomes, Khan and colleagues examined the development of liver-related complications of chronic HCV in a large cohort of patients who were heterogeneous in age, country of birth, mode of HCV acquisition, HCV genotype, and histological and functional severity of liver disease. Patients were followed up for five years. These authors found that the major independent predictors of liver-related complications were sporadic transmission, advanced liver fibrosis at entry and low albumin<sup>[43]</sup>.

## **HBV**

The WHO has reported HBV to be second only to tobacco as a known human carcinogen<sup>[51]</sup>. Many studies on HCC risk following chronic HBV infection have been conducted in the East Asian countries, where most patients acquired HBV as newborn infants<sup>[52,53]</sup>. The incidence of HCC in HBV-related cirrhosis in this area of the world has been reported to be 2.7%<sup>[53]</sup>. The annual risk of HCC is 0.5% for asymptomatic HBsAg carriers and 0.8% for patients with chronic hepatitis B<sup>[53,54]</sup>, while patients with HBV-cirrhosis have 1000 times higher risk of developing HCC, compared to HBsAg negative individuals<sup>[53,55]</sup>. Thus, it is likely that the probability of acquiring HCC increases with severity of underlying liver disease<sup>[53]</sup>. In Japan, the mean interval between HBV initial infection and the occurrence of HCC is 50 years. As most people are infected at birth, HBV-related cirrhosis usually develops earlier than in Western Europe or North America<sup>[35,53]</sup>.

Few adequate studies have been performed in Europe or North America to address the issue of the incidence of HCC in individuals who are positive for HBsAg. Most of the studies in Western countries are based on small

numbers of HBsAg positive patients and/or have not specifically analysed the group of HBsAg carriers. Additionally there is lack of uniformity in the timing of initiation of follow-up monitoring. In a cohort of 350 Western European patients with compensated cirrhosis, followed for about 6 years, the 5-year cumulative incidence of HCC was 6%<sup>[53,56,57]</sup>. A retrospective analysis of European patients with HBV-related cirrhosis found the 5-year incidence of HCC was 9%, irrespective of HBeAg or HBV DNA status at the time of diagnosis of cirrhosis<sup>[53,58]</sup>.

HCC has been the first human cancer amenable to prevention using mass vaccination programmes. From a global perspective, the burden of chronic HBV infection is expected to decline because of the increasing utilisation of HBV immunization, since the early 1980s<sup>[20,59,60]</sup>. The Taiwanese mass vaccination program against HBV has considerably reduced the rate of HBsAg carrier in children and adolescents and consequently the incidence of childhood HCC<sup>[20,61,62]</sup>. The average annual incidence of HCC in children aged 6-14 years declined gradually (0.70 per 100 000 children in 1981-1986, 0.57 in 1986-1990 and 0.36 in 1990-1994). A significant decrease in HCC incidence in adults was also observed, 3-4 decades later<sup>[20,63]</sup>.

## **HBV factors in HBV-related HCC**

The mechanisms of carcinogenesis in HBV infection have been extensively studied, and a major factor is chronic necroinflammation with subsequent fibrosis and hepatocyte proliferation. However, HCC may occur in HBsAg carriers without cirrhosis. Both HBV and host hepatocytes may contribute to the final pathogenic outcomes, either individually or synergistically. Therefore, it is reasonable to consider that apart from host factors, viral factors are likely involved in HBV-related hepatocarcinogenesis<sup>[20]</sup>.

## **Viral proteins in hepatocarcinogenesis**

HBV may encode oncogenic viral proteins that may contribute to hepatocarcinogenesis<sup>[20]</sup>. For example, HBx is a well-known viral non-structural gene that has roles as a multifunctional regulator modulating gene transcription, as well as controlling cell responses to genotoxic stress, protein degradation, apoptosis, and several signalling pathways<sup>[20,64-67]</sup>. Although the specific mechanisms are still unknown, its critical role in liver malignant transformation has been demonstrated in studies of transgenic mice with HBx overexpression<sup>[20,68]</sup>. HBx protein has been shown to complex the tumor suppressor p53 protein and to suppress its function<sup>[53,69,70]</sup>.

## **HBV genotype, basal core promoter (BCP) mutation and viral load in hepatocarcinogenesis**

Several viral factors other than viral proteins as viral genotype, BCP mutations in the viral genome and viral load have been associated with hepatocarcinogenesis<sup>[20]</sup>. Eight HBV genotypes (A-H) have been described, based on genomic sequence divergence<sup>[20,71,72]</sup>. These have distinct geographical and ethnic distributions: genotypes A and D prevail in Africa, Europe, and India; genotypes B and C in Asia; genotype E only in West Africa; and genotype

F in Central and South America<sup>[20,73]</sup>. It is reported that HBV genotype affects clinical outcome and treatment responses. For example, in Asia, genotype C is found to be commonly associated with more severe liver disease, cirrhosis and the development of HCC, compared to genotype B<sup>[20,65,68,74-78]</sup> whereas in Western Europe and North America, genotype D is more associated with severe liver disease and a higher incidence of HCC, than genotype A<sup>[20,79]</sup>. In addition to viral genotype, specific viral genomic mutations, particularly the BCP T1762/A1764 mutation, also correlate with HCC risk<sup>[20,80,81]</sup>.

A prospective cohort study with 11 years of follow-up assessed the relationship between HBV viral load and mortality. Viral load was found to be associated with increased mortality from HCC and chronic liver disease in HBV-infected subjects. The relative risk (RR) for HCC mortality in patients with viral load < 10<sup>5</sup> copies/mL was 1.7 (95% CI, 0.5-5.7), whereas it was 11.2 (95% CI, 3.6-35.0) in patients with viral load > 10<sup>5</sup> copies/mL<sup>[74-76]</sup>. Viral load may thus be a useful prognostic tool in HBV infection.

### **HBV factors in young-onset HCC**

Viral factors in association with the development of HBV-related HCC in young patients seem to be different from their old-aged counterparts<sup>[20,82]</sup>. Tsai and colleagues compared serum viral loads in young (less than 40 years of age) and older (over 40 years) patient groups in 183 HBV-related HCC patients and 202 HBV carriers. These authors found high serum HBV DNA levels were associated with the development of HCC in older patients, rather than those under 40 years<sup>[20,83]</sup>. Another study from Taiwan demonstrated that genotype B was significantly more common in patients with HCC, aged under 50 years, compared to age-matched inactive carriers (80% vs 52%,  $P = 0.03$ )<sup>[20,80]</sup>. This predominance was even more striking in younger patients with HCC, with 90% in those under 35 years. Most of these patients did not have cirrhosis. A further Taiwanese study reported that 26 children with HBV-related HCC were documented among 460 HBV carriers during 15 years follow up and genotype B was the major genotype (74%)<sup>[20,84]</sup>. These data suggest that genotype B-HBV may be associated with the development of HCC in young carriers without cirrhosis<sup>[20]</sup>.

### **Viral factors in HCC in the absence of cirrhosis**

Studies of HBV-related HCC in patients without cirrhosis have helped to explain the effect of viral factors in HCC development. Liu *et al* (2006) examined the role of BCP T1762/A1764 mutation, pre-core A1896 mutation and serum viral load in liver cancer, presenting in the absence of cirrhosis, by comparing 44 patients without cirrhosis, but with HBV-related HCC, to 42 individuals with cirrhosis and HBV-related HCC. These authors found that male gender, BCP T1762/A1764 mutation and viral load greater than 10<sup>5</sup> copies/mL were independently associated with the risk of HCC development in the absence of cirrhosis. They suggested that viral features predisposing to HCC might be similar between cirrhotic and non-cirrhotic groups<sup>[20,85]</sup>.

### **Pre-S deletion in HCC**

Recently, pre-S deletion of HBV has been found to be associated with the progression of liver disease and development of HCC in HBV carriers<sup>[20,86]</sup>. PreS deletion mutants hasten the storage of large envelope proteins in hepatocyte cytoplasm which can stimulate cellular promoters by inducing endoplasmic reticulum stress<sup>[53,87,88]</sup>.

The interactions between pre-S deletion, PC mutation and BCP mutation of various stages of chronic HBV infection were investigated in 46 chronic HBV carriers and 106 age-matched carriers with different stages of liver diseases; 38 with chronic hepatitis, 18 with cirrhosis, and 50 individuals with HCC<sup>[87]</sup>. Logistic regression analysis demonstrated that pre-S deletion and BCP mutation were significantly associated with the development of progressive liver disease. Combinations of mutations, especially the pre-S deletion, rather than single mutation were correlated with a greater risk of progressive liver disease. Sequencing analysis showed that the deleted regions were more common in the 3' terminus of pre-S1 and the 5' terminus of pre-S2<sup>[20,86]</sup>.

### **Combined hepatitis B and hepatitis C**

Follow up studies have shown that patients with combined HCV and HBV infection have a higher risk of developing HCC than those with a HCV or HBV alone<sup>[53,89]</sup>. The cumulative risk of developing HCC was 10%, 21%, and 23%, respectively, after 5 years and 16%, 28% and 45%, respectively, after 10 years<sup>[5,90]</sup>.

The HCC risk in subjects with both infections was investigated in a meta-analysis of 32 epidemiological studies between 1993 and 1997<sup>[53,91]</sup>. The OR for development of HCC in HBsAg positive, anti-HCV/HCV RNA negative subjects was 20.4; in HBsAg negative, anti-HCV/HCV RNA positive subjects, 23.6; and subjects positive for both markers, the OR was 135. These data suggest a more than additive effect of HBV and HCV coinfection on HCC risk. The two viruses may possibly act through common, as well as different, pathways in the carcinogenic process. Given that HBV acts as a cofactor in the development of HCV related cirrhosis and HCC, vaccination of patients with chronic hepatitis C against HBV has been recommended aiming to avoid further liver injury<sup>[53,92,93]</sup>.

### **Coinfection of HBV and hepatitis D virus (HDV)**

HDV coinfection with HBV is associated with increased liver damage. Verme and coworkers showed that HBsAg positive patients with HDV superinfection develop cirrhosis and HCC at an earlier stage (mean age 48 years), compared to HBsAg carriers without HDV infection (mean age 62 years)<sup>[53,94]</sup>.

### **Coinfection with HIV**

Chronic hepatitis C is more aggressive in HIV positive subjects, leading to cirrhosis and liver failure in a shorter time period<sup>[53,95]</sup>. Coinfection with HIV is a frequent occurrence because of shared routes of transmission. A recent study of HCC in HIV-HCV coinfecting patients indicated rapid development of HCC in these patients<sup>[53,96]</sup>.

### **Role of schistosomiasis**

Schistosomiasis is a common parasitic infestation in some parts of the world. In Egypt, Schistosomiasis is a major public health problem and infection with *Schistosoma mansoni* constitutes the major cause of liver disease. From 1950s until 1980s, the Egyptian Ministry of Health (MOH) conducted a community-wide therapy campaign using parenteral tarter emetic to control the Schistosomiasis infestation. However, this unfortunately established a large reservoir of HCV infection in the country through needle re-usage at the time of treatment<sup>[97]</sup>. There is some epidemiological evidence that the presence of schistosomal infection may modify the course of hepatitis C genotype 4 co-infection and may lead to significantly more complications, such as portal hypertension at an earlier stage with accelerated progression to hepatitis C-associated fibrosis and thus quicker progression to HCC, than those patients who do not have a parasite burden<sup>[13,31-34]</sup>.

### **Role of aflatoxin B1 (AFB1)**

AFB1 is produced by a fungus of the genus, *Aspergillus* spp, in Asia and sub-Saharan Africa in which climatic factors and storage techniques favour the fungus to be a common contaminant of foods, such as grain, corn, peanuts and legumes. Areas with high exposure of AFB1 coincide with areas with a high prevalence of HCC. It has also been suggested that a high intake of AFB1 in HBV-infected patients is an added risk factor for HCC development<sup>[3,73,98,99]</sup>. It has been observed that areas with a high prevalence of HCC and high aflatoxin intake also correspond to areas with endemic HBV infection, and that patients at highest risk of developing HCC are those who are exposed to both HBV and AFB1<sup>[3,98]</sup>.

Somatic mutations of the tumor suppressor p53 gene are the commonest genetic abnormality in human cancer and evidence supports a high level of p53 alterations in HCC. El Far and colleagues investigated p53 mutations in Egyptian patients with HCC and its relation to other prognostic factors, such as tumor grade,  $\alpha$ -fetoprotein (AFP) and liver function tests to elucidate their implication in HCC pathogenesis. These authors found that p53 detection increased the frequency of HCC prediction from 79.5% to 86.3%. Moreover, significant positive correlation between p53 mutation and tumor size for tumor grade II and III was identified. Thus, serum concentration of p53 protein may be a potential non-invasive screening test for predicting risk of HCC<sup>[100]</sup>.

It has been suggested that AFB1 can lead to HCC through inciting a specific mutation of codon 249 of the p53 tumor suppressor gene<sup>[101]</sup>. However, this mutation has also been found in patients who had previous contact with the HBV<sup>[3,78]</sup>.

### **Pesticides**

Pesticides exposure is one of the environmental factors hypothesized to increase the risk of HCC. Pesticides are considered to be possible epigenetic carcinogens through one or several mechanisms, such as spontaneous initiation of genetic changes, cytotoxicity with per-

sistent cell proliferation, oxidative stress, inhibition of apoptosis, suppression of intracellular communication and construction of activated receptors<sup>[102,103]</sup>.

A case-control study of HCC in HBV and/or HCV infected patients from Egypt suggested pesticides had an additive effect on the risk of HCC in rural males, amongst whom the use of carbamate and organophosphate compounds is commonplace<sup>[103]</sup>.

### **Diabetes mellitus**

A population-based study from the USA found diabetes to be an independent risk factor for HCC, regardless of chronic HCV or HBV infection, alcoholic liver disease, or non-specific cirrhosis. Diabetes was associated with a two- to threefold increase in HCC risk. About 60% of patients with HCC in this study were not diagnosed with chronic HCV-related or HBV-related hepatitis, alcoholic liver disease, or other known causes of chronic liver disease. Among these patients, 47% had diabetes, which was higher than those with other risk factors (41%). This suggests that diabetes may represent a considerable proportion of patients with idiopathic HCC<sup>[104]</sup>.

An increased risk of HCC among patients with diabetes alone was also reported in a population based study using data obtained from the Denmark cancer registry<sup>[104,105]</sup>. Also, it is reported a threefold increased risk of liver cancer among patients hospitalized with diabetes, as well as and a fourfold risk in the presence of hepatitis, cirrhosis, and alcoholism in a Swedish study<sup>[104,106]</sup>.

Diabetes, as part of the insulin resistance syndrome, has been implicated as a risk factor for non-alcoholic fatty liver disease (NAFLD), including in its most severe form, non-alcoholic steatohepatitis (NASH). NASH has been identified as a cause of both "cryptogenic cirrhosis" and HCC<sup>[104,107-112]</sup>.

### **Diet**

Many epidemiological studies have examined the relationship between diet and HCC risk<sup>[113]</sup>. The results are somewhat conflicting. Some studies have shown an inverse relationship between HCC and diets which are high in milk, wheat, vegetable, fish and fruit content. Other studies have shown no association.

With regard to egg consumption, two studies reported an inverse relation with HCC risk<sup>[114,115]</sup>, while three others reported an increased risk<sup>[115-117]</sup>. Similarly, two studies<sup>[118,119]</sup> demonstrated that meat and animal protein consumption were associated with increased risk of HCC, although other studies<sup>[114-116]</sup> did not support this finding<sup>[113]</sup>.

To verify if consumption of soya foods reduce the HCC risk, Sharp and his colleagues conducted a case-control study within a cohort of Japanese A-bomb survivors. They compared the pre-diagnosis intake of isoflavone-rich miso soup and tofu to HCC risk, adjusting for hepatitis B (HBV) and C (HCV) viral infections. They concluded that consumption of miso soup and other soya foods may reduce HCC risk and this is consistent with the results of epidemiological, animal and laboratory-based studies, as well as some clinical trials<sup>[120]</sup>.

This is explained by the opposing effect of isoflavones on oestrogen and testosterone levels which reduce HCC risk, possibly by modifying the hormonal profile and reducing cell proliferation, associated with increased cancer risk. An alternative explanation may be that isoflavones provide an independent anti-tumour effect, such as suppression of angiogenesis, or stimulation of apoptosis<sup>[120]</sup>.

A 41% reduction in HCC risk among coffee drinkers, compared to non-drinkers, has been observed in a meta-analysis study<sup>[121]</sup>. This favourable effect of coffee drinking was established both in studies from southern Europe<sup>[122-124]</sup>, where coffee is widely consumed, and from Japan<sup>[125,126]</sup>, where coffee intake is less frequent, and in subjects with chronic liver disease<sup>[121]</sup>. Some compounds in coffee, including diterpenes, cafestol, and kahweol, may act as blocking agents *via* modulation of multiple enzymes involved in carcinogenic detoxification as demonstrated in animal models and cell culture systems<sup>[119,127,128]</sup>. Moreover, coffee components modify the xenotoxic metabolism *via* induction of glutathione-S-transferase and inhibition of N-acetyltransferase<sup>[121,129]</sup>. Other components of coffee, including caffeine and antioxidant substances from coffee beans, have been related to favorable modifications in liver enzymes such as  $\gamma$ -glutamyltransferase and aminotransferase activities<sup>[119,130-133]</sup>.

## CONCLUSION

HCC is one of the commonest cancers worldwide. It is a major health problem and its incidence is increasing. The presence of cirrhosis is the major risk factor and worldwide this is largely due to chronic HCV and HBV infection. HCC carcinogenesis is likely to involve interplay of viral, environmental and host factors. The advent of mass-vaccination programmes for hepatitis B, particularly in East Asia is beginning to reduce prevalence rates for HCC in some countries, but for the most part, HCV-related HCC is increasing. Concerted strategies need to be developed for HCC surveillance in at risk populations.

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## Essential oil of *Curcuma wenyujin* induces apoptosis in human hepatoma cells

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observed at 70 µg/mL after 72 h of treatment. During the process, cytosolic HepG2 cytochrome C staining showed a markedly stronger green fluorescence than in control cells in a dose-dependent fashion, and CWO also caused mitochondrial transmembrane depolarization. Furthermore, the results clearly demonstrated that both, activity of caspase-3 enzyme and protein levels of cleaved PARP, significantly increased in a dose-dependent manner after treatment with CWO.

**CONCLUSION:** CWO exhibits an antiproliferative effect in HepG2 cells by inducing apoptosis. This growth inhibition is associated with cell cycle arrest, cytochrome C translocation, caspase 3 activation, Poly-ADP-ribose polymerase (PARP) degradation, and loss of mitochondrial membrane potential. This process involves a mitochondria-caspase dependent apoptosis pathway. As apoptosis is an important anti-cancer therapeutic target, these results suggest a potential of CWO as a chemotherapeutic agent.

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**Key words:** Essential oil; *Curcuma wenyujin*; Apoptosis; HepG2; Caspase-3; Mitochondrial; Cytochrome C; Cleaved Poly-ADP-ribose polymerase

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### Abstract

**AIM:** To investigate the effects of the essential oil of *Curcuma wenyujin* (CWO) on growth inhibition and on the induction of apoptosis in human HepG2 cancer cells.

**METHODS:** The cytotoxic effect of drugs on HepG2 cells was measured by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. DNA fragmentation was visualized by agarose gel electrophoresis. Cell cycle and mitochondrial transmembrane potential ( $\Delta\psi_m$ ) were determined by flow cytometry (FCM). Cytochrome C immunostaining was evaluated by fluorescence microscopy. Caspase-3 enzymatic activity was assayed by the cleavage of Ac-DEVD-R110. Cleaved PARP and active caspase-3 protein levels were measured by FCM using BD™ CBA Human Apoptosis Kit.

**RESULTS:** Treatment with CWO inhibited the growth of HepG2 cells in a dose-dependent manner, and the IC<sub>50</sub> of CWO was approximately 70 µg/mL. CWO was found to inhibit the growth of HepG2 cells by inducing a cell cycle arrest at S/G<sub>2</sub>. DNA fragmentation was evidently

### INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer with more than 1 million fatalities occurring annually worldwide<sup>[1]</sup>. Most HCCs, unlike their normal counterparts, are quite resistant to death receptor-mediated apoptosis, because cell surface death receptors are cross-linked with either agonistic antibodies or soluble death ligand proteins<sup>[2,3]</sup>. HCCs also display high resistance to tumor necrosis factor-related

apoptosis-inducing ligand-mediated cell death<sup>[4,5]</sup>, which, together with other apoptosis resistance mechanisms, suggests that alternative approaches are needed to control HCC growth and metastasis.

*Ezhu*, as a Chinese traditional medicine has been used for a long time. It belongs to the family of *Zingiberaceae*. This genus is composed of about 70 species of rhizomatous herbs which are distributed all over the world, and about 20 species could be identified in China. Actually, Chinese Pharmacopoeia indicated that the rhizomes of three species including *Curcuma phaeocaulis*, *C. kwangsiensis*, and *C. wenyujin* are used as *Ezhu*, which has been used for removing blood stasis, alleviating pain, and liver disease protection<sup>[6]</sup>. In order to control the quality of *Ezhu* and develop *Ezhu* as an effective therapeutic agent, we have developed quality control methods and conducted studies comparing the quantities of several chemical components of different types of *Ezhu*<sup>[7]</sup>. Nowadays in China, the essential oil of *Curcuma wenyujin* (CWO) has been used as injection to cure paediatric diseases such as acute upper respiratory infections, viral myocarditis, or acute pneumonia<sup>[8]</sup>. Besides, the essential oil of *Ezhu* has been used as a preparation to treat vaginitis<sup>[9]</sup>. Also, in some other countries, as in France, Japan, or India, the essential oil of *Ezhu* has been reported to possess anti-microbial<sup>[10]</sup>, anti-bacterial, vasorelaxant<sup>[11]</sup>, and anti-inflammatory activities<sup>[12]</sup>. In China, the essential oil of *Ezhu* has shown promising effects in the treatment of liver<sup>[13]</sup>, gastric, lung, and cervical cancers. For instance, inhibitory effects of CWO on the growth of SMMC-7721 cells, cervical cells, L615 cells, and K562 cells have been reported<sup>[14]</sup>. In addition, we recently identified furanodiene, one of *Ezhu*'s ingredients, to activate p38 and to inhibit of ERK mitogen-activated protein kinase (MAPK) signaling in HepG2 cells. The result suggests *Ezhu* as a potential candidate for the treatment of liver diseases<sup>[15]</sup>. *Ezhu* has a long history on treating liver disease and protecting liver functions; however, there is no report about inhibitory effects of the essential oil of *Ezhu* on human HepG2 cell growth and the underlying mechanism of action. This study aimed determining the cytotoxicity of CWO, one species of *Ezhu*, in human hepatoma HepG2 cells and the underlying molecular mechanism of action.

## MATERIALS AND METHODS

### Materials

CWO was purchased from Zhejiang RuiAn Pharmaceutical Company (Lot No. 011001). CWO (0.1 mL) was diluted in 10 mL methanol. The solution thus obtained was filtered through a 0.45 µm Econofilter (Agilent Technologies, Palo Alto, CA, USA) and injected into Agilent Series 1100 (Agilent Technologies, USA) liquid chromatograph, equipped with a vacuum degasser, a quaternary pump, an autosampler, and a diode array detection (DAD) system, and analyzed under the conditions described in a previous report<sup>[7]</sup>. In brief, A Zor-

bax ODS column (250 mm × 4.6 mm I.D., 5 µm) with a Zorbax ODS C18 guard column (20 mm × 3.9 mm I.D., 5 µm) was used. Solvents that constituted the mobile phase were A (water) and B (acetonitrile). The elution conditions applied were: 0-15 min, linear gradient 30%-47% B; 15-30 min, isocratic 47% B; 30-40 min, linear gradient 47%-60% B; 40-50 min, linear gradient 60%-90% B; 50-60 min, linear gradient 90%-100% B; and finally, washing the column with 100% B for 10 min before reconditioning the column for 15 min with 30% B. The flow-rate was 1 mL/min and the injection volume was 10 µL. The column operated at 25°C. The analytes were monitored with DAD at 214 nm and 256 nm. In addition, methanol solutions, containing known concentrations of standards including curcumeneone, curcumenol, neocurdione, curdione, isocurcumenol, furanodieneone, curcumol, germacrone, curzerene, furanodiene, and β-elemene were prepared and subjected to the LC-DAD system for comparison. 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), JC-1 dye, caspase-3 assay kit, and 488 cytochrome C apoptosis detection kit were purchased from Molecular Probes. Human apoptosis kits were purchased from BD Bioscience.

### Methods

**Cell culture and drug treatment:** The human hepatoma cell line HepG2 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Life Technologies Inc., Gaithersburg, MD), 100 µg/mL streptomycin, and 100 U/mL penicillin in 75 cm<sup>2</sup> tissue culture flasks in a humidified atmosphere at 37°C with 5% CO<sub>2</sub>. CWO was dissolved in 1 mL DMSO to make a 1 mmol/L stock solution and diluted to the concentrations as needed. The final volume of drug solution added to medium was 1%. Control samples contained 1% DMSO.

**Growth inhibitory assay:** Cells were seeded in 96-well microplates ( $1 \times 10^5$  cells/well in 100 µL medium). CWO was added to the cultures in serial concentrations and cultures were incubated for 48 h. Medium was discarded and 30 µL tetrazolium dye (MTT) solution (5 mg/mL in PBS) were added to each well. Plates were incubated for additional 4 h. DMSO (10 µL) was added to dissolve the formed formazan crystals. The plate was read in a microplate reader at 570 nm. MTT solution with DMSO (without cells and medium) acted as blank while the DMSO (1%)-treated cells served as control of 100% survival.

**Agarose gel electrophoresis for analysis of DNA fragmentation:** HepG2 cells were treated with the drug for 72 h while the DMSO (1%) containing medium treated cells served as control. Adherent cells ( $2 \times 10^6$ /mL) were harvested, washed once with 400 µL PBS, and

taken up in 400  $\mu$ L lysis buffer (containing 200 mmol/L Tris-HCl (pH 8.3), 100 mmol/L EDTA, and 1% SDS). Twenty  $\mu$ L of 10 mg/mL proteinase K were added for protein digestion, and the tubes were incubated in a 37°C water bath overnight. The samples were allowed to cool down to room temperature before 300  $\mu$ L saturated NaCl solution were added. After centrifugation for 15 min at 9000 r/min, supernatants were collected. DNA fibers were obtained by adding 1 mL cold absolute ethanol (EtOH) and a centrifugation for 20 min at 4°C at 16000 r/min. DNA fibers were washed once with 500  $\mu$ L of -20°C 70% EtOH, and the DNA pellet was dried in a 70°C oven. Fifteen  $\mu$ L TE buffer (10 mmol/L Tris-HCl pH 8.0, 1 mmol/L EDTA) containing 0.2 mg/mL RNase A were added. After an incubation at 37°C for 90 min, 10  $\mu$ L of each sample were loaded on a 1.5% TBE agarose gel to visualize DNA.

**Cell cycle analysis:** Cells were seeded in 6 well plates and treated with CWO at various concentrations for 48 and 72 h. DMSO (1%)-treated cells served as control. After treatment, media were discarded. The adherent cells were washed with PBS, and 300  $\mu$ L trypsin were added for 5 min at room temperature to detach the cells. After centrifugation at 350 g at 4°C for 5 min, the cell pellet was resuspended with 1 mL cold 70% EtOH at 4°C for 12 h and centrifuged again for 5 min at 4°C at 350 g. Finally, 1 mL propidium iodide (PI) staining solution (20  $\mu$ g/mL PI, 8  $\mu$ g/mL DNase free RNase) was added to the samples. The samples were analyzed by flow cytometry (FCM) (BD FACS Canto<sup>TM</sup>). The results were analyzed by Mod Fit LT 3.0 software.

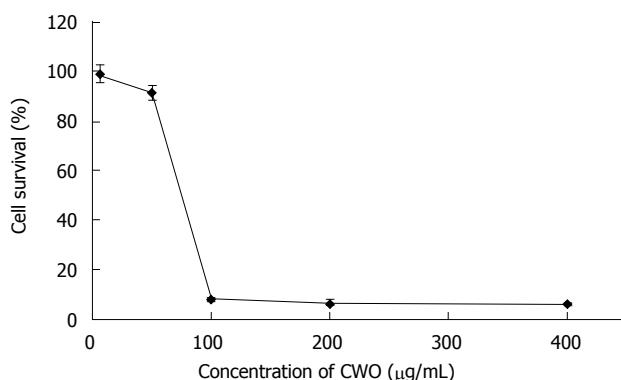
**Measurement of mitochondrial transmembrane potential ( $\Delta\Psi_m$ ):**  $\Delta\Psi_m$  was assessed by JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide), Mitochondrial Potential Sensors (Molecular Probes, Leiden, Netherlands). Red fluorescence J-aggregate form of JC-1 indicates intact mitochondria, whereas green fluorescence shows monomeric form of JC-1 due to breakdown of the mitochondrial membrane potential. Cells were seeded in 6-well plates and then incubated with the desired concentrations of CWO for 48 h. The medium of each well was discarded, cells were treated with 1 mL medium containing 5 mg/mL JC-1 for 15 min at 37°C and 5% CO<sub>2</sub> in the dark, washed twice in PBS, resuspended in 1 mL medium and measured by FCM.

**Immunostaining of cytochrome C:** Cytochrome C release was assessed by SelectFX Alexa Fluor 488 cytochrome C apoptosis detection kit (Molecular Probes, Leiden, Netherlands). Cells were seeded in 24-well plates, and treated with various amounts of CWO in an humidified atmosphere (37°C in 5% CO<sub>2</sub>) for 24 h while the DMSO (1%)-treated cells served as control. Media were discarded and the cells were washed with warm PBS, fixed with freshly prepared 4% formaldehyde in PBS for 15 min at 37°C, and permeabilized with 0.2%

Triton X-100 for 5 min at room temperature. The cells were washed, incubated in a blocking buffer (10% heat-inactivated normal goat serum (NGS) for 30 min at room temperature, and finally for 1 h with 1  $\mu$ g/mL primary antibody (anti-cytochrome C, mouse IgG) at room temperature. Green fluorescence was observed by fluorescence microscopy.

**Caspase-3 enzymatic activity assay:** Caspase-3 enzymatic activity was determined by measuring the cleavage of Ac-DEVD-R110 according to the protocol with the caspase-3 assay kit supplied by Molecular Probes. Cells were treated with various amounts of CWO in an humidified atmosphere (37°C in 5% CO<sub>2</sub>) for 24 h while DMSO (1%)-treated cells served as control. Cells were harvested at a concentration of a minimum of  $1 \times 10^6$ /mL, pellets were collected, appropriate cell lysis buffer was added, and the samples were incubated on ice for 30 min. The samples were then centrifuged and supernatants were collected and transferred to microplates. Cell lysis buffer was used as a no-enzyme control to determine the background fluorescence of the substrate. At the same time, 1  $\mu$ L of 1 mmol/L Ac-DEVD-CHO inhibitor was added to selected samples. One  $\mu$ L of DMSO was added to no-inhibitor samples to serve as control and incubated for 10 min simultaneously. Finally, 0.05 mmol/L Z-DEVD-R110 substrate was added and samples were incubated for 30 min prior to the fluorescence measurement.

**Measurement of cleaved PARP and active caspase-3 protein levels:** The BD<sup>TM</sup> CBA human apoptosis kit (BD, Franklin Lakes, USA) was applied to quantify the active caspase-3 and Poly-ADP-ribose polymerase (PARP) protein levels; cytometric Bead Array (CBA) employs a particle with a discrete fluorescence intensity to detect a soluble analyte. This kit provided two types of bead populations with distinct fluorescence intensities that have been coated with capture antibodies specific for cleaved caspase-3 and PARP. Cells were seeded in 6-well plates and incubated with various concentrations of CWO in an humidified atmosphere (37°C with 5% CO<sub>2</sub>) for 48 h.  $1.0 \times 10^6$  cells per sample were counted, harvested, and washed with PBS. Fifty  $\mu$ L of cell lysis buffer was added to each sample for 30 min on ice and samples were vortexed at 10-min intervals. Pellet cellular debris was removed by centrifugation at 12500 r/min for 10 min. Protein concentrations were measured by 2-D Quant Kit (Amersham Biosciences, Piscataway, USA). Each sample was normalized in a final concentration of 0.2  $\mu$ g/ $\mu$ L. Thirteen standard curves (standard ranging from 0 to 6000 U/mL) were obtained from one set of calibrators. For each sample and the standard mixture of lysate standard (caspase-3 and PARP beads), 50 mL of sample or standard of beads were added to the mixture of 50 mL of 2 mixed capture beads incubated for 1 h, and mixed 50 mL of PE detector bead for another 1 h. After that, samples were washed before data acquisition with a FCM. The results were analyzed by FCAP Array V1.0.



**Figure 1** HepG2 cells were treated with the indicated concentrations of CWO for 48 h. Cell growth was determined by the MTT assay and was directly proportional to the absorbance at a wavelength of 570 nm. Data are expressed as mean  $\pm$  SD from three independent experiments.

### Statistical analysis

The data are expressed as mean  $\pm$  SD from at least 3 independent experiments. Differences between groups were analyzed using a Student's *t*-tests.

## RESULTS

### CWO inhibits cell growth and induces DNA fragmentation in HepG2 cells

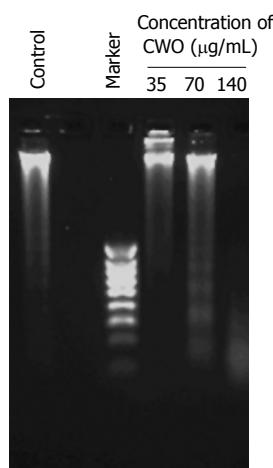
CWO treatment inhibited the growth of HepG2 cells in a dose-dependent manner, and the IC<sub>50</sub> of CWO was approximately 70  $\mu$ g/mL (Figure 1). We then examined whether CWO inhibited HepG2 cell growth through inducing cell death and apoptosis. HepG2 cells were treated with different concentrations of CWO for 72 h. Figure 2 shows that DNA fragmentation was evidently observed at a concentration of 70  $\mu$ g/mL after 72 h of treatment.

### CWO causes S/G<sub>2</sub> cell cycle arrest

The effect of different concentrations of CWO on cell-cycle progression was studied after 48 and 72 h of drug exposure. CWO treatment resulted in the accumulation of cells in S/G<sub>2</sub> phase with concomitant losses from G<sub>0</sub>/G<sub>1</sub> phase (Figure 3). Since substantial proportions of cells were dead in groups treated with 50  $\mu$ g/mL and 70  $\mu$ g/mL CWO, only cultures treated with 35  $\mu$ g/mL were used for the analysis as shown in Figure 4.

### CWO causes mitochondrial transmembrane depolarization in HepG2 cells

Some chemotherapeutic drugs induced apoptosis via mitochondrial pathways by altering  $\Delta\Psi_m$ . To monitor the  $\Delta\Psi_m$ , we used JC-1 probe to determine the  $\Delta\Psi_m$  in cells that were treated with CWO for 48 h at different concentrations. Mitochondria with normal  $\Delta\Psi_m$  concentrate JC-1 into aggregates (red/orange fluorescence), while in depolarized mitochondria, JC-1 forms monomers (green fluorescence). As compared to non-treated HepG2 cells, green fluorescence increased while red/orange fluorescence decreased after CWO exposure. Figure 4 indicates an only minor shift from



**Figure 2** Agarose gel of electrophoresis of genomic DNA obtained from HepG2 cells treated with different concentrations of CWO for 72 h. DNA fragmentation with a ladder pattern is a characteristic for apoptosis.

red/orange to green fluorescence in groups treated with 17.5  $\mu$ g/mL and 35  $\mu$ g/mL CWO, while 50  $\mu$ g/mL and 70  $\mu$ g/mL CWO led to significant changes in  $\Delta\Psi_m$ .

### CWO causes cytochrome C release from mitochondria into cytosol

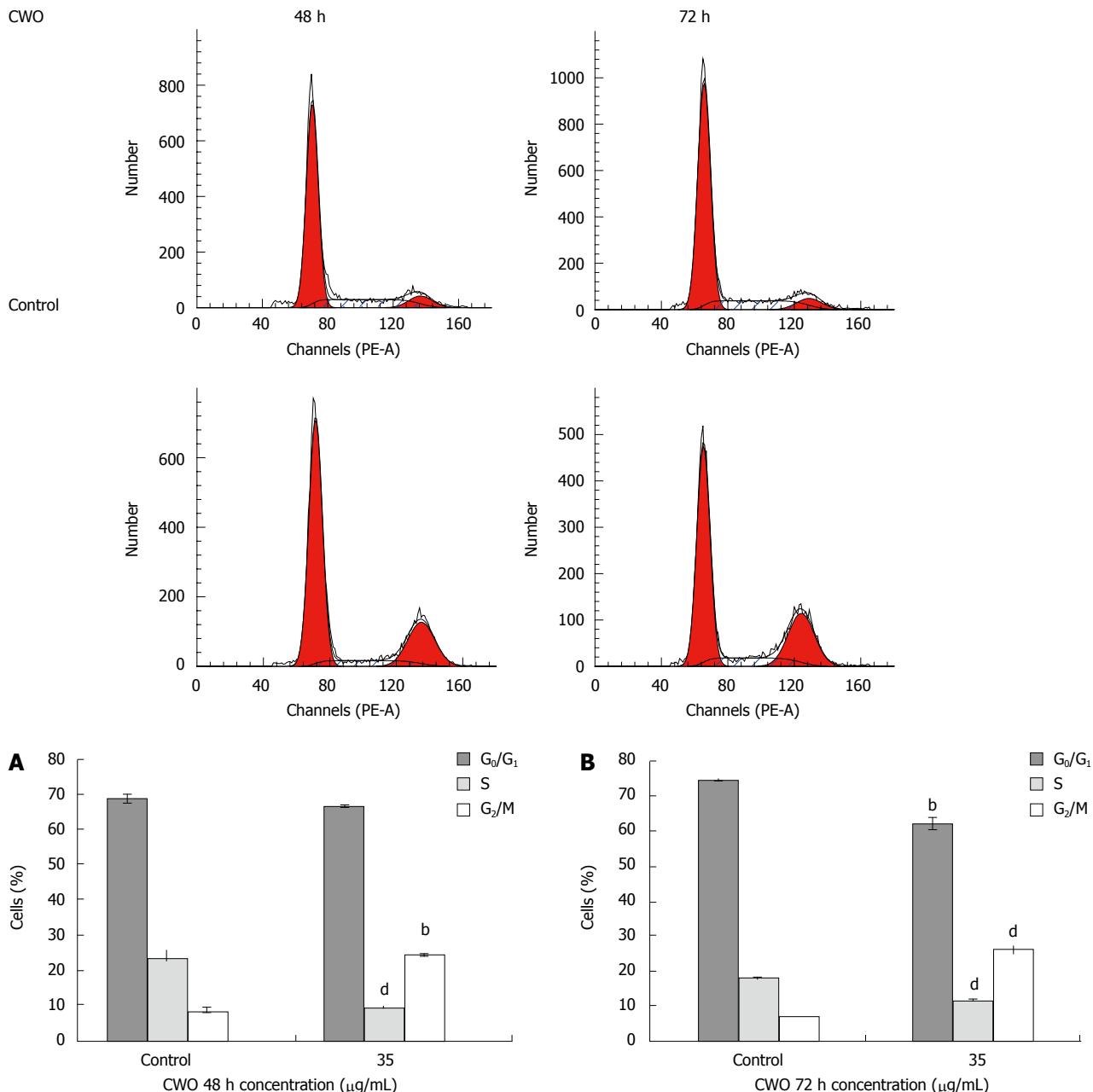
Cytochrome C release from mitochondria to the cytosol is implicated in mitochondria dependent apoptosis<sup>[16]</sup>. Cytochrome C staining in the cytosol of HepG2 cells showed markedly stronger green fluorescence than in control cells in a dose-dependent fashion (Figure 5). CWO treated cells showed obvious punctate green fluorescence staining or appeared to have green fluorescence accumulated in large aggregates compared to the control.

### CWO activates caspase-3 enzymatic activity

Many studies previously have demonstrated that programmed cell death is associated with the activation of caspase as key elements involved in the sequence of events that lead to cell death<sup>[17]</sup>. Caspase-3 particularly, is essential for propagation of the apoptotic signal after exposure to many DNA-damaging agents and anticancer drugs. We examined caspase-3 activity after cells were treated with 17.5-70  $\mu$ g/mL CWO for 24 h. The result clearly demonstrated that caspase-3 activity increased in a dose-dependent manner (Figure 6). Caspase-3 activities were 2 and 5.5 times higher in 35  $\mu$ g/mL and 70  $\mu$ g/mL CWO treated cultures, respectively, when compared to the control. When the cellular samples were incubated with specific Ac-DEVD-CHO inhibitors simultaneously, caspase-3 activity was blocked.

### CWO increases cleaved PARP and active caspase-3 protein levels

Drug-induced cell death via apoptosis pathway, signaling can generally be divided into receptor- and mitochondrial-mediated pathways. These pathways converge at several downstream points including the mitochondria, caspase activation, and substrate cleavage<sup>[18]</sup>. Figure 7 showed that there was significant increase in protein levels of cleaved PARP and active caspase-3 in a dose-dependent manner as determined by BD™ CBA Human



**Figure 3** Effect of CWO on HepG2 cell cycle progression. Flow cytometric analysis of propidium iodide-stained HepG2 cells treated with 35 µg/mL CWO for 48 h (A) and 72 h (B). The results of HepG2 cells treated with CWO were analyzed by Mod Fit LT 3.0. Data expressed as mean ± SD from three independent experiments. \*P < 0.01, \*\*P < 0.001 vs control.

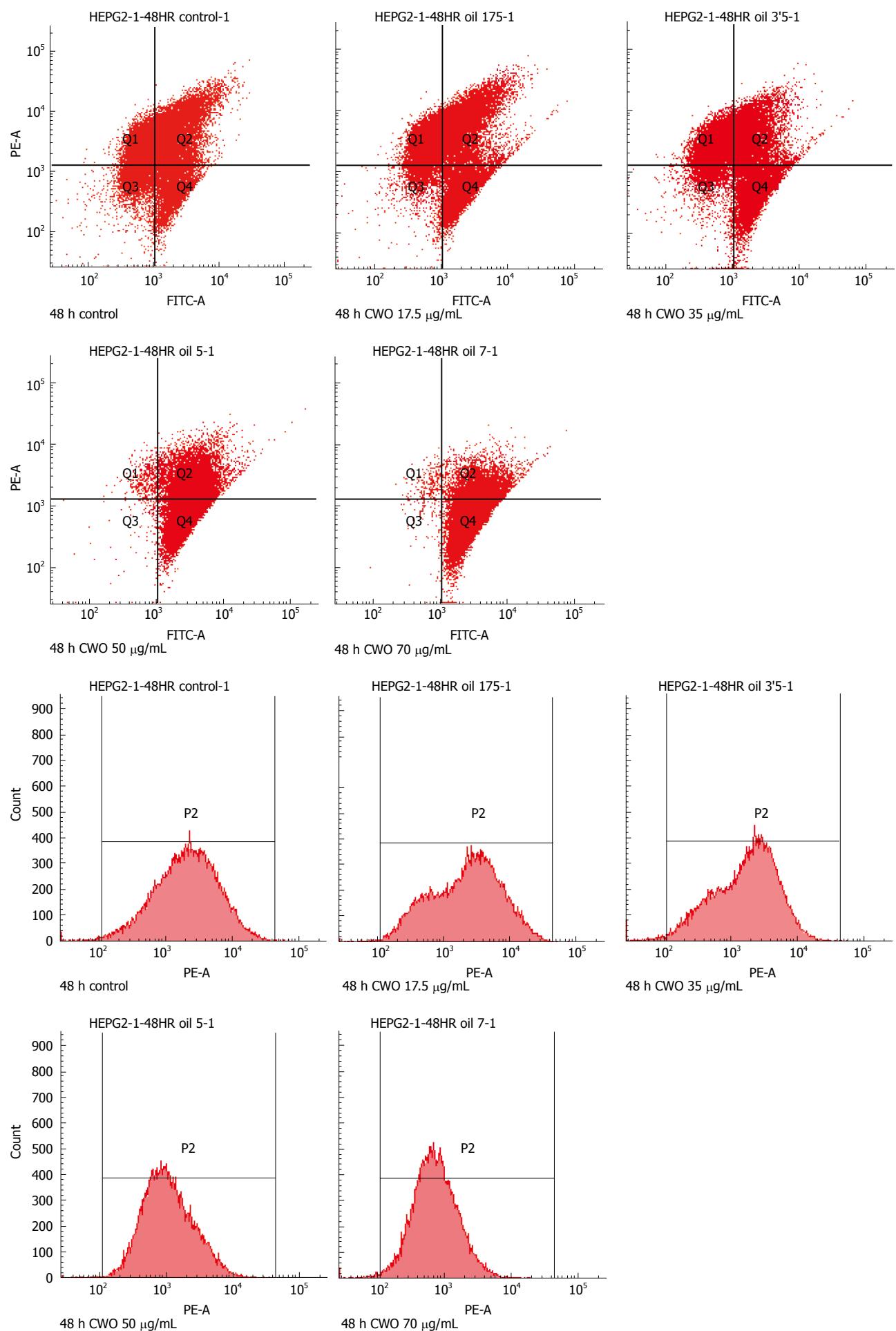
Apoptosis Kit. HepG2 cells treated with 17.5, 35, and 70 µg/mL CWO were 6, 4, and 7 times higher in caspase-3 protein level than control group (Figure 7A). The level of cleavage of PARP had a 2-fold increase in HepG2 cells treated with 70 µg/mL CWO compared to the control (Figure 7B).

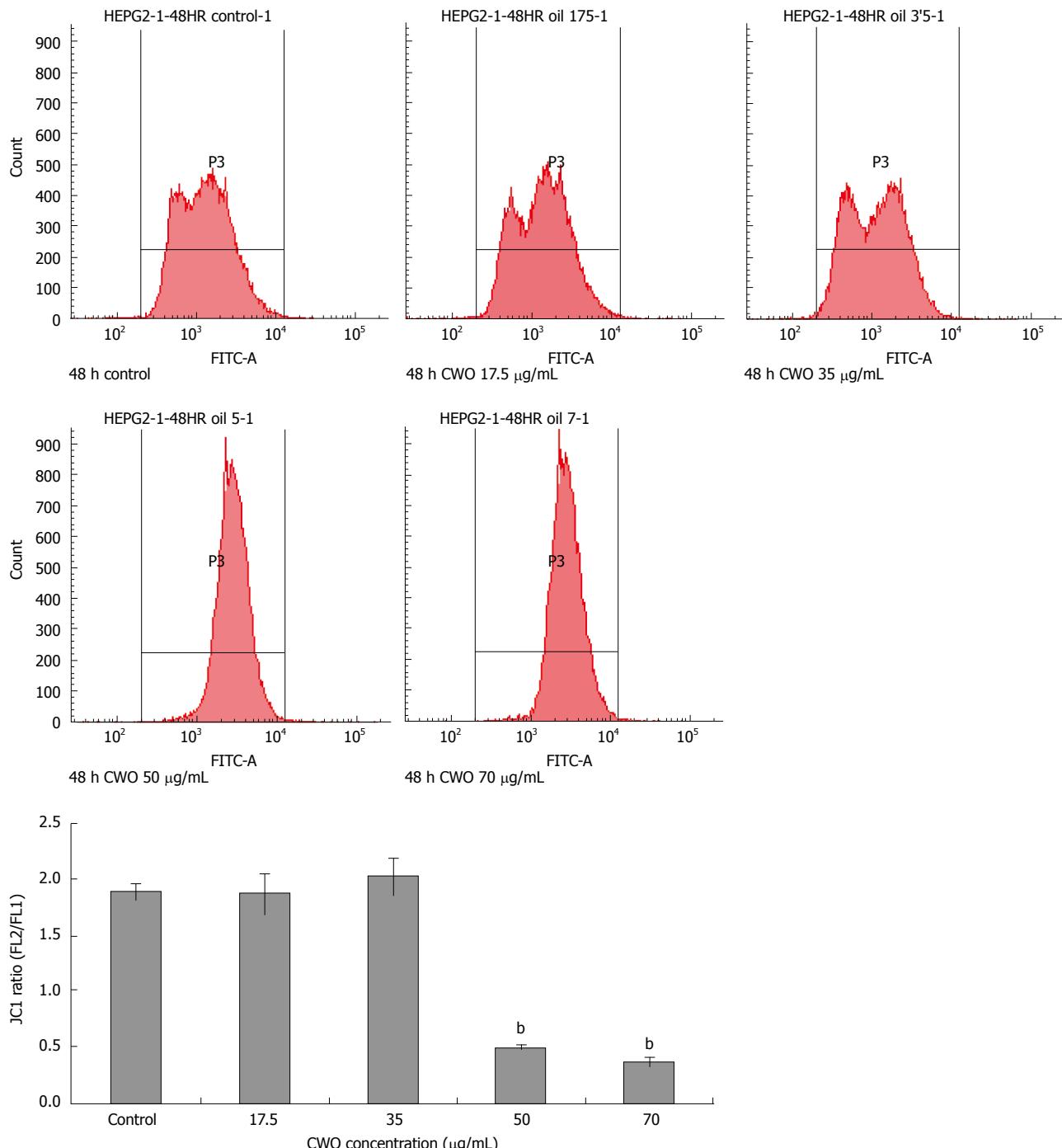
## DISCUSSION

The essential oil of *Ezhu* and its ingredients have been widely used for treatment of malignant tumors in China<sup>[19]</sup> and have been identified to have hepatoprotective effects<sup>[20,21]</sup>. Previously, we have already identified an active ingredient furanodiene, a sesquiterpene compound, which have been isolated from CWO, one of species of

*Ezhu*. Furanodiene has been found to induce apoptosis in HepG2 cells through activation of mitochondrial and caspase dependent pathway which involved activation of P38, and inactivation of ERK1/2 MAPK signaling cascades<sup>[15]</sup>. In the present study, we have found that CWO inhibited HepG2 cells growth with IC<sub>50</sub> at approximately 70 µg/mL and it has been identified to inhibit HepG2 cell growth via inducing apoptosis as evidenced by activation of depolarization of ΔΨ<sub>m</sub>, mitochondrial cytochrome C release, caspase-3, PARP cleavage, S/G<sub>2</sub> cell cycle arrest, and finally DNA fragmentation.

Active caspase-3 has been considered to be indicative of apoptosis<sup>[17]</sup> and another characteristic event of apoptosis is the proteolytic cleavage of PARP, a nuclear enzyme involved in DNA repair, DNA stability, and



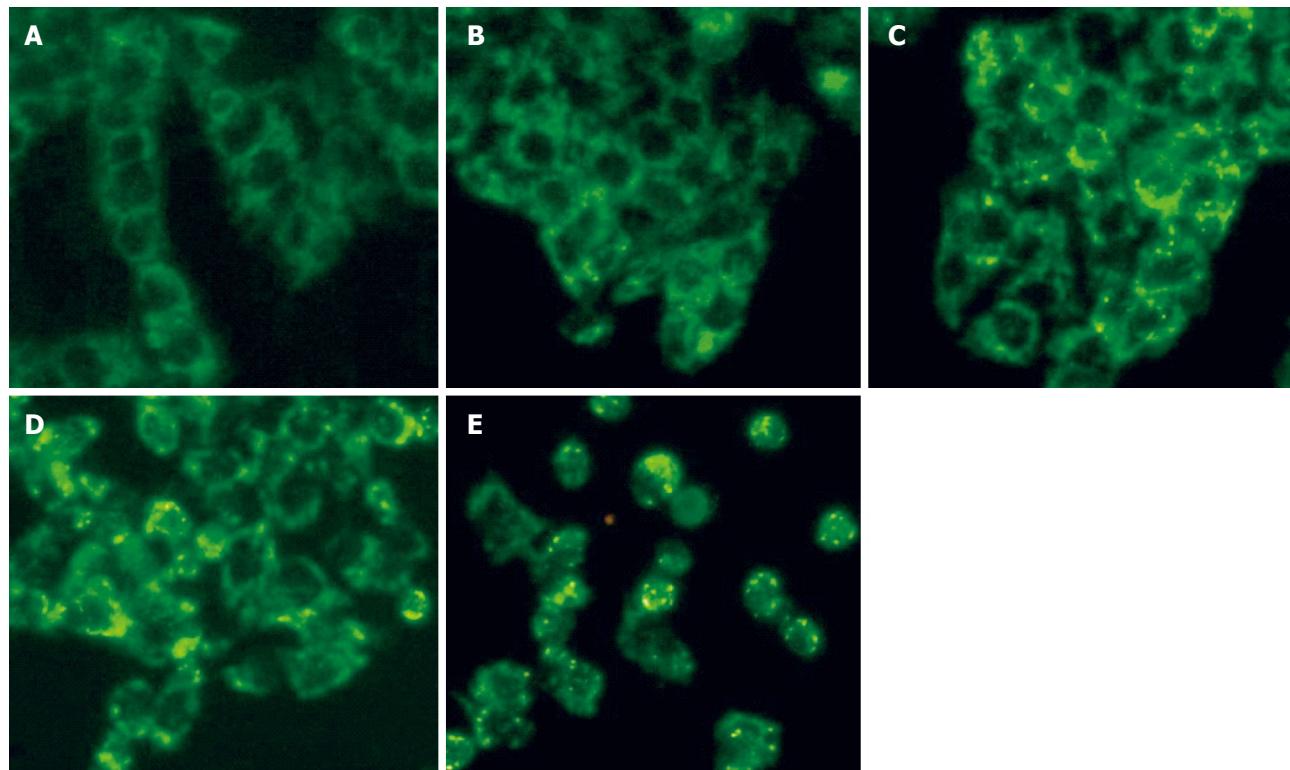


**Figure 4** Analysis of change of  $\Delta\Psi_m$  in HepG2 cells. HepG2 treated with 17.5, 35, 50, and 70  $\mu\text{g/mL}$  for 48 h, were stained with JC-1 probe. The cells were analyzed by FCM. Red and green fluorescence were measured by FL2 and FL1 channel, respectively. Red fluorescence indicates intact mitochondrial potential while green fluorescence indicates breakdown of mitochondrial potential. The ratio of intensity of FL2 to FL1 indicates the change of  $\Delta\Psi_m$ . Data expressed as mean  $\pm$  SD from four independent experiments. <sup>b</sup> $P < 0.001$  vs control.

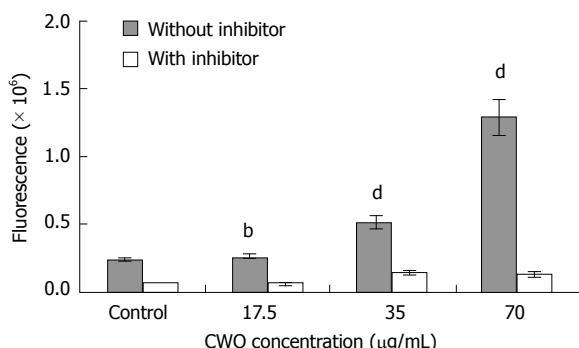
transcriptional regulation<sup>[22]</sup>. An experiment was performed to simultaneously and quantitatively evaluate CWO induced changes of active caspase-3 and cleaved PARP protein levels using CBA technology as demonstrated in Figure 7. As shown, active caspase-3 protein expressions were enhanced approximately 7-fold compared to the control. Meanwhile, we attempted to detect CWO-induced caspase-3 enzymatic activity increases using caspase-3 specific substrate Z-DEVE-R110, and have found that the enhancements disappeared when treated with Ac-DEVD-

CHO inhibitor as shown in Figure 6. This showed the increases in caspase-3 enzymatic activities were specific to CWO treatment and suggested that CWO induces apoptosis via a caspase-3-dependent pathway.

CWO induced significant increase in caspase-3 enzymatic activity as well as the protein levels of active caspase-3 and cleaved PARP (Figure 7). These results suggested that CWO induced apoptosis via caspase pathway. Moreover, whether CWO induced apoptosis in HepG2 cell is mitochondria-dependent is unknown.



**Figure 5** Cytochrome C release into the cytosol in CWO treated HepG2 cells for 12 h. Cytochrome C immunofluorescence was observed with fluorescent microscope. **A:** Control; **B:** 17.5  $\mu\text{g}/\text{mL}$ ; **C:** 35  $\mu\text{g}/\text{mL}$ ; **D:** 70  $\mu\text{g}/\text{mL}$ ; **E:** 120  $\mu\text{g}/\text{mL}$ . Fine punctate/granular stainings for cytochrome C are observed. Cytochrome C release also increases the global cytosolic fluorescent signal. Similar results were obtained for 3 independent experiments ( $\times 20$ ).



**Figure 6** The fluorescence was increased in a dose-dependant manner after 24 h treatment with CWO. Caspase-3 cleaves substrate Ac-DEVD-R110 to emit green fluorescence. Higher fluorescent intensity indicates higher caspase-3 enzymatic activity. Ac-DEVE-CHO inhibitor can inhibit caspase-3 enzymatic activity. Data are expressed as mean  $\pm$  SD from three independent experiments. <sup>b</sup> $P < 0.001$ , <sup>d</sup> $P < 0.01$  vs control.

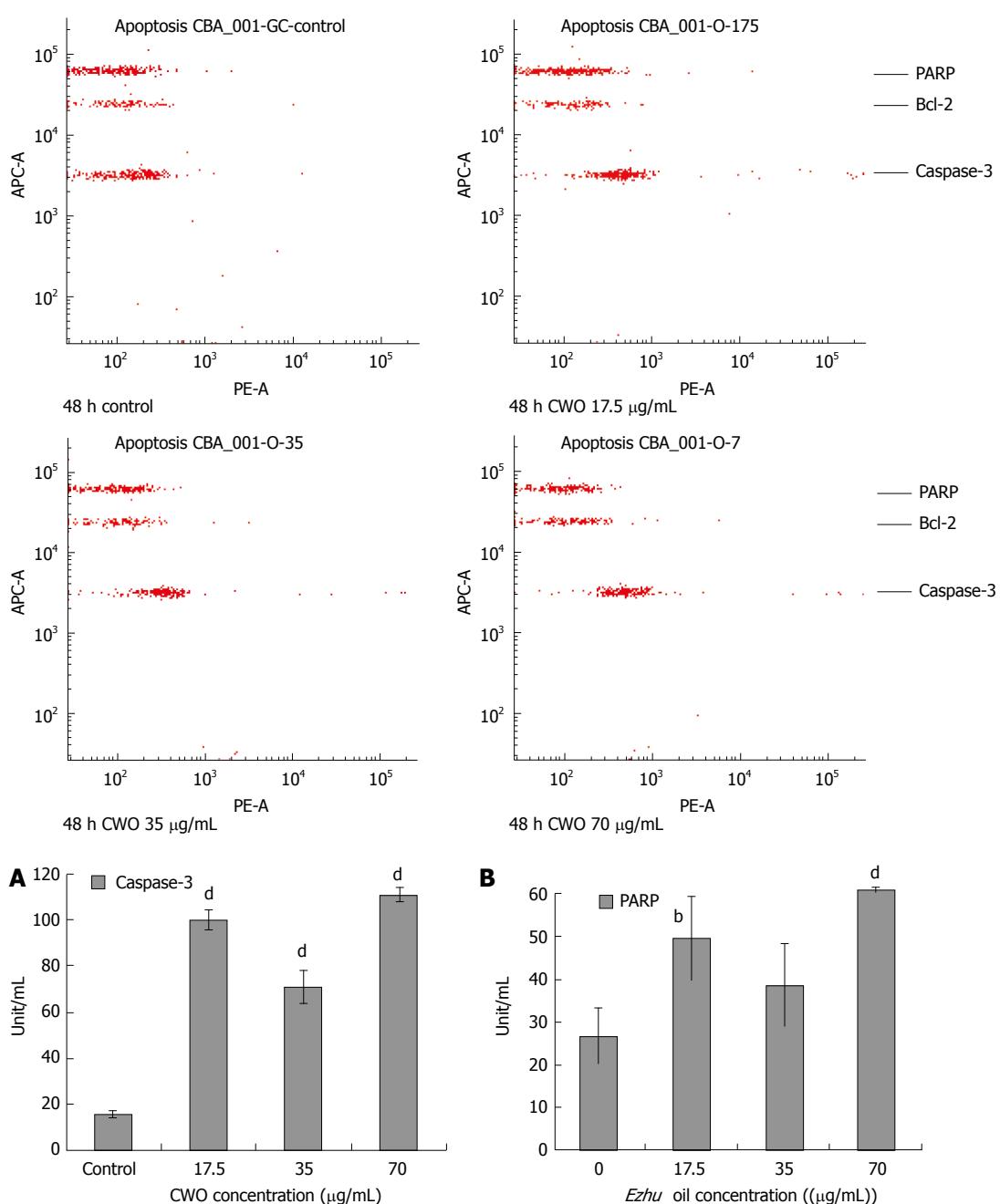
To address this question, the change of  $\Delta\Psi_m$  and mitochondrial cytochrome C release were determined. Figures 4 and 5 suggested that CWO-mediated apoptosis was accompanied with the  $\Delta\Psi_m$  as well as the release of mitochondrial cytochrome C into cytosol. These results demonstrated that a mitochondrial pathway was also involved in CWO-induced apoptosis.

It is now widely believed that p38 and JNK mediate apoptotic signals, while ERK promotes growth, differentiation, and proliferation. Nowadays, many studies have shown that p38 MAPK activation is necessary for cancer cell death initiated by a variety of anti-cancer agents<sup>[23]</sup>. Furthermore, different MAPK

signaling pathways can be coordinately manipulated to enhance the efficacy of anticancer drug. Co-treatment of anticancer drugs with ERK inhibitors has been found to enhance anticancer effects. Anti-cancer drug paclitaxel (Taxol) induces tumor cell apoptosis through activating endogenous JNK in tumor cells<sup>[24]</sup>. When paclitaxel and ERK inhibitor were combined in cancer treatment, ERK inhibitor significantly enhances the JNK activation-mediated cytotoxic effect of paclitaxel. ERK inhibitor also found to enhance docetaxel-induced apoptosis of androgen-independent human prostate cancer cells<sup>[25]</sup>.

CWO, possibly act as chemopreventive agents with respect to inhibition of the growth of human HepG2 cells through the induction of apoptosis. As apoptosis has become a new therapeutic target in cancer research, these results confirm the potential of CWO as an agent of chemotherapeutic and cytostatic activity in human HepG2 cells. Besides, in our previous study, furanodiene, isolated from CWO, has been identified induce HepG2 cell apoptosis through alternating MAPK signaling. Furanodiene obviously elevated phosphorylated form of P38 and reduced phosphorylated form of ERK1/2 in a dose-dependent manner, but a slightly and statistically insignificant change in phosphorylated form of JNK. Therefore, furanodiene induced-apoptosis in HepG2, involve activation of P38 and inhibition of ERK MAPK signaling. Whether CWO acts on these signaling pathways should be an interesting area for further study.

In short, we conclude that CWO induces apoptosis in HepG2 cells through activation of mitochondrial and caspase-3 pathway.



## COMMENTS

### Background

*Ezhu* has a long history on treating liver diseases and protecting liver functions. Chinese Pharmacopoeia indicated that the rhizomes of three species including *Curcuma phaeocaulis*, *C. kwangsiensis* and *C. wenyujin* are used as *Ezhu*. The essential oil of *Ezhu* has been reported to possess various biological roles such as antimicrobial, anti-inflammatory, and anti-tumor activity. Some sesquiterpene compounds isolated from essential oil of *Curcuma wenyujin* (CWO) has been identified to have hepatoprotective effects. The total effects of the complex interactions of different compounds in extracts isolated from CWO are not well characterized.

### Research frontiers

Hepatocellular carcinoma (HCC) is the fifth most commonly diagnosed cancer with more than 1 million deaths reported annually worldwide. Many sesquiterpenes are identified to possess protective effects against carcinogenesis or tumor growth. For example, artemisinin, a sesquiterpene lactones, killed human oral cancer cells through apoptosis and may be useful as an alternative treatment for oral cancer. In order to develop effective means for prevention and treatment of HCC and related liver diseases, extensive research is being

carried out to isolate and identify chemical extracts and pure compounds from Chinese medicine with hepatoprotective, anti-hepatoma, anti-multidrug resistant hepatoma, and anti-viral effects.

### Innovations and breakthroughs

This is the first study to report the biological activity and mechanism of action of CWO on HCC cells.

### Applications

CWO induce apoptosis in HepG2 cells through activation of mitochondrial and caspase-3-pathway and the result suggests the potential value of development of *Ezhu* on treatment of liver diseases and further study on anti-apoptotic effect of CWO may lead to identification of new lead compounds and novel drug targets for treatment of liver cancer and diseases.

### Peer review

This is an interesting study showing that CWO inhibits growth and induces apoptosis in human HepG2 hepatoma cells. These findings are of interest.

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CLINICAL RESEARCH

# Prevalence of microscopic colitis in patients with diarrhea of unknown etiology in Turkey

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**CONCLUSION:** Biopsy of Turkish patients with the diagnosis of chronic non-bloody diarrhea of unexplained etiology and normal colonoscopic findings will reveal microscopic colitis in approximately 10% of the patients. Lymphocytic colitis is 4 times more frequent than collagenous colitis in these patients.

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**Key words:** Diarrhea of unknown etiology; Microscopic colitis; Lymphocytic colitis; Collagenous colitis

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## Abstract

**AIM:** To investigate the prevalence and demography of microscopic colitis in patients with diarrhea of unknown etiology and normal colonoscopy in Turkey.

**METHODS:** Between March, 1998 to July, 2005, 129 patients with chronic non-bloody diarrhea of unexplained etiology who had undergone full colonoscopy with no obvious abnormalities were included in the study. Two biopsies were obtained from all colonic segments and terminal ileum for diagnosis of microscopic colitis. On histopathologic examination, criteria for lymphocytic colitis (intraepithelial lymphocyte  $\geq 20$  per 100 intercryptal epithelial cells, change in surface epithelium, mononuclear infiltration of the lamina propria) and collagenous colitis (subepithelial collagen band thickness  $\geq 10 \mu\text{m}$ ) were explored.

**RESULTS:** Lymphocytic colitis was diagnosed in 12 (9%) patients (Female/Male: 7/5, mean age: 45 year, range: 27-63) and collagenous colitis was diagnosed in only 3 (2.5%) patients (all female, mean age: 60 years, range: 54-65).

## INTRODUCTION

Chronic diarrhea with no obvious reason is one of the challenges of gastroenterology. In 1980, Read *et al*<sup>[1]</sup> introduced microscopic colitis characterized by chronic diarrhea with normal endoscopic and radiologic findings, but with increased colonic mucosal inflammatory cells and epithelial lymphocytic infiltration on histologic examination. Later, Leivonen *et al*<sup>[2]</sup> emphasized that microscopic colitis covered all cases of colitis with normal colonoscopy, but abnormal histopathologic features and described lymphocytic colitis separately. Collagenous colitis, which is a closely related condition, was first described in 1976 as a separate subtype with additional histological finding of increased subepithelial collagen band thickness<sup>[3]</sup>. Thus, microscopic colitis is a condition with two subtypes having similar clinical, but different histological characteristics. In collagenous colitis subepithelial collagenous band thickness is important.

The prevalence of microscopic colitis has been difficult to estimate. The symptoms of microscopic

colitis have been frequently attributed to diarrhea-predominant irritable bowel syndrome, often for many years before diagnosis. Diagnostic awareness of these conditions by physicians in the geographic area of interest significantly effects the likelihood of diagnosis and, therefore, the prevalence.

Clinical and histological characteristics of microscopic colitis have been well established<sup>[4-8]</sup>. However, limited data is available regarding the prevalence, pathogenesis and progress of the disease and its treatment. The diagnosis is made only by histologic examination and most of these patients are treated and followed up erroneously as irritable bowel syndrome. Recently, several studies from Sweden and Iceland reported high prevalence of microscopic colitis<sup>[9-11]</sup>. In this prospective study we aimed to determine the prevalence of lymphocytic and collagenous colitis in Turkey in a subset of patients with chronic non-bloody diarrhea of unknown origin in which colonoscopy was not conclusive.

## MATERIALS AND METHODS

### **Patients**

Between March, 1998 and July, 2005, in three centers around Istanbul (two gastroenterology clinics and one private endoscopy laboratory), 129 consecutive patients with unexplained chronic (at least 3 mo duration), non-bloody diarrhea have undergone colonoscopy with visualization of terminal ileum and normal mucosal appearance noted. These patients were included in the study. Inclusion criteria are shown in Table 1. All patients underwent abdominal ultrasonography and/or computer tomography (CT). Patients who received radiotherapy, chemotherapy, or who had undergone operation related to bowel, stomach or gallbladder or patients with inflammatory bowel disease, chronic liver disease, renal disease or pancreatitis, and patients with the history of long term laxative and antibiotic use were excluded from the study. Stool consistency (liquid, semiliquid, soft), number of daily defecation, duration of diarrhea, and other gastrointestinal symptoms (abdominal pain, weight loss, etc) and previous medication were recorded.

### **Colonoscopy and histology**

Patients were prepared for colonoscopy with 90 mL oral monobasic sodium phosphate and dibasic sodium phosphate. During colonoscopy two biopsies were taken from terminal ileum and all segments of the colon. Specimens were stained with HE and Masson's Trichrome or Van Gieson dyes.

### **Diagnostic criteria**

Increased chronic inflammatory infiltration in the lamina propria, increased intraepithelial lymphocytes (IELs), degeneration of surface epithelium and increased mitosis in crypts were sought for the diagnosis of microscopic colitis. Over 20 IEL per 100 intercryptal epithelial cells (normal < 1-5/100) were deemed necessary for

**Table 1 Inclusion criteria for study**

#### **Inclusion criteria**

Diarrhea without blood (> 3 mo)
Normal stool microscopy
No growth in stool culture
Normal D-Xylose absorption test
Normal biochemical profile
Normal thyroid tests, normal serum gastrin
Negative antigliadin antibodies (IgA, IgG)
Negative <i>Clostridium difficile</i> toxins (A, B)
Negative HIV test
Normal urine 5-HIAA
Normal upper GI endoscopy
Normal abdominal US
Normal small bowel radiology
Normal duodenal biopsy
Normal colonoscopy including terminal ileum

**Table 2 Histopathologic criteria for diagnosis of lymphocytic colitis and collagenous colitis**

#### **Histopathologic criteria**

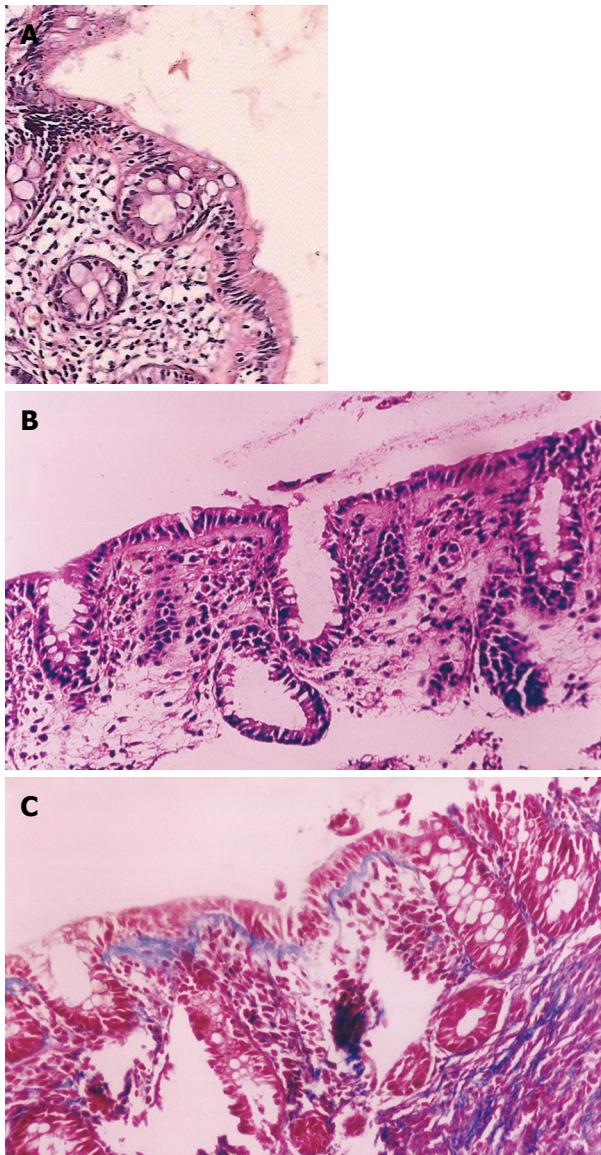
Lymphocytic colitis	Chronic inflammatory infiltration in lamina propria Increased IELs Superficial epithelial degeneration and increased mitosis in crypts IELs/100 intercryptal epithelial cell > 20/100
Collagenous colitis	A diffusely distributed and thickened subepithelial collagen band > 10 µm Chronic inflammatory infiltration in lamina propria

the diagnosis of lymphocytic colitis<sup>[7,8,12]</sup> (Table 2). For collagenous colitis subepithelial collagen band thickness was measured by ocular micrometer in Masson's Trichrome stained specimens. Thickness over 10 µm was required for the diagnosis<sup>[7,8,12]</sup> (Table 2).

## RESULTS

During the mentioned period, colonoscopy was performed in a total of 9862 patients due to various reasons. One hundred and twenty-nine of those patients had chronic non-bloody diarrhea with no apparent cause even after laboratory and radiologic examination and full colonoscopy with terminal ileal visualization. These patients were included in the study. After colonoscopic biopsy of colonic segments in 114 patients, histopathologic examinations of colonic biopsies were normal. In all patients, biopsies from terminal ileum revealed normal epithelial features.

Fifteen patients (11.5%) had microscopic colitis (12 lymphocytic colitis and 3 collagenous colitis; 9%, 2.5%, respectively) (Figure 1A and B). Seven of the lymphocytic colitis patients were female, mean age was  $45 \pm 11.6$  (27-63), mean duration of diarrhea was 22 mo (4-96) and mean number of daily defecation was 5 (3-9). Criteria of lymphocytic colitis were present in specimens obtained from all segments of the colon of these patients. Mean number of IEL per 100 intercryptal



**Figure 1** Pathologic view ( $\times 200$ ). **A:** Lymphocytic colitis. Note the increased number of chronic inflammatory cells in the lamina propria and within the surface epithelium (HE); **B:** Collagenous colitis. Note the subepithelial thick collagenous band (HE); **C:** Collagenous band thickness on Mason trichrome dye.

epithelial cells was  $28.2 \pm 6.8$  (range 20-60) (Figure 1A). All patients with collagenous colitis were female (ages 65, 54 and 61 years) and their subepithelial collagenous band thickness was 31, 21 and  $17.5 \mu\text{m}$  (Figure 1B and C). Mean durations of diarrhea were 34, 11 and 68 mo and mean daily stool frequencies were 5, 8 and 4 times, respectively.

## DISCUSSION

Microscopic colitis, which is characterized by chronic watery diarrhea with normal radiological and endoscopic appearances, is diagnosed only by histopathologic examination. This condition which consists of two main subtypes (lymphocytic and collagenous colitis) is a relatively common cause of chronic watery diarrhea, often accompanied by abdominal pain and weight loss.

Studies from different countries reported microscopic colitis rates between 4%-13% in the cohort of population with non-bloody diarrhea of unknown origin<sup>[10-13]</sup>. In the current study, we found this rate to be 11.5% in Turkey.

Diagnosis of this condition is possible only with the awareness of health workers and careful assessment of the criteria. Therefore, the reported prevalence seems to change within years. In Sweden, microscopic colitis was reported in 4% of patients with non-bloody chronic diarrhea in 1993, but this rate was reported as 10% in 1998<sup>[9,10,13]</sup>. The prevalence of collagenous colitis in Sweden between 1984-1988 was  $0.8/10^5$  inhabitants, but increased to  $6.1/10^5$  inhabitants between 1996-1998<sup>[9,10,13,15]</sup>. Recently, higher prevalence values have been reported from Iceland where the mean annual prevalence of collagenous colitis was  $5.2/10^5$  inhabitants and the mean annual incidence of lymphocytic colitis was  $4.0/10^5$  inhabitants in the period 1995-1999<sup>[11]</sup>. According to various studies prevalence of collagenous colitis and lymphocytic colitis is  $10-15.7/100000$  and  $14.4/100000$ , respectively<sup>[12-14,16]</sup>.

In a study performed in Spain, lymphocytic colitis was found in 9.5% of patients who had undergone colonoscopy because of chronic diarrhea during a period of 5 years<sup>[14]</sup>. In this study, the prevalence of lymphocytic colitis was three times that of the prevalence of collagenous colitis, female/male ratio in lymphocytic and collagenous colitis was found 2.7/1 and 4.7/1, respectively. Female/male ratio were reported as 5/1 from Iceland and 2.1 from Sweden<sup>[10-15]</sup>. In reported series this ratio for collagenous colitis was found as 4/1-20/1<sup>[13-17]</sup>. In our study, female/male ratio for lymphocytic colitis was 1.4/1 and lymphocytic colitis was 4 times more than that of collagenous colitis.

Marshall *et al*<sup>[18]</sup> encountered 13 lymphocytic colitis and 1 collagenous colitis in their 111 chronic-diarrhea patients with unexplained etiology. In another study of 132 consecutive patients who had undergone colonoscopy for chronic diarrhea and abdominal pain, lymphocytic and collagenous colitis found in 21 (16%) and 7 (5%) of patients, respectively<sup>[19]</sup>.

Mean ages of the patients with lymphocytic and collagenous colitis in other studies were between 51-59 years, and 64-68 years, respectively<sup>[13-17]</sup>. In our study, the mean age of the patients with lymphocytic colitis was 45 years (range 27-63). The mean age of our three collagenous colitis patients was 60 years.

In the studies of Lazenby *et al* and Baert *et al*, the mean IEL per 100 intercryptal epithelial cells was 34.7 and 29.4, respectively<sup>[5,8]</sup>. In the current study, the mean IEL per 100 intercryptal epithelial cells was 28.2. Normal subjects may have up to 1 to 5 IEL per 100 intercryptal epithelial cells. Some studies have reported that biopsy specimens from all segments of the colon revealed similar number of IEL and, therefore, biopsy obtained only from sigmoid colon would be enough for diagnosis<sup>[7,12]</sup>. In our study, the number of IEL was increased in all bowel segments.

Since subepithelial band thickness was less than  $8 \mu\text{m}$

in all our cases of lymphocytic colitis, the diagnosis of collagenous colitis and overlapping form was excluded. In normal subjects subepithelial collagen band thickness of 5-7  $\mu\text{m}$  is considered as normal and band thickness of 7-80  $\mu\text{m}$  is found in collagenous colitis<sup>[7,8,12]</sup>. In our 3 collagenous colitis patients the mean band thickness was 23  $\mu\text{m}$ .

As yet, the etiology of lymphocytic colitis has not been well understood. Gastrointestinal infections, autoimmune diseases and various drugs (non-steroidal anti-inflammatory drugs, ranitidine, carbamazepine, simvastatin, ticlopidine, flutamide etc) were reported to be causative factors<sup>[12,16,17,20]</sup>. Some gastrointestinal rheumatologic disorders (celiac sprue, rheumatoid arthritis, uveitis, idiopathic pulmonary fibrosis, diabetes mellitus, pernicious anemia, autoimmune thyroiditis etc) and positivity of some autoantibodies, particularly antinuclear antibody (ANA) may be associated with both lymphocytic and collagenous colitis<sup>[14,21-24]</sup>. Giardiello *et al* found 4 ANA positive patients in their 12 lymphocytic colitis patients<sup>[24]</sup>. We found only one case of ANA positivity in our patients, but none of them were associated with any of the disorders or conditions mentioned above.

Patients with lymphocytic colitis were reported to be effectively treated with medications used in inflammatory bowel disease such as sulfasalazine and 5-ASA. If this regimen fails, bismuth subsalicylate, corticosteroids, azathioprine and cyclosporine may be given<sup>[12,15-17,25,26]</sup>. In the present study sulfasalazine or 5-ASA was used as first line treatment agents. Preliminary results show positive response in terms of symptom relief. Evaluation of long term outcome should wait completion of the study.

In conclusion, considering 11.5% of the patients with chronic diarrhea of unknown etiology and normal colonoscopy would have microscopic colitis, biopsy should be taken during colonoscopy in this subset of patients. Although the number of our cases was not enough to answer the question of how many biopsies should be taken and from which part of the colon, the fact that histopathological criteria were determined on all colonic regions in patients with lymphocytic colitis on whom biopsy was performed is promising in terms of diagnostic convenience. Lymphocytic colitis in Turkish patients was found to be 4 times more frequent than collagenous colitis.

## COMMENTS

### Background

Microscopic colitis is a chronic diarrheal disease with normal colonoscopic, but with abnormal histopathologic features. It is a disease with two subtypes of similar clinical but different histological features: lymphocytic colitis, which is characterized by pronounced colonic mucosal lymphocyte infiltration and collagenous colitis, which is characterized by increased subepithelial collagenous band thickness. In limited number of studies from various countries the rates of microscopic colitis in patients with chronic diarrhea have been reported between 4%-13%.

### Research frontiers

Although the number of the cases was not enough to answer the question of how many biopsies should be taken and from which part of the colon, the fact that histopathological criteria were determined on all colonic regions in patients

with lymphocytic colitis on whom biopsy was performed is promising in terms of diagnostic convenience.

### Applications

Considering 11.5% of the patients with chronic diarrhea of unknown etiology and normal colonoscopy would have microscopic colitis, biopsy should be taken during colonoscopy in this subset of patients.

### Terminology

Microscopic colitis is characterized by chronic watery diarrhea with normal radiological and endoscopic appearances. Lymphocytic colitis has similar characteristics with over 20 intraepithelial lymphocytes (IELs) per 100 intercryptal epithelial cells. Collagenous colitis has same characteristics with additional histological finding of increased subepithelial collagen band thickness.

### Peer review

This is an epidemiologic study confirming findings reported from other countries about the frequency of lymphocytic and collagenous colitis and the importance of biopsies for the diagnosis. It's a nice paper, well written and well designed.

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BASIC RESEARCH

# Effects of ethanol on insulin-like growth factor- I system in primary cultured rat hepatocytes: Implications of JNK1/2 and alcoholdehydrogenase

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viability and p-JNK1/2 activity (at 180 min).

**CONCLUSION:** This study suggests that ethanol-induced p-JNK1/2 activation is associated with the IGF- I system and cell viability in hepatocytes. Furthermore, alcohol dehydrogenase is involved in the relationship between ethanol-induced inactivation of p-JNK1/2 and the changes of the IGF- I system and cell viability.

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**Key words:** Insulin-like growth factor- I ; Insulin-like growth factor- I receptor; C-Jun N-terminal kinase; Hepatocyte; Ethanol

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Oh YI, Kim JH, Kang CW. Effects of ethanol on insulin-like growth factor- I system in primary cultured rat hepatocytes: Implications of JNK1/2 and alcoholdehydrogenase. *World J Gastroenterol* 2008; 14(27): 4324-4331 Available from: URL: <http://www.wjgnet.com/1007-9327/14/4324.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4324>

## Abstract

**AIM:** To evaluate the effects of ethanol on the insulin-like growth factor- I (IGF- I ) system involved in c-Jun N-terminal kinase (JNK1/2) and alcoholdehydrogenase (ADH) activity in primary cultured rat hepatocytes.

**METHODS:** Hepatocytes isolated from male Sprague-Dawley rats were incubated with various concentrations of ethanol for different durations of time. The cells were pretreated with SP600125 (10 μmol/L) and 4-MP (200 μmol/L), and then treated with ethanol (200 mmol/L). We then measured IGF- I secretion, IGF- I mRNA expression, cell viability and JNK1/2 activity by radioimmunoassay, RT-PCR, MTT assay and Western blot, respectively ( $n = 6$ ).

**RESULTS:** Ethanol induced the activity of phospho (p)-JNK1/2, reaching a maximum at 60 min and then decreasing at 180 min. The effects of ethanol on the IGF- I system were increased at 60 min (secretion:  $7.11 \pm 0.59$  ng/mg protein vs  $4.91 \pm 0.51$  ng/mg, mRNA expression:  $150.2\% \pm 10.2\%$  vs  $101.5\% \pm 11.3\%$ ,  $P = 0.045$ ) and then decreased at 180 min (secretion:  $3.89 \pm 0.25$  ng/mg vs  $5.4 \pm 0.54$  ng/mg protein; mRNA expression:  $41.5\% \pm 10.4\%$  vs  $84.7\% \pm 12.1\%$ ,  $P = 0.04$ ), however cell viability was decreased in a dose- and time-dependent manner. SP600125 blocked the ethanol-induced changes (at 60 min). Additionally, 4-methylpyrazole prevented the ethanol-induced decreases in the IGF- I system, cell

## INTRODUCTION

Ethanol abuse exerts deleterious effects on the internal organs of the body, particularly the liver and brain, and alcohol-induced liver damage is one of the major causes of morbidity and mortality in alcoholics<sup>[1]</sup>. Ethanol alters hepatic carbohydrate and lipid metabolism as well as the synthesis of protein and DNA, which leads to hepatic dysfunction and cirrhosis<sup>[2]</sup>. Although the spectrum of ethanol toxicity is well known, the underlying pathophysiology of the signal transduction pathways has not been elucidated.

Ethanol alters cell functions via multiple signaling pathways, particularly those involving mitogen-activated protein kinases (MAPKs), which are involved in a variety of cellular responses including proliferation, differentiation, and apoptosis<sup>[3]</sup>. Several MAPK cascades have been identified, including those involving p42/44

and p38 MAPKs, and c-Jun N-terminal kinase (JNK1/2, also known as stress-activated protein kinase)<sup>[4]</sup>. JNK1/2 activity has been linked to the proliferation and apoptosis of hepatocytes<sup>[5]</sup>.

Ethanol also induces prolonged activation of tumor necrosis factor (TNF)-stimulated JNK1/2 after hepatocytes are stimulated with various agonists, and prolonged activation of JNK1/2 and activator protein 1 (AP-1) is associated with the apoptosis and necrosis of hepatocytes that occurs in response to oxidative stress<sup>[4]</sup> and ischemia/reperfusion injury<sup>[6]</sup>.

Insulin-like growth factor (IGF)- I is a peptide that plays an important role in regulating cell metabolism, growth, and differentiation<sup>[7]</sup>. The dose-dependent effects of ethanol on the IGFs system have been previously described in male rats<sup>[8]</sup>. The cellular action of IGF- I is mediated *via* the insulin-like growth factor- I receptor (IGF-IR), which exhibits tyrosine kinase activity<sup>[7]</sup>. IGF-IR is a key regulator of normal cellular processes, and plays a critical role in the development and progression of many types of cancer<sup>[9]</sup>. It has been reported that the renin-angiotensin system regulates the IGF- I system in hepatocytes<sup>[10]</sup>, and it is known that retinoic acid inhibits growth-hormone-stimulated IGF- I production *via* protein kinase C (PKC)- $\delta$  in breast cancer cells. We recently found that the inhibitory effects of the ethanol-induced IGF- I system are related to p42/44 activity<sup>[11]</sup>. Although the relationships between ethanol-induced cellular action and apoptosis *via* MAPK including JNK1/2 activity have been reported previously, the secretion control mechanisms of the IGF- I system (IGF- I secretion, IGF- I mRNA expression, and IGF-IR activity) remain to be elucidated in primary cultured hepatocytes.

In the present study, we investigated the effects of ethanol on the IGF- I system, with particular attention to the JNK1/2 activity and alcoholdehydrogenase (ADH) in primary cultured rat hepatocytes.

## MATERIALS AND METHODS

### Materials

IGF- I antigen and IGF- I antibodies were purchased from GroPep (Adelaide, Australia), and the JNK1/2 inhibitor SP600125 was purchased from New England Biolabs (Beverly, MA, USA). An enhanced chemiluminescence (ECL) kit was purchased from Cell Signaling (Beverly, MA, USA). All routine culture media were obtained from Gibco-BRL (Grand Island, NY, USA). Aquasol, reflection X-ray film, and  $^{125}\text{I}$  isotope were purchased from Dupont-NEN (Boston, MA, USA). Polyvinylidene difluoride (PVDF) membranes were purchased from BioRad (Hercules, CA, USA). BSA (fraction V), glycine, SDS, acrylamide, glycerol, and Tween-20 were obtained from Sigma (St. Louis, MO, USA).

### IGF- I radioimmunoassay

Recombinant human IGF- I was iodinated to a specific radioactivity of 150-300 Ci/g using the  $^{125}\text{I}$  isotope

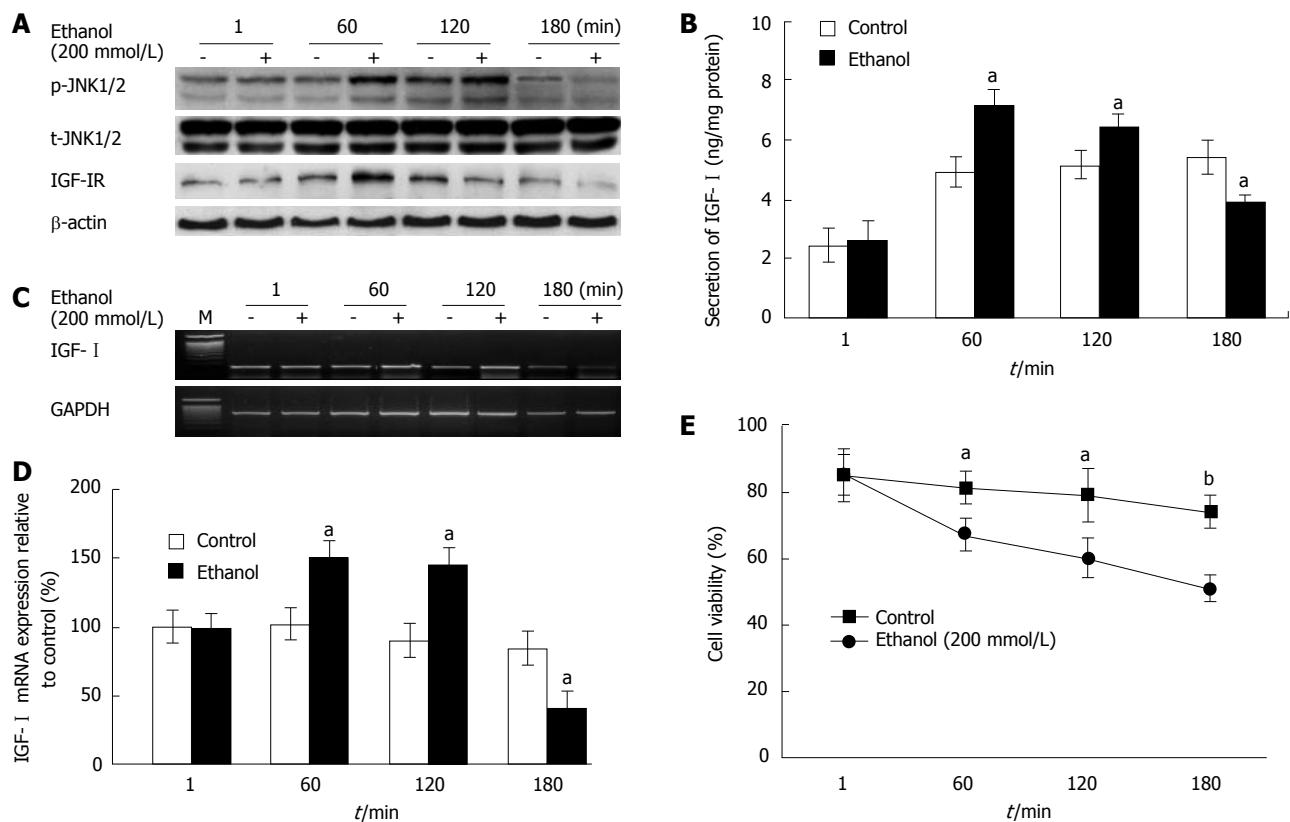
following a modified version of the chloramine-T (Kodak, Grand Island, NY, USA) method. The specific activity of the iodinated IGF- I was typically 60-110 Ci/g protein. The iodination mixture was purified on a Sephadex G-50 column (150 cm) and pre-equilibrated with phosphate-buffered saline (0.1 mol/L, pH 7.4). The samples were then separated, after which the immunoreactive IGF- I was determined as previously described<sup>[11]</sup> with some modifications. All IGF- I data were expressed as nanograms of pure human IGF- I per milliliter, while assuming that equal cross-reactivity occurred between the rat and human IGF- I in the radioimmunoassay. Fifty microliters of rat polyclonal IGF- I antibody diluted to 1:1500 was added to 100  $\mu\text{L}$  of each sample/standard and then incubated for 1 h at room temperature. Next,  $[^{125}\text{I}]$ -IGF- I was added at 20000 cpm, and the samples and standards were then incubated for an additional 18 h at 4°C. Fifty microliters of horse serum (Sigma, St. Louis, MO, USA) was then added to the sample, which was then centrifuged at 3000  $\times g$  for 30 min. After discarding this supernatants, the radioactivities of the precipitates containing the bound  $[^{125}\text{I}]$ -IGF- I were counted with a gamma scintillation counter (Wallac, Finland). The intra- and interassay coefficients of variation for IGFs were 8% and 10%, respectively.

### Isolation and culture of rat hepatocytes

Hepatocytes were isolated from male Sprague-Dawley rats weighing 200-300 g by a two-step perfusion procedure using 0.05% collagenase as described previously<sup>[12,13]</sup>. Cell viability, which was assessed by the exclusion of trypan blue, was 90%  $\pm$  5% (mean  $\pm$  SD). Isolated hepatocytes were then plated onto collagen-coated plastic culture dishes (60 mm in diameter) at a density of  $5 \times 10^4$  cells/cm $^2$  in Williams' medium E containing 10% FBS. The plates were then placed in a 5% CO $_2$  incubator for 3 h at 37°C, after which the medium was changed to FBS-free Williams' medium E. After an additional 30 min, ethanol, SP600125 and 4-methylpyrazole (4-MP) were added at various concentrations to the dishes, which were then immediately sealed with Parafilm. The cells were then incubated for 0-180 min at 37°C.

### Cell lysis and quantification

After incubation, cells were rinsed twice with ice-cold phosphate-buffered saline, followed by the addition of lysis buffer comprising 20 mmol/L HEPES (pH 8.8), 136 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 10 mmol/L KCl, 2 mmol/L MgCl $_2$ , 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 1 mmol/L dithiothreitol, 1 mmol/L benzamidine, 10 mmol/L  $\beta$ -glycerophosphate, 10  $\mu\text{g}/\text{mL}$  aprotinin, 10  $\mu\text{g}/\text{mL}$  leupeptin, and 1  $\mu\text{g}/\text{mL}$  pepstatin A. The cell lysates were then sonicated for 5 min using a Vibra Cell ultrasonic processor (Sonics and Materials, Danbury, USA). After centrifugation of the sonicated samples at 12000  $\times g$  for 10 min at 4°C, the supernatants were



**Figure 1** Time course of effects on the IGF-I system, JNK1/2 activity, and cell viability induced by ethanol in primary cultured rat hepatocytes (mean  $\pm$  SD). The cells were exposed to 200 mmol/L ethanol for 0, 60, 120 and 180 min. **A:** P-JNK1/2, t-JNK1/2, and IGF-IR activities; **B:** IGF-I concentration; **C** and **D:** IGF-I mRNA expression; **E:** Cell viability.  $\beta$ -actin (**A**) and GAPDH (**C**) were used as loading controls. The mRNA expression (as indicated by a band at 180 bp, **C**) was determined by densitometric analysis (**D**) of the amplification products. Data represent percentages relative to control. The cell viability (**D**) was determined by the MTT assay.  $^aP < 0.05$ ,  $^bP < 0.01$  vs control ( $n = 6$ ).

collected, and the protein concentrations were estimated using a bicinchoninic acid (BCA) protein assay kit (Pierce, Bonn, Germany).

#### Western blot

Cell lysates containing equal amounts of protein (20–30  $\mu$ g) were fractionated by 10% SDS-polyacrylamide gel electrophoresis, after which the proteins were transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA) and then washed with 25 mmol/L Tris (pH 7.4) containing 137 mmol/L NaCl and 0.1% Tween-20. The membrane was then blocked with 25 mmol/L Tris (pH 7.4) containing 137 mmol/L NaCl and 0.1% Tween-20 containing 5% nonfat dry milk for 2 h at room temperature. The blots were then incubated with antibodies against p54/46 JNK1/2 and IGF-IR overnight at 4°C, after which they were incubated with antirabbit and antimouse horseradish peroxidase. After being washed, the blots were developed using an ECL kit and exposed to X-ray film to allow detection of the protein bands.

#### Cell viability

Standard MTT assay as described in literature was used with slight modification (2). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] (Sigma Co., MO, USA) was dissolved in isotonic

phosphate buffer (IPB, pH 7.4) solution at 5 mg/mL and filtered to sterilize and remove insoluble residues. Hepatocytes were cultured in containing Williams' medium E containing 10% FBS and incubated for 4 h at 37°C in serum free Williams' medium E. Cell survival was assayed by measuring the conversion of yellow, water-soluble tetrazolium MTT to blue, water-insoluble formazan. The absorbance was measured at 570 nm.

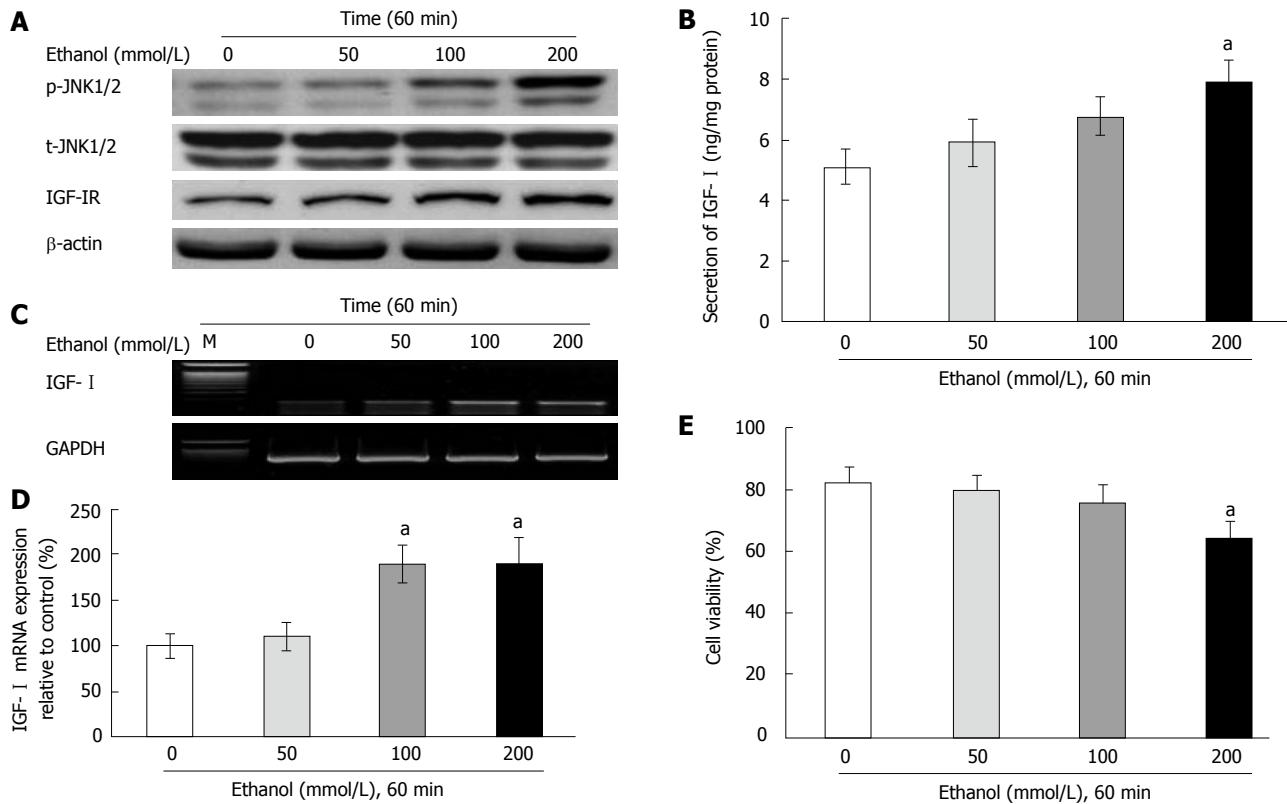
#### Statistical analysis

The statistical significance of differences between groups was determined using a Student's *t* test, with a probability value of  $P < 0.05$  being considered to be indicative of statistical significance. All experiments were performed at least six times.

## RESULTS

#### Time course of the effects of ethanol on the IGF-I system, cell viability, and JNK1/2 activity

To evaluate the time course of the effects of exposure to ethanol on the IGF-I system, cell viability, and JNK1/2 activity, primary cultured rat hepatocytes were exposed to 200 mmol/L ethanol for different times (1, 60, 120 and 180 min). The activity of p-JNK1/2 was observed at 60 and 120 min, and was decreased relative to control at 180 min by ethanol exposure (Figure 1A). However, the total (t)-JNK1/2 activity



**Figure 2** Effects of ethanol on the IGF-I system, JNK1/2 activity, and cell viability at different concentrations (0, 50, 100 and 200 mmol/L) in primary cultured hepatocytes (mean  $\pm$  SD). The cells were exposed to ethanol at different concentrations for 60 min. **A:** P-JNK1/2, t-JNK1/2, and IGF-IR activities; **B:** IGF-I concentration; **C** and **D:** IGF-I mRNA expression; **E:** Cell viability.  $\beta$ -actin (**A**) and GAPDH (**C**) were used as loading controls. The mRNA expression (as indicated by a band at 180 bp, **C**) was determined by densitometric analysis (**D**) of the amplification products. Data represent percentages relative to control. The cell viability (**D**) was determined by the MTT assay.  $^aP < 0.05$ , vs control ( $n = 6$ ).

was not affected. In addition, IGF-IR activity was also observed at 60 min, and it was decreased at 180 min (Figure 1A). The effects of ethanol on the secretion and mRNA expression of IGF-I were similar to changes in p-JNK1/2 activity, which increased at 60 (IGF-I secretion:  $7.11 \pm 0.59$  ng/mg vs  $4.91 \pm 0.51$  ng/mg protein; mRNA expression:  $150.2\% \pm 10.2\%$  vs  $101.5\% \pm 11.3\%$ ,  $P = 0.045$ ) and 120 min and then decreased at 180 min (IGF-I secretion:  $3.89 \pm 0.25$  ng/mg vs  $5.4 \pm 0.54$  ng/mg protein; mRNA expression:  $41.5\% \pm 10.4\%$  vs  $84.7\% \pm 12.1\%$ ,  $P = 0.04$ ; Figure 1B-D). The effects of ethanol on cell viability significantly decreased over time from 60 min onwards (at 60 min:  $66.7\% \pm 5.12\%$  vs  $80.45\% \pm 5.21\%$ ,  $P = 0.035$ ; Figure 1E).

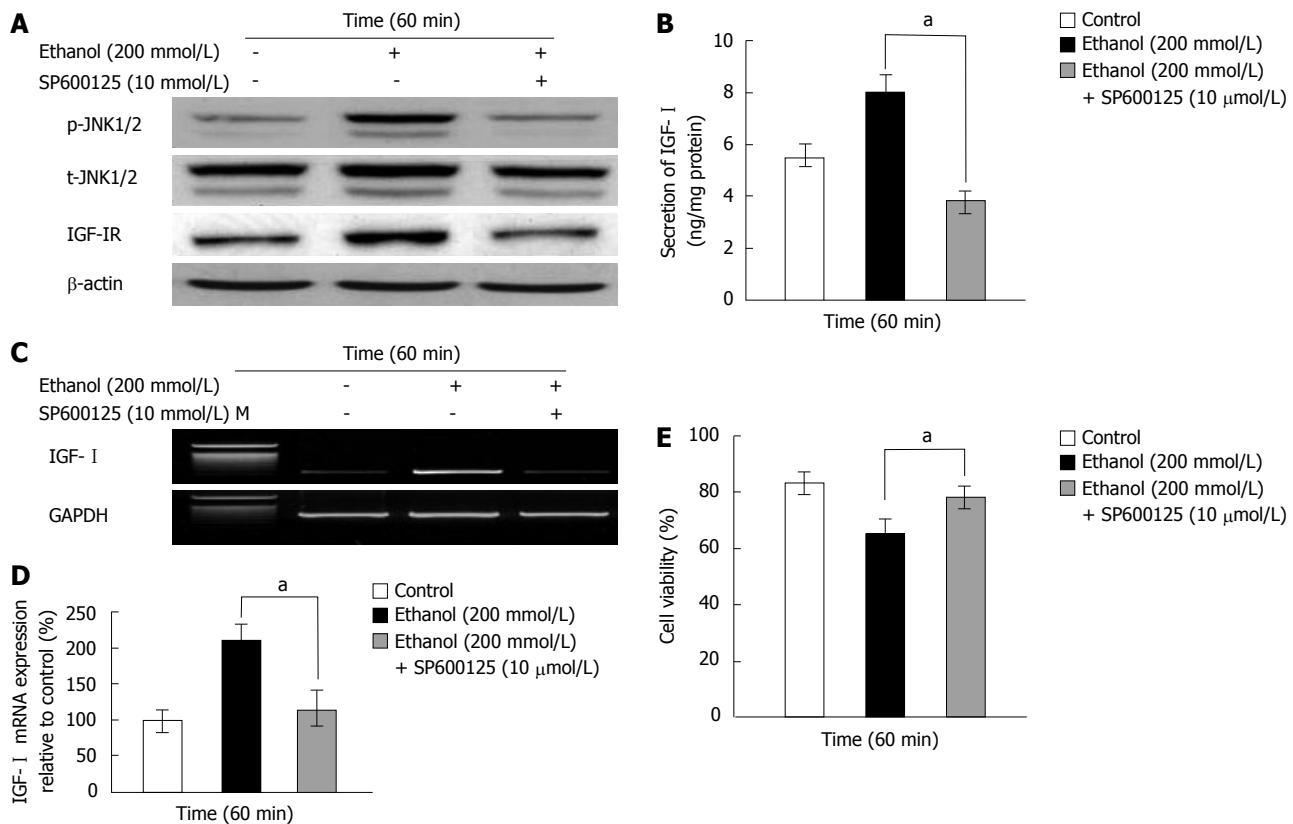
#### Dose-dependent effects of ethanol on the IGF-I system, cell viability, and JNK1/2 activity

To investigate the dose-response effects of ethanol on the IGF-I system and JNK1/2 activity, the cells were exposed to ethanol at different concentrations (0, 50, 100 and 200 mmol/L) for 60 min. The effects of ethanol on the p-JNK1/2 activity increased in a dose-dependent manner (Figure 2A). In addition, the activity of p-JNK1/2 relative to control was maximal in response to exposure to 200 mmol/L ethanol, whereas the t-JNK1/2 activity was not affected (Figure 2A). Furthermore, the changes in IGF-I secretion, mRNA expression, and IGF-IR activity were increased by ethanol in a dose-

dependent manner (Figure 2A-C). The treatment with 200 mmol/L ethanol showed significantly decrease of IGF-I secretion, mRNA expression and IGF-IR activity when compared with the control (IGF-I secretion:  $7.89 \pm 0.71$  ng/mg vs  $5.09 \pm 0.56$  ng/mg protein, mRNA expression:  $191.5\% \pm 27.4\%$  vs  $100\% \pm 13.1\%$ ,  $P = 0.032$ ; Figure 2A-D). These results were similar to those of the ethanol-induced p-JNK1/2 activity. However, cell viability was significantly decreased by exposure to 200 mmol/L ethanol ( $65.2\% \pm 4.9\%$  vs  $83.2\% \pm 4.2\%$ ,  $P = 0.024$ ; Figure 2E).

#### Relationships between ethanol-induced activation of JNK1/2, the IGF-I system, and cell viability

The JNK1/2 inhibitor SP600125 was used to determine if ethanol-induced activation of p-JNK1/2 (at 60 min) was related to the IGF-I system and cell viability. The ethanol-induced activations of p-JNK1/2 and IGF-IR were blocked by treatment with  $10^{-5}$  mol/L SP600125 (Figure 3A). In addition, the temporary increases in secretion ( $8.02 \pm 0.67$  ng/mg protein) and mRNA expression ( $208.8\% \pm 23.4\%$ ) of IGF-I induced by ethanol were also blocked by SP600125 (IGF-I secretion:  $3.78 \pm 0.42$  ng/mg protein, mRNA expression:  $113.87\% \pm 27.5\%$ ,  $P = 0.024$ ; Figure 3B-D), whereas t-JNK1/2 activity was not affected (Figure 3A). The ethanol-induced decrease in cell viability ( $64.2\% \pm 5.5\%$ ) was recovered by SP600125 ( $77.6\% \pm 4.1\%$ ,  $P =$



**Figure 3** Effects of JNK1/2 inhibitor on the IGF-I system, cell viability, and activity of JNK1/2 induced by ethanol at 60 min in primary cultured rat hepatocytes (mean  $\pm$  SD). The cells were pretreated with 10  $\mu$ mol/L SP600125 30 min before being exposed to 200 mmol/L ethanol for 60 min. **A:** p-JNK1/2, t-JNK1/2, and IGF-IR activities; **B:** IGF-I concentration; **C** and **D:** IGF-I mRNA expression; **E:** Cell viability.  $\beta$ -actin (**A**) and GAPDH (**C**) were used as loading controls. The mRNA expression (as indicated by a band at 180 bp, **C**) was determined by densitometric analysis (**D**) of the amplification products. Data represent percentages relative to control. The cell viability (**E**) was determined by the MTT assay.  $^aP < 0.05$  vs control ( $n = 6$ ).

0.045; Figure 3E). These results together demonstrate that the transient changes in the ethanol-induced IGF-I system and the decreased cell viability were related to p-JNK1/2 activity.

#### Relationships between ADH and decreased changes in the IGF-I system by ethanol-induced JNK1/2 activity

To determine the effects of ADH (alcohol dehydrogenase) on the ethanol-induced inactivation of p-JNK1/2 in the IGF-I system and decreased cell viability at 180 min, cells were exposed to 200 mmol/L ethanol after being pretreated with the ADH inhibitor 4-MP (200  $\mu$ mol/L). The ethanol-induced inactivation of p-JNK1/2 and IGF-IR was recovered by 10<sup>-5</sup> mol/L 4-MP, whereas the t-JNK1/2 activity was not affected (Figure 4A). The ethanol-induced decreases in the secretion (4.54  $\pm$  0.52 ng/mg protein) and mRNA expression (24.5%  $\pm$  17.1%) of IGF-I and cell viability (50.3%  $\pm$  5.5%) were also recovered by pretreatment with 4-MP (IGF-I secretion: 10.3  $\pm$  0.79, mRNA expression: 109.4%  $\pm$  21.8%, cell viability: 77.2  $\pm$  7.2,  $P = 0.035$ ; Figure 4B-E). These results together indicate that the decreases in ethanol-induced p-JNK1/2 activity, IGF-I system and cell viability were related to the ADH.

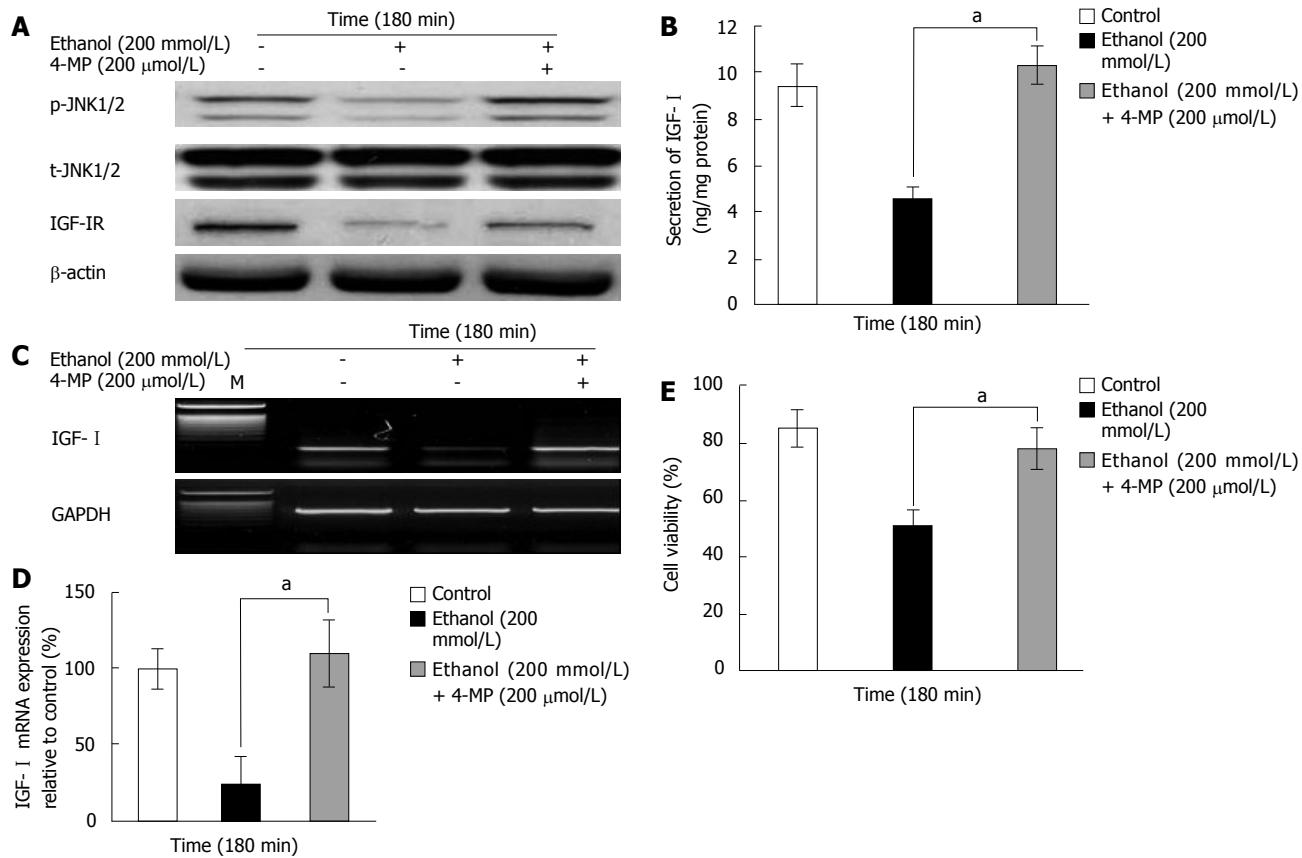
## DISCUSSION

Ethanol exerts toxic effects on almost all organs,

particularly liver and brain. The liver is a major metabolic organ in which most IGF-I is produced and secreted, and its mediators are responsible for alcohol-induced liver injury<sup>[14]</sup>.

In the present study, we showed that ethanol transiently increased p-JNK1/2 activity at 60 min and then decreased it at 180 min. It has been reported that ethanol activates MAPKs<sup>[1,2]</sup>, and acute exposure of primary cultured rat hepatocytes to ethanol for 60 min increases the activities of p42/44 MAPK and p-JNK1/2, with both activities gradually decreasing thereafter<sup>[15]</sup>. Exposure to ethanol has been shown to cause prolonged activation of p-JNK1/2; however, this response was attenuated in hepatocytes obtained from rats chronically exposed to ethanol for 6 wk<sup>[16]</sup>. Our result is similar with those of previous studies mentioned above, in which exposure time to ethanol causes activation and inactivation of p-JNK1/2 activation in rat hepatocytes.

The activation of p-JNK1/2 normally occurs in response to growth stimuli and is involved in cell proliferation<sup>[17]</sup>. This is also related to apoptosis and antiapoptosis, and is activated by cytokines or stress stimuli such as osmotic shock, UV light, and heat<sup>[9,18]</sup>. IGF-I system has been reported to protect against a variety of chemical cellular injuries that induce apoptosis<sup>[19]</sup>. We previously showed that ethanol decreased the synthesis and secretion of IGF-I and the activity of IGF-IR, an effect that is related to cell



**Figure 4** Effects of ADH inhibitor on the IGF-I system, cell viability, and JNK1/2 activity induced by ethanol at 180 min in primary cultured rat hepatocytes (mean  $\pm$  SD). The cells were pretreated with 200  $\mu$ mol/L 4-MP 30 min before being exposed to 200 mmol/L ethanol for 180 min. **A:** p-JNK1/2, t-JNK1/2, and IGF-IR activities; **B:** IGF-I concentration; **C** and **D:** IGF-I mRNA expression; **E:** Cell viability. β-actin (**A**) and GAPDH (**C**) were used as loading controls. The mRNA expression (as indicated by a band at 180 bp, **C**) was determined by densitometric analysis (**D**) of the amplification products. Data represent percentages relative to control. The cell viability (**D**) was determined by the MTT assay. <sup>a</sup> $P < 0.05$  vs control ( $n = 6$ ).

proliferation and differentiation<sup>[11]</sup>. In the present study, ethanol-induced transient activation of p-JNK1/2 increased in the IGF-I system, but this decreased when p-JNK1/2 was inactivated. Furthermore, IGF-IR activity also regulates ethanol-induced secretion and synthesis of IGF-I. These results are consistent with our previous study that ethanol-induced p42/44 activity was related to the secretion and synthesis of IGF-I in primary cultured rat hepatocytes<sup>[11]</sup>. In our previous study, although there were different doses (5%-20%), chronic ethanol treatment caused dose-dependent decreases in the secretion and synthesis of IGF-I in liver and blood *in vivo*<sup>[8]</sup>.

We also used a JNK1/2 inhibitor to verify whether the ethanol-induced transient activation of p-JNK1/2 may alter the IGF-I system. The results reported here confirmed that the effects of ethanol on p-JNK1/2 activation, the IGF-I system, and cell viability were recovered by an inhibitor of the JNK1/2 activity. We suggest that acute exposure to ethanol affects not only p42/44 MAPK but also p-JNK1/2 activities, which in turn alter the IGF-I system. It has been reported that ethanol-induced transient activation of p-JNK1/2 indicates pro-apoptosis, while a prolonged activation induces anti-apoptosis in hepatocytes<sup>[20]</sup>. Hepatocytes express two JNK genes (JNK1 and JNK2) and bile acids

cause activation of both JNK1 and JNK2, but JNK1 activation causes apoptosis whereas JNK2 activation protects against apoptosis<sup>[21]</sup>. It has been reported that ethanol causes more pronounced activation of JNK 1 compared to JNK 2, suggesting a role for this preferential activation of JNK 1 in ethanol-induced apoptosis of hepatocytes<sup>[15]</sup>.

Interestingly, we found that cell viability is always decreased by ethanol. However, there was a transient activation of p-JNK1/2 and the subsequent inactivation of p-JNK1/2 in parallel with changes of the IGF-I system. These results suggest that transient activation of p-JNK1/2 with increment of the IGF-I system lead to pro-apoptotic events and transient resistance of hepatocytes. Also, the ethanol-induced inactivation of p-JNK upon decrease of the IGF-I system indicate that the cells have already passed the threshold for proliferation or survival against the ethanol-induced toxicity.

The ADH is an enzyme involved in ethanol metabolism that appears to provide the link between the effects of ethanol-induced p-JNK1/2 activity on the IGF-I system and cell viability at 180 min but not at 60 min (data not shown). It has been reported that the level of 4-MP decreased by approximately 90% in rat hepatocytes following exposure to ADH and ethanol<sup>[22]</sup>.

Ethanol rapidly activates p-JNK1/2, which is associated with the response of the endoplasmic reticulum to stress, which in turn causes inhibition of ADH<sup>[23]</sup>. Acute exposure to 200 mmol/L ethanol may also activate p-JNK1/2 via acetaldehyde-dependent<sup>[15]</sup> and acetaldehyde-independent<sup>[24]</sup> pathways in rat hepatocytes. It was reported that acetaldehyde produced by ethanol oxidation activates p42/44 MAPK and p-JNK1/2 in rat hepatocytes<sup>[1,2,15]</sup>. We previously reported that ethanol-induced changes of the IGF-I system are related to ADH activity<sup>[11]</sup>. These results suggest that the decrease in p-JNK1/2 activity induced by exposure to ethanol for 180 min regulates the decrease of IGF-I system and cell viability, with these effects being related to ADH. However, the effects of the ethanol-induced transient activation of p-JNK1/2 on the increment of IGF-I system were not due to ADH.

In conclusion, this study suggest that ethanol-induced p-JNK1/2 activation is related to changes in the IGF-I system and cell viability in hepatocytes. Furthermore, ethanol-induced inactivation of p-JNK1/2 is involved in the IGF-I system and cell viability via ADH. These findings might be helpful to understand the pathogenesis of liver damage induced by ethanol, and may lead to a rational therapeutic intervention against ethanol toxicity.

## COMMENTS

### Background

Ethanol-induced liver damage is unavoidable upon exposure to alcohol. Moreover, enhanced c-Jun N-terminal kinase (JNK1/2) and alcohol dehydrogenase (ADH) activity have been linked to the ethanol induced hepatotoxicity. Insulin-like growth factor-I (IGF-I) system has been reported to protect against a variety of chemical cellular injuries that induce apoptosis; however it has not been well defined whether the IGF-I system is associated with the ethanol-induced JNK and ADH activity.

### Research frontiers

It was reported that acetaldehyde produced by ethanol oxidation activates p42/44 MAPK and p-JNK1/2 in rat hepatocytes. We previously reported that ethanol-induced changes of the IGF-I system are related to p42/44 activity. To investigate the importance of IGF-I system via JNK and ADH, we performed this study using specific inhibitors.

### Innovations and breakthroughs

We found that there was increase and then decrease in the IGF-I secretion and mRNA expression during ethanol treatment. The activity of JNK was also temporary increased and then decreased by ethanol. However, cell viability was monotonically decreased. Both JNK and ADH inhibitors blocked ethanol-induced changes of IGF-I system and cell viability.

### Applications

The present study evaluated the changes of the IGF-I system indicating that the potential value of IGF-I system for patients with ethanol-induced liver damage. Moreover, this study demonstrates that ethanol-induced IGF-I system is involved in the activities of JNK1/2 and ADH.

### Terminology

IGF-I system has been reported to protect against a variety of chemical cellular injuries and promote proliferation of hepatocytes. The liver is a major metabolic organ in which most IGF-I is produced and secreted, and its mediators are responsible for alcohol-induced liver injury.

### Peer review

This manuscript describes the activation of JNK1/2 activity by ethanol treatment of rat hepatocytes and subsequent changes in IGF expression and secretion as well as changes in proliferation. These effects could be inhibited by the specific inhibitors of JNK1/2 as well as an inhibitor of ADH. This manuscript is well-written, clear and concise with a thorough results section.

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BASIC RESEARCH

## Protective effect of prednisolone on ischemia-induced liver injury in rats

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Wang M, Shen F, Shi LH, Xi T, Li XF, Chen X, Wu MC. Protective effect of prednisolone on ischemia-induced liver injury in rats. *World J Gastroenterol* 2008; 14(27): 4332-4337 Available from: URL: <http://www.wjgnet.com/1007-9327/14/4332.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4332>

### Abstract

**AIM:** To investigate the effects of prednisolone on cell membrane bleb formation, calpain  $\mu$  activation and talin degradation during hepatic ischemia-reperfusion injury in rats.

**METHODS:** The hilar area of the left lateral and median lobes of rat liver (68%) was clamped for 60 min and followed by 120 min reperfusion. Prednisolone was administered at 1.0, 3.0, or 10 mg/kg at 30 min before ischemia. In addition to biochemical and microscopic analyses, activation of calpain  $\mu$  was determined using specific antibodies against the intermediate (activated) form of calpain  $\mu$ . Degradation of talin was also studied by Western blotting.

**RESULTS:** In the control and prednisolone (1.0 mg/kg) groups, serum aspartate transaminase (AST) and alanine transaminase (ALT) level were elevated, and cell membrane bleb formation was observed after 120 min of reperfusion. Moreover, calpain  $\mu$  activation and talin degradation were detected. Infusion of prednisolone at 3.0 or 10 mg/kg significantly suppressed serum AST and ALT, and prevented cell membrane bleb formation. At 10 mg/kg, prednisolone markedly suppressed calpain  $\mu$  activation and talin degradation.

**CONCLUSION:** Prednisolone can suppress ischemia-reperfusion injury of the rat liver. Its cytoprotective effect is closely associated with the suppression of calpain  $\mu$  activation and talin degradation.

### INTRODUCTION

Hepatic ischemia-reperfusion injury is a serious complication but unavoidable problem in liver surgery including liver transplantation and hepatic resection<sup>[1]</sup>. The most important consequence of this pathological process is multiple organ failure with a high mortality rate. Therefore, there is a considerable interest in the prevention of hepatic ischemia-reperfusion injury. Steroid therapy suppresses liver injury by a variety of mechanisms, including increased tissue blood flow and suppression of oxygen free radicals, arachidonic acid derivatives, lysosomal proteases (cathepsins) and cytokine production<sup>[2-5]</sup>. However, the exact intracellular mechanisms of steroid action on hepatic ischemia-reperfusion injury remains unknown.

Exposure of hepatocytes to hypoxia or oxidative stress is thought to result in a rise in intracellular Ca<sup>2+</sup> concentrations ([Ca<sup>2+</sup>]<sub>i</sub>), initiating cell membrane bleb formation, an early event leading to cell death<sup>[6,7]</sup>. Although the molecular mechanisms of bleb formation are unknown at present, Ca<sup>2+</sup>-dependent disruption of the cytoskeleton is considered to play an important role in the blebbing of plasma membrane<sup>[8,9]</sup>. Calpain  $\mu$ , a Ca<sup>2+</sup>-sensitive form of Ca<sup>2+</sup>-activated neutral protease (EC 3, 4, 22, 17), has been shown to degrade various cytoskeletal proteins such as talin,  $\alpha$ -actinin and

filamin<sup>[10-14]</sup>.

Steroids are the most potent anti-inflammatory and immunosuppressive agents<sup>[15]</sup>. They inhibit the synthesis of almost all known cytokines and cell surface molecules required for immune function, and suppress the activity of nuclear factor kappa B (NF-κB)<sup>[16]</sup>. This inhibition is mediated by the induction of IκBα inhibitory protein, which traps activated NF-κB in inactive cytoplasmic complexes. On the other hand, inflammatory cytokines, such as interleukin-1β and tumor necrosis factor-α, are involved in the pathophysiology of hepatic ischemia-reperfusion injury<sup>[17]</sup>. The liver also plays a central role in the metabolism of these acute reactant cytokines. In the liver, these mediators are produced in large amounts by Kupffer cells or endothelial cells<sup>[18]</sup>, and are released rapidly under various insults like hypoxia<sup>[17]</sup>.

In the present study, we investigated the cytoprotective mechanisms of prednisolone using an experimental model of ischemia-reperfusion injury of rat liver, with special emphasis on the activation of calpain μ and degradation of talin.

## MATERIALS AND METHODS

### Animals

Thirty-seven adult male Wistar (Aburabi Laboratory, Shiga, Japan) rats weighing 220-260 g were used in the study. All procedures were carried out in accordance with the guidelines set forth in the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health. Rats were anesthetized by an interperitoneal injection of pentobarbital sodium (50 mg/kg body weight), and underwent laparotomy. Before vascular clamping, heparin sodium (50 units) was intravenously injected to prevent blood coagulation.

### Prednisolone

A highly potent antagonist of prednisolone was obtained from Shionogi Pharmaceutical Co., Japan. Its molecular formula is C<sub>25</sub>H<sub>31</sub>NaO<sub>8</sub> and molecular weight is 482.51 (Figure 1). Animals were divided into two groups: rats treated with 0.9% normal saline solution were assigned as the control group ( $n = 10$ ), and those treated with intravenous injection of prednisolone (1.0, 3.0, or 10 mg/kg) as the prednisolone group ( $n = 27$ ) (1.0, 3.0 and 10 mg/kg) (Figure 2).

### Partial liver ischemia

An intravenous catheter was placed in the tail vein, through which prednisolone was infused at 30 min before vascular clamping. Partial (68%) hepatic ischemia was induced by clamping the branches of the portal vein, and hepatic artery feeding the left lateral and median lobes, the branches of the right lateral (24%) and caudate (8%) lobes were not clamped<sup>[13,14]</sup>. In this condition, intestinal congestion or other complications did not occur throughout the experiment (Figure 3).

Chemical Name: monosodium 11 β, 17, 21-trihydroxy-1, 4-pregnadiene-3, 20-dione 21-succinate  
Molecular Formula: C<sub>25</sub>H<sub>31</sub>NaO<sub>8</sub>  
Molecular Weight: 482.51

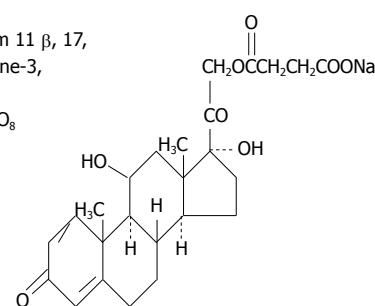


Figure 1 Structure of prednisolone.

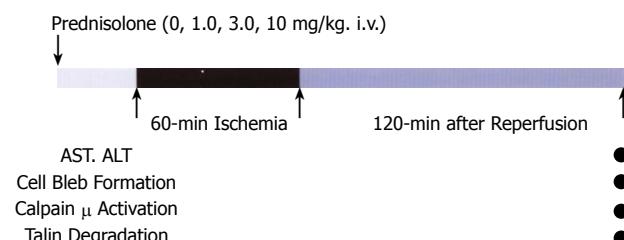


Figure 2 Experimental model.

### Measurement of serum aspartate transaminase (AST) and alanine transaminase (ALT)

Blood samples were collected at 120 min after declamping, and serum samples were stored at -80°C until biochemical analysis. Serum AST and ALT concentrations were determined by a Spot Chem kit (Spotchem Co., Kyoto, Japan).

### Cell membrane bleb formation

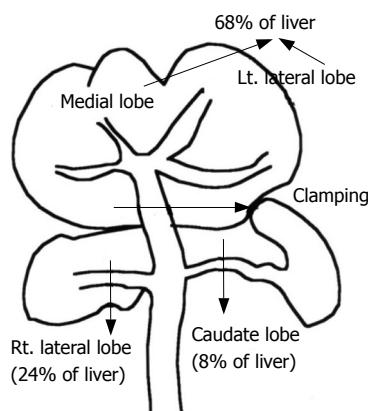
In a separate set of experiments similar to those described above, tissue samples were obtained for histological examination after *in situ* perfusion and fixation with 0.1% glutaraldehyde and 4% paraformaldehyde, which were infused via the portal vein. Biopsy specimens were also processed for routine histopathologic examination<sup>[13,14]</sup>.

### Antibodies against intermediate (activated) form of calpain μ

The preparation and characteristics of an antibody that specifically recognizes the N-terminal peptide of intermediate (activated) (NH<sub>2</sub>-AQVQKQC-COOH) form of calpain μ (78 kDa) have been described previously<sup>[13,14]</sup>.

### Western blot analysis

Liver tissues were immediately frozen by liquid nitrogen and stored at -80°C for 3 d. For Western blot analysis, samples were homogenized in an ice-water bath using a radioimmune protein assay buffer [1.0% Nonidet P-40 (Iwai Kagaku Co Ltd, Tokyo, Japan); 0.1% deoxycholic acid; 150 mmol/L sodium chloride; 50 mmol/L Tris hydrochloride; 1.0 mmol/L phenylmethylsulfonyl fluoride, pH 7.5] containing 5 mmol/L ethylene glycol-bis (β-aminoethyl ether)-N, N, N', N'-tetra acetic acid (EGTA) and 5 μmol/L leupeptin. After centrifugation



**Figure 3** Partial liver ischemia by clamping hilar area of left lateral and medial lobes without causing intestinal congestion. Lt.: Left; Rt.: Right.

at 3000 r/min for 20 min at 4°C, the supernatant (25 µg of protein) was subjected to Western blot analysis using an antibody against the intermediate form of calpain µ or an anti-talin antibody (Sigma Chemical Co., St. Louis, MO). The amount of these polypeptides was determined by a densitometric analysis.

#### Statistical analysis

Data were expressed as means  $\pm$  SD. Differences in transaminase levels, calpain µ activation and talin degradation among various groups were tested for statistical significance using the Student's *t* test and Dunnett's multiple comparison test. A *P* value of less than 0.05 denoted the presence of a statistically significant difference.

## RESULTS

### Serum AST and ALT

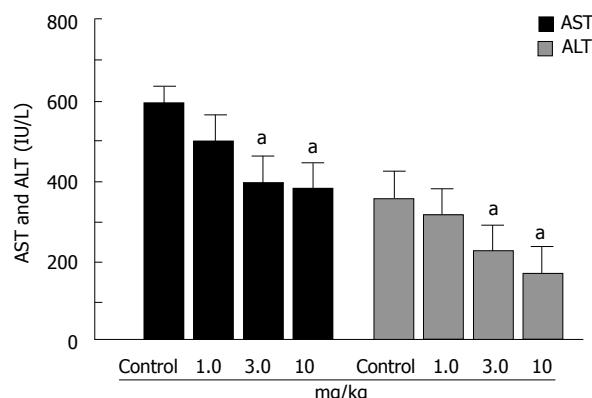
Serum AST and ALT concentrations were increased at 120 min after declamping in the control group. Prednisolone reduced the concentration of both transaminases in a dose-dependent manner. Differences between control and prednisolone levels were significant at prednisolone dose of 3.0 and 10 mg/kg (Figure 4).

### Histological findings

To further investigate the cytoprotective effect of prednisolone, liver tissues were examined histologically (Figure 5). In the control group, at 120 min after declamping, the cell structure of hepatocytes was not clear due to membrane bleb formation. Furthermore, numerous membrane microparticles were present in the sinusoidal space. In contrast, membrane blebbing was rarely seen and the cell structure was well preserved in prednisolone-treated (3.0 and 10 mg/kg) rats.

### Activation of calpain µ

Using a specific antibody to the intermediate (78 kDa) form of calpain µ, Western blotting was performed to examine the relationship between calpain µ activation and cell injury of the liver (Figure 6). The activated form



**Figure 4** Effects of prednisolone on the AST and ALT levels in animals with 60 min of partial hepatic ischemia. \**P* < 0.05.

of calpain µ appeared in all groups at 120 min after vascular declamping. However, prednisolone inhibited calpain µ activation in a dose-dependent manner. The difference in calpain µ activation was significant between control and high dose prednisolone (10 mg/kg) rats.

### Degradation of talin

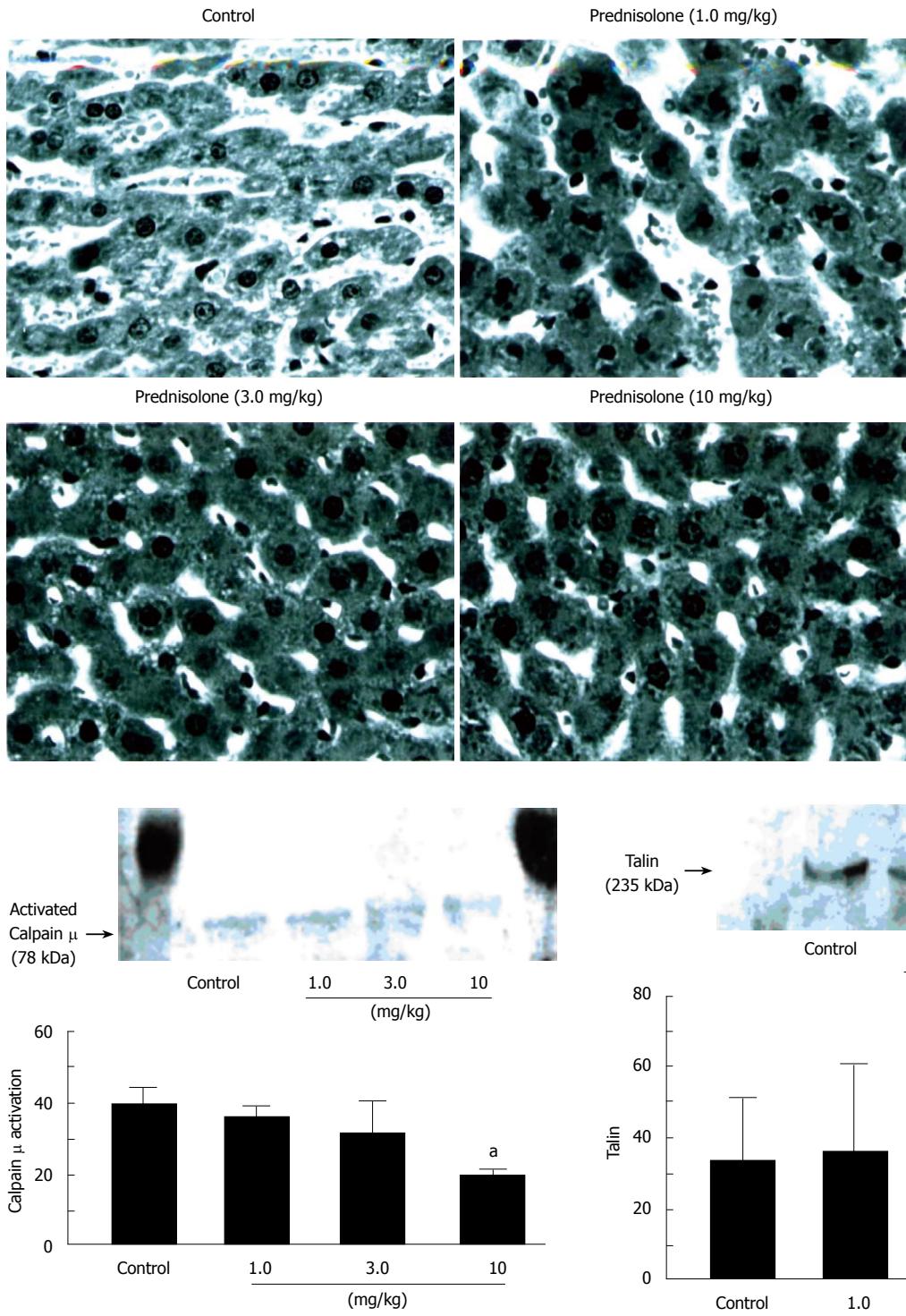
Proteolysis of talin, a favorable intracellular substrate of calpain µ was also investigated by Western blotting (Figure 7). Talin was markedly degraded in control and low-dose prednisolone (1.0 mg/kg) rats 120 min after vascular declamping. However, at 3.0 and 10 mg/kg, prednisolone significantly suppressed talin degradation, compared to the control group.

## DISCUSSION

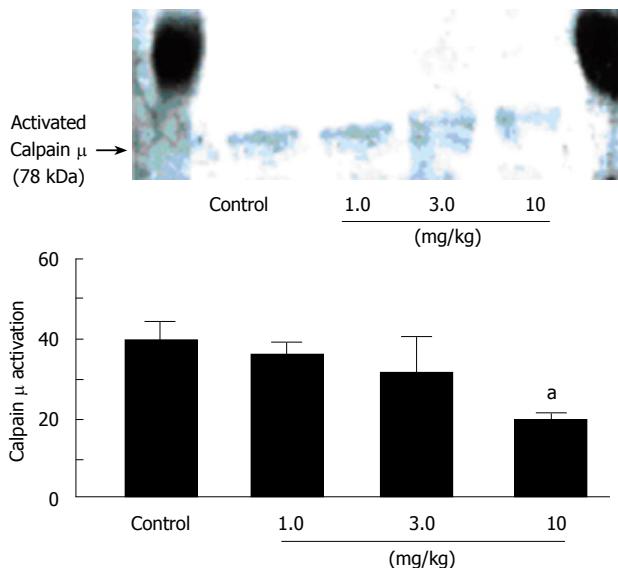
The major findings of our study were that prednisolone inhibited calpain µ activation in ischemia-reperfusion injury of the rat liver and that the degree of calpain µ activation closely correlated to the morphological changes in hepatocytes, i.e., cell membrane bleb formation. Furthermore, we also showed that prednisolone reduced the level of talin degradation in ischemic liver tissues. These changes were associated with improved overall liver function as reflected by lowering of serum AST and ALT concentrations, relative to the control.

Calpain µ is a major  $\text{Ca}^{2+}$ -dependent cytosolic protease so far described<sup>[13,14]</sup> and is activated following increased  $[\text{Ca}^{2+}]_i$  in hepatocyte injury. Since cell membrane bleb formation is an irreversible phenomenon leading to cell necrosis, the role of calpain activation in cell membrane blebbing through the proteolysis of cytoskeletal proteins<sup>[15]</sup> is considered to be particularly important. Furthermore, we have previously reported that talin and  $\alpha$ -actinin were degraded simultaneously with calpain µ activation in oxidative stress-induced hepatocyte injury, and that all these events were suppressed by a specific calpain inhibitor, calpeptin<sup>[10-14]</sup>. Thus, the beneficial effects of prednisolone on hepatic ischemia-reperfusion injury may be at least due to inhibition of calpain µ activation.

Recent studies have demonstrated that hepatocyte

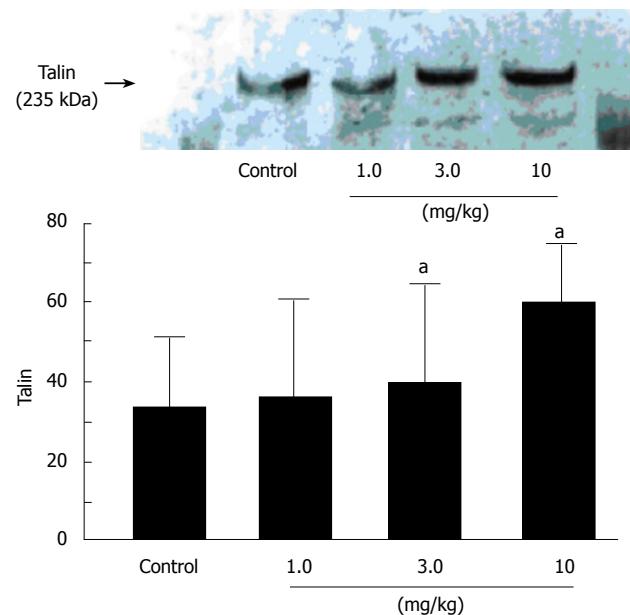


**Figure 5** Effects of prednisolone on histopathological findings in animals with 60 min partial hepatic ischemia. T (HE,  $\times 400$ ).



**Figure 6** Effects of prednisolone on calpain  $\mu$  activation in animals with 60 min of partial hepatic ischemia.  $^aP < 0.05$ .

injury is initiated by a rise in  $[Ca^{2+}]_i$ , which results from  $Ca^{2+}$  release from the internal storage sites and  $Ca^{2+}$  influx<sup>[7,11,12]</sup>, and is closely correlated with the magnitude of ischemic insult. On the other hand, the pathogenesis of hepatic ischemia-reperfusion injury is complex and multifactorial. Various factors such as the reactive oxygen species<sup>[19]</sup>, platelet-activating factor<sup>[20]</sup>, thromboxane A<sub>2</sub><sup>[21]</sup>, leukotriene B<sub>4</sub><sup>[22]</sup> and endothelin-1<sup>[23]</sup> have been identified. In addition, inflammatory cytokines like interleukin-1 $\beta$  and tumor necrosis factor- $\alpha$  also



**Figure 7** Effects of prednisolone on the degradation of talin in animals with 60 min of partial hepatic ischemia.  $^aP < 0.05$ .

play an important role in hepatic ischemia-reperfusion injury<sup>[17]</sup>. All these substances induce a rise in  $[Ca^{2+}]_i$ . These findings indicate that in addition to prostaglandin E1 and prostacyclin<sup>[13]</sup> which directly suppress the rise in  $[Ca^{2+}]_i$ , other agents like corticosteroids may inhibit hepatic ischemia-reperfusion injury by suppressing the production of these extracellular mediators. In fact, corticosteroids have been shown to inhibit the generation of oxygen free radicals<sup>[24]</sup>, and reduce

hepatic ischemia-reperfusion injury<sup>[25]</sup>. In the present study, we demonstrated that at a dose of 10 mg/kg, prednisolone inhibited degradation of talin, as well as calpain  $\mu$  activation in the ischemic liver. Since the increase in  $[Ca^{2+}]_i$  induces calpain  $\mu$  activation, inhibition of calpain  $\mu$  activation by prednisolone suggests a possible suppression of increased  $[Ca^{2+}]_i$  in hepatocytes. Therefore, it is possible that prednisolone inhibits hepatic ischemia-reperfusion injury by suppressing the generation of extracellular mediators with subsequent abrogation of the rise in  $[Ca^{2+}]_i$ .

Consistent with the beneficial effects on extra- and intracellular mediators, these findings suggest that preoperative administration of glucocorticoids may prevent hepatic ischemia-reperfusion injury and subsequent systemic reactions. However, the inhibitory effect of prednisolone on the calpain  $\mu$  activation was not completely similar to the results of the serum transaminase levels, and membrane bleb formation. From the clinical point of view, a complete inhibition of hepatic ischemia-reperfusion injury may be an ultimate goal in liver surgery. To accomplish this, treatment with a combination of several agents that inhibit various steps of hepatic ischemia-reperfusion injury is probably necessary since all agents reported so far produce only a partial inhibitory effect. The multiplicity approach for hepatic ischemia-reperfusion injury (e.g. prostaglandin E1 and prednisolone) is now under investigation.

In conclusion, we demonstrated in the present study that prednisolone suppressed ischemia-reperfusion injury of the rat liver. Its cytoprotective effect was partial, but was closely associated with inhibition of calpain  $\mu$  activation and suppression of talin degradation. The effects of a combination of two or more agents of different inhibitory mechanisms should be examined in the future to reduce the complications encountered during hepatic surgery.

## COMMENTS

### Background

Hepatic ischemia-reperfusion injury is a serious complication but unavoidable problem in liver surgery including liver transplantation and hepatic resection. The most important consequence of this pathological process is multiple organ failure with a high mortality rate. Steroid therapy suppresses liver injury by a variety of mechanisms, including increased tissue blood flow and suppression of oxygen free radicals, arachidonic acid derivatives, lysosomal proteases (cathepsins) and cytokine production. In this study, authors investigated the cytoprotective mechanisms of prednisolone using an experimental model of ischemia-reperfusion injury of rat liver, with special emphasis on the activation of calpain  $\mu$  and degradation of talin.

### Research frontiers

Steroids are the most potent anti-inflammatory and immunosuppressive agents. They inhibit the synthesis of almost all known cytokines and cell surface molecules required for immune function, and suppress the activity of nuclear factor kappa B (NF- $\kappa$ B). This inhibition is mediated by the induction of I $\kappa$ B $\alpha$  inhibitory protein, which traps activated NF- $\kappa$ B in inactive cytoplasmic complexes. On the other hand, inflammatory cytokines, such as interleukin-1 $\beta$  and tumor necrosis factor- $\alpha$ , are involved in the pathophysiology of hepatic ischemia-reperfusion injury. The liver also plays a central role in the metabolism of these acute reactant cytokines. In the liver, these mediators are produced in large amounts by Kupffer cells or endothelial cells, and are released rapidly under various insults like hypoxia.

### Innovations and breakthroughs

Recent studies have demonstrated that hepatocyte injury is initiated by a rise in  $[Ca^{2+}]_i$ , which results from  $Ca^{2+}$  release from the internal storage sites and  $Ca^{2+}$  influx, and is closely correlated with the magnitude of ischemic insult. On the other hand, the pathogenesis of hepatic ischemia-reperfusion injury is complex and multifactorial. All these reactive oxygen species induce a rise in  $[Ca^{2+}]_i$ . This study found that at a dose of 10 mg/kg, prednisolone inhibited degradation of talin, as well as calpain  $\mu$  activation in the ischemic liver.

### Applications

In this research, authors found calpain  $\mu$  is an important enzyme related to hepatic ischemia-reperfusion injury. The mechanism of calpain  $\mu$  is first reported in living organism in this research.

### Peer review

The study deals with protective effect of prednisolone antagonist on ischemia-induced liver injury. The authors have shown that the compound used offered protection for the liver. This is a well written and interesting study.

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RAPID COMMUNICATION

# Univariate and multivariate analysis of risk factors for severe *Clostridium difficile*-associated diarrhoea: Importance of co-morbidity and serum C-reactive protein

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## INTRODUCTION

*Clostridium difficile* associated diarrhoea (CDAD) is the most common cause of healthcare-associated diarrhoea and results in a wide spectrum of disease severity ranging from asymptomatic carriage to life-threatening enterocolitis and death<sup>[1-5]</sup>. Recently, a new epidemic strain producing higher levels of toxin has emerged in Canada and the US<sup>[5-8]</sup> as well as in some European countries which results in CDAD with higher morbidity and mortality<sup>[9-13]</sup>. Many studies have investigated risk factors for infection with *Clostridium difficile* (*C. difficile*) and subsequent development of CDAD. Thus, advanced age, severe comorbidity<sup>[14]</sup>, hospitalisation<sup>[15]</sup>, antibiotic exposure, immunosuppressive therapy<sup>[16,17]</sup> and treatment with motility influencing or acid-suppressive drugs have all been reported as risk factors for CDAD<sup>[18-21]</sup>. In contrast, less is known about risk factors associated with a severe course of CDAD in hospitalized patients.

## MATERIALS AND METHODS

We conducted a retrospective analysis of CDAD in hospitalized patients to identify possible risk factors for a severe clinical course. Our institution is a community hospital treating approximately 19 000 in-patients per year. Using a computer-based search, we identified 186 positive stool tests for *C. difficile* toxin B from 142 patients who fulfilled the case definition for CDAD between October 2003 and August 2006. After chart review 18 cases were excluded: 5 patients had multiple admissions and only the first admission was included, 5 patients were younger than 18 years and in 8 patients

## Abstract

**AIM:** To investigate risk factors for severe *Clostridium difficile* associated diarrhoea (CDAD) in hospitalized patients.

**METHODS:** We analysed risk factors for severe CDAD (associated with systemic signs of hypovolemia) in 124 hospitalized patients by retrospective chart review.

**RESULTS:** Severe CDAD was present in 27 patients (22%). Statistical analysis showed a significant association with a higher 30-d mortality (33% vs 4%,  $P < 0.001$ ) and a higher proportion of longer hospital stay exceeding 14 d (74% vs 52%,  $P = 0.048$ ). Charlson co-morbidity score (OR 1.29 for 1 point increment,  $P < 0.05$ ) and serum C-reactive protein at diagnosis (OR 1.15 for 10 mg/L increment,  $P < 0.001$ ) were independent predictors of severe CDAD.

**CONCLUSION:** Patients with a severe level of co-morbidity and high serum C-reactive protein levels at the time of diagnosis should receive particular attention.

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**Key words:** *Clostridium difficile*; Nosocomial diarrhoea; Co-morbidity; C-reactive protein; 30-day mortality

**Peer reviewer:** Hitoshi Asakura, Director, Emeritus Professor,

**Table 1** Patient characteristics

Patient characteristics	Data
Age <sup>1</sup> (yr)	76 (18-93)
Sex	
Female	71 (57%)
Male	53 (43%)
Nursing home residency	19 (15%)
Charlson's comorbidity score	4 (0-10)
GI procedures including PEG and surgery	13 (10%)
Previous medication:	
Antibiotic therapy within 6 wk prior to onset CDAD	101 (81%)
Acid-suppressive therapy	66 (53%)
Immunosuppressive therapy	25 (20%)
Opioid use	57 (46%)
Laxative use	30 (24%)
Clinical features of CDAD	
Hospital-acquired CDAD	101 (81%)
Interval onset of diarrhoea to CDAD therapy $\geq 7$ d	45 (37%)
Body temperature $\geq 38^\circ\text{C}$	56 (45%)
Severe CDAD	27 (22%)
Laboratory at diagnosis:	
White blood cell count (G/L)	14.1 (4.6-81.3)
CRP (mg/L)	118 (2-413)
Creatinine (mg/L)	11.5 (3.1-110.5)
Sodium (mmol/L)	136 (114-145)
Potassium (mmol/L)	3.52 (2.43-5.07)
Continuation of initial antibiotic therapy despite CDAD	71 (57%)
Antibiotic therapy for CDAD	113 (91%)
Length of hospital stay > 14 d	70 (56%)
30-d mortality	13 (10%)

<sup>1</sup>Data are given as median (range) or number (percentage).

data were incomplete, leaving 124 patients for further analysis. We recorded patient age, sex, nursing home residency, comorbidity to calculate the Charlson comorbidity score<sup>[22,23]</sup> previous and concomitant medication (systemic antibiotic treatment within 6 wk preceding diagnosis, continuation of the initial antibiotic therapy after diagnosis of CDAD, use of opioids or laxatives), predisposing medical or surgical procedures (endoscopy, percutaneous gastrostomy, nasogastric tubes, chemotherapy or radiotherapy) as well as vital signs (heart rate, blood pressure and body temperature) and laboratory parameters (white blood cells, C-reactive protein, sodium, potassium, creatinine) at the time of diagnosis. In addition, we recorded the length of hospital stay, the period until beginning therapy for CDAD after the onset of diarrhoea, whether a specific antibiotic therapy for CDAD was instituted or not and the 30-d mortality after initial diagnosis of CDAD.

Patients with more than three loose stools per day on more than two consecutive days with a positive stool test for *C. difficile* toxin were diagnosed as CDAD<sup>[16,24]</sup>. Hospital-acquired CDAD was assumed if the onset of diarrhoea was > 72 h after hospital admission or if there had been a hospital admission for CDAD within the previous 6 wk. Severe CDAD was defined as profuse diarrhoea associated with a positive shock index (heart rate bpm/systolic blood pressure mmHg > 1.5) at initial diagnosis<sup>[10]</sup>. All other patients were classified as non-severe CDAD.

Comparisons between the two groups of severe and

**Table 2** Univariate analysis of risk factors for severe CDAD

Variable	Non-severe CDAD (n = 97)	Severe CDAD (n = 27)	P
Male sex	41/42	12/44	
Nursing home residency	12/12	7/26	
Hospital-acquired CDAD	76/78	25/93	
Immunosuppressive therapy	15/15	10/37	< 0.05
Previous antibiotic therapy	78/80	23/85	
Acid-suppressive therapy	47/48	19/70	
Therapy with opioids	40/41	17/63	
Laxative use	19/20	11/41	< 0.05
GI procedures including PEG and surgery	13/13	0/0	
Continuation of initial antibiotic therapy	52/54	19/70	
Antibiotic treatment for CDAD	88/91	25/93	
Body temperature $\geq 38^\circ\text{C}$	38/39	18/67	< 0.05
Therapy $\geq 7$ d after onset diarrhoea	33/34	12/44	
Length of hospital stay > 14 d	50/52	20/74	< 0.05
30-d mortality	4/4	9/33	< 0.001
Age (yr)	74 ± 12	77 ± 12	
Charlson's score (points)	3.4 ± 2.2	5 ± 2.6	< 0.001
White blood cell count (G/L)	15.3 ± 9.9	21.6 ± 10.4	< 0.01
C-reactive protein (mg/L)	109 ± 79	223 ± 92	< 0.001
Creatinine (mg/L)	14 ± 13	24 ± 17	< 0.01
Sodium (mmol/L)	135 ± 5	133 ± 7	
Potassium (mmol/L)	3.6 ± 0.5	3.4 ± 0.5	

non-severe CDAD were performed by Student *t* test for normally distributed data, proportions were analysed by  $\chi^2$  or *F* test as appropriate. A two-sided error level of *P* < 0.05 was considered statistically significant. Variables significantly associated with severe CDAD in univariate analysis together with risk factors reported in the literature were entered into a multivariate analysis. Statistical analysis was computed with SPSS version 14.0 (SPSS, Inc., Chicago, IL, USA).

## RESULTS

### Demographics and results of initial evaluation

Patient characteristics are summarised in Table 1. Many patients had a comorbidity resulting in a median Charlson comorbidity score of 4. The majority of patients had hospital-acquired CDAD, 27 patients (22%) had severe CDAD, the overall 30-d mortality was 10% (13/124); all patients who died were >70 years.

### Analysis of possible risk factors

Univariate analysis for comparison of patients with non-severe (*n* = 97) and severe CDAD (*n* = 27) revealed that immunosuppressive therapy, laxative use, body temperature  $\geq 38^\circ\text{C}$ , length of hospital stay > 14 d, 30-d mortality, Charlson comorbidity score, white blood cell count, serum levels of C-reactive protein and creatinine were all significantly associated with severe CDAD (Table 2).

**Table 3 Multivariate analysis of possible risk factors for severe CDAD**

Variable (unit)	P	OR; 95% CI
Charlson's score (points; 1-point increments)	< 0.05	1.39; 1.06-1.83
Body temperature $\geq 38^{\circ}\text{C}$		1.15; 0.35-3.83
Immunosuppressive therapy		1.84; 0.45-7.49
Acid-suppressive therapy		1.28; 0.39-4.21
Opioid use		2.50; 0.80-7.84
Laxative use		2.66; 0.79-8.97
C-reactive protein (mg/L; 10 mg/L increments)	< 0.01	11.2; 10.3-12.1
White blood cell count (G/L; 1 G/L increments)		1.01; 0.96-1.06
Creatinine level (mg/L; 10 mg/L increments)		12.5; 9.2-16.8
Reduced Model		
Charlson's score (points; 1-point increments)	< 0.05	1.29; 1.02-1.61
C-reactive protein (mg/L; 10 mg/L increments)	< 0.001	1.15; 1.08-1.22

A borderline statistically significant association was found for comedication with acid-suppressive therapy or opioids. By contrast, severe CDAD was not associated with nursing home residency, presence of hospital-acquired CDAD, continuation of the initial antibiotic therapy after diagnosis or increasing age. Multiple logistic regression analysis confirmed a significant association of severe CDAD and Charlson comorbidity score (OR 1.29 for 1 point increment,  $P < 0.05$ ) and levels of serum C-reactive protein (OR 1.15 for 10 mg/L increment,  $P < 0.001$ ; Tables 2 and 3).

## DISCUSSION

The major findings in this retrospective analysis were a 22% rate of severe CDAD significantly associated with relatively high 30-d mortality (33% vs 4%,  $P < 0.001$ ) and a higher proportion of a hospital stay exceeding 14 d (74% vs 52%,  $P < 0.05$ ). In addition, comorbidity assessed by the Charlson comorbidity score ( $P < 0.05$ ) and serum C-reactive protein at the time of diagnosis ( $P < 0.001$ ) were identified as independent risk factors for severe CDAD in multivariate analysis.

The rate of severe CDAD and associated 30-d mortality in this study are relatively high. Infection with the recently emerging strain BI/NAP1 associated with severe courses of CDAD<sup>[2,8-11]</sup> is an unlikely explanation, since this strain had not been documented in Germany at the time of our retrospective analysis<sup>[25]</sup>. Therefore the most likely explanation are advanced age (median 76 years) and high comorbidity (median Charlson score of 4) of our cohort. The observed association of disease severity with comorbidity assessed by the Charlson comorbidity score is in line with reports on an association of severe CDAD with cognitive impairment<sup>[16]</sup>, number of chronically affected organ systems<sup>[26]</sup>, cardiac disease, malignancy, chronic obstructive pulmonary disease, pre-existing renal failure and other severe disease<sup>[27,28]</sup>. Our data support the hypothesis that comorbidity is an important risk factor for severe CDAD and the Charlson comorbidity

score, which includes most of these conditions, might be a useful tool to identify patients at particular risk for severe CDAD. We also identified serum levels of C-reactive protein as independently associated with severe CDAD. In fact, serum C-reactive protein was a far better predictor of severe CDAD than white blood cell count, which has been described by others<sup>[28,29]</sup>. Thus, at the median Charlson comorbidity score of our cohort (4 points) a C-reactive protein level of 250 mg/L at diagnosis predicted a higher than 50% probability for severe CDAD. Perhaps more sensitive markers of inflammation such as procalcitonin might be even more useful in the evaluation of disease severity.

Other known risk factors for CDAD<sup>[2,18,30]</sup> might also be relevant for severe CDAD. In line with these data we found comedication with laxatives, opioids and acid-suppressive therapy associated with severe CDAD in univariate analysis although these risk factors could not be confirmed in multivariate analysis. In contrast, a variety of other putative risk factors for severe CDAD could not be confirmed. Thus, we did not detect an association of severe CDAD with increased age<sup>[27,30]</sup>, which is probably due to the already advanced median age of our cohort. Moreover, prolonged antibiotic use *per se*, continuation of the antibiotic therapy after the diagnosis of CDAD, gastrointestinal procedures or surgery, which have all been reported as risk factors for *C. difficile* colonisation and CDAD<sup>[2,17]</sup> were not associated with severe disease in this study.

In conclusion, comorbidity and serum levels of serum C-reactive protein were identified as predictors of severe CDAD. Patients with strong comorbidity and high serum C-reactive protein levels at the time of diagnosis should be treated with particular attention.

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## COMMENTS

### Background

*Clostridium difficile* associated diarrhoea (CDAD) is the most common cause of healthcare-associated diarrhoea. It results in a wide spectrum of disease severity ranging from asymptomatic carriage to life-threatening enterocolitis and death with associated health care costs.

### Research frontiers

A variety of studies has investigated risk factors for the development of CDAD. Thus, advanced age, severe comorbidity, hospitalisation, antibiotic exposure, immunosuppressive therapy as well as treatment with motility influencing or acid-suppressive drugs were identified as risk factors for CDAD. However, little is known about risk factors for associated with a severe course of CDAD in hospitalized patients.

### Innovations and breakthroughs

The major findings reported are a 22% rate of severe CDAD which was significantly associated with relatively high 30-d mortality and a higher proportion of a hospital stay exceeding 14 d. Moreover, comorbidity assessed by the Charlson comorbidity score and levels of serum C-reactive protein at the time of diagnosis were identified as independent risk factors for severe CDAD

in multivariate analysis.

### Applications

The major findings of this study should help to identify hospitalized patients with a particular risk for a severe course of CDAD. An early identification of patients at risk would allow a more timely intervention probably improving both morbidity and mortality.

### Peer review

The paper describes important risk factors for a severe course of CDAD in hospitalized patients which have a potential for everyday clinical practice. It's an interesting paper.

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RAPID COMMUNICATION

## Efficacy of 6-mercaptopurine treatment after azathioprine hypersensitivity in inflammatory bowel disease

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### Abstract

**AIM:** To investigate the efficacy of 6-mercaptopurine (6-MP) in cases of azathioprine (AZA) hypersensitivity in patients with inflammatory bowel disease.

**METHODS:** Twenty nine previously confirmed Crohn's disease (CD) ( $n = 14$ ) and ulcerative colitis (UC) ( $n = 15$ ) patients with a known previous (AZA) hypersensitivity reaction were studied prospectively. The 6-MP doses were gradually increased from 0.5 up to 1.0-1.5 mg/kg per day. Clinical activity indices (CDAI/CAI), laboratory variables and daily doses of oral 5-ASA, corticosteroids, and 6-MP were assessed before and in the first, sixth and twelfth months of treatment.

**RESULTS:** In 9 patients, 6-MP was withdrawn in the first 2 wk due to an early hypersensitivity reaction. Medication was ineffective within 6 mo in 6 CD patients, and myelotoxic reaction was observed in two. Data were evaluated at the end of the sixth month in 12 (8 UC, 4 CD) patients, and after the first year in 9 (6 UC, 3 CD) patients. CDAI decreased transiently at the end of the sixth month, but no significant changes were observed in the CDAI or the CAI values at the end of the year. Leukocyte counts ( $P = 0.01$ ), CRP ( $P = 0.02$ ), and serum iron ( $P = 0.05$ ) values indicated decreased inflammatory reactions, especially in the UC patients at the end of the year, making the possibility to taper oral steroid doses.

**CONCLUSION:** About one-third of the previously AZA-intolerant patients showed adverse effects on taking 6MP. In our series, 20 patients tolerated 6MP, but it was ineffective in 8 CD cases, and valuable mainly in ulcerative colitis patients.

### INTRODUCTION

5-aminosalicylate is usually ineffective in the maintenance treatment of steroid induced remission in idiopathic inflammatory bowel (IBD) diseases, i.e. ulcerative colitis (UC) and Crohn's disease (CD)<sup>[1]</sup>. In the vast majority of cases immunosuppressive treatment is necessary to maintain the remission. Azathioprine (AZA) has been advised in the treatment of UC and CD since the middle of 1960's<sup>[2]</sup>. It is worth starting if the patient is corticosteroid resistant (the effective dose does not lead to remission) or dependent (discontinuation of the corticosteroid causes relapse)<sup>[3]</sup>. Azathioprine and its first metabolite 6-mercaptopurine are effective immunomodulators, but contrary to the corticosteroids, purine analogues have a late onset of action<sup>[4]</sup>. Their maximum effect can only be expected after 3-6 mo<sup>[5]</sup>. Azathioprine is offered as first choice, but it can cause early hypersensitivity reaction (fever), or gastrointestinal side effects (nausea, vomiting, and diarrhoea) in the first two weeks in 5%-10% of patients. In these cases, 6-mercaptopurine (6-MP) may be effective without side effects<sup>[6]</sup>. However, there are few data about the clinical efficacy of changing the AZA to 6-MP therapy in cases having hypersensitivity reactions after the first AZA medication.

### MATERIALS AND METHODS

Between 2002 and 2005, 29 IBD patients (15 women and 14 men) were treated with 6-mercaptopurine due to azathioprine hypersensitivity. The drug Purinethol, 50 mg (Laboratoire GlaxoSmithKline) was approved by the National Institute of Pharmacy. The mean age of the

**Table 1** The number of IBD patients in the three groups, according to extents, fistulas and surgeries

Disease	No. of patients	A	B	C	D	E	F
Group1 UC	7	1	2	4	-	-	-
Group1 CD	2	-	2	-	-	-	-
Group2 CD	8	3	2	2	1	8	16
Group3 UC	8	1	-	7			1
Group3 CD	4	-	3	1	-	4	4
	29	4	9	14	1	12	21

UC-A: Distal; UC-B: Left sided; UC-C: Pancolitis; CD-A: Small bowel only; CD-B: Colon only; CD-C: Small bowel + colon, CD-D: Upper GI + small bowel + colon; CD-E: Number of fistulas; CD-F: Number of surgeries.

patient's was 40.1 years (range 19-66 years), and mean time to 6-MP treatment from the IBD diagnosis was 5.4 years (range 0.1-16.4 years). Fifteen patients had UC and 14 patients had CD. Table 1 contains the number of IBD patients in the 3 groups, according to extents, fistulas and surgeries. 6-MP test dose (50 mg/d for 7-10 d) was administered first. The therapy had to be discontinued in the first two weeks because of the same or similar hypersensitivity reactions as taking azathioprine during the initial doses in 9/29 patients (7/9 UC, 2/9 CD). Among the 9 patients, 4 had hypersensitivity reactions, including fever, and 5 were intolerant due to GI side-effects. During the very short interval (7-10 d) between the AZA start and the appearance of adverse events, we did not observe side-effects, such as leucopenia, abnormal LFTs or pancreatitis. Medication had to be suspended in 8/20 patients during the first 6 mo because it was ineffective. Decreasing the CDAI score not more than 70, and at least 3 score values in the CAI, was considered to be a treatment failure. All of them were CD patients; their treatment was continued by methotrexate and/or infliximab, if required.

Twelve patients tolerated 6-mercaptopurine without side-effects for more than six months with clinical efficiency. Four of 12 had Crohn's disease, 8/12 had UC. During the study 9 patients (6 UC, 3 CD) were treated for more than a year. The initial dose of 6-MP was 50 mg/d and it was increased, if possible, up to 1.5 mg/kg per day. The clinical activity, 6-MP and corticosteroid (prednisolone, methylprednisolone, separately) as well as the laboratory variables of acute inflammatory process were recorded. The medication was initiated at the first visit, and follow-up visits after the first, third, and sixth, twelfth months after the initial therapy. Crohn's Disease Activity Index (CDAI) and Clinical Activity Index of Ulcerative Colitis (CAI) were scored and calculated at each visit. According to the calculated values, the actual activity was grouped as inactive (1), mild (2), moderate (3) or severe (4). The following blood chemistry variables were determined, erythrocyte sedimentation rate, hematocrit, white cell and platelet counts, blood iron level, CRP, and fibrinogen.

STATA V8 program was used for statistical analysis. The repeated measurements of ANOVA and correlation analysis were used. Associations of variables between

activity groups were analyzed by one way method of ANOVA.

## RESULTS

The baseline means  $\pm$  SD of CDAI/CAI, CRP, ESR, WBC, platelets, and the changes over time demonstrated decreased inflammatory reactions (Table 2). Relationships between time (at six months and one year), activity, and laboratory variables are presented in Table 3. Results of the analysis corresponded to 12 patients at 6 mo (8 UC, 4 CD), and 9 mo (6 UC, 3 CD) at the end of the first year (repeated measurement of ANOVA). CDAI decreased significantly only at the end of the sixth month in 4 of the 12 patients in whom 6-MP treatment was successful, but a similar decrease could not be demonstrated in the CAI of UC. At the end of the year, such an alteration could not be detected in any of the activity indices. CRP, referring to the severity of the inflammation, decreased and the iron level increased significantly at the end of the year (Table 3). Correlation between the calculated activity index numeric values (CDAI, CAI) and laboratory variables (correlation analysis) is presented in Table 4. The laboratory variables associated with the numeric values of the activity revealed a decrease in the inflammatory reaction in UC, but not in CD, due to the small number of CD cases. Besides the amelioration of the inflammatory reaction, the direct suppressive effect on the bone marrow may also be associated with reductions in the numbers of leukocytes and platelets. Treatment had to be discontinued temporarily in 1 case because of leukopenia at the end of the year. In another case, a significant bone marrow depression developed, necessitating surgical intervention (ileal-pouch anal anastomosis - IPAA) after drug cessation. Hepatotoxic side-effects and pancreatitis were not observed during the year. Table 5 shows the correlation between the activity groups - inactive (1), mild (2), moderate (3), severe (4) - and the values of laboratory variables in UC and CD patients (one way ANOVA). The laboratory variables correlated substantially better with the group classification than with the activity index numeric values.

The average doses of 6-MP and corticosteroids administered at the time of the visits is presented as mg/kg (Figure 1). Prednisolone treatment could be omitted and the dose of methylprednisolone could be tapered to one third at the end of the year. At the end of the first year 5/9 patients (3 UC, and 2 CD) became steroid free.

## DISCUSSION

Systemic corticosteroid treatment is often needed in relapses of idiopathic inflammatory bowel diseases (UC, CD)<sup>[7]</sup>. The dose depends on the activity of the disease with severe cases treated intravenously<sup>[8]</sup>. If the patient responds to the intravenous regime, treatment should be switched to oral administration, and oral doses should be tapered gradually and finally terminated. If

**Table 2 Changes of CDAI/CAI, CRP, ESR, WBC, PLT variables in the third group (mean  $\pm$  SD)**

Disease	Months	CDAI	CAI	CRP	ESR	WBC	PLT
UC	0	-	7.8 $\pm$ 6.3	16 $\pm$ 10	30.4 $\pm$ 16.2	9.3 $\pm$ 3.3	384 $\pm$ 175
UC	3	-	6.9 $\pm$ 6	8.3 $\pm$ 3.5	28.3 $\pm$ 16.5	10.3 $\pm$ 4.5	336 $\pm$ 119
UC	6	-	6.1 $\pm$ 6.7	11 $\pm$ 19	31.9 $\pm$ 32.7	6.8 $\pm$ 2	348 $\pm$ 197
UC	12	-	1.1 $\pm$ 1	4.6 $\pm$ 3.5	27 $\pm$ 17.2	6.1 $\pm$ 2.6	283 $\pm$ 87
CD	0	146 $\pm$ 128	-	39.8 $\pm$ 26.7	41.3 $\pm$ 11.6	9.9 $\pm$ 2.7	431 $\pm$ 136
CD	3	145 $\pm$ 124	-	23.3 $\pm$ 10.7	29.2 $\pm$ 14.1	7 $\pm$ 3	332 $\pm$ 66
CD	6	167 $\pm$ 68	-	21 $\pm$ 15	17.6 $\pm$ 28.6	9.9 $\pm$ 4.2	317 $\pm$ 89
CD	12	46 $\pm$ 56	-	6 $\pm$ 4	18 $\pm$ 14.7	6.2 $\pm$ 1.3	247 $\pm$ 80

**Table 3 Significant changes after six and twelve months of 6-MP treatment**

Variable	6 mo	12 mo
CDAI	P = 0.0144 (n = 4)	NS n = 3
CAI	NS n = 8	NS n = 6
Leukocyte	NS	P = 0.0057
CRP	NS	P = 0.0206
Serum iron	NS	P = 0.0459

NS: Not significant.

**Table 4 Correlation of activity index numbers with the laboratory variables**

Variable	CAI/UC	CDAI/CD
Thrombocyte	P < 0.001	NS
Serum iron	P = 0.006	NS
CRP	P = 0.008	NS
Haematocrit	P = 0.022	NS
Leukocyte	P = 0.071 (BS)	NS
ESR 1st h	P = 0.077 (BS)	NS

NS: Not significant; BS: Borderline significance.

**Table 5 Correlation between activity groups and laboratory variables**

Variable	1-4 UC (P)	1-4 CD (P)
Fibrinogen	0.0046	NS
Thrombocyte	0.0060	0.0350
CRP	0.0061	NS
Leukocyte	0.0144	0.071 (BS)
Haematocrit	0.0270	NS
Serum iron	0.0808 (BS)	NS

BS: Borderline significance.

the patient proves to be steroid resistant, or dependent, immunosuppressive treatment is suggested<sup>[9]</sup>. Azathioprine is recommended first, where it is the most frequently used immunosuppressive drug for IBD<sup>[10]</sup>. An oral dose of 1.5-2.5 mg/kg per day is usually effective, but requires monitoring<sup>[11]</sup>. Therapeutic effects and side-effects of AZA show great variability among the patients due to the various concentrations of the therapeutic and toxic metabolites<sup>[12]</sup>. The application is hampered in 9%-25% of the patients due to its toxic effects. Hypersensitivity reactions (fever), or gastrointestinal side effects (nausea, diarrhea), can occur during the first weeks of the treatment<sup>[13]</sup>. According to McGovern *et al*, imidazole that is cleaved off the AZA molecule can be responsible for the development of this process<sup>[14]</sup>. AZA is a pro-drug that is converted to 6-MP through a non-enzymatic step. Further metabolism of 6-MP depends on three competing enzyme pathways. Hepatotoxic 6-methylmercaptopurine (6-MMP) is produced by thiopurine methyltransferase (TPMT), a key enzyme of toxic and therapeutic metabolites. Measurement of its activity helps determine individual doses<sup>[14,15]</sup>. Catabolism, *via* xanthine oxidase (XO), forms inactive 6-thiouric acid which is eliminated through the urine. Metabolism, *via* hypoxanthin-guanine phosphoribosyl

**Figure 1 Changes of the 6-MP and oral steroid mean doses.**

Visit (mo)	6-MP (●)	Methylprednisolone (■)	Prednisolone (▲)
0	0.00	0.35	0.15
1	0.75	0.20	0.20
6	0.75	0.15	0.20
12	0.75	0.10	0.05

transferase (HPRT), leads to the formation of cytoactive 6-thioguanine nucleotide (6-TGN) that binds to the DNA and RNA, and is the active molecule responsible for the late side-effects in a dose-dependent manner. The proportion of these three enzymes determines the effective 6-TGN level. In case of low TPMT activity (due to enzyme polymorphisms) metabolism shifts to the production of 6-TGN, where high concentrations are associated with efficacy, but above a certain level ( $> 450$  pmol/108 erythrocyte), 6-TGN has a myelotoxic effects<sup>[16]</sup>. Bone marrow depression that is a late toxic reaction usually occurs in the first three months<sup>[17]</sup>. Other mechanisms participate in the development of this process as well since this side-effect is noticed with normal and high TPMT activity. During the occurrence of immunosuppressor activity in IBD, a decrease in the number of lamina propria plasma cells and altered function of the lymphocytes and killer cells is detected. According to previous studies that need further

confirmation, the therapeutic effect of AZA is explained by its apoptosis inducing property that is independent from 6-TGN. Tiede *et al* showed that azathioprine and its 6-thioGTP metabolite alters apoptosis of T cells<sup>[18]</sup>. The activity of certain genes in the cells is repressed by the metabolite which induces apoptosis in a mitochondrial manner.

In the view of the clinical efficacy, results achieved by 6-MP treatment can be compared with azathioprine. Considering bioequivalence, 6-MP shows the same efficacy as half (0.75-1.5 mg/kg per day) of the daily dose of AZA (1.5-2.5 mg/kg per day)<sup>[19]</sup>. There are few data about the clinical use of 6-MP<sup>[20]</sup> and only some investigated treatment results of 6-MP in AZA-intolerant patients suffering from IBD. Boulton-Jones *et al* reported a cohort of 19 patients who had failed AZA therapy. Ten had CD and 9 UC<sup>[21]</sup>. The AZA dose prior to discontinuation ranged from 50 to 150 mg. Eleven of 19 patients were able to tolerate 6-MP in a dose ranging from 50 to 100 mg (median 100 mg). Two of 8 patients developed fever; other adverse events were vomiting, leucopenia, skin rash, headache, and abdominal pain. The treatment could be continued in 6 patients from the 11 in the study performed by Bowen *et al* and were successful only in 4 cases<sup>[22]</sup>. McGovern *et al* achieved steroid independent remission in 15 of 29 patients<sup>[23]</sup>. Similar results were achieved by Doménech *et al*<sup>[24]</sup>.

In our CD patients, favourable effects were attained only in 4/14 cases, at the end of the first 6 mo with 6-MP (average: 0.75 mg/kg per day). Eight CD patients were withdrawn due to ineffective therapy within the same time. Later, at the end of the first year, the benefit of the therapy was lost in all patients. In these cases, higher doses (more than 1.5 mg/kg per day) might increase the response and perhaps the number of adverse events<sup>[25]</sup>. Decreased inflammatory reactions were more significant in UC. The efficacy of the drug was indicated by the improvement of biological parameters associated with inflammation, which permitted a gradual reduction in the steroid. It is worth mentioning that clinical activity indexes (CDAI and CAI) were less sensitive than the laboratory variables at presenting decreased inflammation<sup>[26]</sup>. The correlation between activity groups and the laboratory variables showed substantially better statistical association. Considering the efficacy in case of our patients without 6-MP intolerance, it should be mentioned that more significant improvement could be achieved by using higher doses (mainly in CD). Measurement of the 6-TGN and 6-MMP metabolites and TPMT activity is recommended for the safe dosage of thiopurine analogues, and to avoid the development of late toxic effects, although none of these parameters ensure total safety<sup>[27]</sup>. In patients with early AZA intolerance treated with 6-MP thereafter, our results showed wide individual variation of 6-MP doses and responses. Patients on effective doses may develop adverse reactions at any time; therefore, blood counts and liver function tests have to be checked in every second week at the beginning, then every month, and later on in every third month. Because of the

potential for life-threatening side-effects, the drug is recommended in cooperating patients only<sup>[28]</sup>.

AZA and 6-MP have been accepted as maintenance therapies in IBD, but controversy exists regarding optimal dosing and benefits of therapeutic drug monitoring of metabolites<sup>[29]</sup>. The AZA baseline dose (1.7 mg/kg per day) proved to be effective in maintaining remission in a French cohort of CD patients, which was lower than the dose used in clinical trials (2.5 mg/kg per day). Nielsen *et al* in their review in the year 2001, suggested a  $0.25 \pm 0.5$  mg/kg daily initial (AZA equivalent) dose for the 6-MP, increasing to  $1.0 \pm 1.5$  mg/kg daily<sup>[30]</sup>. Su and Lichtenstein, three years later, advocated "the optimal dose for the treatment of active CD is generally considered 2.5 mg/kg per day for azathioprine and 1.5 mg/kg per day for 6-MP"<sup>[17]</sup>. In our AZA intolerant patients, the initial dose was 50 mg/d with a stepwise increase after the third and sixth month with 25 or 50 mg/d up to 1.5 mg/kg per day according to the tolerability of the patients. The low dose and the step up policy may play a role in the poor therapeutic response, especially in very active Crohn disease patients in the second group.

It is worth starting with 6-MP therapy in patients with hypersensitivity reactions to AZA. One third of the patients will be intolerant, and another third will not gain any benefit, especially those who had serious active CD. However, patients with mild or moderate UC/CD might have the advantage of being able to tolerate the daily dose of more than 1 mg/kg.

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RAPID COMMUNICATION

# Epstein-Barr virus is associated with gastric carcinoma: The question is what is the significance?

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## INTRODUCTION

Gastric adenocarcinoma (GC) is the second most common cause of cancer-related mortality and a major public health problem worldwide<sup>[1]</sup>. GC has a distinct geographical distribution with the highest incidence rates in Asia and South America and the lowest incidence in western countries<sup>[1-3]</sup>.

The Epstein-Barr virus (EBV) is a ubiquitous virus, with carcinogenic properties, which has been linked to the development of several malignancies including nasopharyngeal carcinoma and Burkitt's lymphoma<sup>[4]</sup>. It has been estimated that over 90% of the population worldwide has been exposed to EBV, although, not all infected individuals develop EBV-related disease<sup>[4]</sup>. After infection, EBV remains in a latent state in B-cell lymphocytes, at a rate of 1 in 10<sup>6</sup> circulating cells. The reason why it is difficult to identify EBV is perhaps due to the fact that expression of only a small number of viral proteins allows the maintenance and control of cell proliferation<sup>[5,6]</sup>.

EBV is a common co-infection in several diseases and some authors have suggested that it may represent a late event in the GC carcinogenesis, after *H pylori* infection<sup>[7]</sup>. However, there is lack of specific information implicating EBV in the pathogenesis of GC. The worldwide occurrence of EBV positive GC is estimated at >50 000 cases/year, although, it remains unclear whether the presence of EBV is the cause or a consequence of GC<sup>[8]</sup>.

## Abstract

**AIM:** To examine the possible role of the Epstein-Barr Virus (EBV) in the development of gastric adenocarcinoma (GC). It is unclear whether EBV is involved in GC development or is a consequence of gastric inflammation secondary to immunosuppressive treatments.

**METHODS:** A systematic review was carried out of all published observational studies on the temporal association between EBV and GC, with a view to determine a causal relationship.

**RESULTS:** The present study showed that the worldwide crude prevalence of EBV in gastric adenocarcinoma was 8.29%. The prevalence varied from 7.08% for intestinal type and 9.82% for diffuse type of GC. It was observed that Western and Central Asian countries had a significantly higher frequency of EBV positive cases compared to South-Eastern countries. America had the highest EBV-GC prevalence whereas Europe had the lowest.

**CONCLUSION:** The present review has demonstrated a high prevalence of EBV in gastric adenocarcinoma. However, studies designed to assess a temporal relationship and histological association using sensitive techniques should be carried out to establish the role of EBV in GC carcinogenesis.

Therefore, we carried out a systematic analysis of all observational studies on EBV and GC with a view to assess a possible temporal and statistical association.

## MATERIAL AND METHODS

### Articles search

All studies included in the present review ( $n = 494$ ) were selected from the Medline database using the following subject headings: (1) EBV or Epstein-Barr or “EBV-associated membrane antigen, Epstein-Barr virus” [Substance Name] or “Epstein-Barr Virus Infections” [MeSH] or “Epstein-Barr viral capsid antigen” [Substance Name] or “Epstein-Barr virus early antigen” [Substance Name]; (2) virus OR (“Viruses” [MeSH] or “Virus Activation” [MeSH] or “DNA Tumor Viruses” [MeSH] or “Tumor Virus Infections” [MeSH]); (3) gastric or stomach or (“Stomach” [MeSH] or “Stomach Diseases” [MeSH]); (4) cancer or neoplasms OR “Neoplasms” [MeSH]. Furthermore, we also searched the reference list of all published articles.

### Inclusion/exclusion criteria

All original published studies in English, French, Spanish and Portuguese on EBV infection and gastric carcinoma were included in the analysis ( $n = 494$ ). Studies were excluded for the following reasons (Figure 1): studies referring to other types of cancer ( $n = 140$ ); use of languages such as Russian, Chinese, Japanese or Italian ( $n = 30$ ); articles other than those containing research data, such as reviews, letters to the editor and case-reports ( $n = 155$ ); *in vitro* and *in vivo* experiments ( $n = 81$ ); and studies referring to histological types other than gastric adenocarcinoma ( $n = 44$ ).

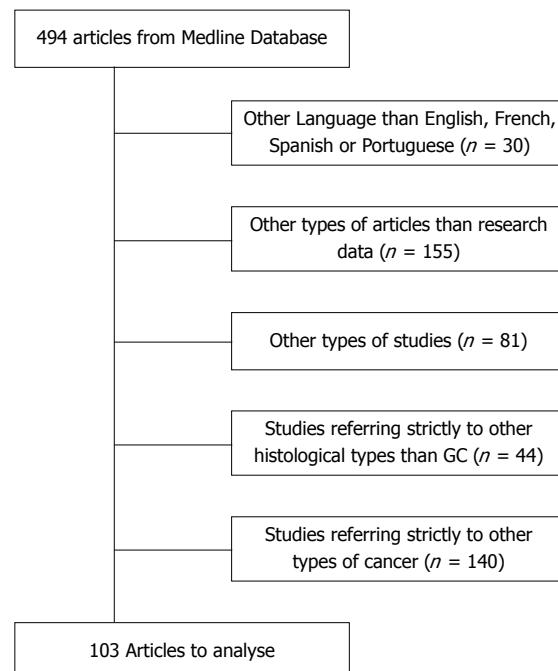
### Data extraction and statistical analysis

The studies included in the present analysis ( $n = 103$ ) were reviewed by all the authors, in order to obtain the necessary information, such as: design of study (case-control or retrospective), source of tissue sample (formalin embedded tissues, fresh tissues or peripheral blood samples), study population, geographical distribution, histological type of the lesion and EBV diagnostic methodology (*in situ* hybridization, Polymerase Chain Reaction, immunohistochemistry and PCR followed by Enzyme Linked Immunoassay). Pooled frequencies were estimated by country, region and continent and were adjusted for the type of cancer, sample source and EBV diagnostic methodology.

## RESULTS

### General observations

Our study included a total of 103 published articles which analysed the frequency of EBV in GC in 21 different countries: 9 from Asia (China, India, Japan, Taiwan, Korea, Papua New Guinea, Pakistan, Kazakhstan and Turkey), 7 from Europe (UK, France, Germany, Italy, Netherlands, Poland and Russia) and



**Figure 1** Application of Inclusion/exclusion criteria.

5 from America (USA, Mexico, Brazil, Chile and Colombia).

### Study design

Our study material consisted of 96 retrospective studies, 6 prospective studies and one case-control study.

### Sample source

The study samples comprised of the following: 92 studies were conducted on formalin embedded tissues, 10 on fresh tissues and one on peripheral blood samples.

### EBV diagnostic methodology

The most common methodology used for the diagnosis of EBV was *In Situ* Hybridization (ISH) for EBER1 ( $n = 88$ ), followed by Polymerase Chain Reaction (PCR) for the BamHI-W region of EBV ( $n = 6$ ), PCR for EBNA1 ( $n = 3$ ), immuno-histochemistry for LMP1 ( $n = 2$ ), PCR followed by Enzyme Immunoassay (PCR-EIA) for BamHI-W, and ISH for BamHI-W in one study each. In two studies, it was not possible to determine the methodology used for EBV detection.

### Statistical analysis

A total of 114 studies, comprising of 33471 cases were evaluated. The great majority of studies were conducted in Asian countries ( $n = 77$ ) with a total of 29076 individuals, followed by Europe ( $n = 18$ ) with 2296 individuals and America ( $n = 19$ ) with 2099 individuals (Table 1). With respect to the diffuse type of GC, a total of 5846 cases were analyzed, of which 4862 individuals were from Asian countries, 173 from Europe and 811 from America (Table 1). By contrast, intestinal type of GC was reported in 7786 cases, including 6367 individuals from Asia, 338 from Europe and 1082 from

**Table 1** Distribution of Epstein-Barr virus-association with gastric adenocarcinoma

	Gastric adenocarcinoma				Gastric adenocarcinoma intestinal type				Gastric adenocarcinoma diffuse type						
	n	Total	EBV +	Crude frequency (%)	Adjusted frequency (%)	n	Total	EBV +	Crude frequency (%)	Adjusted frequency (%)	n	Total	EBV +	Crude frequency (%)	Adjusted frequency (%)
EUROPE	18	2296	201	8.75	7.96	4	338	27	7.99	2.94	4	173	9	5.2	7.83
East Europe	4	293	43	14.7	26.3	1	13	0	0	---	1	19	4	21.1	---
Poland	3	87	25	28.7	40	1	13	0	0	---	1	19	4	21.1	---
Russia	1	206	18	8.74	---	---	---	---	---	---	---	---	---	---	---
Central Europe	13	1938	154	7.95	7.58	3	325	27	8.31	2.94	3	154	5	3.25	4.55
United Kingdom	4	682	12	1.76	1.7	---	---	---	---	---	---	---	---	---	---
France	1	59	5	8.47	---	1	21	0	0	---	1	22	1	4.55	---
Germany	1	35	3	8.57	---	1	17	1	5.88	---	1	18	2	11.1	---
Netherlands	7	1162	134	11.5	7.58	1	287	16	9.06	---	1	114	2	1.75	---
South Europe	1	65	4	6.15	---	---	---	---	---	---	---	---	---	---	---
Italy															
ASIA	77	29076	2324	7.99	9.65	21	6367	430	6.75	5.82	21	4862	441	9.07	11.2
South-East Asia	72	28760	2253	7.83	9.56	19	6290	414	6.58	5.82	18	4665	398	8.53	6.89
Taiwan	3	486	77	15.8	20	2	129	23	17.8	19	2	103	24	23.3	21.2
Papua New Guinea	1	150	2	1.33	---	1	72	0	0	---	1	78	2	1.33	---
Korea	13	4784	300	6.27	5.7	2	233	4	1.72	1.71	2	319	22	6.9	6.89
Japan	45	21956	1750	7.97	11.6	11	5480	353	6.44	5.82	11	4011	346	8.63	11.2
China	10	1384	124	8.96	7.74	3	376	34	9.04	10.7	2	154	4	3.25	1.43
South Asia	2	112	21	18.8	17.6	---	---	---	---	---	1	70	8	11.4	---
India	1	60	20	33.3	---	---	---	---	---	---	1	70	8	11.4	---
Pakistan	1	52	1	1.92	---	---	---	---	---	---	---	---	---	---	---
Central-western Asia	3	204	51	25	10.8	2	77	16	20.8	26.9	2	127	35	27.6	37.7
Kazakhstan	2	139	14	10.1	9.89	1	48	1	2.08	---	1	91	13	14.3	---
Turkey	1	65	37	56.9	---	1	29	15	51.7	---	1	36	22	61.1	---
AMERICA	19	2099	249	11.9	11.3	14	2082	95	4.56	8.3	13	811	124	15.3	13.3
South America	8	927	124	13.4	12.2	7	503	47	9.34	8.77	6	381	75	19.7	18.8
Colombia	2	294	31	10.5	9.92	1	91	11	10.5	---	1	86	12	14	---
Chile	1	278	53	20.2	---	2	178	23	12.9	14.5	3	193	52	26.9	29.6
Brazil	4	355	40	11.3	11.3	4	234	13	5.56	4.35	2	102	11	10.8	6.55
Central America	4	630	55	8.73	9.86	3	241	8	3.32	2.84	3	319	38	11.9	12.8
Mexico															
North America	7	542	70	12.9	10.2	4	338	40	11.8	10.8	4	111	11	9.91	8.63
United States															
GLOBAL ANALYSIS	114	33471	2775	8.29	9.67	39	7786	552	7.08	5.88	38	5846	574	9.82	12.1

Distribution of EBV association with Gastric Adenocarcinoma.

America (Table 1).

The largest sample size for gastric adenocarcinoma was obtained from South-east Asia (28760 cases), followed by Central Europe (1938 cases) and South America (927 cases). With respect to the intestinal and diffuse types of GC, the largest sample was from South-east Asia (6290 and 4665 cases respectively), followed by South America (503 and 381 studies respectively) and Central Europe (325 and 154 studies, respectively).

Table 1 shows the crude and adjusted frequency of positive EBV results in different countries considering the world as a discrimination factor. The worldwide crude EBV positive prevalence was 8.29% for gastric adenocarcinoma, with 7.08% for intestinal type and 9.82% for diffuse type. The corresponding adjusted prevalence was estimated as 9.67%, 5.88% and 12.1%, respectively. The adjustment of EBV positive prevalence resulted in minor changes from the crude estimates. America registered the highest adjusted EBV positive prevalence for both gastric adenocarcinomas and the diffuse type of GC (11.3% and 15.3%, respectively). On the other hand, Europe had the lowest adjusted EBV

positive prevalence for gastric adenocarcinoma, and intestinal and diffuse types of GC (7.96%, 2.94%, and 7.83%, respectively).

## DISCUSSION

As a Group I carcinogenic agent, EBV has been intensively studied over the past 40 years<sup>[9]</sup>. It has been demonstrated consistently that EBV has an association with nearly 100% of gastric lymphoepithelioma-like carcinomas<sup>[10-12]</sup>, which are believed to have similar pathogenic mechanism as nasopharyngeal carcinoma<sup>[7]</sup>.

Despite the presence of several hundred published studies demonstrating an association between EBV and GC, the pathogenic role of EBV in gastric carcinogenesis remains to be established<sup>[7]</sup>. Nevertheless, there is strong evidence to directly implicate EBV in GC development: presence of EBV in all cancer cells detected by *in situ* hybridization of EBER1, but not in the surrounding epithelial cells<sup>[13]</sup>; monoclonality of the viruses in neoplastic cells as judged by the analysis of single terminal repeats of EBV DNA<sup>[14]</sup>; and elevation of immunoglobulin A and B antibodies against viral

capsid antigen several months before the clinical presentation of the disease<sup>[15]</sup>.

The pathogenic mechanism of EBV-GC is not well understood. EBV is transmitted through the saliva, and primary infection occurs at the oropharyngeal mucosa, with the involvement of the Waldeyer's ring followed by infection of naive B-cells as they circulate near the infected cells<sup>[16]</sup>. After infection, EBV establishes a persistent state in the B-cells, and is maintained *ad eternum*. From time to time, the infected B-cells become activated as a result of immune suppression of the host, and the infected cells are able to enter other tissues<sup>[16]</sup>. Since the main receptor (CD21/CR2, the receptor for the C3d component of complement) for EBV is normally absent from gastric epithelial cells<sup>[17,18]</sup>, EBV infection has been suggested to be accomplished by the binding of EBV particles with IgA antibodies which are then engaged by gastric epithelial cells<sup>[7,19]</sup>. Once inside the gastric cells, EBV activates its latency state by expressing latency proteins which induce cell proliferation and viral maintenance. Recent studies have shown that in gastric carcinoma, EBV expresses a different pattern of proteins than those seen in Burkitt's lymphoma and nasopharyngeal carcinoma, suggesting a different oncogenic mechanism in gastric carcinoma<sup>[20,21]</sup>.

The development of gastric carcinoma is a multistep event, progressing from normal to preneoplastic lesions to highly malignant tumours<sup>[20]</sup>. Despite strong evidence implicating *H pylori* as the main etiological factor for GC development, several other environmental and genetic co-factors have been reported<sup>[22]</sup>. At present, it is generally accepted that EBV infects gastric cells depending on a permissive environment and a genetically susceptible host, upon an inflammatory state of gastric epithelium, which is in agreement with the findings that EBV-GC are monoclonal tumours<sup>[22]</sup>. Moreover, there are clinico-pathological features that are seen consistently in EBV-GC, such as: higher prevalence in males, with no impact on stromal invasion or survival<sup>[11,12,23]</sup>; tumour histology varying from moderately differentiated tubular to poorly differentiated solid type<sup>[17,24-29]</sup>; and a predisposition for the upper two-thirds of the stomach.

By performing a systematic review of all published studies worldwide, we have attempted to analyze the role of EBV in GC development, by estimating and comparing the worldwide occurrence of EBV-GC and by elucidating their temporal relationship and statistical association.

Our study included 103 published reports from 21 different countries distributed over 3 continents (Europe, Asia and America). Gastric adenocarcinoma is the second most common cause of cancer-related mortality worldwide and is the 14th overall cause of death<sup>[2]</sup>. Worldwide, there are widespread differences in gastric cancer rates, with the highest rates seen in Japan, China and South America, and much lower rates in Western Europe and the United States<sup>[2,22]</sup>. As expected, the great majority of studies were conducted in Asian countries, with 80 general reports on EBV and gastric adenocarcinoma, 20 on diffuse type and 21 on intestinal

type of GC. The crude frequency of EBV positive cases in Asian countries was 8.07%, 9.13% and 6.73% for GC, diffuse type and intestinal type, respectively. Moreover, western and central Asian countries had significantly higher frequency of EBV positive cases than south-eastern countries.

Despite these studies, the exact association between EBV and GC remains to be established. Moreover, recent data reinforces the importance of the differences in the association of EBV with gastric carcinoma among different ethnic communities<sup>[30,31]</sup>, with respect to temporal relationship, histological association and the application of sensitive methodologies for EBV diagnosis in GC. Nevertheless, the present review emphasizes the need for further studies designed to elucidate EBV-GC carcinogenesis model.

## COMMENTS

### Background

Gastric adenocarcinoma is a major public health problem worldwide. Epstein-Barr Virus (EBV) infects over 90% of population worldwide and has been linked as a late event in the gastric carcinogenesis process, after *H pylori* infection.

### Research frontiers

Despite evidence to suggest the existence of biological plausibility, there is lack of information about the role of EBV in gastric cancer, either as a risk factor for the development of cancer or as a predictive marker for major outcomes after medical therapy.

### Innovations and breakthroughs

A systematic review was conducted on 103 studies from 21 different countries representing 33471 cases of cancer. Most of the studies ( $n = 96$ ) were retrospective and used formalin embedded tissues ( $n = 92$ ) and used In Situ Hybridization (ISH) for EBER1. The adjusted prevalence of EBV was 9.67%, 5.88% and 12.1%, for gastric adenocarcinoma, intestinal type and diffuse type, respectively. America registered the highest adjusted EBV prevalence (11.3% and 15.3%, respectively) for both gastric adenocarcinomas and diffuse type. By contrast, Europe had the lowest adjusted EBV prevalence (7.96%, 2.94%, and 7.83%, respectively), for gastric adenocarcinoma, intestinal and diffuse type.

### Applications

Differences in EBV prevalence may provide further insight into research on gastric carcinogenesis and the natural history of this disease.

### Peer review

The present study is relevant as it emphasizes the lack of prospective studies and the absence of methodologies that can discriminate past from current viral infection. The focus of research and clinical application may become relevant in future studies.

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RAPID COMMUNICATION

# Postoperative change of anti-Thomsen-Friedenreich and Tn IgG level: The follow-up study of gastrointestinal cancer patients

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## Abstract

**AIM:** To study the influence of tumor removal on the serum level of IgG antibodies to tumor-associated Thomsen-Friedenreich (TF), Tn carbohydrate epitopes and xenogeneic  $\alpha$ Gal, and to elucidate on the change of the level during the follow-up as well as its association with the stage and morphology of the tumor and the values of blood parameters in gastrointestinal cancer.

**METHODS:** Sixty patients with gastric cancer and 34 patients with colorectal cancer in stages I -IV without distant metastases were subjected to follow-up. The level of antibodies in serum was determined by the enzyme-linked immunosorbent assay (ELISA) using synthetic polyacrylamide (PAA) glycoconjugates. Biochemical and haematological analyses were performed using automated equipment.

**RESULTS:** In gastrointestinal cancer, the TF antibody level was found to have elevated significantly after the removal of G3 tumors as compared with the preoperative level ( $\mu = 278.5, P < 0.05$ ). After surgery, the TF and Tn antibody level was elevated in the majority of gastric cancer patients (sign test, 20 vs 8,  $P < 0.05$ , and 21 vs 8,  $P < 0.05$ , respectively). In gastrointestinal cancer, the elevated postoperative level of TF, Tn and  $\alpha$ Gal antibodies was noted in most patients with G3 tumors (sign test, 22 vs 5,  $P < 0.01$ ; 19 vs 6,  $P < 0.05$ ; 24 vs 8,

$P < 0.01$ , respectively), but the elevation was not significant in patients with G1 + G2 resected tumors. The postoperative follow-up showed that the percentage of patients with G3 resected tumors of the digestive tract, who had a mean level of anti-TF IgG above the cut-off value (1.53), was significantly higher than that of patients with G1 + G2 resected tumors ( $\chi^2 = 3.89$ , all patients;  $\chi^2 = 5.34$ , patients without regional lymph node metastases;  $P < 0.05$ ). The percentage of patients with a tumor in stage I, whose mean anti-TF IgG level remained above the cut-off value (1.26), was significantly higher than that of patients with the cancer in stages III-IV ( $\chi^2 = 4.71$ , gastric cancer;  $\chi^2 = 4.11$ , gastrointestinal cancer;  $P < 0.05$ ). The correlation was observed to exist between the level of anti-TF IgG and the count of lymphocytes ( $r = 0.517, P < 0.01$ ), as well as between the level of anti-Tn IgG and that of serum CA 19-9 ( $r = 0.481, P < 0.05$ ). No positive delayed-type hypersensitivity reaction in skin test challenges with TF-PAA in any of the fifteen patients, including those with a high level of anti-TF IgG, was observed.

**CONCLUSION:** The surgical operation raises the level of anti-carbohydrate IgG in most patients, especially in those with the G3 tumor of the gastrointestinal tract. The follow-up demonstrates that after surgery the low preoperative level of TF antibodies may be considerably increased in patients with the carcinoma in its early stage but remains low in its terminal stages. The stage-and morphology-dependent immunosuppression affects the TF-antibody response and may be one of the reasons for unresponsiveness to the immunization with TF-antigens.

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**Key words:** IgG antibodies; Thomsen-Friedenreich; Tn;  $\alpha$ Gal; Gastrointestinal cancer; Immunosuppression; CA 19-9

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## INTRODUCTION

Cancer immune surveillance is considered to be important in the anti-tumor protection of the host. However, immunity not only protects the host from cancer but also may promote tumor growth, creating an immuno-resistant cancer cell phenotype ("cancer immunoediting"). In the advanced stages of cancer, tumor escapes the immune control under the immunosuppressive conditions<sup>[1]</sup>. The removal of the tumor appears to disturb the immunoediting process and may reverse immunosuppression. The suppressed antigen-specific antibody responses in tumor-bearing mice may be reversed after the surgical removal of the primary tumor even in the existence of a disseminated metastatic disease<sup>[2]</sup>.

Mucin-type tumor-associated carbohydrate antigens (TACA), the Thomsen-Friedenreich (TF) antigen and its precursor, Tn, are frequently expressed in malignant tumor cells. They deserve to be studied as targets for active specific immunotherapy<sup>[3]</sup>. Human blood serum contains natural TF- and Tn antibodies whose subpopulations may bind the corresponding antigens on human tumor cell lines<sup>[3-5]</sup>. It is not yet clear which role antibodies play in the natural anti-cancer defense system. The level of TF- and Tn antibodies was significantly decreased in the serum of primary (not operated) patients with cancer, including patients with the disease in its early stage<sup>[6-9]</sup>. Furthermore, the low level of anti-TF IgG was associated with a lower differentiated carcinoma and advanced gastric cancer that suggests an implication for antibodies in the progression and pathology of the tumor<sup>[10]</sup>. The high level of anti-TF IgG in the serum of primary patients with gastric cancer is closely associated with survival<sup>[11]</sup>. The dynamic changes of the level of TF- and Tn-antibodies in the serum of patients with cancer and its association with survival have been insufficiently studied. Investigations have mainly focused on clinical trials of antigen-specific immunotherapy<sup>[12,13]</sup>. The authors have undertaken a long-term follow-up of cancer patients to determine changes in the postoperative level of TF- and Tn antibodies, as well as to elucidate the association of this level with the progression of cancer, and survival. For comparison, the level of xenoreactive antibodies to the  $\alpha$ Gal epitope was determined. In humans, the  $\alpha$ Gal epitope is absent because the  $\alpha$ 1-3 galactosyltransferase gene was inactivated in evolution, but approximately 1% of immunoglobulins have an anti- $\alpha$ Gal specificity<sup>[14]</sup>.

In this work, the influence of the surgical removal of the tumor on the level of antibodies and its change during the follow-up was investigated. The association of the

level of antibodies with the stage and morphology of the tumor, as well as values of blood parameters was studied. Also, the delayed-type hypersensitivity reaction to TF-PAA conjugates in skin testing was examined.

## MATERIALS AND METHODS

### Subjects

The investigation was carried out in accordance with the ICH GCP Standards and approved by Tallinn Medical Research Ethics Committee. Informed consent was obtained from subjects under study. The follow-up study was undertaken of patients with a verified diagnosis of gastric ( $n = 60$ ) and colorectal cancer ( $n = 34$ ) of stages I-IV by using the pTNM system<sup>[15]</sup>. Patients with distant metastases of the cancer or those who received chemo- and X-ray therapy were not subjected to study. The median age of the patients was 60 years (the age ranging from 30 to 75 years). The venous blood samples were taken before and after surgery at intervals from three to sixteen months, with a further follow-up during two to twelve years. The extended D2 gastrectomy with lymphadenectomy or, additionally, with the splenectomy in gastric cancer, as well as the resection of local lesions of colorectal cancer was performed. In advanced cancer regional lymph node metastases were also removed. In some patients concomitant diseases were documented. Breast cancer was diagnosed in three, benign diseases, in five, anaemia and diabetes mellitus, in two cases. The other sporadic manifestations were Parkinson's disease, carcinoma of the uterus and chronic hepatitis.

### Glycoconjugates

Synthetic polyacrylamide (PAA) glycoconjugates with a single reiterated epitope were used in comparative immunoassays<sup>[16]</sup>. The homogeneity of PAA-conjugates enables a precise detection of epitope-specific antibodies. The following PAA-conjugates were used: the TF disaccharide, Gal $\beta$ 1-3GalNAc $\alpha$ ; T $\beta$  $\beta$ , Gal $\beta$ 1-3GalNAc $\beta$ ; Tn, GalNAc $\alpha$ ;  $\alpha$ Gal or a B-blood group disaccharide, Gal $\alpha$ 1-3Gal $\beta$ ; SiaLe<sup>a</sup> (CA 19-9 tetrasaccharide), Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-3 (Fuc $\alpha$ 1-4) GlcNAc $\beta$ . Tris-PAA, tris (hydroxymethyl) aminomethane-PAA, was used as a negative control because of its low background and good reproducibility in immunoassay<sup>[8]</sup>. The TF-PAA as a substituted PAA containing 0.1 mol of TF per 1 mol of PAA was used because of its elevated binding to human IgG antibodies. The rest of the polyacrylethanolamide-conjugates had 0.2 mol of a saccharide per 1 mol of PAA. All PAA-conjugates were received from Lectinity, Russia.

### The determination of the level of epitope-specific IgG antibodies in sera by enzyme-linked immunosorbent assay (ELISA)

The method has been described elsewhere<sup>[8]</sup>. The dilution of serum was 1:50-1:200. The antibody levels were calculated as a ratio  $A_{\text{test}}/A_{\text{control}}$  where  $A_{\text{test}}$  is the absorbance with PAA-glycoconjugate and  $A_{\text{control}}$ , with Tris-PAA. The variation coefficient was 3%. To diminish

**Table 1** The effect of gastrectomy on the antibody level in patients with gastric cancer

Detection of antibodies	Mean $\pm$ SD	Increase	Median of difference <sup>1</sup>	P	
				+	-
TF ( <i>n</i> = 28)			20	8	
Preoperative level	1.95 $\pm$ 1.86				
Postoperative level	2.66 $\pm$ 2.61	36.4%	0.28		< 0.05
Tn ( <i>n</i> = 29)			21	8	
Preoperative level	2.64 $\pm$ 2.24				
Postoperative level	3.36 $\pm$ 2.84	27.3%	0.38		< 0.05

<sup>1</sup>Values of the postoperative (the first analysis after surgery) minus preoperative antibody level; <sup>2</sup>The number of patients having the positive or negative difference.

the variation of the test, the serum samples taken before and after surgery were analyzed using the same plate.

### Clinical analysis of blood samples

Biochemical and hematological analyses were performed at the Oncological Centre of the North-Estonian Regional Hospital. The following automatic equipment was used: a Hitachi 912 and Elecsys 2010, Roche Diagnostics; a Sysmex XE-2100, Sysmex Corporation.

Blood samples were taken before and after surgical operation during the planned visits to the physician for health control. The antibody levels were correlated with those of the C-reactive protein (CRP), tumor markers (CA19-9, CEA), the alanine aminotransferase, glucose, haemoglobin, circulating red blood cells (count), leukocytes (count), neutrophils (%), monocytes (%), lymphocytes (%), platelets (count) and eosinophils (%). The concentration of CRP was determined by a turbidimetric method and that of tumor markers, by an electrochemiluminescence immunoassay.

### Skin test

Antigens: TF-PAA, *Mr* 30 and 1000 ku; T $\beta\beta$ -PAA, *Mr* 1000 ku. The antigens (50-100  $\mu$ g) were injected intradermally and the delayed-type hypersensitivity reaction was monitored twice: through 24 and 48 h. The reaction was considered positive if erythema > 5 mm was developed.

### Statistical analysis

The Mann-Whitney (U-test), the sign test with a null hypothesis (median = 0) for a paired-sample comparison, the Chi-squared test and the regression analysis were used in the study. The differences were considered significant when *P* < 0.05. The graphs were plotted by means of a SigmaPlot 2000 program and Statgraphics Plus 5.1.

## RESULTS

### The influence of the removal of the tumor on antibody levels

To investigate the effect of the surgical removal of the

tumor on antibody levels, the serum samples taken before and after surgery were analyzed. The subtracted values of the postoperative minus preoperative level of antibodies varied and showed an abnormal distribution, however, were mostly positive. The median of differences was also positive (Tables 1 and 2). A sign test for a paired-sample comparison showed the postoperative level of TF- and Tn antibodies to have significantly increased in patients with gastric cancer. Patients with gastric cancer, whose postoperative level of TF- and Tn antibodies was higher than the preoperative one, predominated significantly over those having a lower postoperative level (Table 1, *P* < 0.05). In gastrointestinal cancer, the TF antibody level was found to have elevated significantly after the removal of G3 tumors as compared with preoperative level (median 1.42 and 1.23, respectively, *n* = 278.5, *P* < 0.05). The elevation of the level of anti-Tn and  $\alpha$ Gal IgG after surgery was not significant in U-test owing to the variation in levels. However, the paired-sample sign test demonstrated a significant (more than 75%) predominance of patients having a postoperative elevation of TF, Tn or  $\alpha$ Gal antibody level over those having a lower postoperative level after resection of G3 tumors. These differences were not significant in patients with G1 and G2 resected tumors (Table 2). The level of all three antibodies was increased after surgery in 12% (mainly patients having G3 tumors) and reduced in 4% of patients with gastrointestinal cancer. The change of antibody levels was not associated with the transfusion of erythrocytes after surgery.

### The postoperative follow-up

The clustered distribution of the combined pre- and postoperative level of antibodies was observed. The clusters were separated with the following cut-off values: TF 1.26, 1.53; Tn 1.88, 2.38;  $\alpha$ Gal 2.18; 2.80. Patients whose level of antibodies was higher than the first cut-off value were considered responders. The follow-up showed that the percentage of patients with G3 resected tumors of the digestive tract, whose individual mean level of anti-TF IgG remained above the cut-off value, exceeded significantly the percentage of patients with G1 + G2 resected tumors (Table 3). In the case of the other antibodies these differences were not observed.

In gastric or gastrointestinal cancer, the percentage of anti-TF IgG responders was significantly higher in stage I than in stages III-IV (Table 4). In gastric cancer without metastases (N0 in stages I-II vs N1 + N2) the percentage of anti-TF IgG responders tended to increase. The proportion of Tn- or  $\alpha$ Gal-responders was not increased in stage I vs stages III-IV of the disease.

It was established that despite the elevation of anti-TF IgG level after surgery of patients with advanced cancer, its level during the follow-up varied only slightly, remaining mostly low (Figure 1).

### The relation of antibody levels to the values of blood parameters

Linear regression analysis showed an existing correlation

**Table 2** Postoperative changes of the level of antibodies in gastrointestinal tumor of different grades

Grade <sup>1</sup>	Detection of antibodies	Mean ± SD	Increase	Median of difference	Difference	P (sign test)
					+	-
G1 + G2 (n = 24)	TF, preoperative	2.03 ± 2.04				
	postoperative	2.43 ± 2.86	19.7%	0.05	15	9
G3 (n = 27)	TF, preoperative	1.84 ± 1.71				
	postoperative	3.03 ± 3.20	64.7%	0.29	22	5
G1 + G2 (n = 30)	Tn, preoperative	2.46 ± 1.80				
	postoperative	2.99 ± 2.45	21.5%	0.12	18	12
G3 (n = 25)	Tn, preoperative	2.45 ± 2.18				
	postoperative	3.15 ± 2.92	28.6%	0.42	19	6
G1 + G2 (n = 32)	αGal, preoperative	4.10 ± 2.55				
	postoperative	4.62 ± 3.20	12.7%	0.17	20	12
G3 (n = 32)	αGal, preoperative	5.09 ± 3.34				
	postoperative	6.61 ± 3.70	29.9%	1.20	24	8

<sup>1</sup>G1+G2-well-and moderately-differentiated carcinomas.

**Table 3** The percentage of patients with the mean level of anti-TF IgG above the cut-off value (1.53) by histological grading: The postoperative follow-up study

Cancer	G1 + G2		G3		$\chi^2$	P
	n	> cut-off	n	> cut-off		
Gastric, all	22	9.1%	38	26.3%	2.58	0.108
Gastric, without metastases (N0)	16	6.2%	22	31.8%	3.64	0.056
Gastrointestinal, all	47	8.5%	47	23.4%	3.89	0.049
Gastrointestinal (N0)	35	5.7%	26	26.9%	5.34	0.021

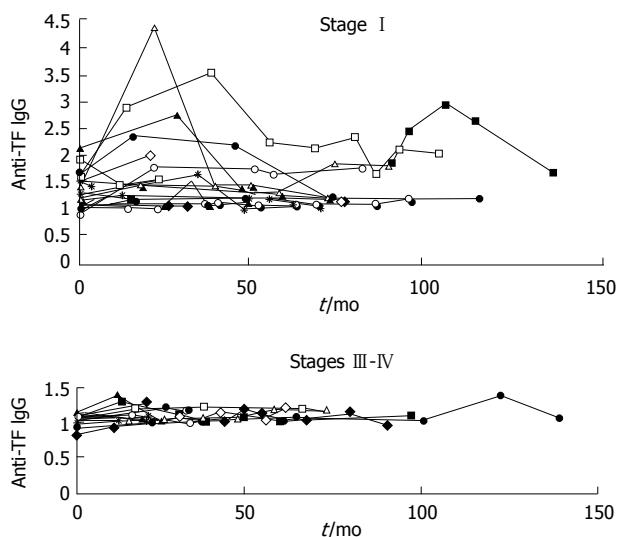
**Table 4** The percentage of patients with the mean level of anti-TF IgG above the cut-off value of 1.26 (responders) in relation to cancer progression: The postoperative follow-up study

Cancer	n	> cut-off	$\chi^2$	P
Gastric, N0	38	39.5%	2.92	0.088
Gastric, N1 + N2	22	18.9%		
Gastric, stage I	24	41.7%	4.71	0.03
Gastric, stages III-IV	18	11.1%		
Gastrointestinal, stage I	29	37.9%	4.11	0.043
Gastrointestinal, stages III-IV	28	14.3%		

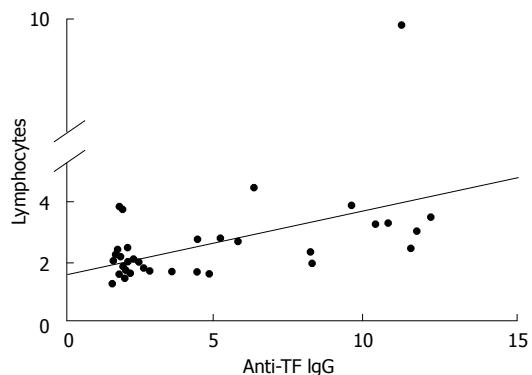
between the level of anti-TF IgG and the count of lymphocytes (Figure 2). As well, the correlation between the level of anti-Tn IgG and the serum level of tumor marker CA 19-9 was established:  $r = 0.481$ ,  $P = 0.011$ ,  $n = 27$  (Tn-responders);  $r = 0.495$ ,  $P = 0.002$ ,  $n = 36$  (all patients). No correlation between the level of antibodies and the values of other biochemical or hematological parameters was established.

### Skin test

No positive delayed-type hypersensitivity reaction in skin test challenges with TF-PAA in any of the fifteen patients, including those with a high level of anti-TF IgG, was observed. In some cases the weak erythema did not exceed 3 mm. Neither side effects nor complications were recorded.



**Figure 1** Changes of the level of anti-TF IgG in gastric cancer in stages I and III-IV during follow-up.



**Figure 2** The correlation between the level of anti-TF IgG and the count of lymphocytes ( $10^9/L$ ) in gastrointestinal cancer patients, whose anti-TF IgG level exceeded a cut-off value of 1.53.  $r = 0.517$ ,  $P = 0.001$ ,  $n = 37$ ,  $y = 0.215x + 1.380$ .

### DISCUSSION

The investigation of the level of serum IgG antibodies to tumor-associated carbohydrate epitopes (TF, Tn) and xenogeneic for human αGal epitope has been carried out earlier. The preoperative level of TF and Tn antibodies

was significantly lower in patients with gastrointestinal cancer, including stage I, than in blood donors, whereas the difference in the level of  $\alpha$ Gal IgG between cancer patients and blood donors was not significant<sup>[8]</sup>. The preoperative level of anti-TF IgG antibodies in patients with gastric carcinoma having regional lymph node metastases was found to be significantly lower than that in patients without metastases<sup>[10]</sup>.

In gastrointestinal cancer the level of TF antibodies in responders with regional metastases was increased after surgery but the follow-up showed the percentage of TF-responders to be significantly higher among patients with cancer in stage I (Table 4). This is due to a minor and short-term postoperative elevation of the antibody level in the serum of patients with lymph node metastases in stages III-IV of the disease. The level of anti-TF IgG in the serum of five patients with gastrointestinal cancer in stages II-IV, which was high prior to surgery rose even more after surgery and remained high for a long time (not shown). However, in most patients the TF IgG immune response in the terminal stages of the disease was suppressed whereas in stage I both the stimulation and suppression of the immune response took place (Figure 1). Since with cancer progression no significant differences were observed in the level of Tn and  $\alpha$ Gal antibodies, the immunosuppression associated with advanced cancer concerns TF antibodies mainly. In this study, the level of  $\alpha$ Gal antibodies was determined because their low level may be indicative of humoral immunodeficiency disorders<sup>[17]</sup>. However, the immunosuppression associated with advanced gastrointestinal cancer appeared not to affect the level of  $\alpha$ Gal antibodies. In gastric cancer the higher level of anti- $\alpha$ Gal IgG was observed in patients with a larger tumor<sup>[10]</sup>. In patients with pancreatic cancer, who often suffer from severe immunosuppression<sup>[18]</sup>, a high level of anti- $\alpha$ Gal IgG was observed as well.

The positive correlation between the level of TF antibodies and the count of lymphocytes in TF-responders ( $n = 57$ ,  $r = 0.400$ ,  $P = 0.002$ ) appeared to reflect the adaptive immune response and provided a further explanation for the involvement of anti-TF IgG in cancer-associated immunosuppression. In patients whose level of anti-TF IgG was higher than the second cut-off value (1.53, Figure 2) this correlation was more pronounced. However, during the follow-up of some patients no such correlation was observed. This is indicative of the existence of a complex relationship between both the parameters which depend on pathological conditions. A decreased preoperative count of lymphocytes and postoperative surgery-related lymphocytopenia occurred in patients with gastrointestinal cancer. Besides, the T helper deficiency was more frequently observed in patients with regional nodal metastases<sup>[18]</sup>.

The above findings are supportive of the idea that patients with a disease of an early stage and its minimal residue after surgery are more responsive to active immunotherapy. The pre- and postoperative level of TF-antibodies, the pattern of TF-expression in tumor, and the individual profile of immune response to the immunization with TF-antigen should be taken into

account when selecting the contingent for immunotherapy.

The postoperative elevation of the level of carbohydrate-specific antibodies, viz. Forssman antibodies, has been documented earlier<sup>[19,20]</sup>. In our study, the postoperative elevation of antibody levels was found to be related to the low-differentiated carcinoma. Thus, the levels of all carbohydrate-specific antibodies, including those investigated earlier<sup>[21]</sup>, were elevated in most patients after the surgical removal of G3 tumors, while in patients with G1 and G2 resected tumors, the differences in antibody levels before and after surgery were insignificant (Table 2).

In the preoperative examination of patients with gastrointestinal cancer, significant differences were established in the level of anti-TF IgG between them, namely, its lower level was associated with a lower-differentiated carcinoma in stages I-II<sup>[10]</sup>. These lower levels were increased significantly after surgery as shown in the present study. Moreover, the significant preponderance of patients with an elevated postoperative level of anti-TF IgG in the case of G3 resected tumors over those with G1 + G2 resected tumors was observed during the follow-up.

In general, the study shows that in most patients with the low-differentiated carcinoma the lower preoperative level of TF antibodies increases after surgery and has a tendency to remain elevated in the early stages of the cancer.

Taken together, the above results may be interpreted as follows: (1) A specific suppressive influence of the tumor on the production of TF antibodies is associated with the stage and grade of the tumor; the surgical removal of the primary tumor (especially G3 tumors) with lymphadenectomy may reverse the suppression of TF antibodies in the early stage of the disease; (2) The mainly low-differentiated carcinoma has an unspecific suppressive influence on the production of anti-carbohydrate antibodies. This influence may also be reversed by the removal of the tumor.

The specificity of human anticarbohydrate antibodies and their natural targets have been poorly studied. Natural anti-carbohydrate immunoglobulins are mostly antibodies of IgM-class. The high level of anti-TF and Tn IgG observed in some patients with cancer may be a sign of an acquired immune response which is indicative of the switching of antibody to the IgG-class. The anti-TF and anti-Tn IgG were affinity-purified from the serum of patients by using synthetic TF- and Tn-PAA sorbents<sup>[22,23]</sup>. TF antibodies demonstrated a high activity of binding to mucins isolated from human malignant tumors, but only in 15% of tumor extracts, whereas the high activity of Tn antibodies was not observed. The analysis of the specificity of purified anti-TF IgG, the mutual and complete inhibition of serum antibodies by TF $\alpha$  and TF $\beta$  conjugates, and a good correlation between the levels of anti-TF $\alpha$  and anti-TF $\beta$  IgG in sera manifest that human anti-TF IgG is specific to both TF $\alpha$  and  $\beta$  anomers, with preference to the latter<sup>[23]</sup>. In this respect, antibodies resemble human monoclonal antibodies, which are able to bind different carcinoma cell lines and immunostained

mucin-related tumor tissues<sup>[24]</sup>.

The level of anti-Tn IgG is correlated with that of CA 19-9 in the serum. This seems to be unusual because the CA 19-9 antigen (SiaLe<sup>a</sup>) differs structurally from Tn and contains no GalNAc residues. The correlation is not due to the cross-reactivity of antigens because the affinity-purified anti-Tn IgG did not react with SiaLe<sup>a</sup>-PAA in the ELISA. This correlation may be explained by a similar relationship between both independent parameters and pathological conditions. Cancer progression or disorders of excretion (cholestasis and other disorders) may provoke the elevation of the level of CA 19-9<sup>[25]</sup> and Tn antibodies, respectively. Since the tumor marker CA 19-9 is a prognostic factor in colorectal cancer<sup>[26]</sup>, the correlation between parameters may be indicative of the possible prognostic significance of antibodies as well.

Synthetic TF and Tn glycoconjugates deserve to be studied from a viewpoint of development of anti-cancer vaccines<sup>[12,13]</sup>. Synthetic PAA-glycoconjugates may be promising preparations because they have been described well and can be modified by epitope density or supplemented with the amplifier of immune response and synthesized in necessary quantities. The results of the skin test performed by the authors show the TF-PAA to be a safe and non-toxic glycoconjugate. The lack of the delayed-type hypersensitivity reaction indicates that the TF antibody response took place through the T-cell independent mechanism, which is typical of carbohydrate antigens.

The high preoperative level of anti-TF as well as anti-MUC1 IgG was closely associated with the survival of patients with gastric cancer<sup>[11]</sup>. However, the possible protective mechanism of TF antibodies in cancer has yet remained unclear. The TF antigen seems to play a crucial role in the adhesion of cancer cells to the endothelium through the interaction of galectin-3<sup>[27,28]</sup>. We suppose that even if TF antibodies are not cytotoxic for TF-expressed tumor cells, they may exhibit an anti-adhesive effect by blocking the TF- galectin-3 mediated metastatic spread. Whether anti-TF and anti-Tn IgG are prognostic factors and how their level in the follow-up is associated with survival will be shown by further investigations.

## ACKNOWLEDGMENTS

We are grateful to Professor N Bovin for consultations and for providing us with synthetic glycoconjugates, to Dr. A Lipping for providing us with histological data, to R Syld for correcting the English and to A Kull for technical assistance.

## COMMENTS

### Background

Tumor-associated carbohydrate antigens (TACA), the Thomsen-Friedenreich (TF) antigen and Tn are frequently expressed in malignant tumors. Human blood serum contains TF- and Tn antibodies but it is not yet clear which role antibodies play in the natural anti-cancer defense system. The TF antigen seems to play a crucial role in the metastatic mechanism due to adhesion of cancer cells to the endothelium. The high level of TF antibodies in the serum may be favourable if antibodies could block the TF- mediated metastatic spread. The level of TF- and

Tn antibodies is significantly decreased in the serum of primary (not operated) patients with cancer. The high level of anti-TF IgG in the serum of primary patients with gastric cancer is closely associated with survival.

### Research frontiers

The relationship of the immune system with the tumor is far from a clear understanding. Cancer immune surveillance is considered to be important in the anti-tumor protection of the host. The growing tumor escapes the immune control under the immunosuppressive conditions. The surgical removal of the tumor may reverse the immunosuppression.

### Innovations and breakthroughs

The dynamic changes of the level of TF- and Tn-antibodies in the serum of patients with cancer have been insufficiently studied. Investigations have mainly focused on short-term clinical trials of antigen-specific immunotherapy. Authors have undertaken a long-term follow-up of cancer patients to determine changes in the postoperative level of TF- and Tn antibodies, as well as to elucidate the association of this level with the progression of cancer, and survival. The association of the level of antibodies with the stage and morphology of the tumor, as well as values of blood parameters was studied. Also, the delayed-type hypersensitivity reaction to TF- polyacrylamide (PAA) conjugates in skin testing was examined.

### Applications

The patients with a disease of an early stage may be more responsive to TF-specific active immunotherapy. Synthetic TF and Tn glycoconjugates deserve to be studied from a viewpoint of development of anti-cancer vaccines and in diagnostic aims.

### Terminology

The epitope is an antigenic determinant, viz. saccharide. The PAA glycoconjugate is a polymeric molecule with covalently bound saccharide. The anomer ( $\alpha$  or  $\beta$ ) is a spatial structure of saccharides.

### Peer review

This is interesting and provocative work. The authors present most of their results in terms of changes in antibody levels. It would be important for them to state what the basal level of these antibodies is, and to give us some idea of what percentage change is represented.

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RAPID COMMUNICATION

## Procedure-related musculoskeletal symptoms in gastrointestinal endoscopists in Korea

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analogue score greater than 5.5. Factors related to the development of severe pain were (1) standing position during upper endoscopy, (2) specific posture/habit during endoscopic procedures, and (3) multiple symptomatic areas. Finger pain was more common in beginners, whereas shoulder pain was more common in experienced endoscopists. Sixteen percent of symptomatic endoscopists have modified their practice or reduced the number of endoscopic examinations. Only a few symptomatic endoscopists had sought professional consultation with related specialists.

**CONCLUSION:** The prevalence of musculoskeletal pain in endoscopists is very high. The location of pain was different between beginners and experienced endoscopists. Measures for the prevention and adequate management of endoscopy-related musculoskeletal symptoms are necessary.

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**Key words:** Endoscopy; Endoscopist; Musculoskeletal symptom

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### Abstract

**AIM:** To determine the prevalence and risk factors of work-related musculoskeletal disorders in gastrointestinal endoscopists in Korea.

**METHODS:** A survey of musculoskeletal symptoms, using a self-administered questionnaire, was conducted on 55 endoscopists practicing in general hospitals or health promotion centers.

**RESULTS:** Forty-nine (89.1%) endoscopists reported musculoskeletal pain on at least one anatomic location and 37 (67.3%) endoscopists complained of pain at rest. Twenty-six (47.3%) endoscopists had severe musculoskeletal pain defined as a visual

### INTRODUCTION

Muscle and joint pains are common complaints in individuals whose job requires repetitive isometric maneuvers or awkward body positions<sup>[1]</sup>. Musculoskeletal pains have been reported in individuals with different occupations such as bus drivers, unskilled laborers,

musicians, physical therapists, and computer keyboard operators<sup>[1-7]</sup>. Recently, ergonomic mechanisms related to the development of work-related musculoskeletal disorders (MSD) have drawn substantial interest<sup>[8-10]</sup>.

Work-related musculoskeletal symptoms are common in certain medical specialists such as laparoscopic surgeons and dentists<sup>[11,12]</sup>. However, there are very few studies on MSD in gastrointestinal endoscopists. The incidence of musculoskeletal injuries has been variously reported from as low as 13% for neck pain<sup>[13]</sup> to as high as 57% for back pain<sup>[14]</sup>. To the best of our knowledge, a detailed study in endoscopists' on the severity of musculoskeletal symptoms, symptom-related risk factors, and doctor's response to their own symptoms has not been performed in eastern countries. The purpose of the present study was to assess the prevalence, severity, risk factors, and clinical impact of work-related MSD in gastrointestinal endoscopists in Korea.

## MATERIALS AND METHODS

From June 2006 to September 2006, 55 endoscopists practicing in 4 general hospitals and 2 health promotion centers were included in the present study. Endoscopists expressed their willingness to participate and completed a self-reported questionnaire. The questionnaire was largely structured; although some questions were kept open. Data were collected on the age, gender, duration of endoscopy practice, underlying musculoskeletal disease, and postures and habits during endoscopy. Workload parameters included total duration of endoscopy practice, weekly working hours and monthly number of endoscopic procedures. The areas of musculoskeletal pain were marked on a figure of the human body, and the severity of pain at each site was expressed using a 100 mm visual analogue scale (VAS), a standard measurement tool in pain research. The presence of severe pain was defined as a VAS value greater than 55 mm<sup>[15,16]</sup>. The participants were also asked as to whether their symptoms affected their ability to perform endoscopic procedures, how they managed their symptoms, and whether they had endoscopy-related symptoms other than MSD.

Continuous data were expressed as mean  $\pm$  standard deviation (SD) or median with range. Categorical data analysis was conducted using the chi-square test. Continuous data were analyzed using the independent *t* test. All *P* values were 2-tailed and *P* values less than 0.05 were considered statistically significant.

## RESULTS

### General characteristics of the endoscopists

Fifty-five endoscopists (male 37, female 18) participated in the study. The median age was 39 years (range, 28-47 years), and the median duration of practicing endoscopy was 39 mo (range, 1-228 mo). The average procedure time per week was  $19.5 \pm 7.7$  h. The average number of endoscopies performed per month was  $270.2 \pm 153.2$ .

Eighty-three percent of the endoscopists reported possible endoscopy-related non-musculoskeletal symptoms, such as decreased visual acuity (63.6%), chronic fatigue (60%), depressive mood (18.2%), dizziness (14.5%), headache (12.7%), and skin allergy (1.8%).

### Prevalence of musculoskeletal pain among the endoscopists

Forty-nine (89.1%) endoscopists reported musculoskeletal pain on at least one anatomic location. The average number of symptomatic areas was  $3.9 \pm 2.8$ . Forty endoscopists (72.7%) had pain at more than one anatomic location. Thirty-seven endoscopists (67.3%) had pain at rest. The VAS value of the most painful area was  $5.4 \pm 2.2$ . The musculoskeletal pain developed at  $27.5 \pm 37.9$  mo (range, 1-156 mo) after starting endoscopy.

The location and incidence of musculoskeletal pain during endoscopic procedures or at rest are shown in Figure 1A. The most commonly reported painful area during endoscopic procedures was right shoulder, followed by left shoulder and left finger. There was little difference in the overall distribution of the painful areas at rest. However, neck pain and upper back pain were relatively frequent.

The most painful site during endoscopic procedures was the left finger (16.4%), followed by left shoulder and right wrist (14.5%), left wrist (9.1%) and right shoulder (7.3%). The left and right shoulder (12.7%) were the most painful areas at rest, followed by neck (9.1%), right upper back and lower back (5.5%).

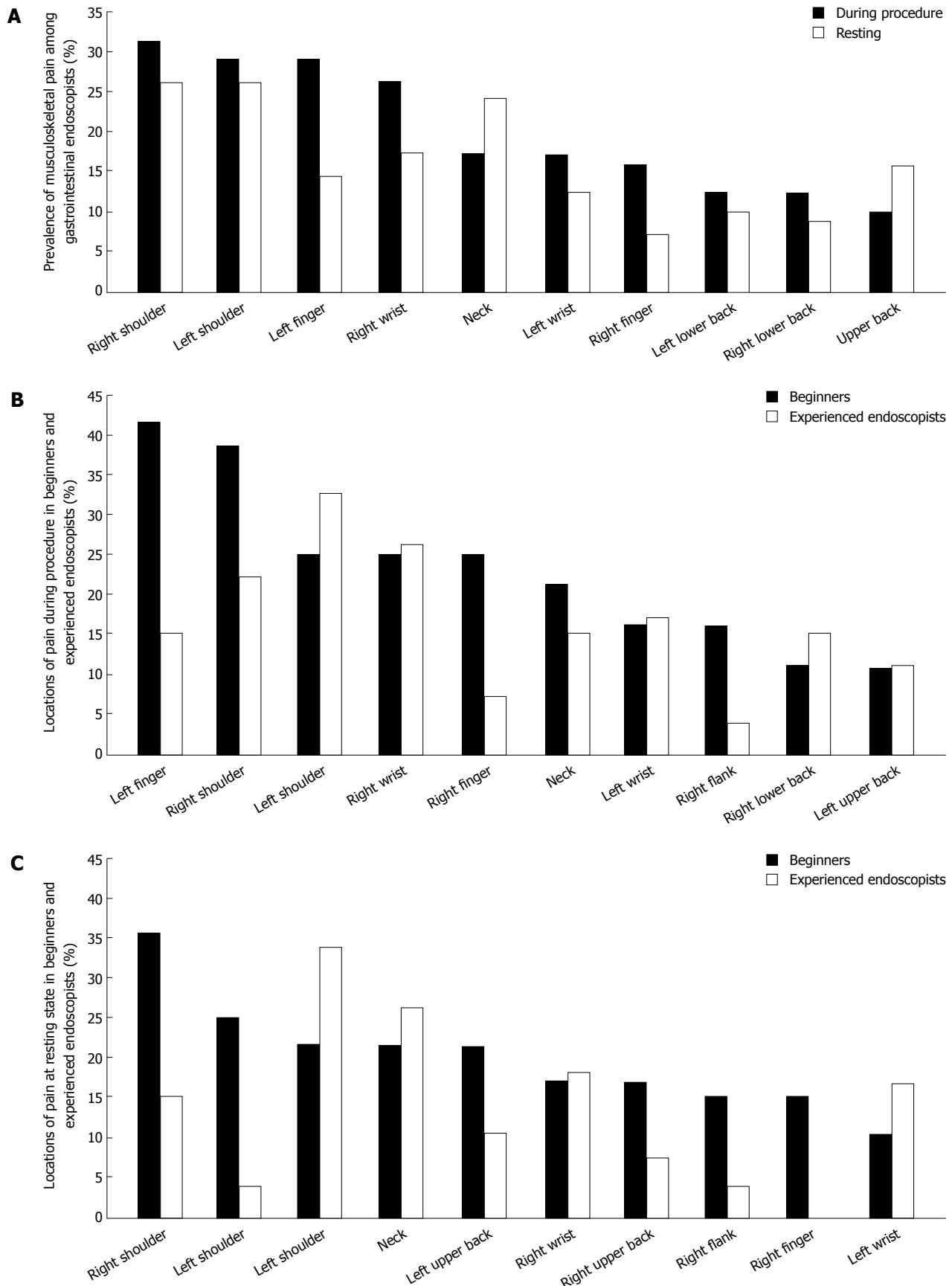
### Risk factors associated with severe musculoskeletal pain

Twenty-six endoscopists (47.3%) had severe musculoskeletal pain with a VAS value greater than 55 mm<sup>[15,16]</sup>. Three factors were statistically related to the development of severe musculoskeletal pain: (1) standing position during upper endoscopic procedures, (2) specific posture or habit during endoscopic procedures, and (3) multiple symptomatic areas (Table 1). The proportion of endoscopists with severe musculoskeletal pain was slightly higher in female than in male endoscopists (61.1% and 40.5%, respectively; *P* = 0.152).

### Comparison between beginners and experienced endoscopists

Endoscopists were divided into two groups (beginner versus experienced) by the total duration of practicing endoscopy (39 mo, Table 2). In the beginner group, the weekly procedure time was longer and the number of endoscopic examinations was greater. However, there was no significant difference in the prevalence of musculoskeletal pain, number of symptomatic areas, and VAS value of the most painful area between the two groups.

By contrast, the location of pain was different between the two groups (Figure 1B and C). During a



**Figure 1** A: Prevalence of musculoskeletal pain in gastrointestinal endoscopists in Korea. Right shoulder pain was the most common symptom both during endoscopic procedures and at rest; B: Prevalence of musculoskeletal pain in beginners and experienced endoscopists during endoscopic procedures ( $^aP < 0.05$ ); C: Prevalence of musculoskeletal pain in beginners and experienced endoscopists at rest.

procedure, the left finger was the most commonly reported painful area in beginners (42.9%, 12/28), whereas the

**Table 1 Comparison of demographic and procedure-related variables between endoscopists with or without severe (VAS > 5.5) musculoskeletal pain**

	<b>With severe musculoskeletal pain (n = 26)</b>	<b>Without severe musculoskeletal pain (n = 29)</b>	<b>P</b>
Age (median, range, yr)	34 (29-47)	36 (28-45)	
Sex			
Male	15	22	
Female	11	7	
Career			
Full time faculty	16	15	
Trainee	10	14	
Duration of practicing endoscopy (mean, range, mo)	40.5 (1-204)	39 (1-228)	
Posture in upper endoscopy			0.027
Mainly standing	25	21	
Mainly sitting	1	8	
Posture in colonoscopy			
Mainly standing	11	12	
Mainly sitting	15	17	
Specific posture or habit during endoscopy (%)	11 (68.8)	5 (31.3)	0.041
Number of endoscopies per month (mean ± SD)	280.5 ± 159.3	260.1 ± 149.7	
Time of endoscopic procedure per week (mean ± SD, h)	20.5 ± 8.1	17.9 ± 7.2	
Number of painful areas (mean ± SD)	4.8 ± 3.2	2.3 ± 2.1	0.002

**Table 2 Comparison of musculoskeletal pain and endoscopic workload between beginners and experienced endoscopists**

	<b>Beginners (n = 28)</b>	<b>Experienced endoscopists (n = 27)</b>	<b>P</b>
Pain over at least one anatomic location (%)	26 (92.8)	23 (85.2)	
Severe pain with VAS value > 5.5 (%)	15 (53.6)	13 (48.1)	
Multiple painful areas (%)	22 (78.6)	18 (66.7)	
Number of painful areas (mean ± SD)	3.9 ± 3.0	3.1 ± 2.9	
VAS value of the most painful area (mean ± SD)	4.9 ± 2.3	4.7 ± 3.1	
Duration of endoscopic procedure per week (mean ± SD, h)	23.7 ± 7.6	14.9 ± 5.2	0.001
Number of endoscopies per month (mean ± SD)	322.4 ± 159.9	216.1 ± 127.4	0.009

VAS: Visual analogue scale.

left shoulder was the most commonly reported painful area by experienced endoscopists (33.3%, 9/27) (Figure 1B). The distribution of the most painful area during endoscopic procedures was also different in the two groups. The most painful area was the left finger (21.4%, 6/28) in beginners, followed by right wrist (10.7%, 3/28), left shoulder (7.1%, 2/28) and left wrist (7.1%, 2/28). However, the most painful locations in experienced endoscopists were the left shoulder (22.2%, 6/27), followed by right wrist (18.5%, 5/27), left finger (11.1%, 3/27) and left wrist (11.1%, 3/27). The distribution of pain and the most painful areas at rest were very similar in the two groups (data not shown).

#### **Response of endoscopists to the musculoskeletal pain**

Among endoscopists with musculoskeletal pain, 16.3% (8/49) reported that they had modified their practice or reduced the number of the endoscopic procedures. A majority (81.6%) of endoscopists with musculoskeletal pain managed their symptoms by themselves using stretching (67.3%), exercising (8.2%) and rest (6.1%). Fourteen endoscopists (28.6%) reported the use of medications such as nonsteroidal anti-inflammatory drugs or topical analgesic patches.

Only 14.3% (7/49) of symptomatic endoscopists had sought advice from specialists on musculoskeletal

disorders or had undergone a specific diagnostic work-up. Three endoscopists were diagnosed to have a sprain ( $n = 1$ ) or a cervical intervertebral disk herniation ( $n = 2$ ). In the two endoscopists with herniated disc, one required a 4-wk sick leave until the symptoms improved and the other had modified the practice and reduced the number of endoscopic procedures.

#### **DISCUSSION**

Work-related MSDs or overuse syndrome are a group of diseases resulting from repetitive action at the work place<sup>[17]</sup>. Collagen failure and connective tissue damage results in inflammation, pain, and further weakening of the tissues. Such a vicious cycle can lead to permanent injury and disability if the tissues are not allowed to heal properly<sup>[13]</sup>. Work-related injury is an important cause of missed workdays and impaired performance at work.

We observed that MSD is very prevalent in gastrointestinal endoscopists in Korea. A majority of endoscopists experienced pain at multiple anatomic areas (72.7%) and two-third (67.3%) complained of the pain at rest. The prevalence of MSD observed in the present study is higher than that noted in previous reports by other groups<sup>[13,14]</sup>. One possible explanation is that the endoscopic workload of the doctors in the present study

was very high. In this regard, a previous study reported that the endoscopic volume measured in terms of hours per week, number per week, or percentage of working time was strongly associated with the development of musculoskeletal injuries among endoscopists<sup>[13]</sup>. Workload-associated factors such as the number or duration of work were also related to the prevalence of MSD in other occupations<sup>[1,3,18]</sup>.

In the present study, 26 (47.3%) endoscopists complained of severe musculoskeletal pain, defined as a VAS value greater than 5.5 (Table 1). Endoscopists with severe pain were more likely to have multiple painful areas than those without severe pain. Contrary to our expectations, there was no significant difference in the endoscopic workload between them. This may be due to the small number of participants of the present study. However, ergonomic factors like bad posture during the procedure may be important in the development of endoscopy-related musculoskeletal symptoms. With respect to this hypothesis, we observed that ergonomic factors, such as specific posture/habit during the procedure and standing position in upper gastrointestinal endoscopy, correlated significantly with the development of severe musculoskeletal pain.

In the present study, the experience level of endoscopists was not related to the prevalence of musculoskeletal pain, the number of symptomatic areas, and the severity of the most painful regions. However, there were differences in the distribution of the symptomatic areas (Figure 1B and C). For example, finger pain was more common in beginners, whereas shoulder pain was more common in experienced endoscopists. This was especially true when the endoscopist was performing a procedure. The exact reason of this difference is unclear, but muscles and/or joints frequently used during an endoscopic procedure may differ with the experience of the endoscopist. In this respect, it should be noted that beginners tend to depend more on the movement of the knob during endoscopy.

The best approach to MSD must be preventive. As noted in a previous report<sup>[13]</sup>, endoscopists in the present study tended to neglect or tried to alleviate their symptoms by themselves without resorting to professional help. However, we found that a small proportion (16.3%) of symptomatic endoscopists modified the procedure patterns or reduced the number of endoscopic procedures. The overall efficacy of an endoscopy unit may be negatively influenced by these factors. Measures for the prevention of work-related MSD may improve the productivity of healthcare institutions. The importance of ergonomics in work-related MSDs has been studied in a certain fields<sup>[19,20]</sup>. Endoscopists should also take advantage of such studies.

A major limitation of our study was that it was difficult to determine whether a particular symptom was related to endoscopy or not. This was related to our study design using a self-administered questionnaire without an objective assessment of the symptoms. However, we presumed that a great proportion of

the symptoms in the present study were endoscopy-related. Another limitation was that the participants were working in either general hospitals or health promotion centers, where the volume of endoscopic procedures exceeds the average endoscopic workload. About one-half of endoscopies, in general, were performed under sedation, the proportion of upper to lower endoscopy was approximately three to two, and that of therapeutic endoscopies was almost twenty percent, although this aspect was not investigated in the present survey. Finally, there was no follow-up data in the present study. Large-scaled follow-up studies in various clinical settings are needed.

In conclusion, the prevalence of musculoskeletal symptoms in endoscopists is very high, and the majority of symptomatic endoscopists do not seek professional consultation. The pattern of musculoskeletal pain among beginners and experienced endoscopists was different, suggesting multiple ergonomic mechanisms for symptom development. Measures for the prevention and adequate management of endoscopy-related musculoskeletal symptoms are necessary.

## COMMENTS

### Background

Work related musculoskeletal disorder (MSD) is a common problem in individuals whose job requires repetitive isometric maneuvers or awkward body positions. However, the prevalence of MSD among endoscopists is not well known. The present study was designed to investigate the prevalence and risk factors of work-related MSD in gastrointestinal endoscopist in Korea.

### Research frontiers

We investigated the incidence, severity, location and clinical impact of work-related MSD during endoscopic procedures and at rest in gastrointestinal endoscopists. The present study is the first detailed study designed to investigate MSD in gastrointestinal endoscopists in eastern countries.

### Innovations and breakthroughs

We used a self reported questionnaire in 55 endoscopists practicing in 4 general hospitals and 2 health promotion centers. The severity of MSD was assessed by the visual analogue scale, a standard measurement tool in pain research.

### Applications

In the present study, the prevalence of musculoskeletal symptoms among endoscopists was very high, and the majority of symptomatic endoscopists did not seek professional consultation. The pattern of musculoskeletal pain in beginners and in experienced endoscopists was different, suggesting multiple ergonomic mechanisms for symptom development. We hope the present study would lead to interest in work-related MSD and ergonomics in gastrointestinal endoscopists.

### Peer review

The present study has shown that the development of severe pain in gastrointestinal endoscopists is related to ergonomic factors, such as specific posture/habit and the standing position during endoscopic procedures. The study suggests that endoscopists need to focus on teaching a beginner about proper posture and manipulation techniques in order to prevent musculoskeletal symptoms.

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RAPID COMMUNICATION

# Resting energy expenditure and glucose, protein and fat oxidation in severe chronic virus hepatitis B patients

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cantly decreased, and a high proportion of energy is provided by fat in severe chronic hepatitis. These results warrant a large clinical trial to assess the optimal nutritive support therapy for patients with severe liver disease.

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**Key words:** Chronic severe viral hepatitis; Energy metabolism; Respiratory quotient; Malnutrition

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Fan CL, Wu YJ, Duan ZP, Zhang B, Dong PL, Ding HG. Resting energy expenditure and glucose, protein and fat oxidation in severe chronic virus hepatitis B patients. *World J Gastroenterol* 2008; 14(27): 4365-4369 Available from: URL: <http://www.wjnet.com/1007-9327/14/4365.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4365>

## Abstract

**AIM:** To study and determine the resting energy expenditure (REE) and oxidation rates of glucose, fat and protein in severe chronic hepatitis B patients.

**METHODS:** A total of 100 patients with liver diseases were categorized into three groups: 16 in the acute hepatitis group, 56 in the severe chronic hepatitis group, and 28 in the cirrhosis group. The REE and the oxidation rates of glucose, fat and protein were assessed by indirect heat measurement using the CCM-D nutritive metabolic investigation system.

**RESULTS:** The REE of the severe chronic hepatitis group ( $20.7 \pm 6.1$  kcal/d per kg) was significantly lower than that of the acute hepatitis group ( $P = 0.014$ ). The respiratory quotient (RQ) of the severe chronic hepatitis group ( $0.84 \pm 0.06$ ) was significantly lower than that of the acute hepatitis and cirrhosis groups ( $P = 0.001$ ). The glucose oxidation rate of the severe hepatitis group (39.2%) was significantly lower than that of the acute hepatitis group and the cirrhosis group ( $P < 0.05$ ), while the fat oxidation rate (39.8%) in the severe hepatitis group was markedly higher than that of the other two groups ( $P < 0.05$ ). With improvement of liver function, the glucose oxidation rate increased from 41.7% to 60.1%, while the fat oxidation rate decreased from 26.3% to 7.6%.

**CONCLUSION:** The glucose oxidation rate is signifi-

## INTRODUCTION

The liver plays a pivotal role in controlling carbohydrate metabolism by maintaining glucose concentrations in the normal range, as well as in protein and fat metabolism. This is achieved by a tightly regulated system of enzymes and kinases that regulate nutrient breakdown and synthesis in hepatocytes. Many studies have shown that cirrhotic patients have nutrient and energy metabolism imbalance, which lead to malnutrition and seriously affect their prognosis<sup>[1,2]</sup>. On multivariate regression analysis, the Child-Pugh's score is a good independent predictor of malnutrition. With respect to energy metabolism, there is no consensus on energy expenditure, but there is a consensus that respiratory quotients are lower in liver cirrhosis patients than in healthy subjects. Therefore, resting energy expenditure (REE), calorie intake, and energy balance should be routinely assessed in cirrhotic patients in order to identify hypermetabolic and hypometabolic patients. The nutritional and metabolic parameters in these patients are indispensable for design-

ing and prescribing personalized nutritional strategies for the treatment of muscle malnutrition, which can thus improve their morbidity and mortality rates<sup>[3]</sup>. Protein-energy malnutrition is frequently observed in liver cirrhosis patients; disorders of protein metabolism and energy metabolism are closely correlated with protein-energy malnutrition. It has been shown that, in protein metabolism disorders, the synthesis and degradation rates of albumin decreased and the serum half-life of albumin became longer<sup>[4]</sup>. These changes are closely correlated with the prognosis of cirrhosis patients. Therefore, it is important for clinicians to identify and treat metabolic disorders in liver cirrhosis patients. Currently, it is thought that supplementation with branched-chain amino acids (BCAAs) is useful for improving protein metabolism disorders, and that a late evening snack (LES) improves the catabolic state of advanced liver cirrhosis patients. Long-term oral supplementation with a BCAA mixture was found to be better than an ordinary diet<sup>[5,6]</sup>. Recently, it was shown that the ingestion of a 200 kcal rice LES can improve nutritional metabolism in cirrhosis patients. Furthermore, a short course of recombinant human growth hormone (rhGH) and insulin-like growth factor-I (IGF-I) raised albumin levels and tended to improve energy metabolism in liver cirrhosis patients. The exogenous administration of IGF-I has hepatoprotective and antifibrogenic actions in experimental liver cirrhosis<sup>[7-11]</sup>. Therefore, proper nutritional support is very important in promoting the recovery of liver disease patients. However, the characteristics of the energy metabolism of severe chronic hepatitis patients are not clear. Thus, the aim of the present study was to explore the characteristics of the energy metabolism of severe chronic hepatitis patients so as to provide data that could be used for optimal nutritional support.

## MATERIALS AND METHODS

### Subjects

One hundred patients with liver diseases were enrolled from July 2006 to September 2007. They were categorized into 3 groups according to their disease recovery stage: acute hepatitis group ( $n = 16$ ); hepatitis A,  $n = 10$ ; hepatitis E,  $n = 6$ ), severe chronic hepatitis B-related hepatitis group ( $n = 56$ ), and hepatitis B-related cirrhosis group ( $n = 28$ , Table 1). Of the severe chronic hepatitis B-related hepatitis patients, 14 patients (10 males, 4 females), whose status changed from the acute severe stage to the recovery stage within 8 wk, were randomly selected for assessment twice. The patients' average age was 42.5 years (range, 36-62 years). The patients all met the diagnostic criteria of the "Symposium on Viral Hepatitis and Liver Diseases in 2000"<sup>[12]</sup>. Briefly, the severe chronic hepatitis was defined based on the following inclusion criteria: (1) a history of chronic hepatitis or liver cirrhosis; (2) severe asthenia and a serum total bilirubin more than 171  $\mu\text{mol/L}$ ; and (3) prothrombin time activity (PTA) less than 60%. All enrolled patients had not received nutritional support and rational regime treatment, anti-viral agents

and steroid. The study was explained to the patients and/or their relatives, and written informed consent was obtained. The study was approved by the Ethics Committee of the Beijing You'an Hospital of Capital Medical University.

### REE and glucose, protein and fat oxidation rates

The REE and the carbohydrate, protein and fat oxidation rates were determined by indirect heat measurement. The REE was determined using the CCM-D nutrition metabolism investigation system (Medgraphics Company, United States). The average  $\text{O}_2$  amount consumed and the  $\text{CO}_2$  amount produced per minute by the subjects were used to calculate the actual REE using the Weir formula; subsequently, the respiratory quotient, the ratio of the average  $\text{O}_2$  amount consumed to the  $\text{CO}_2$  amount produced per minute by the subjects, was calculated. Twenty-four-hour urine samples were collected from all subjects for the determination of the urea nitrogen level using the HITACHI7170 automatic biochemistry analyzer (Japan). The protein oxidation rate, non-protein-respiratory quotient (npRQ), the carbohydrate (CHO) and the fat oxidation rates were calculated from the collected data using a computer program. The predicted REE value was calculated using the Harris-Benedict formula based on the subject's height, weight and age.

The subjects were required to fast for at least 8 h prior to testing. In order to avoid muscle activation; they stayed in bed in the morning for at least 30 min. The room temperature was kept between 24°C and 26°C, with a humidity of 45%-60%. The volume and gas were calibrated for the CCM-D nutrition metabolism investigation system; and the subject's data on height, weight, gender and age were put into the system. The value of energy metabolism may be related to body weight. Therefore, the REE/kg of all patients were also evaluated.

### Liver function assessment

Serum samples were collected from all subjects, and the following liver function indices were determined by the HITACHI7170 automatic biochemistry analyzer (Japan): alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin (TBIL), albumin (ALB), proalbumin (PA), cholinesterase (CHE), and cholesterol (CH).

### Statistical analysis

The data is presented as mean  $\pm$  SD. The means were compared between groups using variance analysis. Data from severe virus hepatitis patients in the severe stage and the recovery stage were compared using the paired *t* test. SPSS 10.0 statistical software was used for all analyses.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Energy metabolism characteristics

The REE of the severe chronic hepatitis group and the

**Table 1** Clinical characteristics of the patients

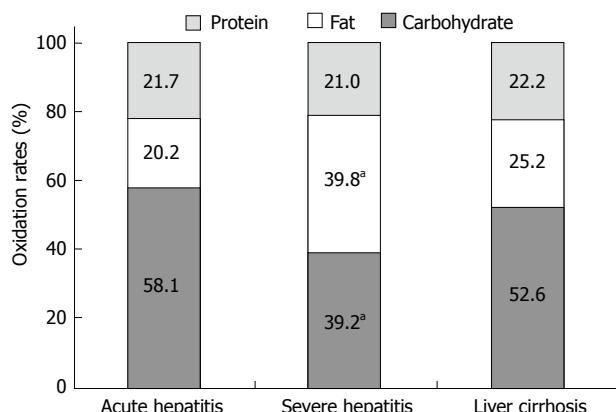
Group	<i>n</i>	Age (yr)	Male/Female	ALT (U/L)	AST (U/L)	T-Bil (mg/dL)	ALB (g/L)	PA (g/L)	CHE (g/L)	CH (g/L)
1	16	34 ± 16	10/6	154.3 ± 184.8	68.3 ± 58.4	4.4 ± 6.4	36.6 ± 4.4	142.0 ± 66.0	6050.9 ± 2243.6	164.9 ± 49.4
2	56	46 ± 13	49/9	189.2 ± 374.9	156.8 ± 159.4	20.4 ± 9.8	29.8 ± 4.6	52.5 ± 27.5	2723.6 ± 1422.2	52.3 ± 36.2
3	28	53 ± 10	24/4	68.3 ± 118.9	80.2 ± 80.6	5.8 ± 7.2	29.5 ± 7.2	62.2 ± 34	2628.6 ± 1416.3	114.9 ± 42.7

Group 1: Acute hepatitis group; Group 2: Chronic severe hepatitis group; Group 3: Cirrhosis group. PTA: Prothrombin activity. PTA = (control PT-8.7)/(patients PT-8.7) × 100%.

**Table 2** REE and oxidation rates in different groups of patients with liver disease (mean ± SD)

Group	REE (kcal/d)	REE/kg (kcal/d)	RQ	npRQ	CHO (g/d per kg)	Fat (g/d per kg)	Protein (g/d per kg)
1	1586.7 ± 783.0	25.8 ± 11.3	0.90 ± 0.05	0.92 ± 0.21	3.8 ± 2.1	0.5 ± 0.5	1.1 ± 0.6
2	1388.5 ± 334.5	20.7 ± 6.1	0.84 ± 0.06 <sup>a</sup>	0.88 ± 0.18	1.8 ± 1.0 <sup>a</sup>	0.9 ± 0.7	0.9 ± 0.6
3	1317.9 ± 266.3	19.8 ± 3.6	0.88 ± 0.08	0.91 ± 0.09	2.7 ± 1.4	0.9 ± 2.0	1.0 ± 0.6
<i>P</i> value	0.126	0.014	0.001	0.441	0	0.721	0.525

Group 1: Acute hepatitis group; Group 2: Chronic severe hepatitis group; Group 3: Cirrhosis group. <sup>a</sup>*P* < 0.05, 2 vs 1 and 3 vs 1.



**Figure 1** Oxidation rates of protein, carbohydrate and fat in the severe hepatitis group, the acute hepatitis group, and the cirrhosis group. <sup>a</sup>*P* < 0.05, the acute hepatitis group vs the cirrhosis group. There is no significant difference between groups of severe hepatitis and liver cirrhosis.

acute hepatitis group was not significantly different from that of the cirrhosis group. However, the REE per kg (REE/kg) of the acute hepatitis group was significantly higher than that of the chronic severe hepatitis group and the cirrhosis group (*P* = 0.014), but the difference in the REE/kg between the chronic severe hepatitis group and the cirrhosis group was not significant. The respiratory quotient of the severe chronic hepatitis group (0.84 ± 0.06) was significantly lower than that of the acute hepatitis group and the cirrhosis group (*P* = 0.001). The respiratory quotients of the chronic hepatitis group (0.90 ± 0.05) and the cirrhosis group (0.88 ± 0.08) were not significantly different (Table 2).

The proportion of energy supplied by the three major substrates (carbohydrate, fat and protein) differed among the groups. Protein oxidation rates were not significantly different among the groups; they ranged from 21.0% to 22.2%. The carbohydrate oxidation rate of the severe hepatitis group (39.2%) was significantly lower than that of the acute hepatitis group and the cirrhosis group (*P* = 0.048). The fat oxidation rate of the severe hepa-

titis group (39.8%) was significantly higher than that of the acute hepatitis group and the cirrhosis group (*P* = 0.01). The carbohydrate and fat oxidation rates of the acute hepatitis group and the cirrhosis group were not significantly different (*P* = 0.472). Energy supplied by carbohydrate oxidation accounted for 50% or more of the total energy supplies (Figure 1). The actual consumptions per kg weight per day of protein and fat did not differ significantly among the three groups. However, the carbohydrate consumption per kg weight per day was highest in the acute hepatitis group and lowest in the chronic severe hepatitis group; there were significant differences among the groups in carbohydrate consumption (*P* < 0.0001, Table 2).

#### Energy metabolism differences between severe hepatitis patients at severe and recovery stages

The energy metabolism of the 14 patients with severe chronic hepatitis whose disease improved from the severe stage to the recovery stage was assessed twice. Four of these patients had been given growth hormone (4.5 IU/d for 2 wk). Significant improvement in the biochemical indices was seen in the patients at the recovery stage: ALT decreased from 561.2 ± 818.5 U/L to ALT 35.7 ± 9.37 U/L, T-Bil decreased from 17.4 ± 5.5 mg/dL to 5.3 ± 2.7 mg/dL, and PTA increased from 38.2% ± 29% to 72.7% ± 35.9%. After the improvement of liver function, the REE was not significantly changed compared to that obtained prior to the improvement; there was no significant change in the REE per kg weight. However, the carbohydrate oxidation rate increased from 41.7% to 60.1%, while the fat oxidation rate decreased from 26.3% to 7.6%. There was a non-significant trend for the respiratory quotient (RQ) value to increase (*P* = 0.105, Table 3).

#### DISCUSSION

The liver plays a unique role in carbohydrate metabolism by maintaining glucose concentration levels in the normal

**Table 3** Dynamic determination of REE and oxidation rates in severe chronic hepatitis patients ( $n = 14$ , mean  $\pm$  SD)

Patient	REE (kcal/d)	REE/kg (kcal/d per kg)	RQ	CHO (%)	Fat (%)	Protein (%)
Severe stage	1366.1 $\pm$ 140.7	19.1 $\pm$ 3.3	0.86 $\pm$ 0.05	41.7 $\pm$ 18.3	26.3 $\pm$ 27.2	32.1 $\pm$ 24.8
Recovery stage	1306.7 $\pm$ 497.8	17.9 $\pm$ 6.2	0.91 $\pm$ 0.06	60.1 $\pm$ 11.7	7.6 $\pm$ 38.6	32.3 $\pm$ 30.9
P value	0.761	0.638	0.105	0.075	0.232	0.990

range. This is achieved by a tightly regulated system of enzymes and kinases that regulate glucose breakdown and synthesis in hepatocytes. This process is under the control of glucoregulatory mediators, of which insulin plays a key role. Therefore, the liver is the major organ of substrate and energy metabolism. It has recently been noted that the energy metabolism of patients with end-stage liver diseases, such as cirrhosis, was altered compared to normal controls, they showed evidence of malnutrition, a high metabolism, a lower RQ value, and a relatively higher fat oxidation rate<sup>[2,4,6,11]</sup>. Long-term oral supplementation with a BCAA mixture is better than ordinary food taken as a late evening snack for improving the serum albumin level and the energy metabolism of cirrhosis patients<sup>[5,10]</sup>.

Severe chronic hepatitis is a severe liver disease in which extended liver tissue necrosis caused by chronic viral hepatitis or hepatitis cirrhosis may lead to liver failure. Nutritional support is an important component of comprehensive therapy. However, the characteristics of the energy metabolism of severe chronic hepatitis patients have never been previously reported. The present study found that the energy metabolism of patients with severe chronic hepatitis had several unique characteristics.

First, the REE of severe chronic hepatitis patients was not significantly different from that of acute hepatitis and cirrhosis patients, who did not have increased energy metabolism. The REE per kg weight was similar in the severe chronic hepatitis group to that in the cirrhosis group, and both were lower than that in the acute hepatitis group. This may be due to the fact that the REE/kg value in the severe chronic hepatitis patients was lower than that in normal controls. This is different from the high energy metabolism condition found in cirrhosis patients, which is widely acknowledged. Tajika<sup>[2]</sup> found that energy metabolism was normal in 58% patients, and only 12% of patients had low energy metabolism. This can be related to the high energy metabolism of acute hepatitis patients. Plauth<sup>[6]</sup> showed that chronic hepatitis C patients had a high energy metabolism related to their viral load; and their high energy metabolism resolved with anti-viral treatment. Therefore, it is likely that high energy metabolism is related to the presence of acute hepatitis.

Second, severe the chronic hepatitis group had a notably higher fat oxidation rate than the acute hepatitis group and the cirrhosis group, and a lower glucose oxidation rate than the acute hepatitis group and the cirrhosis group. There was no difference in the protein oxidation rate among the three groups.

Third, the REE before and after liver function improvement in the severe hepatitis patients was compared. The REE of these patients was not changed, but they had a lower fat oxidation rate and a higher carbohydrate oxidation rate; however, these differences were not statistically

significant, perhaps as a result of the small sample size. Recently, Plauth<sup>[6]</sup> showed that TIPS could improve energy metabolism and malnutrition in cirrhosis patients. Growth hormone treatment can improve the liver function and energy metabolism of severe hepatitis patients. From these results, it would appear that severe chronic hepatitis patients cannot utilize carbohydrate. With a notable decrease in the glucose oxidation rate, they can only obtain energy by increased fat utilization. Glucose utilization returns to normal when the patients recover. The inability to use glucose may be due to insulin resistance in chronic liver disease patients<sup>[8]</sup>. An increase in the RQ value can be used as a marker of recovery in these patients. Tajika<sup>[2]</sup> also found that, in cirrhosis patients, the non-protein respiratory quotient (npRQ) was an independent risk factor for survival; and patients with a lower npRQ had a worse prognosis.

In the present study, substrate and energy metabolism of cirrhosis patients was not significantly different from that of acute hepatitis patients. This may be related to patient selection. The cirrhotic patients selected were in Child grade A or B, resulting in no significant difference between the cirrhosis patient group and the acute hepatitis group. Multivariate regression analysis confirmed that the Child-Pugh's score is a better independent predictor of malnutrition than the other variables. However, the REE, TEE, calorie intake and energy balance need to be routinely assessed in cirrhotic patients in order to identify hypermetabolic and hypometabolic patients, which account for approximately 30% of patients. In these patients, the nutritional and metabolic parameters are indispensable for designing and prescribing personalized nutritional strategies for the treatment of the patients' muscle malnutrition, thus improving their morbidity and mortality rates.

In conclusion, in the present study, severe chronic hepatitis patients had a lower resting energy metabolism per kg weight than acute hepatitis patients, but similar to cirrhosis patients. Severe chronic hepatitis patients had a significantly higher fat oxidation rate and a significantly lower glucose oxidation rate than acute hepatitis and cirrhosis patients. As the severe chronic hepatitis patients' condition improved, the glucose oxidation rate increased. An increase in the RQ value can be used as a marker of recovery in these patients. The REE and the oxidation rates of various substrates determined by indirect heat measurement can be used to determine the optimal nutritive support therapy for severe liver disease patients.

## COMMENTS

### Background

The liver plays a pivotal role in glucose, fat, protein and energy metabolism.

Many studies have shown that patients with liver cirrhosis have nutrient and energy metabolism imbalance, which lead to malnutrition and seriously affect their prognosis. However, the characteristics of the glucose, fat, protein and energy metabolism in patients with severe chronic hepatitis are not clear.

### Research frontiers

It has recently been noted that the energy metabolism of patients with end-stage liver diseases such as cirrhosis was altered compared to normal controls. They showed evidence of malnutrition, a high metabolism, a lower respiratory quotient (RQ) value, and a relatively higher fat oxidation rate. Long-term oral supplementation with a branched-chain amino acids (BCAA) mixture is better than ordinary food taken as a late evening snack for improving the serum albumin level and the energy metabolism of cirrhotic patients. Severe chronic hepatitis, which extended hepatic cell necrosis caused by chronic viral hepatitis B, leads to liver failure. Nutritional support is an important component of comprehensive therapy. However, the characteristics of the glucose, fat, protein and energy metabolism in patients with severe chronic hepatitis remain unclear.

### Innovations and breakthroughs

The characteristics of the energy metabolism of severe chronic hepatitis patients have not been previously reported. The authors studied the disturbed homeostasis of energy, carbohydrate, fat and protein metabolism in severe chronic viral hepatitis B patients, and found that these patients had increased fat oxidation and reduced carbohydrate metabolism, which intended to improve when the liver function became normal. An increase in the RQ value can be used as a marker of recovery in these patients.

### Applications

The measurement of resting energy expenditure (REE) and the oxidation rates of various substrates can be used to determine the optimal nutritive support therapy for severe liver disease patients. This research is expected to warrant a large clinical trial to assess the optimal nutritive support therapy for severe liver disease patients.

### Peer review

The authors have estimated REE and RQ of patients with severe chronic hepatitis B. And most of energy consumed by these patients is provided by fat, not carbohydrate. The study is interesting and valuable.

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RAPID COMMUNICATION

# Orthotopic liver transplantation as a rescue operation for recurrent hepatocellular carcinoma after partial hepatectomy

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## Abstract

**AIM:** To compare post-orthotopic liver transplantation (OLT) survival between patients with recurrent hepatocellular carcinoma (HCC) after partial hepatectomy and those who received *de novo* OLT for HCC and to assess the risk factors associated with post-OLT mortality.

**METHODS:** From July 2003 to August 2005, 77 consecutive HCC patients underwent OLT, including 15 patients with recurrent HCC after partial hepatectomy for tumor resection (the rescue OLT group) and 62 patients with *de novo* OLT for HCC (the *de novo* OLT group). Thirty-three demographic, clinical, histological, laboratory, intra-operative and post-operative variables were analyzed. Survival was calculated by the Kaplan-Meier method. Univariable and multivariable analyses were also performed.

**RESULTS:** The median age of the patients was 49.0 years. The median follow-up was 20 mo. Three patients (20.0%) in the rescue OLT group and 15 patients (24.2%) in the *de novo* OLT group died during the follow-up period ( $P = 0.73$ ). The 30-day mortality of OLT was 6.7% for the rescue OLT group vs 1.6% for the *de novo* OLT group ( $P = 0.27$ ). Cox proportional hazards model showed that pre-OLT hyperbilirubinemia, the requirement of post-OLT transfusion, the size of the tumor, and family history of HCC were significantly associated with a higher hazard for mortality.

**CONCLUSION:** There are no significant differences

in survival/mortality rates between OLT as *de novo* therapy and OLT as a rescue therapy for patients with HCC. Pre-OLT hyperbilirubinemia, post-OLT requirement of transfusion, large tumor size and family history of HCC are associated with a poor survival outcome.

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**Key words:** Orthotopic liver transplantation; Liver cancer; Resection; Recurrence; Survival

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. An estimated number of 372000 new cases of HCC are diagnosed each year, constituting 4.6% of all new cancers (6.3% in men, 2.7% in women)<sup>[1,2]</sup>. Endemic areas include the Far East and Africa. The number of patients with HCC in China comprises approximately 40%-50% of all HCC patients of the world and HCC is the 2nd leading cause of death among cancer mortalities in the country<sup>[3]</sup>. While the etiology and pathogenesis of HCC are not entirely clear, the major contributing factors for the high prevalence of HCC in the country include chronic hepatitis B infection and dietary contamination of aflatoxin<sup>[4]</sup>.

Partial hepatectomy with tumor resection and orthotopic liver transplantation (OLT) are the two commonly used surgical modalities for HCC treatment. Partial hepatectomy with tumor resection may be performed as a definitive or bridging therapy. Post-operative recurrence of HCC is common in patients undergoing hepatectomy, affecting long-term outcome. For patients with recurrent HCC after partial hepatectomy, rescue treatment options include percutaneous ethanol injection (PEI), trans-catheter

arterial chemoembolization (TACE), and surgical resection of recurrent tumor<sup>[5,6]</sup>. In addition, OLT appears to be a valid rescue option for these patients<sup>[7]</sup>. Poon *et al*<sup>[7]</sup> reported that OLT as a salvage operation is feasible and effective in patients with transplantable small HCC (solitary ≤ 5 cm, or 2 or 3 tumors ≤ 3 cm) who initially had primary resection and had preserved liver function. However, OLT has not been routinely performed in patients with recurrent advanced HCC<sup>[8]</sup>. In our clinical practice, we found that these patients with recurrent HCC, even at an advanced stage, might still benefit from OLT as a rescue operation. We hypothesized that OLT as a rescue operation for patients with recurrent HCC after initial resection surgery may have a similar outcome to these patients with HCC who had *de novo* OLT. The aims of the study were to compare post-OLT survival rates between patients who have recurrent HCC and those who have no history of resection surgery, and to evaluate the risk factors associated with postoperative mortalities.

## MATERIALS AND METHODS

### Patients

This is an historical cohort study involving 77 consecutive patients who underwent OLT for HCC from July 2003 to August 2005 in Eastern Hepatobiliary Surgery Hospital, Shanghai, China, a tertiary referral center specializing in surgical treatment of HCC and a variety of other liver disorders. The data were extracted from a prospectively maintained database for OLT which was approved by the Institutional Ethics Committee. The 77 eligible patients were divided into two groups: the study group with OLT performed as a rescue operation for recurrent HCC after initial partial hepatectomy for tumor resection (the rescue OLT group,  $n = 15$ ) and the control group with OLT performed as the 1st line *de novo* therapy for HCC (the *de novo* OLT group,  $n = 62$ ).

### Inclusion and exclusion criteria

The inclusion criteria were patients aged > 18 years and those with HCC who underwent OLT. The exclusion criteria were patients who had OLT for etiologies other than HCC and patients who had OLT combined with transplantation of other organ(s).

### Criteria for diagnosis and selection

The diagnosis of HCC was based on a combined assessment of clinical presentations, history of HBV infection and liver cirrhosis, imaging data (ultrasound, CT scan, and/or MRI), preoperative laboratory evaluation (alpha-fetoprotein), and pre- and/or postoperative histopathology. Patients considered to be candidates for OLT met the following criteria: (1) primary or recurrent HCC met Shanghai Criteria for OLT<sup>[9]</sup>, i.e., tumor size ≤ 9 cm, number of tumors ≤ 3, the absence of macrovascular tumor embolism, and the absence of extrahepatic metastasis; (2) the ability to take anti-rejection medications after OLT; and (3) the absence

of significant comorbidities. Written informed consent was obtained from all patients before the surgery.

### Demographic and clinical variables

A panel of demographic and clinical variables were evaluated, including age, sex, excessive alcohol use, tobacco use, family history of HCC, HBV and/or hepatitis C infection, liver cirrhosis, diabetes, cardiopulmonary diseases, renal insufficiency, the time from the tumor detection to OLT, donor source, preoperative blood biochemistry, pre-OLT Child-Pugh scores<sup>[10]</sup>, post-OLT pTNM tumor staging (UICC, International Union Against Cancer, 1953), intraoperative variables, postoperative histopathology and postoperative course.

### Clinical outcomes

The primary outcomes were estimated based on the 30-d postoperative mortality, overall survival, and tumor-free survival, and the secondary outcomes were risk factors associated with the mortality.

### Statistical analysis

Descriptive statistics were computed for all factors. These include medians and percentiles for continuous factors and frequencies for categorical factors. Time of follow-up is defined as either months from OLT to death, or months from OLT to last follow-up visit. A Kaplan-Meier plot was used for graphical representation of survival probabilities by recurrence of the liver cancer. Univariable and multivariable Cox proportional hazards models were used to estimate the hazard rates for several factors of interest. A stepwise selection method was used to choose the final multivariable model using a 0.50 and 0.25 significance criterions for entering and remaining in the model, respectively. A significance level of 0.05 was considered for all analyses. SAS version 9.1 software (SAS Institute, Cary, NC) and R 2.0.1 software (The R Foundation for Statistical Computing) were used to perform all analyses.

## RESULTS

### Procedure

In a median waiting period of 2 mo (ranging from 4 d to 3 mo), all 77 patients underwent cadaver OLT. Two patients (13.3%) in the rescue OLT group and 11 patients (17.7%) in the *de novo* OLT group underwent TACE or PEI therapy before OLT operation. As a part of our routine clinical protocol, end-to-end anastomoses of the inferior vena cava, portal veins, hepatic arteries, and bile ducts of the donors and recipients were performed. At anhepatic phase, 4000 units of hepatitis B immunoglobulin (HBIG) were injected intramuscularly. At the completion of the operation, methylprednisolone 500 mg was infused intravenously. As a part of the post-OLT protocol, 74 patients (96.1%) received one to three 6-d courses of intravenous 5-fluorouracil 500 mg on post-operative day 1 and day 4, mitomycin 2 mg

**Table 1 Demographic and clinical data**

Factors	Rescue OLT group ( <i>n</i> = 15)	De novo OLT group ( <i>n</i> = 62)
Age (yr) <sup>1</sup>	50.0 (46.0-55.0)	49.0 (44.0-55.0)
Male, No. (%)	14 (93.3)	51 (82.3)
Excessive alcohol use, No. (%)	5 (33.3)	13 (21.0)
Tobacco use, No. (%)	7 (46.7)	52 (83.9)
Family history of liver cancer, No. (%)	3 (20.0)	8 (12.9)
Hepatitis B infection, No. (%)	15 (100.0)	57 (91.9)
Diabetes, No. (%)	2 (13.3)	7 (11.3)
Liver cirrhosis, No. (%)	15 (100.0)	60 (96.8)
Months from tumor detection to OLT <sup>1</sup>	1.0 (1.0, 3.0)	2.0 (1.0, 3.0)
Child-Pugh Score, No. (%)		
A	7 (46.7)	39 (62.9)
B	6 (40.0)	23 (37.1)
C	2 (13.3)	0 (0.0)
UICC pTNM tumor staging <sup>1</sup>	3.0 (3.0, 3.0)	3.0 (3.0, 3.0)
Total bilirubin (μmol/L) <sup>1</sup>	22.8 (15.0, 54.6)	32.1 (23.0, 55.4)
Direct bilirubin (μmol/L) <sup>1</sup>	10.4 (5.8, 35.3)	14.4 (9.8, 29.5)
Albumin (g/L) <sup>1</sup>	35.3 (32.1, 42.1)	34.5 (32.0, 37.6)
Prealbumin (g/L) <sup>1</sup>	12.7 (9.3, 15.3)	10.3 (6.9, 14.5)
Alanine aminotransferase (U/L) <sup>1</sup>	90.1 (37.7, 183.9)	48.9 (37.8, 91.7)
Aspartate aminotransferase (U/L) <sup>1</sup>	85.4 (36.5, 150.0)	65.5 (47.1, 99.4)
Prothrombin time (s) <sup>1</sup>	14.5 (13.3, 17.9)	15.2 (13.4, 17.8)
Activated partial thromboplastin time (s) <sup>1</sup>	34.7 (31.8, 38.0)	34.8 (30.7, 41.3)
OLT operating room time (h) <sup>1</sup>	8.0 (7.2, 8.1)	6.8 (5.7, 7.7)
OLT anhepatic time (min) <sup>1</sup>	65.0 (60.0, 81.0)	62.0 (56.0, 74.0)
OLT intra-op bleeding (L) <sup>1</sup>	2.0 (1.5, 2.3)	1.5 (1.2, 2.0)
OLT intra-op transfusion (L) <sup>1</sup>	1.6 (1.0, 2.0)	0.8 (0.4, 1.4)
OLT post-op transfusion (L) <sup>1</sup>	1.2 (0.8, 2.0)	0.4 (0.0, 1.4)
Pre-OLT α-fetoprotein (U/L) <sup>1</sup>	7.5 (4.9, 27.1)	362.6 (20.4, 3480.0)
Post-OLT α-fetoprotein (U/L) <sup>1</sup>	5.3 (4.1, 10.9)	15.7 (5.4, 120.4)
Change in α-fetoprotein after OLT <sup>1</sup>	4.2 (0.8, 21.8)	337.9 (13.3, 3023.5)
Postoperative histopathology <sup>1</sup>		
Size of largest tumor in diameter (cm) <sup>1</sup>	3.0 (1.5, 6.0)	3.6 (2.5, 6.0)
Number of tumor <sup>1</sup>	1.0 (1.0, 4.0)	1.0 (1.0, 2.0)
Histology differentiation of tumor, No. (%)		
Moderate	8 (53.3)	9 (14.5)
Poor	7 (46.7)	53 (85.5)
Tumor vascular invasion, No. (%)		
None	5 (33.3)	26 (41.9)
Microvascular	8 (53.3)	33 (53.2)
Macrovascular	2 (13.3)	3 (4.8)
Tumor in right lobe, No. (%)	13 (86.7)	53 (85.5)
Post-OLT treatment, No. (%)		
None	1 (6.7)	2 (3.2)
Chemotherapy	14 (93.3)	60 (96.8)
Time of follow-up (mo) <sup>1</sup>	18.0 (12.0, 24.0)	21.0 (14.0, 32.0)

<sup>1</sup>Statistics presented are medians (Q25, Q75).

on post-operative day 2 and day 5, and carboplatin 100 mg on postoperative day 3 and day 6. Post-operative anti-rejection regimens included tacrolimus, methylprednisolone, and mycophenolate mofetil. The blood levels of tacrolimus were maintained at 10-15 ng/mL for 3 mo after OLT and 5-10 ng/mL thereafter. Intravenous methylprednisolone was started on postoperative day 1, at 50 mg per 6 h, and tapered to 20 mg per day. On discharge from the hospital, intravenous methylprednisolone was switched to oral prednisone 15 mg per day with tapering. Prednisone was discontinued at 3 mo post-operatively.

Table 1 summarizes descriptive statistics for all 77 patients in the rescue OLT (*n* = 15) and *de novo* OLT (*n* = 62) groups.

### Clinical data on the rescue OLT group

Table 2 presents detailed information about the tumor, at the time of tumor resection in the study group. All patients in the rescue OLT group underwent radical tumor resection because of the lack of a donor liver, even though the patients met Shanghai Criteria for OLT. The patients had partial hepatectomy with a tumor-free margin of  $\geq 1.5$  cm which was confirmed by postoperative histopathologic evaluation. The majority of patients underwent postoperative adjunctive therapy, including TACE in 12 (80.0%) patients and PEI in 2 (13.3%) patients.

### Survival data

Within a median follow-up of 20 mo (interquartile

**Table 2** Initial clinical data of HCC at time of tumor resection in the study group (*n* = 15)

Factors	<i>n</i>
Years since tumor detection <sup>1</sup>	4.0 (3.0, 5.0)
Mean diameter of tumor (cm) <sup>1</sup>	4.5 (3.0, 8.0)
Tumor-free interval after resection (mo) <sup>1</sup>	26.0 (6.0, 38.0)
Microsatellite lesions (%)	
0	11 (73.3)
1	4 (26.7)
Number of post-resection trans-catheter Arterial chemoembolization	
0	3 (20)
1	4 (26.7)
2	5 (33.3)
3	2 (13.3)
6	1 (6.7)
Number of post-resection percutaneous ethanol injection	
0	13 (86.7)
3	1 (6.7)
16	1 (6.7)
Tumor in the left lobe (%)	12 (80)
Unifocal tumor (%)	15 (100)
Vascular invasion (%)	
None	10 (66.7)
Microvascular	3 (20)
Macrovascular	2 (13.3)
Non-encapsulated tumor (%)	9 (60)

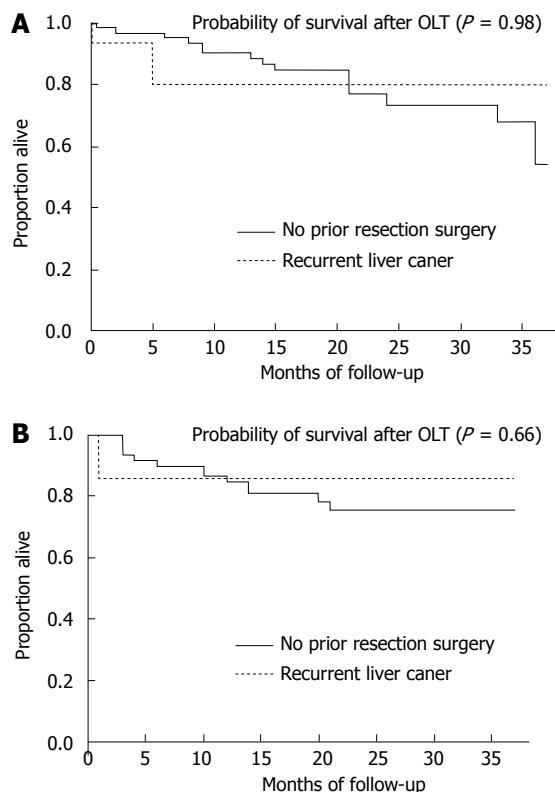
<sup>1</sup>Statistics presented are median (Q25, Q75).

range: 14–29 mo), 18 (23.4%) patients died: 3 (20%) in the rescue OLT group and 15 (24.2%) in the *de novo* OLT group. One patient from each group died in 30 d of OLT (6.7% vs 1.6%) because of disseminated intravascular coagulation and pneumonia, respectively. One patient in the control group died of graft-versus-host disease 2 mo after OLT. The main cause of the death was recurrent tumor and metastasis, and 15 patients died more than 30 d after OLT.

### Risk factors associated with survival outcome

Table 3 presents univariable hazard rates for the association between several factors of interest and overall survival rate. Younger age, pre-operative hyperbilirubinemia, large tumor size, family history of liver cancer, and tumor microembolism were found to significantly influence the hazard for post-OLT mortality. Figure 1 presents the Kaplan-Meier curves of overall survival and tumor-free survival by the two groups, respectively. Interaction between time and the study groups was verified as the two curves cross each other suggested non-proportionality of the hazards; but this was not found statistically significant.

Table 4 shows the results of the multivariable Cox proportional hazards model. Total bilirubin, the requirement for post-operative transfusion, the size of the largest tumor, family history of HCC, and microembolisms remained in the final model. Recurrence of HCC was kept in the final model because of clinical importance, even though it was not found significantly associated with survival rate. The hazard of dying after OLT increases by 1% for every 1 unit increase in total bilirubin. Also, for every 1 L increase in



**Figure 1** Probability of overall survival and tumor-free survival after orthotopic liver transplantation- Kaplan-Meier curves. **A:** Interaction between time and groups was verified as the two curves cross each other suggest non-proportionality of the hazards but this is not found statistically significant ( $P = 0.98$ ); **B:** Interaction between time and groups was verified as the two curves cross each other suggest non-proportionality of the hazards, but not statistically significant ( $P = 0.66$ ).

the amount of post-OLT blood transfusion, the hazard of dying increases by 40%. In addition, the hazard of dying increases by 30% for every 1 cm increase in the size of the largest tumor. Finally, subjects with a family history of HCC have 7.7 times of the hazards of dying that of those without a family history of HCC.

## DISCUSSION

Partial hepatectomy and OLT are effective treatment modalities for HCC patients with underlying cirrhosis<sup>[11–13]</sup>. The surgical outcome of the two approaches appeared to be comparable, with each of the treatment modalities having its advantages and disadvantages. For example, operative mortality was lower for partial hepatectomy which may be preferably applied to HCC patients with a well compensated cirrhotic liver, while OLT can be performed selectively in patients with tumor recurrence and/or decompensated liver. Although the decision of choice on partial hepatectomy and OLT can be difficult to make<sup>[14–16]</sup>, it appears that the latter treatment modality may have a survival advantage<sup>[17,18]</sup>. The main barrier for the routine application of OLT in the patient population has been the availability of a donor organ. On the other hand, partial hepatectomy can be offered as a sole surgical treatment or as a bridging procedure for OLT. However,

**Table 3** Univariable Cox proportional hazards models

	Factor	Hazard ratio (95% CI)	P
Demographic	Age	0.92 (0.88-0.97)	< 0.001
	Gender (male vs female)	1.6 (0.36-6.9)	0.54
	Tobacco (yes vs no)	1.7 (0.48-5.8)	0.42
	Excess alcohol use (no vs yes)	1.1 (0.37-3.4)	0.83
	Family history liver cancer (yes vs no)	4.4 (1.6-11.9)	0.004
	Diabetes (yes vs no)	1.4 (0.42-5.0)	0.57
	Interval from tumor detection to OLT in mo	0.99 (0.91-1.07)	0.74
Disease groups	Recurrent liver cancer (yes vs no)	1.02 (0.29-3.5)	0.98
	Total bilirubin	1.01 (1.004-1.01)	< 0.001
Preoperative laboratory tests	Direct bilirubin	1.01 (1.01-1.01)	< 0.001
	Albumin	0.99 (0.90-1.10)	0.91
	Pre-albumin	0.91 (0.82-1.02)	0.11
	Alanine aminotransferase	1.00 (1.00-1.01)	0.57
	Aspartate aminotransferase	1.00 (1.00-1.01)	0.15
	Prothrombin time	1.04 (0.92-1.2)	0.51
	Activated partial thromboplastin time	1.01 (0.96-1.08)	0.62
Tumor histopathology	Size of largest tumor (cm)	1.1 (1.03-1.2)	0.009
	Tumor staging	1.6 (0.43-6.2)	0.47
	Tumor range	1.3 (0.90-1.9)	0.16
	Tumor location (left vs right)	1.2 (0.33-4.0)	0.82
	Microembolism (micro vs none)	2.1 (0.67-6.7)	0.2
Preoperative staging	Microembolism (macro vs none)	10.5 (2.3-48.3)	0.003
	Child-Pugh Score	1.5 (0.67-3.4)	0.31
Intraoperative factors	OLT operative room time (h)	1.1 (0.80-1.6)	0.48
	OLT anhepatic time (min)	0.97 (0.92-1.01)	0.11
	OLT intra-op bleeding (L)	0.47 (0.20-1.1)	0.097
	OLT intra-op transfusion (L)	0.73 (0.43-1.2)	0.24
	OLT post-op transfusion (L)	1.1 (0.90-1.4)	0.33

**Table 4** Multivariable Cox proportional hazards model

Factors	Hazard ratio (95% CI)	P
Recurrent liver cancer (yes vs no)	1.7 (0.38-7.2)	0.5
Total bilirubin	1.01 (1.006-1.02)	< 0.0001
OLT post-op transfusion (L)	1.4 (1.1-1.8)	0.008
Size of largest tumor (cm)	1.3 (1.1-1.4)	0.0004
Family history liver cancer (yes vs no)	7.7 (2.2-26.9)	0.001
Microembolism (yes vs no)	3.4 (0.85-13.5)	0.08

partial hepatectomy can be associated with a short-term risk for postoperative hepatic failure with a 5%-10% mortality and a 30%-50% morbidity<sup>[19,20]</sup> and with a long-term risk for tumor recurrence, affecting 80% of the patients at 5 years<sup>[21-24]</sup>. Since the recurrence of the tumor after partial hepatectomy is common, rescue medical, radiographic, and surgical therapies are often needed.

There are scanty data on the clinical outcome of OLT as a rescue operation for patients with recurrent HCC after the initial tumor resection. We evaluated 77 consecutive patients with OLT, of whom 15 patients had rescue OLT for recurrent HCC. All the 15 patients had concomitant liver cirrhosis. These patients all had postoperative single or multiple sessions of TACE and/or PEI. We found no difference in the overall and tumor-free survivals between the rescue OLT and *de novo* OLT groups, suggesting that OLT may be a valid option for patients with recurrent HCC after the tumor resection.

In addition to its application in advanced stage HCC, OLT can also be a valid treatment option for

patients with early stage tumors if partial hepatectomy is not amenable<sup>[25]</sup>, even though the survival advantage of OLT over partial hepatectomy in these patients has not been confirmed<sup>[26,27]</sup>. When compared with partial hepatectomy, OLT for resectable HCC may offer a survival benefit in a subset of patients as long as the donor organ is available within 6-10 mo<sup>[16]</sup>.

OLT has increasingly been performed for HCC where reported 1- and 2-year cumulative survival rates were 90.0% and 65.6%, and the disease-free survival rates were 77.5% and 62.5%, respectively<sup>[28]</sup>. The 3-year survival reached 77%-80%<sup>[29,30]</sup>. Poon *et al*<sup>[7]</sup> reported that a 5-year overall survival and tumor-free survival of OLT for patients with recurrent small HCC (diameter ≤ 3 cm) after initial tumor resection were 48% and 0%, respectively. The 5-year survival rate of 422 HCC patients with OLT was 44.4%, and histologic grade of HCC and tumor size (> 5 cm) were found associated with tumor-free survival<sup>[31]</sup>. Multiple factors may have contributed to the improvement in survival in HCC patients after OLT, including appropriate selection of candidate patients, the application of surgical techniques minimizing the risk for intraoperative tumor spread, and postoperative adjunct medical therapy.

The most commonly used criteria for transplantation for HCC patients are the Milan criteria: solitary tumor ≤ 5 cm in diameter or 2 or 3 tumor nodules with the largest diameter ≤ 3 cm and the absence of macroscopic vascular invasion or extrahepatic metastasis<sup>[11]</sup>. In the current study, we used the Shanghai Criteria<sup>[9]</sup>, i.e., tumor size ≤ 9 cm, number of tumors ≤ 3, the absence of macrovascular tumor embolism(s), and the absence of

extrahepatic metastasis. The main difference between the Milan Criteria and Shanghai Criteria was the tumor size with cut-off 5 cm vs 9 cm.

A variety of factors were reported to be associated with a poor surgical outcome after OLT, including large tumor size<sup>[13,28,31,32]</sup>, the presence of vascular invasion<sup>[13]</sup>, the presence of portal vein thrombosis<sup>[28]</sup>, and poor histologic differentiation<sup>[31]</sup>. In the current study, the risk factor associated with a poor survival were pre-OLT hyperbilirubinemia, the requirement of post-OLT blood transfusion, large tumor size, family history of liver cancer, and the presence of tumor microembolism. The recurrence of HCC after the tumor resection as an indication of OLT was not found associated with a poor survival outcome after OLT. Therefore, OLT appears to be a valid option as rescue operation for recurrent HCC after tumor resection.

Post-operative corticosteroid use may be associated with a high risk for tumor recurrence in patients with OLT. Mazzaferro *et al*<sup>[11,33]</sup> reported that discontinuation of corticosteroid use for 3-6 mo in HCC patients with OLT had a lower risk for tumor recurrence and post-operative long-term corticosteroid use had a 4-fold increase in tumor recurrence after OLT. In addition, post-operative corticosteroid use appears to pose a higher risk for post-operative infection and metabolic side effects. It appears that post-operative immunosuppression with omission of cortisosteroid use may be safe<sup>[34]</sup>. In our study protocol, all patients had corticosteroid tapering and discontinued the agent at one month after OLT.

There are some limitations to the study. First, this is not a randomized trial with a small sample size in the rescue OLT group ( $n=15$ ), which would have been subjected to a type II error. Second, longer follow-up would be needed. Finally, the study was conducted in a tertiary care center specializing in surgical treatment of HCC and other liver disorders and there might have been a selection bias.

In conclusion, there are no significant differences in survival/mortality rates between OLT as a *de novo* therapy and OLT as a rescue therapy for patients with HCC. Pre-OLT hyperbilirubinemia, post-OLT requirement of transfusion, large tumor size, and family history of HCC are associated with a poor survival outcome.

## COMMENTS

### Background

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. Liver transplantation (LT) appears to be a valid rescue option for these patients. However, LT has not been routinely performed in patients with recurrent advanced HCC. Meanwhile, the risk factors of orthotopic liver transplantation (OLT) for HCC patients are unclear. So we hypothesized that OLT as a rescue operation for patients with recurrent HCC after initial resection surgery may have a similar outcome to these patients with HCC who had *de novo* OLT. The aims of the study were to compare post-OLT survival rates between patients who have recurrent HCC and those who have no history of resection surgery; and to evaluate risk factors associated with postoperative mortalities.

### Research frontiers

Liver transplantation is one of the hotspots in researches on liver tumors. But

few studies have tried to find the risk factors of OLT for HCC using the statistical method.

### Innovations and breakthroughs

No significant differences were found in survival/mortality rates between OLT as a *de novo* therapy and OLT as a rescue therapy for patients with HCC in this study. And Pre-OLT hyperbilirubinemia, post-OLT requirement of transfusion, large tumor size, and family history of HCC were found associated with a poor survival outcome.

### Applications

This study shows that there were no significant differences in survival/mortality rates between OLT as a *de novo* therapy and OLT as a rescue therapy for patients with HCC. And pre-OLT hyperbilirubinemia, post-OLT requirement of transfusion, large tumor size, and family history of HCC were associated with a poor survival outcome. The main limit in this research are the biology and genetics of tumors.

### Peer review

The authors described excellent results of the OLT for HCC patients using statistical methods. This article identified that recurrent HCC was not a risk factor of OLT. Meanwhile, pre-OLT hyperbilirubinemia, post-OLT requirement of transfusion, large tumor size, and family history of HCC play important roles in the prognosis of OLT. The findings are potentially important for planning of further studies.

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RAPID COMMUNICATION

# Effect of antidepressants on body weight, ethology and tumor growth of human pancreatic carcinoma xenografts in nude mice

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the mirtazapine group and the lightest ( $11.39 \pm 1.45$  g) in the fluoxetine group ( $F_{(2,12)} = 11.43, P < 0.01$ ). The behavioral test on d 7 showed that the horizontal and vertical activities were significantly increased in the mirtazapine group compared with the fluoxetine and control groups ( $F_{(2,18)} = 10.89, P < 0.01$ ). These effects disappeared in the mirtazapine and fluoxetine groups during 2-6 wk. The grooming activity was higher in the mirtazapine group than in the fluoxetine group ( $10.1 \pm 2.1$  vs  $7.1 \pm 1.9$ ) ( $t = 2.40, P < 0.05$ ) in the second week. There was no significant difference in tumor volume and tumor weight of the three groups.

**CONCLUSION:** Mirtazapine and fluoxetine have no effect on the growth of pancreatic tumor. However, mirtazapine can significantly increase food intake and improve nutrition compared with fluoxetine in a pancreatic cancer mouse model.

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**Key words:** Pancreatic carcinoma; Mirtazapine; Fluoxetine; Body weight; Nude mice; Locomotor activity; Ethology

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## INTRODUCTION

Pancreatic adenocarcinoma is the fourth leading cause of cancer-related death in the United States<sup>[1]</sup>. However, its incidence has increased steadily in China in recent years. At the time of diagnosis, 80% of patients present with either locally advanced or metastatic disease. In recent years, although gene therapy and biological targeting therapy can significantly inhibit the growth of pancreatic cancer in animal experiments, there is no satisfactory

therapy for pancreatic cancer patients to extend their median survival time and improve their quality of life. The overall five-year survival rate is less than 5%<sup>[2]</sup>.

The stress associated with the diagnosis and treatment of pancreatic cancer can cause significant psychiatric morbidity. It was reported that pancreatic cancer patients have the highest rate of major depression compared with other cancer patients<sup>[3]</sup>. Depression occurs in 47%-71% of patients with pancreatic cancer<sup>[4,5]</sup>. Unfortunately, depression adversely affects many clinical oncology outcomes. It can prolong hospital stay, augment the complication of therapy, decrease the ability to care oneself, reduce the compliance with medical treatment, lead to a poorer quality of life, and even shorten survival time<sup>[6-8]</sup>. Antidepressant medications not only improve depressive symptoms of patients with cancer but also reverse these adverse impacts<sup>[9]</sup>.

Clearly, antidepressant treatment constitutes one of the new strategies of cancer adjuvant therapy. However, data on treatment of depression with antidepressants in cancer patients are relatively scarce. The effect of different agents on distressing symptoms of cancer patients is still a subject for discussion. At present, selective serotonin reuptake inhibitor (SSRI) antidepressants are recommended as the first-line therapy for major depressive patients. Furthermore, mirtazapine is a new noradrenergic and specific serotonergic antidepressant (NaSSA), which stimulates 5-HT<sub>1</sub> receptors, but blocks serotonin 5-HT<sub>2</sub> and 5-HT<sub>3</sub> receptors and histamine H<sub>1</sub> receptors<sup>[10]</sup>, which may be associated with increasing appetite and weight gain. Recent studies have shown that serotonin has been extensively implicated in the regulation of ingestive behavior<sup>[11,12]</sup>.

Therefore, we performed experiments using fluoxetine and mirtazapine as representatives of SSRI and NaSSA, respectively. The aim of the study was to study the effects of oral mirtazapine and fluoxetine on body weight, food intake, locomotion and tumor growth in a subcutaneous pancreatic tumor model.

## MATERIALS AND METHODS

### **Drugs and reagents**

Mirtazapine was kindly provided by Organon, Oss, the Netherlands. Fluoxetine was purchased from Eli Lilly & Co (Indianapolis, IN). RPMI1640 and fetal bovine serum were purchased from Gibco (Grand Island, NY).

### **Experimental animals**

BALB/c nu/nu male and female mice (5 wk old, weighing 17-20 g) of SPF class were purchased from the Experimental Animal Center, Guangzhou University of Chinese Medicine. The mice were housed under pathogen-free conditions in the Animal Center of Sun Yat-Sen University (4-5 mice per cage at 22 ± 1°C room temperature) with free access to water and standard rat chow, in a 12 h light-dark cycle.

### **Pancreatic cancer cell line and culture conditions**

Human pancreatic cancer cell line SW1990 was a kind

gift from the Second Affiliated Hospital of Sun Yat-Sen University. The cell line was maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). Monolayer cultures were maintained on a culture flask and incubated in a mixture of 50 mL/L CO<sub>2</sub> and 950 mL/L oxygen at 37°C. Trypsinization was terminated with a medium containing 10% FBS and the cells were washed once with a serum-free medium and resuspended in Hank's balanced salt solution. Only single-cell suspensions displaying greater than 90% viability were used for injection.

### **Establishment of subcutaneous xenograft model**

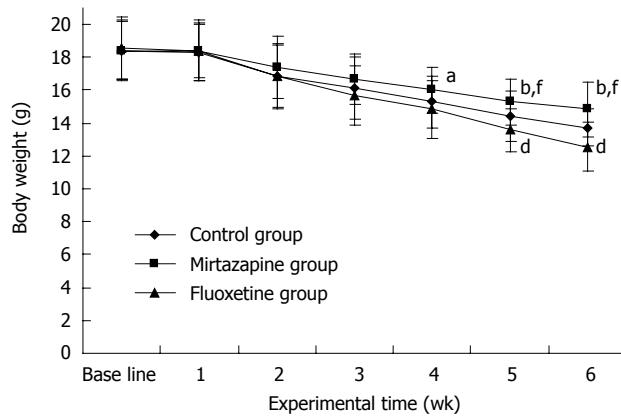
To produce SW1990 donor tumors, 3 × 10<sup>6</sup> cells per animal in a total volume of 0.2 mL were inoculated subcutaneously into the right flank of a nude mouse. Tumor size was measured *via* calliper. When the subcutaneous solid tumor reached approximately 1 cm in diameter and was aseptically removed from the donor animals. Macroscopically necrotic tissues and the remaining healthy tumor tissues were cut with scissors and minced into approximately 1 mm<sup>3</sup> pieces in Hanks' balanced salt solution containing 100 units/mL penicillin and 100 µg/mL streptomycin. A small incision was then made through the right dorsal flank and a tumor tissue piece was implanted subcutaneously beneath the dorsal flank skin of a nude mouse. We established a subcutaneous pancreatic cancer model as previously described<sup>[13]</sup> with certain modifications.

### **Experimental design**

After tumor transplantation, the mice were randomly assigned into three groups (7 mice each group). Treatment was initiated one day after tumor transplantation as the first day experiment. The first group received an equivalent normal saline solution as control. The second and third groups received 10 mg/(kg·d) mirtazapine and 10 mg/(kg·d) fluoxetine, respectively<sup>[14]</sup>, once a day for 42 d. Oral application was chosen as it is the standard application of antidepressants. For the study, the treated mice were closely monitored for any side effects and sacrificed on d 43. The transplanted tumor sizes were measured with a caliper, twice a week, and the tumor volume was calculated using the formula<sup>[15]</sup>: V = W<sup>2</sup> × L/2, where W is the width and L is the length of the tumor. Body was weighed biweekly and food intake was expressed as daily consumption in gram per animal weekly.

### **Open field test (OFT)**

OFT is a widely used test to evaluate the emotion and locomotor activity in rodents. As a test of spontaneous (unconditioned) behavior, it allows the animal to exhibit a wide range of behaviors and is therefore highly suitable for the study of complex phenomena such as anxiety or depression<sup>[16]</sup>. The open field apparatus used is a rectangular chamber (35 cm × 35 cm × 20 cm) made of plexigal, which was built from black walls and white floor. The floor of the open field was divided into 25 identical squares by 4 × 4 black lines<sup>[16]</sup>). A video camera



**Figure 1** Effects of antidepressants on body weight of nude mice. Data are represented as mean  $\pm$  SD. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs fluoxetine group, <sup>c</sup> $P < 0.01$  vs control group, <sup>d</sup> $P < 0.01$  vs control group.

was placed 1.5 m above the apparatus. After each trial, the apparatus was cleaned with water containing 0.1% acetic acid. The behavioral parameters registered during the first 5 min exposure to the open field apparatus were horizontal activity (the number of squares an animal entered), rearing known as vertical activity (the number of times an animal was standing on its hind legs with forelegs in the air or against the wall), grooming activity (the number of paws or tongues used to clean or scratch the body), which could reflect a stable individual trait "nonspecific excitability level". The OFT was performed weekly between 13:00 and 15:30. Any abrupt loud noise could markedly inhibit locomotion and even induce prolonged immobility of the mice. Therefore, the testing room was comprised merely of the background noise.

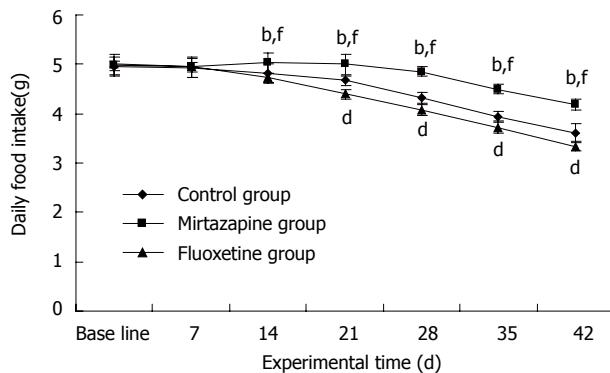
### Statistical analysis

Statistical analysis was performed with the SPSS 13.0 for Windows. Data were expressed as mean  $\pm$  SD. Pancreatic tumor weight, tumor volume and behavioral parameters were compared using one-way ANOVA. Significant differences in body weight and food intake were determined by two-way ANOVA and the Student-Newman-Keuls test for multiple comparisons between groups.  $P < 0.05$  was considered statistically significant based on a two-tailed test.

## RESULTS

### Effects of long-term antidepressant treatment on body weight

The change in body weight during the treatment is shown in Figure 1. The mice had a progressive weight loss. The body weight of mice in the three groups was very close in the first week. In the first 3 wk of treatment, the body weight of mice in the mirtazapine group was greater than that of mice in the other groups. However, no significant difference was observed. In wk 4, the body weight of mice was significantly greater in the mirtazapine group ( $16.00 \pm 1.41$  g) than in the fluoxetine group ( $14.86 \pm 1.77$  g) ( $F_{(2,12)} = 4.2$ ,  $P < 0.05$ ). The effect of mirtazapine lasted until the end of experiment. Nevertheless, the body



**Figure 2** Effects of antidepressants on food intake of mice. Data are represented as mean  $\pm$  SD. <sup>b</sup> $P < 0.01$  vs fluoxetine group, <sup>c</sup> $P < 0.01$  vs control group, <sup>d</sup> $P < 0.01$  vs control group.

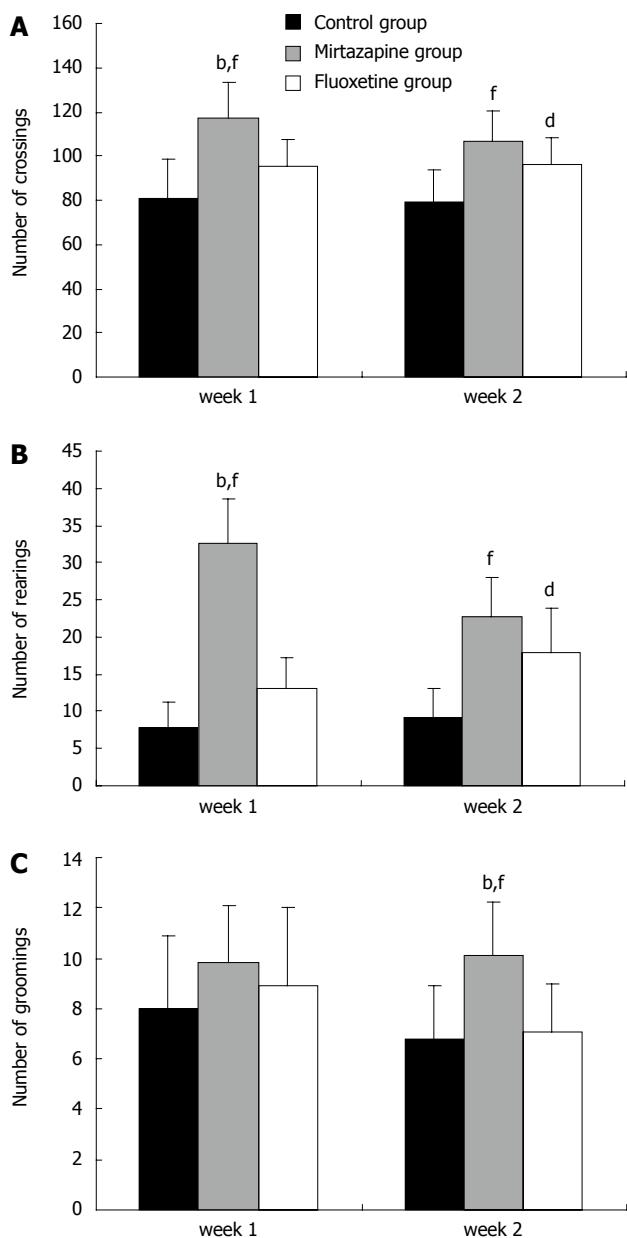
weight was significantly decreased in the fluoxetine group in week 5-6 compared with the control group ( $P < 0.01$ , Figure 1). The body weight of mice after removal of the tumor was also significantly increased in the mirtazapine group ( $13.66 \pm 1.55$  g) but decreased in the fluoxetine group ( $11.39 \pm 1.45$  g) compared with the control group ( $12.56 \pm 1.29$  g) ( $F_{(2,12)} = 11.43$ ,  $P < 0.01$ ).

### Effects of antidepressants on mice ingestive behaviour

Daily food intake of the tumor-bearing mice was gradually reduced over the whole treatment period (Figure 2). At initiation of the study, no difference was observed in ingestive behavior of mice in different groups. On day 14, food consumption of mice was significantly increased in the mirtazapine group ( $5.03 \pm 0.16$  g) compared with the fluoxetine ( $4.73 \pm 0.11$  g) and control groups ( $4.79 \pm 0.16$  g) ( $F_{(2,12)} = 23.31$ ,  $P < 0.01$ ). Mirtazapine exerted its effect to the end of experiment. However, fluoxetine treatment significantly decreased food consumption of mice compared with the control group from day 21 to 42 ( $P < 0.01$ , Figure 2).

### Locomotor behavior in open-field apparatus

Mirtazapine and fluoxetine significantly increased the locomotor activity of mice in the OFT. In the first week of behavioral test, the horizontal activity and vertical activity were significantly increased in the mirtazapine group ( $117.3 \pm 16.4$ ,  $95.3 \pm 13.6$ ) compared with the fluoxetine group ( $95.3 \pm 13.6$ ,  $13.0 \pm 4.2$ ) and control group ( $80.6 \pm 18.0$ ,  $7.9 \pm 3.4$ ) ( $F_{(2,18)} = 10.89$ ,  $F_{(2,18)} = 97.09$ ,  $P < 0.01$ , Figure 3A and B). There was no difference in grooming activity among the three groups. However, the grooming activity was significantly higher in the mirtazapine group ( $10.1 \pm 2.1$ ) than in the fluoxetine group ( $7.1 \pm 1.9$ ) ( $F_{(2,18)} = 4.90$ ,  $P < 0.01$ , Figure 3C) in the second week. Meanwhile, the horizontal and vertical activities were significantly increased in the mirtazapine and fluoxetine groups compared with the control group ( $P < 0.01$ , Figure 3A and B). Nevertheless, these parameters obtained from the mice treated with mirtazapine did not differ from those treated with fluoxetine during week 3-6.



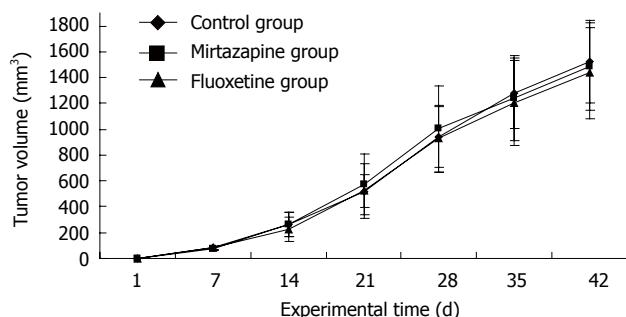
**Figure 3** Horizontal (A), vertical (B) and grooming (C) activity in pancreatic tumor-bearing mice in the OFT. Data are represented as mean  $\pm$  SD. <sup>b</sup> $P < 0.01$  vs fluoxetine group, <sup>d</sup> $P < 0.01$  vs control group, <sup>f</sup> $P < 0.01$  vs control group.

#### Effects of antidepressants on growth of pancreatic cancer *in vivo*

As shown in Figure 4, the tumor xenograft grew very rapidly with the prolongation of experiment. Nevertheless, no significant difference was observed in tumor volume of each group at any time point during the whole experiment. After 6-wk treatment, the animals were killed when the tumors were removed and weighed. However, no significant difference in tumor weight was detected in the mirtazapine group ( $1.18 \pm 0.20$  g), fluoxetine group ( $1.20 \pm 0.28$  g) and control group ( $1.23 \pm 0.34$  g) ( $F_{(2,18)} = 0.06$ ,  $P > 0.05$ ).

#### DISCUSSION

The results of the present study show that daily oral mirtazapine (10 mg/kg) could significantly increase



**Figure 4** Effect of antidepressants on the growth of pancreatic cancer xenografts. Data are represented as mean  $\pm$  SD.

food consumption and attenuated the rate of weight loss. However, treatment with fluoxetine (10 mg/kg) significantly suppressed food intake and promoted weight loss. Mirtazapine and fluoxetine showed their effects on the regulation of food intake and body weight to the end of experiment, suggesting that there is an extensive implication between serotonin and food intake. One explanation for the effects may be that the plasma half life of the two drugs is very long. It was reported that pharmacological agents that increase the levels of 5-HT in the central nervous system (CNS) suppress food intake, whereas drugs that antagonize the actions of 5-HT increase food intake<sup>[17]</sup>. Mirtazapine is a potent antagonist at postsynaptic 5HT<sub>2</sub> and 5HT<sub>3</sub> receptors which may potentially increase the appetite and body weight<sup>[18-20]</sup>. However, fluoxetine augments serotonergic activity by selectively inhibiting the reuptake of neurotransmitter, and reduces food intake and body weight in both animals<sup>[21, 22]</sup> and human beings<sup>[23]</sup>, and is thus used in the treatment of obesity<sup>[24]</sup>.

We examined the behavioral effects of mirtazapine and fluoxetine in the OFT. The horizontal activity fully reflected the animal activity, rearing the degree of curiosity to the novel surroundings, and grooming the level of alert against the novel environment. Mirtazapine and fluoxetine significantly increased the locomotor activity of pancreatic tumor-bearing mice compared with the control group. In the initial behavioral test, the horizontal activity was significantly increased in the mirtazapine group compared with the fluoxetine and control groups. Rearings were also significantly increased in the mirtazapine group compared with the fluoxetine and control groups (Figure 3A and B). Grooming activities increased earlier in the mirtazapine group than in the fluoxetine group. The results of the present study are consistent with the reported data<sup>[25, 26]</sup>, showing that antidepressants increase locomotor activity in a depressed model of normal rats. Nevertheless, mirtazapine adapted faster to the new environment and elevated earlier the alert of tumor-bearing mice against the novel environment. These findings indicate that mirtazapine is better than fluoxetine to tolerate the stress associated with the diagnosis and treatment of pancreatic cancer.

To our knowledge, the effect of mirtazapine and fluoxetine on the growth of pancreatic cancer in nude mice has not been reported. In the present study, the

tumor volume at any time points and tumor weight were not significantly different in the three groups. Interestingly, a previous study demonstrated that fluoxetine is neither a complete carcinogen nor a tumor promoter<sup>[27]</sup>, which is in agreement with our results obtained from pancreatic tumor-bearing mice. Moreover, Abdul *et al*<sup>[28]</sup> reported that the growth of subcutaneous PC-3 xenografts in athymic nude mice is significantly inhibited by antidepressants. Mirtazapine and fluoxetine also did not exhibit any toxicity throughout the whole treatment, suggesting that mirtazapine and fluoxetine can be safely used in the treatment of depression in pancreatic cancer patients.

Pancreatic cancer patients have not only distressing symptoms such as appetite loss, nausea, vomiting, weight loss, sleep disturbances and pain, but also psychiatric comorbidities such as adjustment disorder, depression frequently accompanying the disease process. It was reported that patients with pancreatic cancer have a weight loss of 83%-87% and approximately 30% of the patients have a weight loss of over 10%<sup>[29]</sup>.

In summary, mirtazapine as an adjuvant therapy is beneficial to the pancreatic cancer patients with depression<sup>[30]</sup>. Mirtazapine as the first-line therapy for depressed patients with advanced pancreatic cancer has a bright future. Nevertheless, further investigation and evaluation of mirtazapine are needed before it is widely used in clinical practice.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

The treatment of pancreatic cancer remains a great challenge. The majority of patients with pancreatic cancer develop major depression. Depression adversely affects many clinical outcomes. Antidepressant treatment has been accepted as one of the new strategies in cancer adjuvant therapy. However, systemic studies on the treatment of depression in patients with cancer have not been well documented. The effect of different antidepressants on distressing symptoms of cancer patients is a subject for further evaluation.

### Research frontiers

At present, fluoxetine is one of the selective serotonin reuptake inhibitor (SSRI) antidepressants which are recommended as the first-line therapy for depression. Mirtazapine belongs to a new family of noradrenergic and specific serotonergic antidepressants (NaSSA) used in the treatment of major depression.

### Innovations and breakthroughs

On the basis of previous data, this was the first study examining the effects of mirtazapine and fluoxetine on the growth of pancreatic cancer in nude mice. The results of the present study show that mirtazapine could significantly increase food intake and attenuate the rate of weight loss in experimental mice. However, fluoxetine could significantly suppress food intake and promote weight loss in tumor-bearing mice.

### Applications

To summarize the actual application values, mirtazapine neither inhibits nor promotes pancreatic tumor growth according to the findings from this study. The results support the hypothesis that mirtazapine as an adjuvant therapy is superior to fluoxetine for pancreatic cancer patients with depression.

### Peer review

The title accurately reflects the major contents of the article. On the basis of

previous researches, this paper is an original research article on the effect of mirtazapine and fluoxetine on the growth of human pancreatic carcinoma in nude mice. The findings are of great interest and provide a foundation for their application in clinical practice. The conclusions are reliable and valuable.

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RAPID COMMUNICATION

# Prognostic impact of metastatic lymph node ratio in advanced gastric cancer from cardia and fundus

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category ( $\chi^2 = 30.82, P < 0.001$ ) and MLR ( $\chi^2 = 69.39, P < 0.001$ ) independently influenced the prognosis. A linear correlation between MLR and the 5-year survival was statistically significant based on the multiple linear regression ( $\beta = -0.63, P < 0.001$ ). Hypothetically, the 5-year survival would surpass 50% when MLR was lower than 10%.

**CONCLUSION:** The MLR is an independent prognostic factor for patients with advanced gastric cancer from the cardia and fundus. The decrease of MLR due to adequate number of total resected lymph nodes can improve the survival.

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**Key words:** Stomach neoplasms; Lymph node metastasis; Metastatic lymph node ratio; Lymphadenectomy; Prognosis

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## Abstract

**AIM:** To investigate the prognostic impact of the metastatic lymph node ratio (MLR) in advanced gastric cancer from the cardia and fundus.

**METHODS:** Two hundred and thirty-six patients with gastric cancer from the cardia and fundus who underwent D2 curative resection were analyzed retrospectively. The correlations between MLR and the total lymph nodes, positive nodes and the total lymph nodes were analyzed respectively. The influence of MLR on the survival time of patients was determined with univariate Kaplan-Meier survival analysis and multivariate Cox proportional hazard model analysis. And the multiple linear regression was used to identify the relation between MLR and the 5-year survival rate of the patients.

**RESULTS:** The MLR did not correlate with the total lymph nodes resected ( $r = -0.093, P = 0.057$ ). The 5-year overall survival rate of the whole cohort was 37.5%. Kaplan-Meier survival analysis identified that the following eight factors influenced the survival time of the patients postoperatively: gender ( $\chi^2 = 4.26, P = 0.0389$ ), tumor size ( $\chi^2 = 18.48, P < 0.001$ ), Borrmann type ( $\chi^2 = 7.41, P = 0.0065$ ), histological grade ( $\chi^2 = 5.07, P = 0.0243$ ), pT category ( $\chi^2 = 49.42, P < 0.001$ ), pN category ( $\chi^2 = 87.7, P < 0.001$ ), total number of retrieved lymph nodes ( $\chi^2 = 8.22, P = 0.0042$ ) and MLR ( $\chi^2 = 34.3, P < 0.001$ ). Cox proportional hazard model showed that tumor size ( $\chi^2 = 7.985, P = 0.018$ ), pT

## INTRODUCTION

At present, patients with advanced gastric cancer from the cardia and fundus still have a poor prognosis despite some therapeutic modalities. Lymph node metastasis is considered one of the most important prognostic factors<sup>[1-3]</sup>. And lymphadenectomy is fundamentally critical in radical surgery. Its standardization highly depends on the accuracy of prognosis evaluation according to the classification of lymph node metastasis. The current American Joint Committee on Cancer/International Union Against Cancer (AJCC/UICC) TNM system (1997) has established the classification

system based on the number of metastatic lymph nodes. D2 curative resection, which includes gastrectomy and D2 lymphadenectomy, required dissection of all the Group 1 and Group 2 nodes classified by anatomical location. However, with the development of D2 lymphadenectomy, larger lymph nodes dissected may enable to find larger metastatic lymph nodes, which induces a migration in the staging system. The ratio of the number of metastatic lymph nodes over the total number of resected lymph nodes is introduced to prognosis evaluation. It was reported that metastatic lymph node ratio (MLR) can minimize the stage migration effect caused by increasing total dissected lymph nodes, also can help refine the current TNM stage system<sup>[4,5]</sup>. Though many studies on the prognostic significance of MLR in gastric cancer have been carried out, relevant researches on advanced gastric cancer from the cardia and fundus are still rare. Therefore, the aim of this retrospective study was to discuss the clinical impact of MLR in patients with gastric cancer from the cardia and fundus, and provide further evidence for rational lymphadenectomy.

## MATERIALS AND METHODS

### Materials

Two hundred and thirty-six cases, diagnosed as primary gastric cancer from the cardia and fundus were treated with curative resection at the Department of Oncology, Affiliated Union Hospital of Fujian Medical University, Fuzhou China, between January 1996 and June 2002. The surgical procedure was defined as curative when no grossly visible tumor tissue (metastasis or lymph node involvement) remained after the resection and the resection margins were histologically normal. There were 197 male (83.5%) and 39 female (16.5%) patients aged from 30 to 79 years with a mean of  $58.8 \pm 9.8$  years. All patients received a D2 or more extended dissection of all the Group 1 and Group 2 lymph nodes or more according to the Japanese Classification of Gastric Carcinoma (JCGC)<sup>[6]</sup>. Among the 236 patients, total gastrectomy (TG) was performed in 190 patients, and proximal subtotal gastrectomy (PSG) in 46. Lymph nodes were meticulously dissected from the en bloc specimens, and the classification of the dissected lymph nodes was determined by specialized surgeons who reviewed the excised specimens after surgery based on the JCGC. Clinical and histopathologic data of each patient were collected and recorded in a specifically designed data collection form. The histopathologic spectrum included papillary adenocarcinomas (47/236, 20%), tubular adenocarcinomas (101/236, 43%), mucinous adenocarcinomas (29/236, 12%), poorly differentiated adenocarcinomas (36/236, 15%), undifferentiated carcinomas (8/236, 3%) and others (15/236, 6%) according to the World Health Organization classification system. Based on the 5th Edition of UICC TNM system<sup>[7]</sup>, T category is defined as follows: T2: tumor invades muscularis propria or submucosa; T3: tumor penetrates serosa without invasion of adjacent structure; T4: tumor invades adjacent structures. N

category is defined as follows: N0: no regional lymph node metastasis; N1: metastasis in 1 to 6 regional lymph nodes; N2: metastasis in 7 to 15 regional lymph nodes; N3: metastasis in more than 15 regional lymph nodes. Among our patients, there were 25 at stage pT2, 118 at pT3 and 93 at pT4, respectively, while there were 42 pN0, 97 pN1, 68 pN2 and 29 pN3 respectively. Finally, 48 cases (20%) were categorized as stage II, 128 (54%) as stage III and 60 (25%) as stage IV. All the patients received postoperative chemotherapy, using 5-FU as the dominant agent. No patient received postoperative radiotherapy. The follow-up was carried out by specialized investigators, who were trained about the follow-up system for clinical observation. The median follow-up for the entire cohort was 44 mo (range: 1-136 mo). A total of 222 cases were followed up with a rate of 94.0%.

### Methods

All calculations were performed using the SPSS 11.5 statistical package. Correlation analysis was made to find the relationship between MLR and the total lymph nodes, positive nodes and the total lymph nodes. Cumulative survival was determined via the Kaplan-Meier method, with univariate comparisons between groups through the log-rank test. Covariates that remained significant through the univariate analysis were selected for multivariate analysis. Cox regression was used for multivariate analysis, with a forward stepwise elimination model. A multiple linear regression model to correlate MLR with 5-year survival was obtained based on Kaplan-Meier 5-year survival estimates for each MLR interval, using midpoint of MLR interval as the independent variate. Significance of differences was assumed at  $P$  values of less than 0.05.

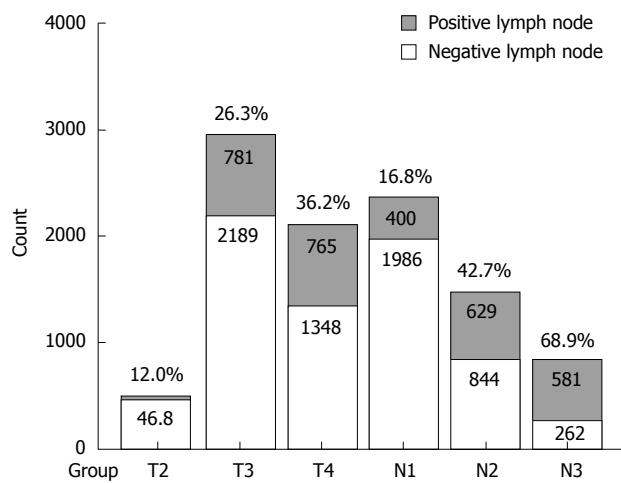
## RESULTS

### MLR of different pT/pN subcategories

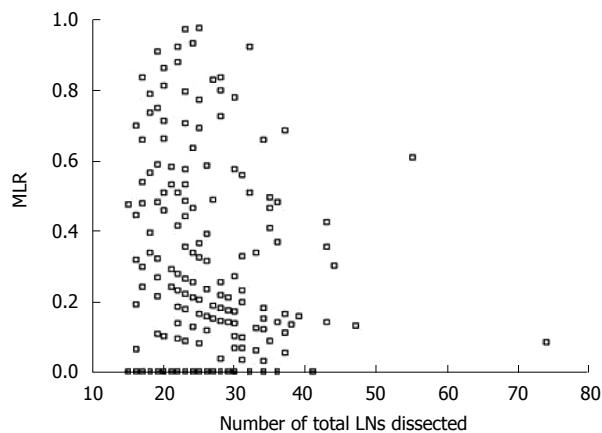
From the 236 cases, a total of 5615 lymph nodes were picked up and histologically examined, with 1610 positive and 4005 negative. The median total LN number was 23 (range 7-74, mean  $23.8 \pm 8.8$  per patient), the median number of positive LNs was 5 (range 0-44, mean  $6.8 \pm 6.8$  per patient), and that of negative LNs was 16 (range 0-48, mean  $16.9 \pm 9.3$  per patient). The MLRs were 12.0%, 26.3% and 36.2% in cases with pT2, pT3 and pT4, ( $\chi^2 = 138.9$ ,  $P < 0.001$ ), and 16.8%, 42.7% and 68.9% in cases with pN1, pN2, and pN3, respectively ( $\chi^2 = 820.7$ ,  $P < 0.001$ ). Figure 1 shows a trend of MLR according to different pT/pN subcategories. The MLR ascended as the invasion deepened, or the number of metastatic nodes increased.

### Correlation analysis between MLR and total lymph nodes, positive lymph nodes and total lymph nodes

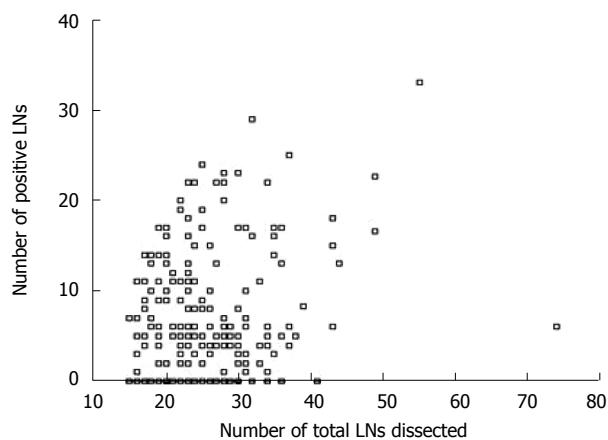
The MLR did not correlate with the total lymph nodes dissected ( $r = -0.093$ ,  $P = 0.057$ ), whereas positive lymph nodes did ( $r = 0.173$ ,  $P = 0.008$ ). Figures 2 and 3 show the scatters of these two groups. The results revealed that, in the same extent of lymphadenectomy, MLR



**Figure 1** MLR of different pT/pN subcategories.



**Figure 2** Scatter of MLR and total LNs dissected.



**Figure 3** Scatter of positive LNs and total LNs dissected.

would not increase with the number of total lymph nodes, but the number of positive lymph nodes would.

#### Univariate survival analysis

The 5-year overall survival rate of the entire cohort was 37.5%. The clinicopathological variables tested in the univariate analysis are shown in Table 1. Factors influencing the 5-year survival rate were patient gender

**Table 1** Univariate survival analysis of MLR and clinicopathological characteristics in 236 patients undergoing curative surgery

Characteristics	n	Median survival (mo)	5-yr survival (%)	$\chi^2$	P
Gender				4.26	0.0389
Male	197	45	36.3		
Female	39	51	43.6		
Age (yr)				1.18	0.2777
< 60	103	45	39.0		
≥ 60	133	46	36.6		
Tumor size (cm)				18.48	0.0000
< 3	88	60	45.5		
3-6	97	47	39.7		
> 6	51	32	18.1		
Borrmann's type <sup>1</sup>				7.41	0.0065
Borrmann I, II	172	50	41.7		
Borrmann III, IV	64	41	26.5		
Histological type				5.07	0.0243
Differentiated	213	47	38.9		
Undifferentiated	23	35	19.9		
pT category				49.42	0.0000
pT2	25	72	84.0		
pT3	118	54	40.2		
pT4	93	29	20.8		
pN category				87.7	0.0000
pN0	42	65	73.2		
pN1	97	55	42.4		
pN2	68	27	19.9		
pN3	29	17	0.0		
Num of dissected LNs				8.22	0.0042
< 15	36	27	22.4		
≥ 15	200	49	39.1		
MLR				34.3	0.0000
< 10%	59	63	60.5		
~ 20%	58	46	34.1		
~ 30%	33	44	33.5		
> 30%	86	28	21.0		
Type of gastrectomy				2.98	0.0844
TG	190	46	39.3		
PSG	46	45	31.3		

<sup>1</sup>Borrmann's type: Macroscopic appearances of primary tumor, classified as Type I : polypoid tumors; Type II: ulcerated carcinomas with demarcated and raised margins; Type III: ulcerated carcinomas without definite limits, infiltrating into the surrounding wall; Type IV: diffusely infiltrating carcinomas.

( $P = 0.0389$ ), tumor size ( $P < 0.001$ ), Borrmann type ( $P = 0.0065$ ), histological grade ( $P = 0.0243$ ), pT category ( $P < 0.001$ ), pN category ( $P < 0.001$ ), total number of dissected lymph nodes ( $P = 0.0042$ ) and MLR ( $P < 0.001$ ). Patient age ( $P = 0.2777$ ) and type of gastrectomy ( $P = 0.0844$ ) had no significant influence on the survival.

#### Multivariate survival analysis

Multiple survival analysis was calculated by the Cox's proportional hazard regression model. In order to confirm the influence of MLR, the prognostic factors considered at univariate analysis were analyzed first by stepwise regression, including gender, tumor size, Borrmann type, histological grade, pT category, pN category and total number of dissected lymph nodes except MLR. As a result, there were four independent, statistically significant prognostic parameters: tumor

**Table 2 Multiple stepwise regression analysis with Cox proportional hazards model**

Characteristics	$\beta$	Wald	$P$	RR	95% CI for RR	
					Low	High
MLR excluded						
Tumor size		6.665	0.039			
3-6 cm vs < 3 cm	0.222	1.938	0.164	1.249	0.913	1.707
> 6 cm vs < 3 cm	0.501	6.636	0.010	1.650	1.127	2.414
pT category		27.747	0.002			
pT3 vs pT2	0.761	9.184	0.000	2.140	1.308	3.499
pT4 vs pT2	1.286	24.369	0.000	3.617	2.171	6.026
pN category		65.066	0.000			
pN1 vs pN0	0.618	9.792	0.002	1.855	1.260	2.733
pN2 vs pN0	1.224	30.727	0.000	3.402	2.206	5.244
pN3 vs pN0	2.156	57.470	0.000	8.639	4.947	15.090
Num of total LNs	-0.682	10.684	0.001	0.506	0.336	0.761
MLR included						
Tumor size		7.985	0.018			
3-6 cm vs < 3 cm	0.307	3.863	0.049	1.359	1.001	1.845
> 6 cm vs < 3 cm	0.512	7.353	0.007	1.668	1.152	2.415
pT category		30.821	0.000			
pT3 vs pT2	0.772	9.940	0.002	2.165	1.339	3.500
pT4 vs pT2	1.343	26.759	0.000	3.832	2.303	6.375
MLR	2.569	69.390	0.000	13.06	7.134	23.900

size ( $P = 0.039$ ), pT category ( $P = 0.002$ ), pN category ( $P < 0.001$ ) and total number of dissected lymph nodes ( $P = 0.001$ ). When MLR was included in the Cox's regression, the overall fit of the Cox model increased (likelihood ratio test with and without MLR: 397 and 305, respectively). Tumor size ( $P = 0.018$ ), pT category ( $P < 0.001$ ) and MLR ( $P < 0.001$ ) were remained to be independent prognostic factors, with MLR being the most significantly independent factor. The risk ratios and their 95% confident interval are listed in Table 2.

#### MLR impact on overall survival

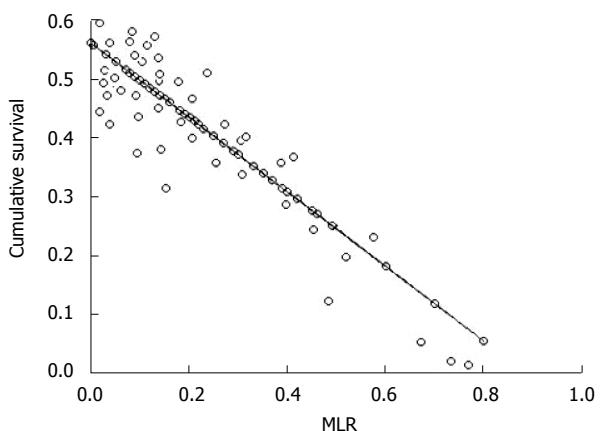
Linear trend test found that tumor size and MLR both had a linear correlation to the 5-year overall survival rate, and the correlation coefficients ( $r$ ) were  $-0.157$  ( $P = 0.019$ ) and  $-0.655$  ( $P < 0.0001$ ), respectively. Obviously, MLR showed a much stronger correlation than tumor size. Despite tumor size and overall survival related, only MLR can reach statistically significant differences according to the multiple linear regressions ( $P < 0.0001$ ). As shown in Table 3, the regression equation of the assumed statistically linearity was  $y = -0.63x + 0.56$ . Figure 4 presents a regression line of this calculated MLR effect on the 5-year survival. The hypothetical baseline 5-year survival (based on the  $y$ -intercept, i.e. MLR 0%) was 56%. For every 10% added to MLR, the calculated 5-year survival rate decreased by 6.3%. Hypothetically, the 5-year survival would surpass 50% when MLR was lower than 10%.

## DISCUSSION

Over the last few decades, the rising trends in incidence rates for upper third gastric cancer have been reported by many investigators around the world<sup>[8,9]</sup>. Cancer of the gastric cardia and fundus is commonly found in late

**Table 3 Estimation based on multiple linear regression model (stepwise method)**

Parameters	$\beta$	$t$	$P$	95% CI for $\beta$
Tumor size	-0.11	-1.97	0.051	
MLR	-0.63	-10.4	0.000	-0.71 to -0.51
Intercept (constant)	0.56	19.0	0.000	0.48 to 0.59

**Figure 4** Regression line of MLR impact on the 5-year survival rate.

or the advanced stage at the initial diagnosis<sup>[10,11]</sup>. It was warranted to link the poor prognosis to lymph node metastases for cancer of this anatomical location reported with a higher frequency of perigastric lymph nodes and higher proportion of overall lymph node metastasis<sup>[12,13]</sup>. A prospective study conducted by de Manzoni *et al*<sup>[14]</sup> showed that 56.9% of patients with types II and III cardia cancer had nodal spread. Di *et al*<sup>[15]</sup> set up a research on lymph node involvement in gastric cancer for different sites, showing an involvement in 80.4% of cases for upper third cancers. In the present study, 82.2% of cases were found to have lymph node metastasis. And the overall MLR was 28.7%. These data confirmed the cited reports.

Lymph node metastasis is one of the most important prognostic factors for gastric cancer after curative resection. Methods of metastatic lymph nodes evaluation are still under investigation and being continuously improved, including some immunohistochemical methods, in order to predict the prognosis and guide the therapy. But few special proteins expressed in gastric cardia cancer, compared with non-cardia cancer. For example, with regard to the mucin phenotype, MUC1 and MUC5AC expression was less frequent in cardia carcinomas than in non-cardia carcinomas<sup>[16]</sup>. Therefore, lymph node status mainly depends on routine pathological examinations, previously based on the anatomical station of metastatic lymph nodes, and is classified by the number of metastatic regional lymph nodes<sup>[17-19]</sup>. The problem of lymph node classification based on number of metastatic ones is the stage migration, induced by larger lymph nodes dissected. Researches on prognostic impact of MLR have been done in patients with colon cancer, pancreatic cancer, breast cancer and other carcinomas<sup>[20-23]</sup> to find its advantage

in predicting survival outcome after curative resection. MLR was introduced as a more reliable prognostic factor for gastric cancer<sup>[24,25]</sup>. In our study, a greater MLR was associated with poorer survival by univariate analysis. Multivariate analysis further identified that the MLR was a most important independent prognostic factor among the other factors evaluated, including pN category. This phenomenon was in agreement with those reported by some other investigators. It may be a superior indicator for lymph node classification system. Relevant data were reported about the grouping of patients with gastric cancer with lymph node metastasis ratios. By imitating the pN category of UICC/AJCC, most researchers selected three or four different groups. Different N ratio cutoffs have been proposed<sup>[26-29]</sup>, such as 0%, 1% to 9%, 10% to 25%, > 25%; 0%, < 25%, < 50%, > 50% and so on. Many authors did not describe a specific method for the selection of the reported cutoffs. Hyung *et al*<sup>[30]</sup> discovered that the cutoff values were 10% for T3N1M0 and 25% for T3N2M0 by analysis of the prognosis according to MLR. In the present study, we found the 5-year survival would surpass 50% when MLR was lower than 0.10.

Our study also showed that MLR can reflect the number of LNs examined and the quality of LN dissection. Curative resection was the determining factor to improve the 5-year survival in this type of tumor<sup>[31]</sup>. Although D2 radical resection for patients with cancer of gastric cardia and fundus is widely accepted<sup>[32]</sup>, how many lymph nodes should be removed to accurately predict clinical outcome has not been determined. Barbour *et al*<sup>[33]</sup> suggested patients with Siewert types II and III adenocarcinoma of the GEJ should undergo adequate lymphadenectomy to permit examination of  $\geq 15$  lymph nodes allowing the accurate identification of prognostic variables. Removal of  $\geq 15$  lymph nodes is associated with more accurate survival estimates for patients with advanced disease. Gee *et al*<sup>[34]</sup> stated that preferably 20-25 lymph nodes were necessary for determining prognosis and treatment for tumors of the gastroesophageal junction. In the present study, MLR did not correlate with the total lymph nodes while the number of metastatic LNs did. This finding indicated that the extent of lymphadenectomy was adequate when MLR value did not fluctuate with the resected number of lymph nodes. Obviously, it requires a certain amount of lymph nodes to be dissected. McKee *et al*<sup>[35]</sup> pointed out, MLR may be confounded by the small number of nodes examined from each patient, it should not be used for prognostic information in patients with fewer than 15 nodes examined. The median total LN number was 23 (mean  $23.8 \pm 8.8$  per patient) in this study, so we suggested D2 lymphadenectomy in order to ensure adequate dissection.

In conclusion, MLR has advantages in providing a more precise prognostic evaluation. We should pay attention to the clinical impact of MLR on prognosis of gastric cancer located in cardia and fundus when performing a D2 radical resection. It is warranted to make efforts to reduce MLR, preferably lower than 10%, in order to achieve better therapeutic efficiency.

## COMMENTS

### Background

The incidence rates of gastric cancer located in cardia and fundus have increased in recent years. Though the staging system of gastric cancer refines step by step, staging techniques never stop updating. So far, few studies have investigated the relative contribution of metastatic lymph node (LN) ratio to the prognosis evaluation with advanced gastric cancer from cardia and fundus.

### Research frontiers

Some researches have shown that metastatic lymph node ratio (MLR) is an excellent predictor for survival outcome in patients with colon cancer, pancreatic cancer, breast cancer and other carcinomas. Some related studies on gastric cancer also found the potential of the MLR on prognostic evaluation, but without a consensus on stratification cutoffs, especially lack of data for advanced gastric cancer located in cardia and fundus.

### Innovations and breakthroughs

The authors retrospectively reviewed 236 patients with gastric cancer from cardia and fundus who were treated with D2 radical resection in a hospital in Fujian between 1996 and 2002, to investigate the validity of metastatic LN ratio as a prognostic factor. The study not only divided MLR into some different grades for survival analysis, but also set up a regression to discover the relation between MLR and survival.

### Applications

The authors suggest that metastatic LN ratio can provide more dependable and accurate information on the extent of LN metastasis and lymphadenectomy for advanced gastric cancer located in cardia and fundus. Moreover, it is an important prognostic factor and can provide further evidence for rational lymphadenectomy.

### Peer review

This is an interesting and well-done study investigating the prognostic role of MLR in cancer of gastric cardia and fundus. This is a well-written and significant paper.

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S- Editor Li DL L- Editor Ma JY E- Editor Ma WH



CASE REPORT

## Evidence for colorectal sarcomatoid carcinoma arising from tubulovillous adenoma

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of sarcomatoid carcinoma remain speculative. To the best of our knowledge, this is the first report of co-existence of sarcomatoid carcinoma and invasive adenocarcinoma with tubulovillous adenoma; all stages represented within the same tumor. This observation supports the "monoclonal theory" of pathogenesis with an adenoma-sarcoma progression with or without an intermediate stage of carcinoma.

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**Key words:** Sarcomatoid carcinoma; Tubulovillous adenoma; Adenocarcinoma; Rectum; Cytokeratin

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## Abstract

Sarcomatoid carcinomas of the colorectum are rare tumors that display both malignant epithelial and stromal components. Clinically, they are aggressive tumors with early metastasis. Due to their infrequent occurrence, the pathogenesis is poorly understood. We report a case of a 52-year-old woman who presented with a rectal mass and intermittent hematochezia. Superficial biopsies during colonoscopy revealed a tubulovillous adenoma with high-grade dysplasia. Endoscopic ultrasound confirmed an invasive nature of the mass, and deeper biopsies revealed the presence of neoplasm with mixed histological components. The surgically-excised specimen demonstrated the presence of poorly differentiated spindle cells underneath the tubulovillous adenoma and an intermediate stage of invasive adenocarcinoma. Based on the histological appearance and immunohistochemical studies, a diagnosis of sarcomatoid carcinoma was made. Only nine cases of sarcomatoid carcinomas of the colorectum have been reported to date. As a result, the terminology and pathogenesis

## INTRODUCTION

Sarcomatoid carcinoma is a rare malignant tumor characterized by a combination of epithelial and mesenchymal elements. Over the past 100 years, sarcomatoid carcinomas have been increasingly recognized at different anatomic locations including the head and neck, respiratory tract, and female reproductive organs<sup>[1-3]</sup>. Within the gastrointestinal tract, the oropharynx and esophagus are the most commonly affected areas<sup>[1,4-7]</sup>. To our knowledge, sarcomatoid carcinomas rarely occur in the colon, with only nine cases reported in the English literature<sup>[8-12]</sup>. A possible reason is likely due to their marked similarity to malignant mesenchymal tumors, such as gastrointestinal stromal tumors, malignant fibrous histiocytoma, and leiomyosarcoma<sup>[12-16]</sup>. As a result, the natural history, pathogenesis, and treatment of these unusual tumors are poorly understood. In general, sarcomatoid carcinoma of the colon has been described as an aggressive neoplasm with an associated poor prognosis.

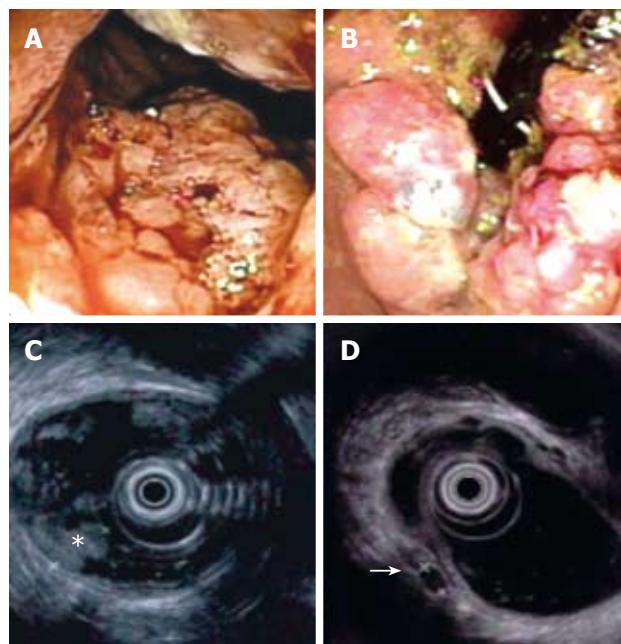
First described by Virchow in 1864, a variety of terms

have been used to describe sarcomatoid carcinomas. They include carcinosarcoma, pseudosarcomatous carcinoma, carcinoma with mesenchymal stroma, and spindle cell carcinoma<sup>[17]</sup>. These varied terminologies for sarcomatoid carcinomas reflect the uncertainty of its histogenesis and classification. Several theories have been proposed to explain the histopathogenesis of sarcomatoid carcinoma; however, these theories remain speculative.

We report an unusual case of a mixed rectal tumor containing a superficial tubulovillous adenoma with deeper areas of high-grade malignant spindle cells and an invasive adenocarcinoma; all stages represented within the same tumor. This could support an adenoma-to-sarcomatoid progression either directly or indirectly via an intermediate stage of adenocarcinoma. In addition, we discuss the basis of pathologic diagnosis, proposed theories regarding its histopathogenesis and review the clinical features of this heterogenous tumor.

## CASE REPORT

A 52-year-old white female presented to the emergency department with a prolapsed rectal mass and intermittent rectal bleeding over the past 10 years. Until presentation, she had attributed her symptoms to hemorrhoidal disease and had performed digital reduction of the prolapsed ‘mass’ from time to time. Increased bleeding and frequency of prolapse made her seek medical attention. Her past medical history was unremarkable; however, there was a family history of colon cancer in her father. Physical examination revealed a large, firm, ulcerated mass that prolapsed from her rectum. Serum levels of carcinoembryonic antigen (CEA) and carbohydrate antigen (CA) 19-9 were not elevated. Colonoscopy confirmed the presence of a large sessile exophytic rectal growth with a velvety, multi-lobulated surface, just proximal to the dentate line (Figure 1A and B). Several biopsies were performed using a jumbo forceps, which revealed a tubulovillous adenoma with high-grade dysplasia. However, the possibility of an adjacent invading tumor beneath the tubulovillous adenoma could not be ruled out given the lack of depth from the biopsy. An extensive staging workup including a CT scan of the chest and abdomen showed no signs of distant metastasis. To further evaluate the tumor for any presence and extent of local invasion, a rectal endoscopic ultrasound (EUS) was performed. At EUS, the invasive nature of the mass became apparent as the tumor invaded into the lamina propria and into the muscularis mucosa (Figure 1C, star). Additionally, an 8-mm hypoechoic well-defined malignant-appearing lymph node was found adjacent to the mass, suggestive of lymphatic metastasis (Figure 1D, arrow). During this procedure, a 1-cm × 1-cm piece of the tumor was snared off to obtain deeper sections of the mass for further histopathological diagnosis, and which demonstrated a neoplasm with mixed histological components consisting of tubulovillous adenoma (Figure 2A and B) and poorly differentiated spindle cells (Figure 2C to E). Subsequently, the patient underwent an abdominoperineal resection of this mass. *Ex-vivo* pathologic analysis of the surgically re-

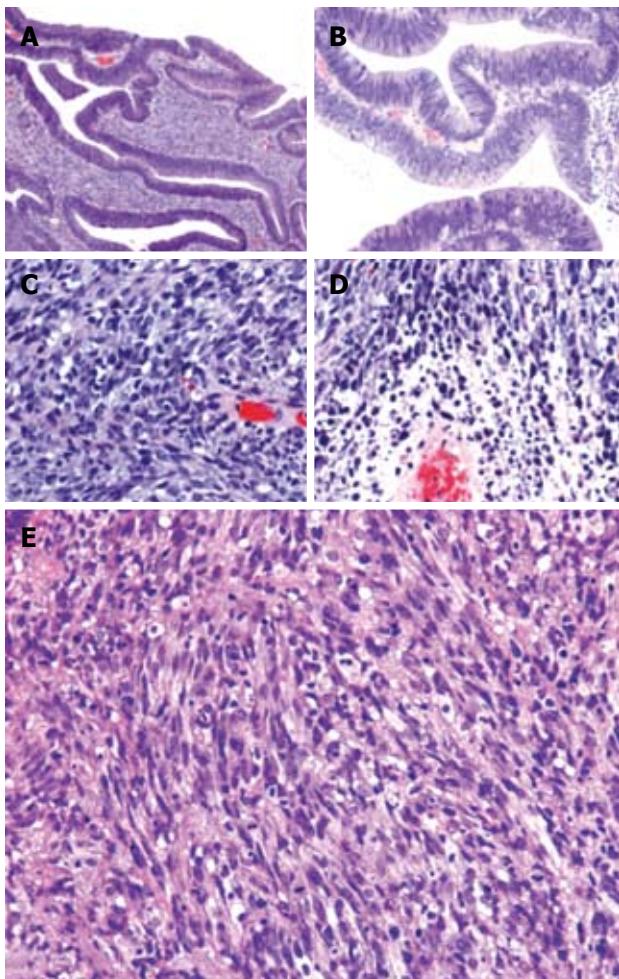


**Figure 1** Colonoscopy demonstrates. **A:** Large sessile polypoid growth with velvety surface and superficial ulceration in the rectum (forward view); **B:** Multilobulated, smooth surfaced exophytic nature of the tumor upon retroflexion. Sonographic images at rectal EUS showing; **C:** An infiltrative mass with invasion of the muscularis mucosa (star); **D:** An 8-mm hypoechoic lymph node suspicious for lymphatic metastasis (arrow).

moved rectum showed a superficial layer of tubulovillous adenoma with high-grade dysplasia arising just proximal to the dentate line and extending 6.2 cm proximally (Figure 3A and B). Its deeper sections featured islands of intermediate stage invasive adenocarcinoma with poorly organized glandular structures (Figure 3C) within a background of poorly differentiated sheets of spindle cells. Immunohistochemical studies showed strong positivity for cytokeratin in both epithelial and stromal components of the tumor (Figure 3D). Histological analysis of all 41 lymph nodes showed no evidence of metastasis including the 8-mm hypoechoic lymph node seen on EUS. Based on the histological appearance and immunohistochemical studies, a diagnosis of sarcomatoid carcinoma was made. The disease was clinically staged according to the American Joint Committee on Cancer as a Stage I (T1N0M0) colorectal cancer. The patient’s postoperative course was uneventful and she remains free of tumor recurrence or metastasis after an 8-mo follow up.

### Case tissue

Tissue from the rectal tumor was fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at 4 µm in thickness, floated onto positively charged slides, and dried overnight at 70°C. From each block, 5 micron thick sections were cut and stained with haematoxylin and eosin (HE). For immunohistochemical analysis, the avidin-biotin complex method was used with the following antibodies: pancytokeratin (Figure 3D), vimentin (Figure 3E), smooth muscle actin (SMA), S100, Bcl-2, CD34, smooth muscle myosin (SMMS), p53 (Figure 4A), CD117 (Figure 4B), desmin, and PDGFR-alpha

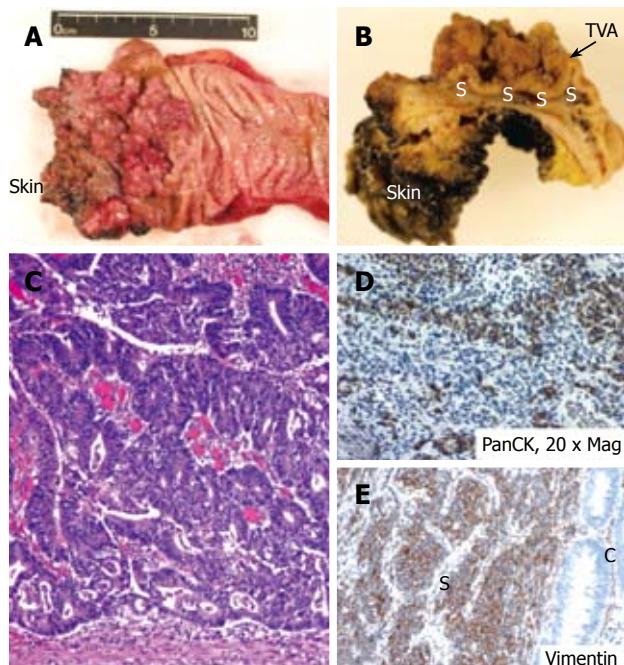


**Figure 2** Histology of the rectal biopsy using HE stains. **A:** Tubulovillous adenoma and underlying spindle cell tumor. The aggressive spindle cell lesion infiltrates directly underneath the adenoma ( $\times 10$ ). **B:** A higher magnification view of the adenomatous component ( $\times 20$ ). High-grade spindle cell lesion ( $\times 40$ ) showing: **C:** Cigar shaped nuclei, nuclear pleomorphism, a high nucleus/cytoplasm ratio within the deeper sections; **D:** Tumor necrosis; **E:** Smooth muscle-like spindle sheets of cells in the sarcomatoid component ( $\times 40$ ).

(Figure 4C). The immunohistochemical profile is listed in Table 1. Appropriate positive and negative control tissues were incubated in parallel with the case slides to confirm the specificity of each antibody.

### Gross findings

The surgical specimen consisted of a sigmoid and rectal segment measuring about 36.5 cm in length with a luminal diameter varying from about 4.8 cm at the proximal end to 8.0 cm at the distal end (Figure 3A and B). Located at the distal end of the specimen was an exophytic, circumferential, and fungating mass measuring about 5.5 cm in length along the gastrointestinal tract, rising approximately 1.3 cm from the luminal surface, with a greatest diameter of 6.2 cm. Just distal to the fungating mass was a squamous anal mucosa measuring about 0.8 cm in length. The remainder of the colonic mucosa was covered by small papules, which were slightly whiter than the gray mucosal background and measured about 0.1-0.3 cm in greatest dimension. These papules covered about 80% of the total surface of the colon and were most prominent in the



**Figure 3** Gross appearance of the surgically-removed rectosigmoid mass (**A** and **B**). **A:** The luminal side view demonstrates the proximity to the dentate line; anal skin is labeled for orientation; **B:** Upon sectioning the sample through the sagittal plane, the lateral view identifies the sarcomatoid smooth component (marked as S) underneath the velvety tubulovillous adenoma component (marked as TVA); **C:** Invasive adenocarcinoma with high nucleus/cytoplasm ratio within the deeper sections; **D:** Pancytokeratin was diffusely positive in both epithelial and mesenchymal components ( $\times 20$ ); **E:** Vimentin showed expression in the sarcomatous (S) component only, with no staining in the carcinoma (C) portion.

**Table 1 Antibodies used for histological evaluation of the tumor, and a summary of the results**

Antibody	Concentration	Company	Result
Smooth muscle actin (SMA)	1:2	Dako	Negative
S100	1:1000	Zymed	Negative
Bcl-2	1:60	Dako	Negative
CD34	1:30	Becton Dickinson	Negative
PDGFA- $\alpha$	1:120	Santa Cruz Biotechnology	Non-contributory
Smooth muscle myosin (SMMS)	1:100	Dako	Negative
CD117	1:150	Dako	Negative
Desmin	1:100	Dako	Negative
Vimentin	1:100	Dako	Negative
p53	1:100	Dako	Positive
Pancytokeratin	1:100	Zymed AE1/AE3 Becton Dickinson Cam 5.2	Patchy positive

distal area near the fungating mass. No ulcers or strictures were noted. Forty-one lymph nodes were isolated from the adipose tissue surrounding the bowel wall and were negative for any evidence of metastasis.

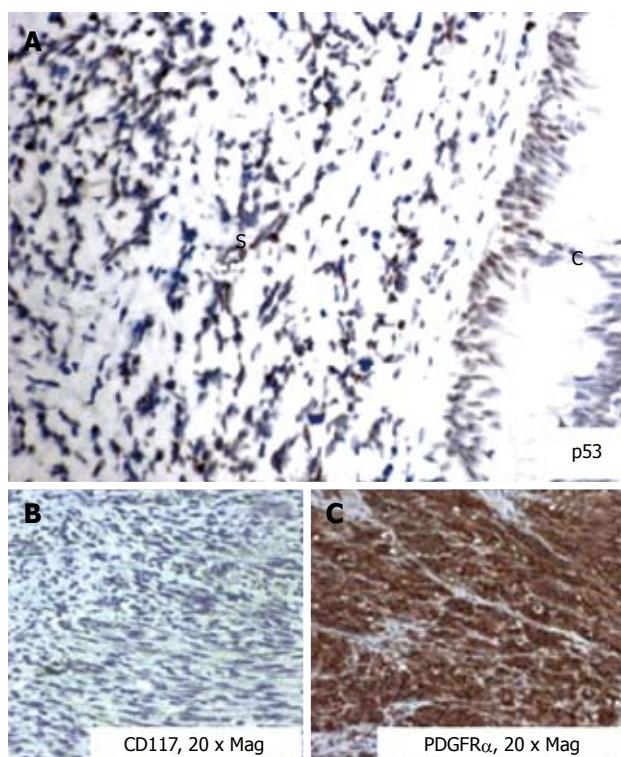
### Histological findings

The initial superficial biopsies from the large rectal mass showed a tubulovillous adenoma with high-grade dysplasia; however, the possibility of adjacent invasive tumor could not be ruled out given the lack of depth from the

**Table 2 Summary of demographics and outcome of cases reported in the English literature with the diagnosis of colorectal sarcomatoid carcinoma**

Case	Author, yr	Age (yr)	Sex	Site	Distant metastasis	Outcome
1	Weidner, 1986	73	M	Sigmoid	On follow up	4 yr, DOD
2	Chetty, 1993	72	F	Cecum	On initial visit	3 mo, DOD
3	Roncaroli, 1995	71	F	Rectum	On follow up	6 mo, DOD
4	Isimbaldi, 1996	86	F	Ascending colon	None	2 yr, disease free
5	Shoji, 1998	78	M	Descending colon	None	16 mo, disease free
6	Takeyoshi, 2000	82	M	Rectum	On follow up	6 mo, DOD
7	Kim, 2001	41	F	Sigmoid	On follow up	4 mo, DOD
8	Di Vizio, 2001	56	F	Descending colon	On follow up	21 mo, DOD
9	Kim, 2005	71	M	Ascending colon	On initial visit	Unspecified
10	Present case	52	F	Rectum	None	8 mo, disease free

DOD: Died of disease.



**Figure 4** Immunohistochemical characteristics of the resected tumor showed. **A:** p53 was expressed in both carcinomatous (C) and sarcomatous (S) components within the tumor, with a relative increase in the latter; **B:** Staining for CD117 was negative; **C:** PDGFR stained diffusely positive in both the components (x 20).

biopsies. Subsequently, a larger piece of tumor tissue, obtained during a EUS procedure, revealed a neoplasm with mixed histological components consisting of high-grade spindle cells and a tubulovillous adenoma (Figure 2). The high-grade spindle cell tumor showed cigar shaped nuclei, nuclear pleomorphism, a high mitotic rate, a high nucleus/cytoplasm ratio, and central necrosis (Figure 2C-E). The spindled cells appear to infiltrate from the deep aspect of the biopsy into the mucosa and surround the adenomatous glands in an aggressive fashion. The tubulovillous adenoma, similar to the previous biopsy, contained abundant dysplastic epithelial glands with very few mucus.

The surgical specimen of the sigmoid colon and rectum revealed an invasive well-differentiated adenocarci-

noma in a background of a large tubulovillous adenoma (Figure 3C). In addition, there was a focal sarcomatous component consisting of high-grade spindle cells. The carcinoma was composed of neoplastic epithelial cells, arranged in nests forming glandular structures with increased nuclear/cytoplasmic ratio and prominent nucleoli. The invasive carcinoma was confined to the submucosa (pT1) without gross or microscopic evidence of muscular invasion. Surgical margins were free of tumor and there was no evidence of metastasis in all 41 lymph nodes. According to the TNM classification, the pathological stage was pT1N0.

#### Immunohistochemistry

Immunohistochemical analysis revealed focal, but strong staining for pancytokeratin (Figure 3D), focal staining for vimentin (Figure 3E), smooth muscle actin, and patchy cell clusters of desmin in the spindle cell component of our specimen. Although staining for p53 revealed immunoreactivity in both the epithelial and sarcomatous components, it was relatively increased in the sarcomatous component (Figure 4A). In addition, the sarcomatous component was weakly positive for CD34 and Bcl-2, but negative for CD117 (Figure 4B). The epithelial component was diffusely immunoreactive to pancytokeratin and negative to smooth muscle actin, desmin, CD34, CD117, and Bcl-2. Since there is a subset of gastrointestinal stromal tumors that are negative for CD117 and positive for PDGFR- $\alpha$ , we performed an immunohistochemical staining for PDGFR- $\alpha$ , which stained diffusely positive in both the spindle and epithelial cells of the overlying tubulovillous adenoma (Figure 4C). Taken together, the strong cytokeratin staining and the focal positivity for smooth muscle markers, the tumor adjacent to the tubulovillous adenoma was diagnosed as sarcomatoid carcinoma rather than a carcinosarcoma or CD117-negative GIST.

#### DISCUSSION

Sarcomatoid carcinoma of the colon is a rare clinical and pathological entity. To the best of our knowledge, only nine cases of colonic sarcomatoid carcinoma have been reported in the English literature (Table 2), the present case being the tenth. Histologically, sarcomatoid carci-

nomas of the colon contain both epithelial and mesenchymal components. The epithelial component of these tumors mainly consists of high-grade adenocarcinoma, while the accompanying sarcomatous component often demonstrates a spindled appearance with varying degrees of mesenchymal-like differentiation.

The nomenclature of these bi-differentiated tumors has been a matter of debate despite the advent of immunohistochemistry and electron microscopy. In the past, the term pseudosarcoma was used to describe the condition in which the epithelial component was malignant and spindle cells were benign<sup>[17-19]</sup>. Later, Matsusaka *et al* showed that pseudosarcoma and sarcomatoid carcinoma were histologically and clinically the same conditions<sup>[20]</sup>. Moreover, several reports have used carcinosarcoma interchangeably to describe sarcomatoid carcinoma, further confusing the diagnosis and classification of these tumors<sup>[13,16,21]</sup>. Rosai J explained that when the sarcomatous component is mainly composed of spindle cells but still identifiable as epithelial ones morphologically or immunohistochemically, the diagnosis should be called sarcomatoid carcinoma<sup>[22]</sup>. However, when sarcomatous components reveal typical specialized differentiation such as obvious striation of rhabdomyosarcoma or osteoid produced by malignant neoplastic cells, the diagnosis should be called carcinosarcoma<sup>[22]</sup>. For cases without any histologically-identifiable differentiation, immunostaining for cytokeratin or other epithelial markers may help in determining whether the neoplasm matches the qualifications for sarcomatoid carcinoma or carcinosarcoma. The common denominator of all sarcomatoid carcinomas is the immunoreactivity of epithelial markers such as cytokeratin for both the sarcomatous and epithelial components. However, if sarcomatous elements do not express epithelial markers, the term carcinosarcoma is the preferred diagnosis<sup>[17,23]</sup>. In our case, both the sarcomatous and epithelial components were immunoreactive to pancytokeratin, confirming our diagnosis of sarcomatoid carcinoma.

The histogenesis of sarcomatoid carcinoma remains unclear and controversial despite more than a century of conjecture. Proposed explanations of the bi-differentiated appearance include the multiclonal hypothesis on one hand, and on the other hand the "collision theory", suggesting that the two tumor components are derived from separate and distinct malignant cell clones<sup>[5,24]</sup>. Other investigators suggest a clonal origin (monoclonal hypothesis) of the tumor since common characteristics have been seen between the different cellular populations as well as an observed transitional population<sup>[25,26]</sup>. For example, the presence of cytokeratin in spindle cells within sarcomatoid carcinomas of various anatomical locations supports the epithelial origin of these cells<sup>[17,19,27,28]</sup>. The observed characteristics in the monoclonal hypothesis could either be due to a malignant transformation of a pluripotent stem cell capable of epithelial and mesenchymal differentiation or the sarcomatous element arising from a metaplastic transformation of the carcinomatous element. Some postulate that this sarcomatous transition from carcinomatous cells could be related to retrovirus infection<sup>[29]</sup>. Sarcomatoid carcinomas demonstrated very rarely to be

"collision tumors" with sharply defined sarcomatous and carcinomatous components without any shared or transitional features. There is, however, strong molecular evidence that supports the monoclonal origin of most sarcomatoid carcinomas<sup>[30]</sup>. Several genetic studies involving loss of heterozygosity (LOH) with microsatellite markers and pattern of X chromosome inactivation have demonstrated a common origin of the admixed components<sup>[31,32]</sup>. In addition, Delahunt *et al* described progressive accumulation of p53 proteins in the phenotypic conversion of carcinoma into sarcomatoid phenotype, thus indicating an increasing clonal dominance of dedifferentiated tumor cells carrying p53 mutations<sup>[33]</sup>.

In our case, the surgically removed rectal tumor demonstrated both tubulovillous adenoma and adenocarcinoma adjacent to each other with sarcomatous elements right beneath the tubulovillous adenoma. This histological finding strongly suggests that the two components originated from a multipotent epithelial cell, and that the sarcomatous components originated with differentiation from adenoma to sarcomatoid phenotype during tumor progression through an intermediate stage of adenocarcinoma. To further support a monoclonal origin and a possible tumor progression sequence in our case, immunohistochemistry for p53 protein showed increasing accumulation of p53 from tubulovillous adenoma to adenocarcinoma and finally to the sarcomatous area (Figure 4A). The adenoma-to-carcinoma sequence is well known; however little is known whether the sarcomatous phenotype is part of this tumor progression sequence. To our knowledge, this is the first case that demonstrates a clear histological adenoma-adenocarcinoma-sarcomatoid phenotype sequence progression all in one image.

Clinical features of all ten cases (including our present report) showed a mean age of 68 years (range 41-86) and a slight predilection for females (Table 2). Sarcomatoid carcinomas can be located anywhere in the large bowel, but a preference for the distal colon (including the descending colon, sigmoid colon, and rectum) is seen in most cases. Lymph nodes and distant sites of metastasis disclose a predominance of the malignant epithelial component. However, only one case has shown metastasis from the sarcomatous element, indicating the aggressive nature of the epithelial component<sup>[13]</sup>. Despite radical surgery, chemotherapy, or radiotherapy, prognosis for sarcomatoid carcinoma of the colon remains poor. Seven cases revealed distant metastasis either at initial or follow-up visit, and six of the ten cases died within five years of diagnosis. Due to the rarity of cases, no specific conclusions can be made regarding prognostic factors.

Treatment for sarcomatoid carcinomas of the colon should follow similar guidelines for colonic adenocarcinomas, as no specific treatment guidelines are available for the management of this tumor. In addition, extensive follow-up should be warranted given the poor prognosis associated with this rare clinical entity. Nine of the ten reported cases (including ours) of colonic sarcomatoid carcinomas have undergone resection of the primary tumor; however, only two of the ten cases have survived over the

past two years despite adjuvant therapy. In a recent 8-mo follow-up, our patient is still alive with no evidence of metastasis after surgical resection of the primary tumor.

In summary, sarcomatoid carcinoma of the colon is a highly aggressive neoplasm that leads to a poor patient outcome despite clinical intervention. Endoscopic biopsy specimens containing lesions with spindle cell morphology should raise the differential diagnosis of sarcomatoid carcinoma with immunostaining for cytokeratin to help confirm the diagnosis. The histogenesis of sarcomatoid carcinoma is still unclear; however, the morphological appearance and immunohistochemical analysis of our case strongly suggests an adenoma-carcinoma-sarcomatoid phenotype sequence progression as the likely pathogenesis of this rare tumor. Clinical management should follow the diagnostic and therapeutic guidelines for colorectal adenocarcinomas given the paucity of cases. Further studies and collection of cases are needed to establish proper therapeutic interventions.

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# AIDS-associated plasmablastic lymphoma presenting as a poorly differentiated esophageal tumor: A diagnostic dilemma

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## Abstract

Plasmablastic lymphoma (PBL) is a rare form of diffuse large B-cell lymphoma characterized by weak/absent expression of conventional B-cell markers and strong expression of plasma cell markers. It is strongly associated with human immunodeficiency virus (HIV) and Epstein Barr virus infection, and shows an unusual tropism to the oral cavity. Herein we describe a patient with AIDS who presented with weight loss and dysphagia owing to a large gastroesophageal mass. His radiographic and endoscopic findings and long history of cigarette consumption suggested carcinoma. Biopsy demonstrated a poorly differentiated tumor stained negatively to routine lymphoid markers including CD20. However, gene rearrangement studies confirmed a B-cell process and a more detailed immunohistochemical analysis revealed the cells stained positively for CD138 (plasma cell antigen). These findings were diagnostic of PBL. Our report reviews the wide differential diagnosis of PBL and underscores the importance of a broad array of viral and molecular studies needed to establish this diagnosis.

## INTRODUCTION

An increasingly large number of HIV-infected patients are developing HIV-associated, but not AIDS-defining neoplasms including esophageal cancer, head and neck malignancies, lung cancer, liver and anal neoplasms and renal cell carcinoma<sup>[1]</sup>. The pathogenesis of these tumors is complex and is in part related to alcohol and cigarette consumption, the consequences of chronic inflammation including hepatitis C virus (HCV)-associated cirrhotic liver disease and the presence of oncogenic viruses such as human papilloma virus, Epstein Barr virus (EBV) and hepatitis B virus (HBV). Many of these patients are receiving highly active anti-retroviral therapy (HAART) and are no longer destined to die from AIDS-related complications.

Plasmablastic lymphomas (PBLs) were originally described in HIV-infected patients as an aggressive variant of diffuse large B-cell lymphoma (DLBCL), with a peculiar tropism for the oral cavity<sup>[2]</sup>. PBLs are composed of rapidly growing, large neoplastic cells displaying some degree of plasma cell differentiation. Phenotypically, PBLs display an unusual immunohistochemical profile characterized by weak or absent expression of conventional B-cell markers

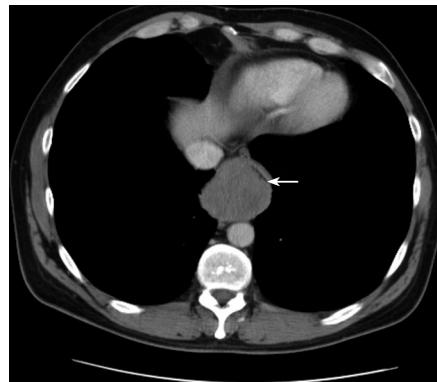
coupled with strong expression of plasma cell markers. Recent reports have identified this neoplasm in extra oral sites in both HIV seropositive and seronegative individuals.

Herein, we describe the clinical course of a patient with AIDS who presented with a constrictive and ulcerating esophageal mass which was thought initially to be a poorly differentiated carcinoma, but after a more detailed immunohistochemical evaluation, proved to be PBL. We emphasize the unusual clinical features of this rare form of non-Hodgkin's lymphoma (NHL) and the diagnostic challenges associated with its identification.

## CASE REPORT

A 40-year-old Caucasian male with a 25-pack-year history of cigarette consumption but no alcohol or illicit drug use, sought medical attention. He had lost 20 lbs over a period of 2 mo, and complained of loss of appetite and progressive odynophagia (solids > liquids). He also experienced low-grade fevers and drenching night sweat that was not ameliorated by acetaminophen. His past medical history was significant for HIV and chronic active HBV co-infection, diagnosed a year earlier when, while homeless, he presented with muscle weakness and altered mental status. His initial CD4+ count was < 50 cells/ $\mu$ L and his HIV viral load was > 100 000 copies/mL. Neurological evaluation led to a diagnosis of AIDS-associated encephalopathy and myelopathy. He was transferred to a skilled nursing facility where he received rehabilitatory care along with once daily HAART consisting of a fixed dose coformulation of tenofovir 300 mg and emtricitabine 200 mg, ritonavir 100 mg and atazanavir 300 mg. His condition gradually improved and 6 mo later his CD4+ count increased to 180 cells/ $\mu$ L and his HIV viral load fell to < 75 copies/mL.

On physical examination he appeared disheveled and emaciated with dry mucous membranes, proximal muscle wasting but no hairy leukoplakia or oral candidiasis. There was no lymphadenopathy or hepatosplenomegaly, and his myelopathy-associated spastic gait and lower extremity hyperreflexia were stable. Laboratory tests included: white blood count 7600 cells/mm<sup>3</sup>; hemoglobin 12.1 mg/dL; platelet count  $303 \times 10^3/\mu$ L; total protein 7.2 mg/dL; albumin 4 mg/dL; lactate dehydrogenase 496 IU/L (normal range, 125-243 IU/L); normal electrolyte and hepatic transaminase levels; positive HBV surface antigen and HBV e antigen; HBV DNA 9360 copies/mL and negative Hepatitis A virus and HCV serologies. His CD4+ count had dipped to 103 cells/ $\mu$ L, but his HIV viral load remained non-detectable. A chest roentgenogram demonstrated a retrocardiac soft tissue density. Chest and abdominal computed tomogram (CT) further showed the density to be a 4.9 cm × 5.1 cm concentric distal esophageal mass associated with extensive gastric wall thickening (Figure 1). The dominant mass corresponded to an area of intensely increased metabolic activity (SUV = 40.3) and was associated with right iliac adenopathy



**Figure 1** CT scan of chest highlighting the large distal esophageal mass. Note the impressive constriction of the esophageal lumen (arrow).



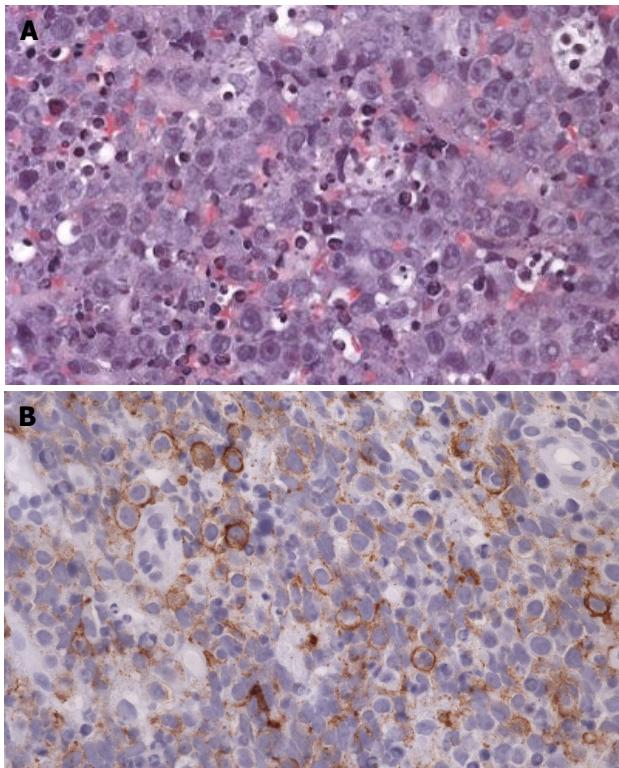
**Figure 2** Whole body PET scan demonstrates intensely increased metabolic activity corresponding to the large esophagogastric mass. There is also focal increased activity in a right iliac node.



**Figure 3** Upper Endoscopic evaluation shows esophageal mass with ulcerative features which extended into the gastric fundus.

(SUV = 40.6) on a whole body F-18 Fluorodeoxyglucose positron emission tomography (PET) scan (Figure 2).

Upper endoscopic evaluation revealed that the mass constricted 40% of the esophageal lumen and extended into the proximal stomach where a large ulcerative lesion was identified (Figure 3). Biopsies of the tumor showed a poorly differentiated, malignant neoplasm composed of irregular sheets of cells, which in many areas were largely necrotic. Cells were cytologically atypical with somewhat eccentric vesicular nuclei and prominent nucleoli (Figure 4A). On initial review of the biopsy, a poorly differentiated carcinoma of gastroesophageal origin was favored. However, immunohistochemical evaluation showed that the tumor cells stained negatively



**Figure 4** **A:** HE image shows a poorly differentiated, malignant neoplasm composed of irregular sheets of cells. Cells are cytologically atypical with somewhat eccentric vesicular nuclei and prominent nucleoli ( $\times 40$ ). **B:** Immunohistochemistry shows patchy but strong staining of tumor cells for CD138 ( $\times 40$ ).

for epithelial markers (cytokeratin), lymphoid markers (CD20, CD3, CD30 and PAX-5) and melanocytic markers (HMB-45, melan-A and S100). Based on these inconclusive results, additional immunohistochemical stains for markers of lymphoid and plasmacytoid differentiation was performed. The tumor cells were stained strongly positive for CD-45 (leukocyte common antigen) and CD-138 (plasma cell marker) and weakly positive for CD-79a (pan B-cell marker) (Figure 4B). Tumor cells were also positive for the B-cell transcription factors Bob-1 (focal) and Oct-2 (focal weak). In situ hybridization studies were positive for EBV but negative for human herpes virus type-8 (HHV-8, Table 1). A clonal rearrangement of the immunoglobulin heavy chain gene was identified on polymerase chain reaction analysis.

Following a diagnosis of PBL, the patient was treated with combined chemotherapy consisting of liposomal doxorubicin, cyclophosphamide and etoposide (LACE regimen) in addition to HAART<sup>[3]</sup>. He tolerated the therapy well, with mild nausea with each treatment cycle, alopecia and a single episode of culture negative neutropenic fever. A follow-up CT scan taken after the second treatment cycle showed dramatic improvement with just mild residual wall thickening of the distal esophagus. After completing a total of six cycles of chemotherapy, his dysphagia and weight loss resolved and a CT-PET scan showed complete resolution of the abnormal activity in esophagus and right iliac region.

**Table 1** Immunohistochemical findings of esophagogastric mass

Type	Antigen	Immunoreactivity
Epithelial markers	Cytokeratin 35BH11	Negative
	EMA	Diffusely positive
	BER-EP4	Negative
Lymphoid markers	CD-45	Positive
	CD-3	Negative
	CD20	Negative
	CD79a	Equivocally positive in some cells
	PAX-5	Negative
	CD30	Negative
	CD138	Positive (patchy, strong)
B-cell transcription factors	Bob-1	Positive
	Oct-2	Positive
Melanocyte markers	Melan-A	Negative
	HMB-45	Negative
	S100	Negative
Viral markers	HHV-8	Negative
	EBV ( <i>in situ</i> hybridization)	Positive

EMA: Epithelial membrane antigen; PAX-5: Paired box gene-5; HHV-8: Human Herpes Virus type-8; EBV: Epstein Barr virus.

His post-chemotherapy upper endoscopic evaluation did not reveal persistent NHL and 6 mo later, he remains in remission.

## DISCUSSION

In 1997, Delecluse and colleagues were the first to describe in HIV-infected patients, the occurrence of a high-grade malignant lymphoma subtype named PBL which exclusively involved the oral cavity<sup>[2]</sup>. These tumors possessed a unique immunohistochemical phenotype characterized by their failure to express common lymphoid markers while stained positively for plasma cell markers<sup>[4]</sup>. Over the past decade, the clinical spectrum of this NHL has expanded to include extra oral involvement in patients with and without HIV infection. Unusual sites of PBL involvement have included the skin, nasal and paranasal sinuses, long bones, lungs, stomach, anorectum, omentum, testes, spermatic cord, bone marrow, sacrococcygeal cysts and central nervous system<sup>[5-10]</sup>.

PBL accounts for 2.6 % of all AIDS-related NHLs, and rarely represents the sentinel manifestation of AIDS<sup>[11-13]</sup>. In HIV-negative individuals, PBL is often associated with iatrogenic immunosuppression such as seen with organ transplantation<sup>[14]</sup>. It has also been reported in association with Azathioprine and Infliximab therapy for management of inflammatory bowel disease<sup>[15,16]</sup>. PBL has been diagnosed in children as young as age 7, but the majority of the reported cases involve middle-aged adults.

Patients with PBL can be divided into three distinct categories<sup>[17-20]</sup>. The first and the more common PBL variant is localized to the oral mucosa, although the tumor may also involve nodal or extranodal sites. Histologically, this variant is characterized by a

**Table 2** Differential diagnosis of PBL

Tumor subtype	Carcinoma	PBL	Common DLBCL	BL	Plasmacytoma
Tumor cell size	Large	Large	Large	Intermediate	Intermediate
CD20	-	- to ±	+	+	-
CD45	-	Variable	+	+	-
CD138	-	+	-	-	+
VS38c	-	+	- to ±	-	+
MUM1	-	+	-	-	+
EBV	-	+	-	+	-
HHV-8	-	Variable	-	-	-

PBL: Plasmablastic lymphoma; DLBCL: Diffuse large B cell lymphoma; BL: Burkitt's lymphoma; CD: Cluster of differentiation; MUM-1: Multiple myeloma oncogene-1-protein; EBV: Epstein Barr virus; HHV-8, human Herpes Virus type-8; +: Expression of the antigen in the majority of cells; -: Absence of antigen expression; ±: Weak antigen expression.

monomorphic population of immunoblasts with no or minimal plasmacytic differentiation. The second PBL category is distinguished by its plasmacytic differentiation and extra oral presentation. The tumor cells are composed predominantly of immunoblasts, plasmablasts and mature plasma cells. The third variant of PBL has also been reported in association with HHV-8 and multicentric Castleman's disease. Patients typically present with lymphadenopathy and splenomegaly, often with plasmablasts circulating in the peripheral blood.

Despite morphologically resembling B-cell immunoblasts, PBL is associated with plasma cell immunophenotype, with loss of B cell markers (CD20) and surface immunoglobulin, and acquisition of plasma cell surface markers [VS38c, CD38 (syndecan-1), MUM-1, CD-138]. The plasmablasts are variably immunoreactive for CD45 and CD-79a<sup>[6,11,15]</sup>. They usually lack Bcl-6, the germinal center-associated B-cell antigen and PAX-5/BSAP, a nuclear factor that is present from the precursor B-cell stage and in all mature B cells but lost in terminal differentiation to plasma cells<sup>[11,17]</sup>. Newer B-lineage markers like the transcription factors OCT-2 and BOB-1 may be helpful to confirm the B-cell origin of these tumors<sup>[20]</sup>.

EBV infection is strongly associated with PBL. *In situ* hybridization for EBV Encoded RNA in tumor specimens has reportedly ranged from 60% to 100%, suggesting that EBV plays an important role in PBL pathogenesis<sup>[9,15,17,21]</sup>. In contrast, HHV-8 is not consistently associated with PBL, although rare cases have identified HHV-8 in conjunction with HIV infection, PBL and multicentric Castleman's disease<sup>[9,15,17,22,23]</sup>. Patients with chronic HBV infection are more likely to develop NHLs, but specific cases of PBL have not been documented in this population<sup>[24]</sup>.

The differential diagnosis of PBL may overlap with a variety of other clinicopathologic entities (Table 2). When PBL presents as an oral lesion, it may be confused with periodontal disease like odontogenic cellulitis, KS or melanoma<sup>[12]</sup>. Carcinomas can be distinguished from PBLs based on presence of immunoreactivity for epithelial markers such as cytokeratin. Primary effusion lymphoma, as opposed to PBL, usually presents with

serous effusions without detectable tumor masses, and is strongly associated with both HHV-8 and HIV infection. The presence of serum monoclonal proteins, and/or bone involvement with radiographically evident lytic lesions, favors the diagnosis of plasma cell myeloma rather than PBL<sup>[25]</sup>.

In the pre-HAART era, HIV-infected patients with PBL were destined to die from their disease shortly after their NHL diagnosis. In the initial report by Delecluse and colleagues, 9 of 11 patients with long-term follow-up had a median survival of 6 mo<sup>[2]</sup>. In the HAART era, patient prognosis appears better and prolonged survival is the goal of treatment. Among six patients treated with anthracycline-based multiagent chemotherapy in conjunction with HAART, five were alive and disease free with a median follow-up of 22 mo<sup>[15]</sup>. The importance of an intact immune system in preventing or controlling PBL is underscored by reports of tumor regression with HAART alone and in the absence of chemotherapy<sup>[26-28]</sup>. Ironically, the early phase of immune reconstitution may be a fertile ground for NHL development. Our patient's diagnosis of lymphoma within 1 year of HAART initiation, and in the context of a rapidly improving CD4+ cell count and non-detectable HIV viral load may be another example<sup>[29]</sup>.

Finally, our patient's complaint of dysphagia in the setting of a large esophagogastric mass, together with his long-standing history of smoking was disconcerting for carcinoma. Though the history of fever, night sweat and an elevated LDH raised the possibility of a lymphoma, the finding of poorly differentiated tumor cells stained negatively for routine lymphoid markers of B and T-cell differentiation did not support this diagnosis. But the subsequent demonstration of the plasma cell antigens, which are not typically part of the routine immunohistochemical panel, in conjunction with a broader array of viral and molecular studies helped us to establish the diagnosis of PBL.

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CASE REPORT

## Severe chest pain in a pediatric ulcerative colitis patient after 5-aminosalicylic acid therapy

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### INTRODUCTION

Mesalamine is a well-known treatment for ulcerative colitis. Drug reactions to mesalamine are uncommon, and most often include skin rash and hypereosinophilia<sup>[1]</sup>. Severe reactions to mesalamine products are rarely seen in pediatric patients. Cardiac complications have been reported as a rare extraintestinal manifestation of inflammatory bowel disease (IBD) and mainly manifest as pericarditis<sup>[2-4]</sup>. However, cardiac complications may be associated as a very rare drug reaction to 5-aminosalicylic acid (5-ASA) products<sup>[1,5,6]</sup>. The difficulty lies in distinguishing between these two etiologies. We herein report a case of a 12-year-old boy with ulcerative colitis who had a severe cardiovascular reaction to a mesalamine product, Asacol.

### CASE REPORT

This is a 12-year-old boy with a past medical history significant for ulcerative colitis (UC) diagnosed at 9 years of age, along with psoriasis and arthritis. He was initially placed on steroids and Pentasa when he was first diagnosed. The Pentasa was discontinued after he developed a rash. It was difficult to distinguish his psoriasis from a potential drug reaction as a cause of the rash. He was subsequently weaned from his steroids after approximately 2 mo.

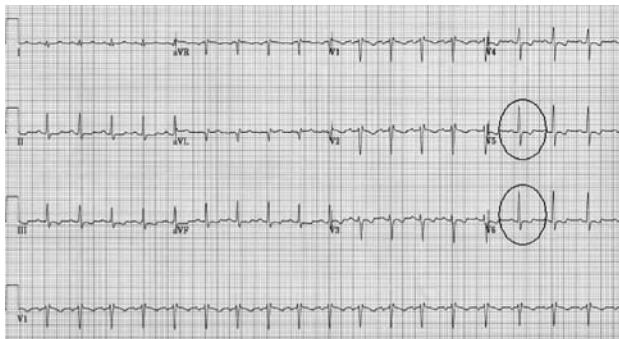
He presented to the hospital three years after his initial diagnosis with a two-month history of exacerbation of his UC. His symptoms continued to progress. He had 6-8 bloody stools per day 2 wk prior to hospital admission. Colonoscopy 6 wk into the flare revealed pancolitis. He was started on Asacol 800 mg three times per day and prednisone 20 mg once a day for his UC flare. The patient was subsequently admitted for worsening abdominal pain, bloody diarrhea as well as fever, fatigue, myalgia, and extremity pain. He also complained of new onset of severe chest pain progressively worsening since

### Abstract

Severe reactions to mesalamine products are rarely seen in pediatric patients. We report a case of a 12-year-old boy who had a severe cardiac reaction to a mesalamine product Asacol. Past medical history is significant for ulcerative colitis (UC) diagnosed at 9 years of age. Colonoscopy one week prior to admission revealed pancolitis. He was treated with Asacol 800 mg three times per day and prednisone 20 mg/d. He was subsequently admitted to the hospital for an exacerbation of his UC and started on intravenous solumedrol. He had improvement of his abdominal pain and diarrhea. The patient complained of new onset of chest pain upon initiating Asacol therapy. Electrocardiogram (ECG) revealed non-specific ST-T wave changes with T-wave inversion in the lateral leads. Echocardiogram (ECHO) revealed low-normal to mildly depressed left ventricular systolic function. The left main coronary artery and left anterior descending artery were mildly prominent measuring 5 mm and 4.7 mm, respectively. His chest pain completely resolved within 24-36 h of discontinuing Asacol. A repeat echocardiogram performed two days later revealed normal left ventricular function with normal coronary arteries (< 3.5 mm). Onset of chest pain after Asacol and immediate improvement of chest pain, as well as improvement of echocardiogram and ECG findings after discontinuing Asacol suggests that our patient suffered from a rare drug-hypersensitivity reaction to Asacol.

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**Key words:** Mesalamine; 5-aminosalicylic acid; Ulcerative colitis; Pericarditis; Drug hypersensitivity reaction



**Figure 1** Non-specific ST-T wave changes with T-wave inversion in the lateral leads (circles).

starting Asacol therapy. The chest pain was not related to exercise and not associated with palpitations, shortness of breath, syncope, pallor, cyanosis or altered by changes in posture or breathing.

On admission, his vitals included a temperature of 38°C, pulse rate of 126 beats per minute, respiratory rate of 18 breaths per minute and a blood pressure of 107/65 mmHg. He was started on intravenous solumedrol 8 mg every 6 h with subsequent improvement of his abdominal pain and diarrhea. Chest pain, however, persisted and the chest pain worsened shortly after ingesting Asacol. Electrocardiogram (ECG) was performed and revealed non-specific ST-T wave changes with T-wave inversion in the lateral leads (Figure 1). Troponin T, CK-MB, Brain Natriuritic Peptide, and CRP levels were ordered to evaluate for possible pericarditis or a myocardial abnormality. The results of this lab work were normal. Echocardiogram (ECHO) revealed low-normal to mildly depressed left ventricular systolic function. Calculated shortening fraction was 26% and left ventricular size was within normal limits. The left main coronary artery and left anterior descending artery were mildly prominent measuring 5 mm and 4.7 mm, respectively (Figure 2). The ECHO also revealed trivial circumferential pericardial effusion that was hemodynamically insignificant (Figure 3). A spiral CT scan ordered to evaluate for possible pulmonary embolism in the setting of an elevated d-dimer was unremarkable except for a small amount of pericardial fluid.

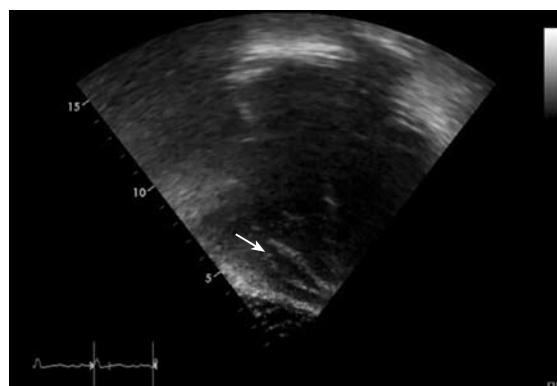
The patient's chest pain completely resolved within 24 h after discontinuation of Asacol. A repeat echocardiogram performed two days later revealed normal left ventricular function with a shortening fraction of 33%, normal coronary arteries (< 3.5 mm) and a trivial pericardial effusion. The patient was subsequently discharged from the hospital on prednisone and methotrexate. His UC remained in remission upon follow-up by his pediatric gastroenterologist 2 mo after hospital discharge. An ECHO and ECG were also repeated in the follow-up 2 mo after hospital discharge, revealing continued resolution of coronary artery dilation (Figure 4). The ECG was normal without the S-T wave abnormality noted 2 mo earlier.

## DISCUSSION

Mesalamine has been found to be a beneficial medication in the treatment of patients with ulcerative colitis. The



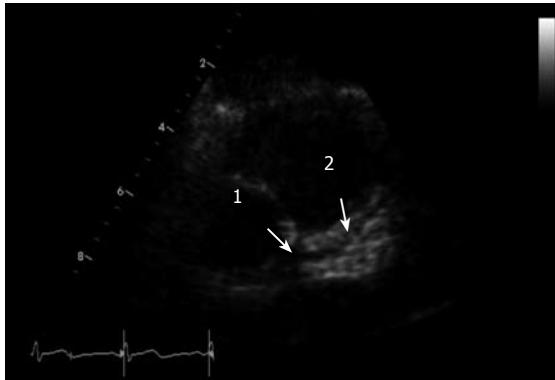
**Figure 2** Echocardiogram-parasternal short-axis view showing mildly dilated left main coronary artery (1), left anterior descending artery (2), left circumflex coronary artery (3).



**Figure 3** Echocardiogram-4-chamber view showing pericardial effusion (arrow).

mechanism of action of mesalamine is unknown; however, it is thought to exert its action topically as opposed to systemically. Mucosal production of arachidonic acid metabolites, through both the cyclo-oxygenase and the lipoxygenase pathways, is thought to be increased in patients with inflammatory bowel disease. Mesalamine acts by a variety of mechanisms which include blocking cyclo-oxygenase thereby inhibiting prostaglandin synthesis, reducing antioxidant and pro-inflammatory cytokine synthesis, reducing lymphocyte metabolism and reducing expression of adhesion molecules. All these work to decrease inflammation in the colon<sup>[7,8]</sup>.

Chest pain along with electrocardiographic and echocardiogram findings in a pediatric patient on a 5-ASA product should alert the physician to the possibility of drug reaction. We have presented a pediatric patient with ulcerative colitis who developed cardiac signs and symptoms associated with pericarditis soon after starting a 5-ASA product. Determining the etiology of pericarditis in this setting can be complex as this presentation has been reported as a rare extraintestinal manifestation of inflammatory bowel disease<sup>[2-4]</sup>. Chest pain may be associated with other more common gastrointestinal conditions such as gastroesophageal reflux disease (GERD). Chest pain in IBD may also be caused by respiratory infection and pleural inflammation which were ruled out in our patient. Patients with IBD have a higher propensity to develop pulmonary embolism (PE) and patients often



**Figure 4** Echocardiogram-parasternal short-axis view showing normal left main coronary artery (1), normal left anterior descending artery (2).

present with acute onset of chest pain<sup>[4]</sup>. The normal spiral chest CT ruled out PE in our patient.

In our case, there was a temporal relationship between the onset of the chest pain and the administration of Asacol. The characteristics of the chest pain were not typical of pericarditis but the ST-T wave changes and the pericardial effusion which resolved after discontinuation of the Asacol were suggestive of the diagnosis. Normal troponin and CKMB levels do not support the diagnosis of myocarditis, although there was a mild but clear change in ventricular systolic function which also resolved after the Asacol was stopped. It is unclear why the coronary arteries were mildly prominent at the time of the diagnosis, which would suggest a vasculitis process such as Kawasaki disease.

This case illustrates the importance of eliciting a

thorough medical history and being aware of the timing when new medications are started. It is imperative that any new onset of chest pain, especially in this setting, should be evaluated via cardiac enzymes, EKG and echocardiogram to quickly diagnose any complication caused by either the inflammatory bowel disease itself or a rare adverse drug reaction.

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CASE REPORT

# Management of rectal foreign bodies: Description of a new technique and clinical practice guidelines

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## INTRODUCTION

Intentional or unintentional insertion of rectal foreign bodies is not uncommon and often poses a serious challenge on the clinician. Objects can be inserted for diagnostic or therapeutic purposes, or self-treatment of anorectal disease, by criminal assault and accident or, most commonly, for sexual purposes. Most patients with rectal foreign bodies present to the emergency room usually after efforts to remove the object at home. Many endoscopic and surgical techniques to remove rectal foreign bodies have been described in the literature and the reported variety in foreign bodies is as large as the number of techniques used to remove them<sup>[1-46]</sup>. The descriptions in the available literature are anecdotic and consist largely of case reports or case series<sup>[1-46]</sup>.

In this report, a novel endoscopic technique to remove rectal foreign bodies using a pneumatic dilatation balloon normally used in achalasia patients is presented. In addition, a systematic review of the literature was performed for non-operative methods to remove foreign bodies from the rectum. These results are summarized, presented as a practical at-a-glance overview and a flow chart is offered to guide the clinician in treatment decisions.

## CASE REPORT

A 19-year-old man presented at the emergency department, 12 h after insertion of a high pressure container with tanning spray into his rectum. A plain abdominal radiograph (Figure 1) showed the container in the rectosigmoid region. There were no signs of perforation. A flexible sigmoidoscopy was performed under conscious sedation. The object was located just above the rectosigmoid junction. The container could not be extracted by bimanual manipulation. An attempt to remove the object with conventional endoscopic instruments, such as polypectomy snares, was unsuccessful.

The sigmoidoscope could be passed alongside the foreign body to its proximal end. A guide wire was left behind with the sigmoidoscope removed. Subsequently, a 40 mm pneumatic dilatation balloon (Rigiflex®, Boston Scientific), normally used in achalasia patients, was inserted over the guide wire and inflated just above the container (Figure 2). For safety purposes, the sigmoidoscope was reintroduced alongside the catheter of the balloon to allow endoscopic visual control of

## Abstract

A number of techniques have been described to remove rectal foreign bodies. In this report, a novel endoscopic technique using a pneumatic dilatation balloon normally used in achalasia patients is presented. In addition, a systematic review of the literature was performed for non-operative methods to remove foreign bodies from the rectum. These results are summarised, presented as a practical at-a-glance overview and a flow chart is offered to guide the clinician in treatment decisions. The design of the flow chart was based on the aims to treat the patient preferably on an outpatient basis with minimally invasive techniques and if possible under conscious sedation rather than general anaesthesia.

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**Key words:** Foreign body; Rectum; Rectal; Removal; Review

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**Figure 1** Plain abdominal radiograph showing the foreign body impacted in the rectosigmoid.



**Figure 3** The removed container.



**Figure 2** Lateral view of abdominal radiograph depicting the foreign body with the achalasia balloon inflated just above the container.

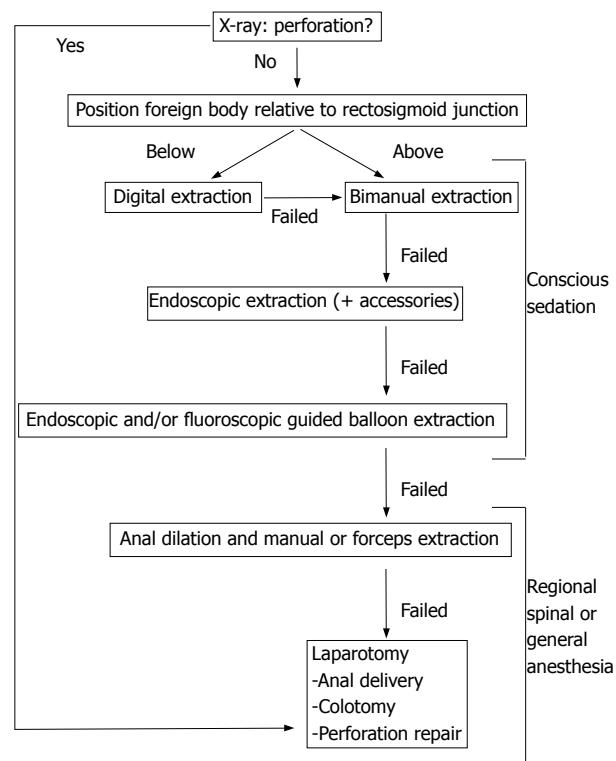
the distal end of the container in the rectum. Gentle traction was exerted on the balloon catheter, and the container was successfully removed under fluoroscopic and endoscopic control (Figure 3).

## DISCUSSION

A large number of surgical and non-surgical techniques have been described to remove rectal foreign bodies<sup>[1-46]</sup>. Our case illustrates that for removal of foreign bodies retained in the rectosigmoid, extraction with a pneumatic dilatation balloon, inflated above the foreign body, may be an elegant and safe alternative when conventional techniques fail. Our technique has not been described before as revealed by a systematic review of the literature. We performed a systematic PubMed search from 1966 to present, using the search terms 'rectal', 'rectum', 'colorectal', 'foreign', 'bodies' and 'endoscopic'. Only reports in English were included. The results of the systematic search of the literature, specified for the type of foreign body, are summarized in Table 1<sup>[1-36]</sup>. Table 1 also summarizes endoscopic techniques and non-endoscopic techniques for removing foreign bodies. In addition to the reports presented in the Table 1, several case series have been published without detailed information on the techniques used to remove various foreign bodies<sup>[18,22,25,37-46]</sup>.

An algorithm was provided to guide the clinician in his or her treatment decisions, partly based on the methods presented in the Table 1 (Figure 4). We included only those methods most commonly used and excluded rare treatment variants.

The first step in the evaluation is that one should



**Figure 4** Algorithm for the removal of a colorectal foreign body.

always be aware of the possibility of a large bowel perforation and perform radiological investigations. Plain abdominal radiography or water soluble contrast enemas may be helpful. An abdominal X-ray will also provide information on the localization of the foreign body, whether it is below or above the rectosigmoid junction. If perforation of the bowel has occurred, immediate laparotomy is warranted. If there are no signs of perforation, several management approaches can be tried. Our aim was to treat the patient on an outpatient basis with minimally invasive techniques and preferably under conscious sedation instead of general anaesthesia.

First, digital removal of the object should be attempted, if necessary with the patient at different positions. If this approach fails, one can try bimanual manipulation. The next step is the insertion of an endoscope with subsequent attempts to grasp the foreign body with regular endoscopy accessories like polypectomy snares. When this fails, it may be helpful to

**Table 1** Overview of reports on endoscopic and non-endoscopic removal of rectal foreign bodies

Type foreign body	Technique	Anaesthesia	Author <sup>[Ref.]</sup>
Ballpoint pen	Polypectomy snare <sup>1</sup>	-	Richter <sup>[1]</sup>
Water filled balloon	Puncture and forceps <sup>1</sup>	-	Wolf <sup>[2]</sup>
Chicken bone	Polypectomy snare <sup>1</sup>	-	Tarnasky <sup>[3]</sup>
Toothpick	Polypectomy snare <sup>1</sup>	-	Over <sup>[4]</sup>
Apple	Defragmentation by APC <sup>1</sup>	None	Glaser <sup>[5]</sup>
Glass bottle	Biopsy forceps <sup>1</sup>	General	Huang <sup>[6]</sup>
Vibrator	Polypectomy snare <sup>1</sup>	None	Huang <sup>[6]</sup>
Glass test tube	Inflated Sengstaken tube <sup>1</sup>	-	Hughes <sup>[7]</sup>
Test tube	Polypectomy snare <sup>1</sup>	-	Kantarian <sup>[8]</sup>
Enema tip	Polypectomy snare <sup>1</sup>	-	Kantarian <sup>[8]</sup>
Vibrator	Polypectomy snare, biopsy forceps <sup>1</sup>	-	Kantarian <sup>[8]</sup>
Pencil	Polypectomy snare <sup>1</sup>	-	Vemula <sup>[9]</sup>
Iron bar	2-channel colonoscope and wires <sup>1</sup>	-	Ahmed <sup>[10]</sup>
Bottle neck	Inflated Foley cathether <sup>1</sup>	General	Humes <sup>[11]</sup>
Spray container	Achalasia balloon <sup>1</sup>	None	Present report
Spongy toy ball	Obstetric vacuum extractor	General	Feigelson <sup>[12]</sup>
Vibrator	Obstetrical forceps, anal dilation	Local	Haft <sup>[13]</sup>
Vibrator	Uterine vulsellum	Local	Levin <sup>[14]</sup>
Aftershave bottle	Rubber-shod bone olding clamp	Spinal	Siroospour <sup>[15]</sup>
Chicken bone	Digitally	None	Davies <sup>[16]</sup>
Aerosol-can Cap	Tenaculum forceps, anal dilatation	General	Aquino <sup>[17]</sup>
Vase	Filling with plaster	General	Couch <sup>[18]</sup>
Glass jar	Extraction with plaster rolls	Spinal	Graves <sup>[19]</sup>
Glass jar	Endotracheal tube, anal dilation	Local	Garber <sup>[20]</sup>
Apple	Bimanual manipulation	Local	Sharma <sup>[21]</sup>
Glass jar	Inflated Foley catheter	General	Yaman <sup>[22]</sup>
Glass bottle	Obstetric vacuum cup	General	MacKinnon <sup>[23]</sup>
Glass bulb	3 inflated Foley catheters	-	Diwan <sup>[24]</sup>
Thermometer	Biopsy forceps	General	Huang <sup>[6]</sup>
Vibrator	Transanal Kocher clamps	Local	Huang <sup>[6]</sup>
Bowling bottle	Obstetric forceps	General	Huang <sup>[6]</sup>
Perfume bottle	Manually	Spinal	Busch <sup>[25]</sup>
Piece of wood	Manually	General	Jansen <sup>[26]</sup>
Toothbrush case	Inflated Fogarty catheter	-	Wigle <sup>[27]</sup>
Oven mitt	Forceps after anal dilation	General	Losanoff <sup>[28]</sup>
Sink waste pipe	Obstetric forceps	General	Peet <sup>[29]</sup>
Metallic boule	Electromagnet	General	Coulson <sup>[30]</sup>
Carrot	Myomectomy screw	-	Vashist <sup>[31]</sup>
Glass	Obstetric vacuum extractor	Spinal	Johnson <sup>[32]</sup>
Rubber ball	Manual extraction, anal dilation	General	Nivatvongs <sup>[33]</sup>
Wooden rod	Bimanually, anal dilation	Spinal	Nivatvongs <sup>[33]</sup>
Bottle	Manually after anal dilation	General	Gopal <sup>[34]</sup>
Dildo	Myomectomy screw	-	Clark <sup>[35]</sup>
Light bulb	Abdominal compression	Spinal	Konishi <sup>[36]</sup>

-: No description; APC: Argon-plasma coagulation; <sup>1</sup>Endoscopic removal of rectal foreign bodies.

use devices that can be inflated in the rectosigmoid, such as a Foley catheter or an achalasia balloon. Such a device prevents a vacuum that might develop upon extraction of the foreign body and may also be directly used to remove the object.

If these interventions fail, we refer the patients to the operating theatre. Full relaxation of the anal sphincter muscles can be achieved by local, spinal or general anaesthesia. Sometimes, bimanual manipulation of the relaxed abdominal wall under spinal or general anaesthesia may evade surgery. Patients should be consented for a laparotomy prior to general anaesthesia should the manual or endoscopic removal fail.

Finally, when conservative measures fail, laparoscopic or laparotomic approaches are indicated. After removal, sigmoidoscopy is generally recommended to rule out perforations. In the largest series of patients with rectal foreign bodies described thus far ( $n = 93$ ), it was found

that objects retained for more than 2 days, those larger than 10 cm and those located proximal to the rectum increase the likelihood of surgery<sup>[37]</sup>.

In conclusion, many techniques are available for the extraction of rectal foreign bodies. If possible, patients should be treated with minimally invasive techniques and preferably on an outpatient basis under conscious sedation.

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CASE REPORT

# Gastric infiltration of diffuse large B-cell lymphoma: Endoscopic diagnosis and improvement of lesions after chemotherapy

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Zepeda-Gómez S, Camacho J, Oviedo-Cárdenas E, Lome-Maldonado C. Gastric infiltration of diffuse large B-cell lymphoma: Endoscopic diagnosis and improvement of lesions after chemotherapy. *World J Gastroenterol* 2008; 14(27): 4407-4409 Available from: URL: <http://www.wjgnet.com/1007-9327/14/4407.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4407>

## INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most common histologic subtype of non-Hodgkin's lymphoma (NHL) accounting for about 40% of all NHLs<sup>[1,2]</sup>. Tumors may be localized or confined to one side of the diaphragm in 20% to 40% of cases (Stage I or II). Stage IV or disseminated disease is observed in approximately 40% of patients and is usually characterized by extranodal extramedullary infiltration<sup>[3,4]</sup>. Sites of extranodal involvement in DLBCL can include the stomach/gastrointestinal system among others<sup>[5-7]</sup>. In this report, we describe a patient with a stage IV DLBCL infiltrating the stomach diagnosed at endoscopic examination and an excellent response after 6 cycles of chemotherapy.

## CASE REPORT

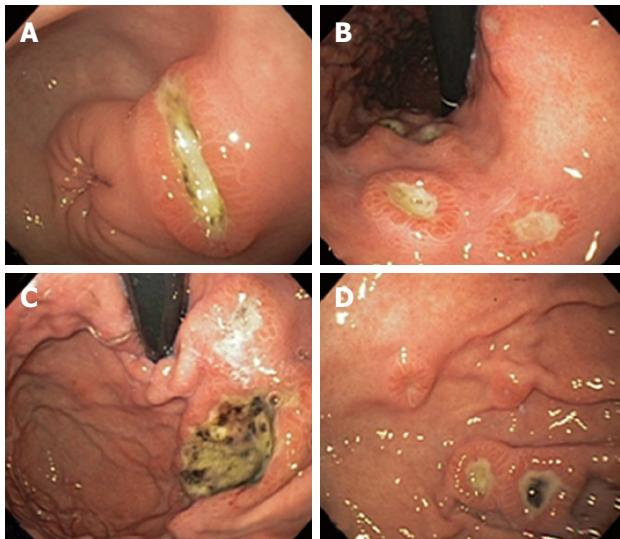
A 39-year-old female was referred for an upper endoscopy because of melena, weight loss and a retroperitoneal mass. She had a 6-mo history of lumbar and left upper quadrant pain. Two months before admission she presented with nausea, early satiety, and intermittent episodes of melena and malaise. At admission she also reported a 5 kg weight loss and nocturnal diaphoresis. Physical examination revealed bilateral supraclavicular lymphadenopathy, hepatosplenomegaly and a palpable tender epigastric mass. A computer tomography (CT) scan showed cervical and mediastinal lymphadenopathy, bilateral pleural effusion, two pulmonary nodules in the upper right lobe, hepatosplenomegaly and a retroperitoneal

## Abstract

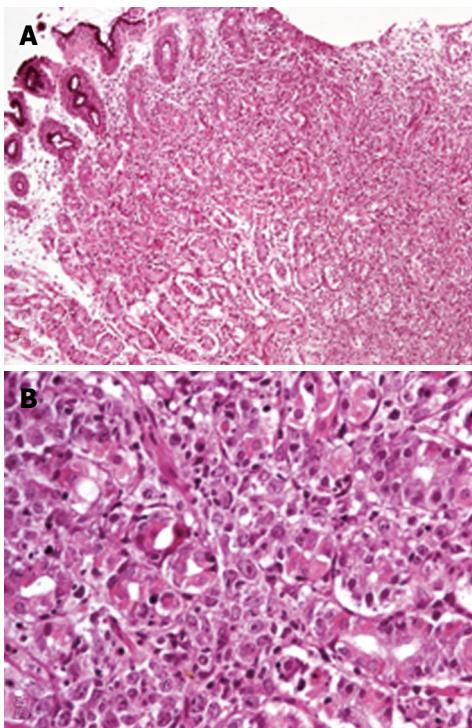
Diffuse large B-cell lymphoma (DLBCL) is the most common histologic subtype of the non-Hodgkin's lymphoma (NHL) accounting for about 40% of all NHLs. This is a case report about the endoscopic appearance of a DLBCL with infiltration to the stomach in a 39-year-old female. She had a 6-mo history of lumbar and left upper quadrant pain with intermittent episodes of melena. A computer tomography (CT) scan showed mural thickening of the gastric antrum. Endoscopic examination revealed multiple gastric ulcers. Definite diagnosis could be made by endoscopic biopsies and the patient had a good response to chemotherapy. This response correlated well with a further endoscopic follow-up. A follow-up endoscopic examination could be considered to evaluate a good response to chemotherapy in DLBCL patients with secondary gastric dissemination.

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**Key words:** Diffuse large B-cell lymphoma; Non-Hodgkin's lymphoma; Gastric infiltration

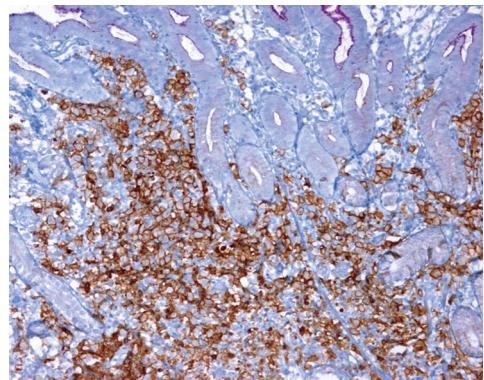


**Figure 1** Endoscopic appearance of DLBCL infiltration of gastric antrum (A), lesser curvature (B), fundus (C), and body (D).

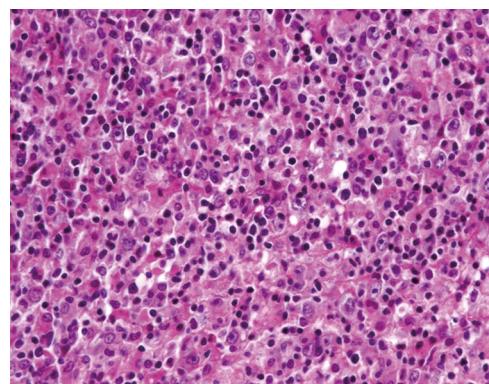


**Figure 2** Gastric mucosa biopsy showing diffuse infiltration of lamina propria with distortion of the glandular architecture (A) and diffuse infiltration by large lymphoid cells (centroblast-type) that surround and destroy the gastric glands (B).

mass with mural thickening of the gastric antrum. A cervical lymph node biopsy was obtained and the patient underwent an upper endoscopic examination. At endoscopy, we observed multiple gastric ulcers without active bleeding. The ulcers had elevated margins, ranging from 5 to 15 mm in diameter and their base was covered by fibrin and/or necrotic material. The margins of the ulcers showed a characteristic erythematous, congestive, mosaic-like pattern suggestive of infiltration (Figure 1). Multiple biopsies were taken from the ulcer margins. Histopathologic analysis revealed a centroblastic DLBCL



**Figure 3** Gastric biopsy showing infiltration by atypical lymphoid cells in the gastric mucosa with intense positivity for CD20 at immunohistochemical analysis.

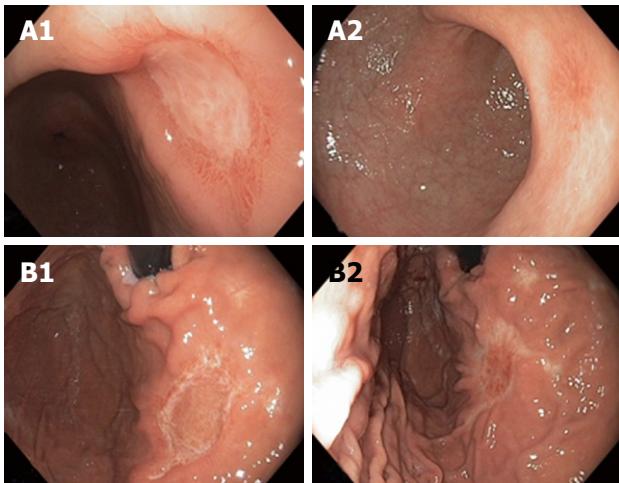


**Figure 4** Lymph node biopsy showing neoplastic lymphocytes with fine nuclear chromatin, some of which have 2 or 3 peripheral nucleoli (centroblast type) and others have prominent central nucleoli (immunoblastic type).

positive for CD20 at immunohistochemical analysis (Figures 2 and 3). The histologic results from the lymph node biopsy confirmed the diagnosis (Figure 4). The patient received rituximab, cyclophosphamide, adriamycin, vincristine and prednisone (R-CHOP) chemotherapy and omeprazole (20 mg *po* twice a day). Her clinical symptoms improved dramatically after 2 cycles of chemotherapy. After 3 cycles of chemotherapy she underwent a follow-up upper endoscopy (6 wk after the first endoscopy) that showed the presence of scarring tissue in majority of the ulcer sites and improvement at the sites where the ulcers with a bigger size were located. A final upper endoscopy performed after 6 cycles of chemotherapy (12 wk after the first endoscopy), showed almost complete resolution of the lesions (Figure 5). She denied early satiety or pain and had no signs of gastrointestinal hemorrhage.

## DISCUSSION

Disseminated DLBCL (stage IV) is seen in approximately 40% of patients and the gastrointestinal tract is the most common site of extranodal NHL accounting for 20% to 60% of newly diagnosed cases<sup>[8-10]</sup>. Disseminated nodal disease requires systemic chemotherapy as opposed to localized primary gastric NHL that is potentially curable



**Figure 5** Endoscopic appearances of gastric antrum (A1, A2) and fundus (B1, B2) after 3 and 6 cycles of chemotherapy respectively.

with local radical treatment<sup>[11]</sup>. Thereby, it is crucial to make an early and reliable identification of the disease to initiate a correct therapy. The characteristics of the lesions at endoscopic examination can differ between primary and secondary gastric NHL. Kolve *et al*<sup>[12]</sup> described the endoscopic differences between 176 patients with primary NHL and 29 with secondary NHL, the lesions had macroscopical polypoid or exulcerative infiltrating changes. Primary low-grade gastric NHL was mainly characterized by diffuse infiltration and unifocal growth pattern with bulky disease in 80% of the cases with high-grade malignancy, whereas the lesions with secondary involvement showed a multifocal growth pattern in 66% of cases with bulky disease in 35%<sup>[12]</sup>. Our case showed a multifocal pattern of ulcerative lesions with elevated margins. Infiltrative disease was suspected on the basis of her clinical presentation, the number of ulcers and the erythematous mosaïc-like pattern at margins of the lesions. Biopsies taken at the margin sites were diagnostic. This description of gastric infiltration by this specific type of neoplasia could help the endoscopist to suspect or identify this entity since appearance of the ulcers at endoscopic examination was very characteristic. The reason to perform the second endoscopic examination was to correlate the endoscopic findings with the improvement in the clinical picture and the radiological follow-up. Obviously, omeprazole treatment could also improve gastric ulcers, but we consider that in such a short period of time and considering the nature of the disease, it is highly improbable that without chemotherapy, we

would have observed the same results. This shows that a follow-up endoscopic examination could also be taken into consideration to evaluate a good response to chemotherapy in DLBCL patients with secondary gastric dissemination.

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CASE REPORT

## Perforation of the duodenum by an ingested toothbrush

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### Abstract

We report a rare case of duodenal perforation caused by an ingested 12-cm long toothbrush handle. A 22-year-old female presented with intermittent epigastric pain for 6 d after swallowing a broken toothbrush. The swallowed toothbrush could not be removed from the second portion of the duodenum by endoscopy. Laparotomy revealed a perforation in the anterior wall of the duodenal bulb. The toothbrush was removed *via* the perforation which was debrided and closed. There were no postoperative complications.

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**Key words:** Duodenum; Endoscopy; Laparotomy; Perforation; Toothbrush

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Chao HH, Chao TC. Perforation of the duodenum by an ingested toothbrush. *World J Gastroenterol* 2008; 14(27): 4410-4412 Available from: URL: <http://www.wjgnet.com/1007-9327/14/4410.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4410>

### INTRODUCTION

Ingestion of various types of foreign bodies, such as toothpicks, fish or meat bones, screws, coins, metal clips,

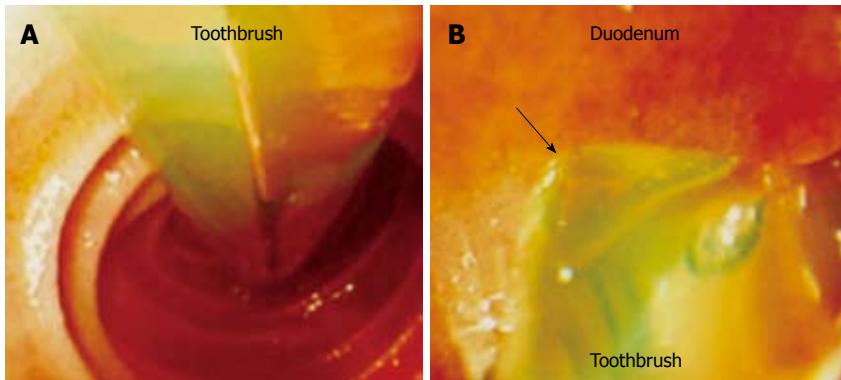
teeth, dental prosthesis, and spoon handles, has been reported<sup>[1-3]</sup>. Most ingested foreign bodies pass through the gastrointestinal tract spontaneously without causing untoward effects. However, sometimes these foreign bodies cause obstruction or perforation of the gastrointestinal tract necessitating surgical intervention<sup>[2-5]</sup>. Here, we report a rare case of duodenal perforation resulting from an ingested toothbrush.

### CASE REPORT

A 22-year-old female presented with intermittent epigastric pain for 6 d. Eight days prior to hospitalization, she experienced nausea and foreign body sensation in the throat. She attempted to induce vomiting by irritating the pharynx with the distal end of a toothbrush handle. The toothbrush broke at the junction of the handle and the brush head. Unfortunately, she accidentally swallowed the handle of the broken toothbrush, which was 12 cm long. She had no passage of tarry noted after swallowing the foreign body. However, she experienced epigastralgia 2 d after ingesting the toothbrush. Endoscopy was performed twice at another hospital. However, the swallowed toothbrush could not be removed. The patient denied any history of excessive alcohol consumption or illicit drug use. Her medical history was otherwise unremarkable.

Upon admission, the patient was afebrile, and her vital signs were normal. Mild tenderness but no rebound pain was noted in the upper abdomen. Laboratory values were as follows: 10.0 g/dL hemoglobin, 30.5% hematocrit, 7100/mm<sup>3</sup> white blood cells (WBC), 30 U/L aspartate aminotransferase (AST), 32 U/L alanine aminotransferase (ALT), and 72 U/L alkaline phosphatase. Chest and plain abdominal X-ray examinations were unremarkable. Endoscopic examination revealed a broken toothbrush handle in the second portion of the duodenum (Figure 1A). The blunt end of the toothbrush faced distally and the broken end, proximally against the duodenal wall (Figure 1B). There was an ulcer on the anterior wall of the duodenal bulb. Endoscopic removal of the toothbrush was reattempted but failed.

Surgery was performed on the second day of hospitalization. Laparotomy revealed a perforation in the anterior wall of the first portion of the duodenum. The perforation had necrotic edges and was sealed off by the infundibulum of the gallbladder. The toothbrush was removed through this perforation which was debrided and then closed primarily with interrupted silk sutures rein-



**Figure 1** A toothbrush handle found in the second portion of the duodenum (A) and the pointed broken end (arrow) of the toothbrush handle impinged on the wall of the duodenal bulb (B).

forced with an omental patch. The patient's postoperative course was uneventful. The patient resumed oral intake on the fourth postoperative day and was discharged from the hospital 7 d after surgery.

## DISCUSSION

Several factors are associated with the ingestion of foreign bodies. Children usually swallow foreign bodies because of carelessness. In adults, poor vision, mental disease, drug addiction, wearing of dentures, and rapid eating have been implicated as the etiologic factors of foreign body ingestion<sup>[2-5]</sup>. In addition, excessive alcohol intake and extremely cold fluids may dull the sensitivity of the palatal surface and increase the risk of swallowing foreign bodies<sup>[6]</sup>. The majority of ingested foreign bodies pass uneventfully through the gastrointestinal tract<sup>[2-5]</sup>. However, in some patients, the ingested foreign body may cause impaction, perforation, or obstruction. Perforation of the gastrointestinal tract may be associated with a considerably high mortality and morbidity. Gastrointestinal tract perforation may cause peritonitis, abscess formation, inflammatory mass formation, obstruction, fistulae, and hemorrhage<sup>[2,6]</sup>. In addition, foreign body perforation of the gastrointestinal tract may involve adjacent structures such as the kidneys, psoas muscle, and inferior vena cava<sup>[7,8]</sup>. Rare cases of foreign body migration to the pleura, heart, kidneys, or liver have been reported<sup>[9-12]</sup>. Most deaths in patients with foreign body perforation of the gastrointestinal tract are due to fulminant sepsis<sup>[2,13,14]</sup>. Therefore, efforts should be made to remove the ingested foreign bodies if they cannot pass through the gastrointestinal tract spontaneously.

In this case, the broken toothbrush handle was entrapped in the duodenal sweep and resulted in perforation of the duodenum. Intestinal injury resulting from an ingested foreign body tends to occur in areas of acute angulation but it may occur in all segments of the gastrointestinal tract<sup>[6]</sup>. The retroperitoneal, relatively immobile, and rigid nature of the duodenum as well as its deep transverse rugae and sharp angulations make it a common site for the entrapment of long and sharp-ended objects. Furthermore, the properties of foreign bodies determine the degree of complications caused by them. Thin and sharp foreign bodies such as chicken and fish bones, toothpicks, and straightened paperclips carry

a higher risk of perforation. Long, slender, sharp-ended items have difficulty in traversing the tortuous gastrointestinal tract<sup>[2,3]</sup>. In a review of 31 cases of toothbrush ingestion, no episodes of spontaneous passage were reported<sup>[15]</sup>. Complications related to pressure necrosis, including gastritis, mucosal tears, and perforations, occurred in several of these cases.

Although a conservative approach toward foreign body ingestion is justified, early endoscopic removal of the ingested foreign body from the stomach is recommended<sup>[2,4,15]</sup>. Ertan *et al*<sup>[16]</sup> reported the first case of successful removal of a swallowed toothbrush. Other authors found the endoscopic approach unsuccessful due to the size and shape of the ingested toothbrush<sup>[17,18]</sup>. Esophageal perforation during the endoscopic extraction of a toothbrush has been reported<sup>[6]</sup>. In addition, objects longer than 6-10 cm have difficulty in passing the duodenal sweep<sup>[19]</sup>. Therefore, in cases of unsuccessful removal of gastric foreign bodies that are longer than 6.0 cm, surgical removal of them should be considered. Wishner and Rogers<sup>[18]</sup> reported a case of successful laparoscopic removal of a toothbrush from the stomach. Laparotomy in this case is justified because it is difficult to remove a toothbrush from the duodenum *via* a laparoscopic approach.

In conclusion, an ingested toothbrush cannot pass spontaneously through the gastrointestinal tract. Early removal of the ingested toothbrush is advised to avoid impaction of the toothbrush at the duodenum and to minimize morbidity. Endoscopic removal should be performed by a skilled endoscopist. If this is not possible or unsuccessful, a surgical approach is recommended.

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## Flare-up of ulcerative colitis after systemic corticosteroids: A strong case for Strongyloides

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### INTRODUCTION

Although exacerbation of ulcerative colitis (UC) usually poses a little diagnostic dilemma, physicians should remain cognizant to the possibility of an alternative cause for patient symptoms.

### CASE REPORT

A 54-year-old practicing physician, native of Brazil who immigrated to Israel 20 years ago, was hospitalized in the Neurology Department with severe cluster headache attack, which responded to oral dexamethasone at 16 mg/d. History was also notable for left-sided UC controlled by 5-aminosalicylates (5-ASA) over the past 11 years. In the previous 2 years, the patient was lost to follow-up but reported he was in clinical remission. Two weeks after his hospital discharge from the Neurology Department, he presented again with non-bloody diarrhea, abdominal pain and muscle pain.

Upon examination a fever of 37.7°C was noted, and right abdominal tenderness was appreciated. The rest of the physical examination was unremarkable. Complete blood count showed that his white blood cell count was 8.4 K/mcl, with a normal differential count. Hemoglobin level was 14.6 g/dL. Chemistry results were all normal except for an albumin of 2.6 g/dL. Urinalysis showed leukocyturia and urine culture-yielded *E.coli*. Abdominal plain film showed small fluid levels.

Although the patient's abdominal pain and diarrhea suggested a possible UC flare-up, the lack of rectal bleeding, the right-sided abdominal tenderness and the muscular complaints raised the suspicion of a superimposed infectious process in addition to a urinary tract

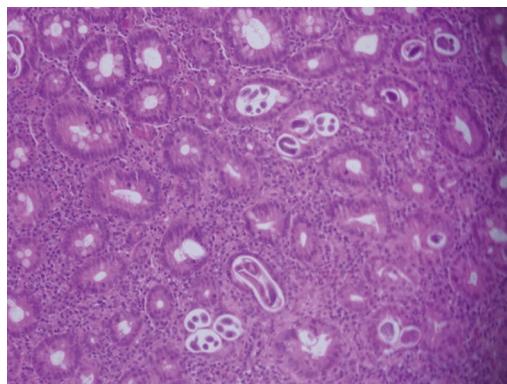
### Abstract

Super-imposed infection with intestinal organisms can mimic a flare-up of underlying disease in patients with inflammatory bowel disease (IBD). We report a case of patient with long standing ulcerative colitis (UC), who presented with abdominal pain, diarrhea and low-grade fever after receiving systemic corticosteroids for an unrelated disorder. Despite a negative stool examination, a peripheral eosinophilia reappeared upon tapering down of a corticosteroid dose. Subsequently, duodenal biopsies showed evidence for Strongyloides, presumably acquired 20 years ago when the patient was residing in Brazil. The patient fully recovered following anti-helminthic therapy. This case underscores the importance of considering Strongyloides in the work-up of flaring-up IBD patients, even if a history of residing or traveling to endemic areas is in the distant past.

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**Key words:** Ulcerative colitis; Infection; Parasites; Eosinophilia; Strongyloides

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**Figure 1** Histology of the duodenum showing chronically and actively inflamed mucosa with rhabditoid developing larvae, developing eggs and adult *Strongyloides*.

infection. Steroid myopathy causing muscles' pain was also considered. Prednisone was tapered to 5 mg over 4 d and ofloxacin was started, but the patients' symptoms persisted and a fever up to 38°C was noted. Stool culture, examination for ova & parasites and *C. difficile* toxin assay were all negative. Following steroid tapering, an elevation of eosinophil count to 15.5% of white blood cells was noted, with an absolute count of 980/mcl. Re-inspection of the blood count obtained in the Neurology Department before initiation of dexamethasone revealed peripheral eosinophilia (1240/mcl), which went unnoticed. The patient underwent gastroduodenoscopy, which showed a hyperemic edematous duodenal wall. Colonoscopy showed edematous mucosa and punctate submucosal hemorrhages extending along the colon from the descending colon to the cecum. The rectum appeared relatively spared.

Histology from the colon revealed chronic eosinophilic inflammation and formation of eosinophilic abscesses. Duodenal histology revealed *Strongyloides* larva (Figure 1). CMV PCR in blood and tissue CMV immunohistochemistry were negative.

Ivermectin at a dose of 12 mg once daily was initiated with prompt resolution of all symptoms. During the 4-year follow-up, the patient was well, but experienced several mild flare-ups of UC, controlled with 5-ASA therapy. Repeat colonoscopies showed left sided colitis, and histology was compatible with UC. Two subsequent gastroduodenoscopies with biopsies did not disclose evidence of *strongyloides*.

## DISCUSSION

*Strongyloides stercoralis* is endemic in tropic and sub-tropic areas of the world, but has also been reported in residents of certain regions of the US and in coal miners in non-endemic areas<sup>[1,2]</sup>. Most cases present with pulmonary and/or upper gastrointestinal symptoms<sup>[3]</sup>. However, *Strongyloides*-associated colitis can occur as part of hyperinfestation following altered host immune status<sup>[4,5]</sup>. In this situation, larvae traveling down from the duodenum penetrate the colon wall rather than continue to be excreted in the stool<sup>[4]</sup>. The ensuing

eosinophilic-predominant colitis may mimic new onset of UC<sup>[2,4]</sup>. Alternatively, it can emerge after corticosteroid treatment of a patient with well established UC, and can be mistaken for a refractory exacerbation<sup>[1,3,6]</sup>.

The diagnosis of *Strongyloides* is often problematic. Stool examination is negative in 50%-70%<sup>[7,8]</sup> of patients. Eosinophilia was present in 84% of patients in one series<sup>[7]</sup>, but in only 32% of corticosteroids-treated patients in another series<sup>[9]</sup>. Our patient had eosinophilia upon his prior admission to the Neurology Department that went unnoticed. Serology testing for *Strongyloides*-specific antibodies is also helpful, although not widely available. Upper endoscopy usually reveals hyperemic edematous duodenal mucosa with white villi<sup>[10]</sup> and colonoscopy may show mucosal edema, erosions and ulcerations<sup>[11]</sup>, but none of these findings is specific. Duodenal aspirates and/or biopsy can assist the diagnosis, but are dependent upon worm burden and amenable to sample error<sup>[7]</sup>. Moreover, even when larvae are present in the intestinal or colonic wall, the diagnosis can be over-looked by the inexperienced or unsuspecting pathologist<sup>[1,12]</sup>, or may be mistaken for eosinophilic gastroenteritis<sup>[13]</sup>.

*Strongyloides* can persist in the host for his or her entire life-time<sup>[14]</sup>. Indeed, in our patient, infection was probably acquired in Brazil, twenty years prior to clinical presentation. Thus, albeit rare, the potential fatal consequences of untreated strongyloidiasis, make it imperative to consider this diagnosis in flaring inflammatory bowel disease (IBD) patients with even a distant history of residence or travel in endemic areas, or in patients failing to respond to standard therapy<sup>[15]</sup>. Eosinophilia should be excluded before initiation of immunosuppression, given the hazards of over-looking a dormant parasitic infection.

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CASE REPORT

## Rapid re-emergence of YMDD mutation of hepatitis B virus with hepatic decompensation after lamivudine retreatment

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### Abstract

Lamivudine has a high rate of antiviral resistance. Sequential treatment of anti-hepatitis B virus (HBV) is commonly used for lamivudine resistance. We report 4 cases of patients with rapid redetection of HBV mutants during the lamivudine retreatment. The four patients received lamivudine as an initial treatment of HBV and adefovir and lamivudine as a rescue therapy consecutively. HBV-DNA level, YMDD mutations and adefovir -resistant mutations (RFMP) were tested every 3 mo during the sequential treatment. All the patients showed YMDD mutations during the initial lamivudine therapy. After adefovir therapy for lamivudine resistance, they showed viral breakthrough. Adefovir was switched to lamivudine, however, it did not induce viral suppression at all, rather increased HBV-DNA with rapid reemergence of the YMDD mutations. All the patients had ALT flares, and hepatic decompensation occurred in two patients. After switching to adefovir combined with entecavir or lamivudine for a rescue therapy, the patients had reduction in HBV-DNA and ALT improvement. These cases demonstrated that lamivudine retreatment of patients with preexposed lamivudine resistance leads to rapid reemergence of YMDD mutation with significant viral rebounds and subsequent hepatic decompensation. Sequential administration of lamivudine in patients with a prior history of YMDD mutation should be abandoned.

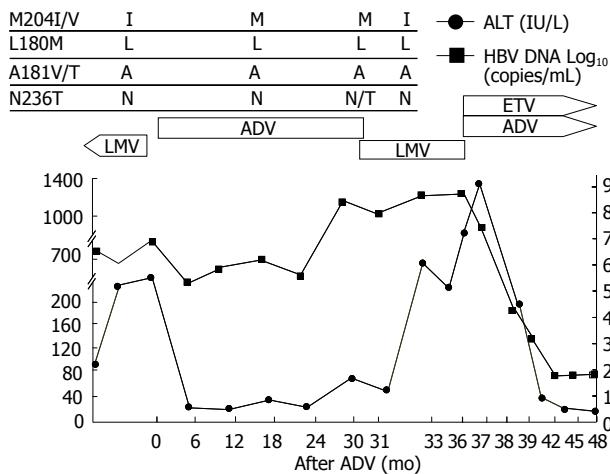
### INTRODUCTION

Lamivudine has been commonly used in the treatment of chronic hepatitis B as a first-line antiviral agent. Long-term lamivudine therapy can usually suppress hepatitis B virus (HBV) replication, however, prolonged monotherapy leads to the emergence of lamivudine-resistant HBV mutants<sup>[1-3]</sup>. The emergence of rtM204I/V (YMDD) mutation of HBV polymerase gene is associated with rebounds in serum HBV DNA and flares of transaminase level<sup>[4]</sup>.

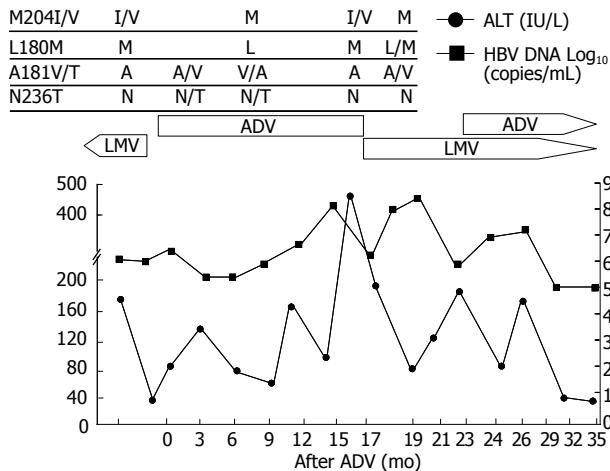
Adefovir dipivoxil is an effective rescue therapy for lamivudine-resistant HBV<sup>[5,6]</sup>. However, adefovir resistance occurs more frequently in second-line treatment of lamivudine-resistant patients than in naïve patients<sup>[7]</sup>. It is well known that sequential monotherapy with antiviral agents induces the sequential occurrence of viral mutations<sup>[8]</sup>. We here report the rapid redetection of YMDD mutants and hepatic decompensation in patients with prior lamivudine resistance during lamivudine retreatment.

### CASE REPORTS

Patient 1 (Figure 1) was a 46-year-old man with HBeAg-positive chronic hepatitis B. Lamivudine was started initially and good virological and biochemical responses were observed in the patient. However, HBV-DNA increased and rtM204I mutation was detected by restriction fragment length polymorphism (RFMP) assay<sup>[9]</sup> after 2 years of lamivudine therapy.



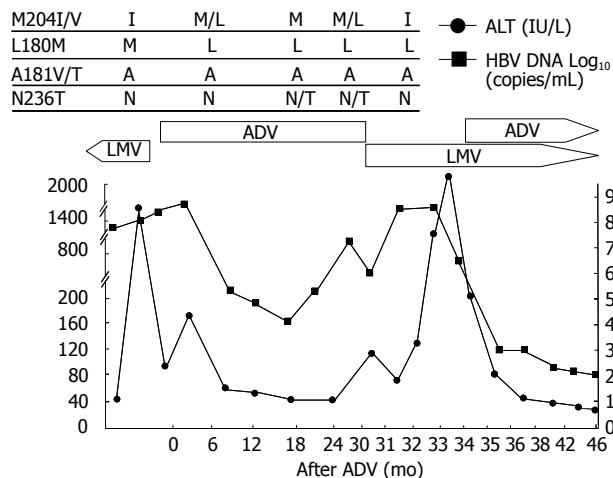
**Figure 1** Viral responses and clinical courses in a 46-year-old man with chronic hepatitis (patient 1). The underline represents sequential evolution of lamivudine- or adefovir- resistant genotypic mutations in HBV polymerase gene. LMV: Lamivudine; ADV: Adefovir; ETV: Entecavir; ALT: Alanine aminotransferase.



**Figure 2** Viral responses and clinical courses in a 49-year-old man with compensated cirrhosis (patient 2). The underline represents sequential evolution of lamivudine- or adefovir- resistant genotypic mutations in HBV polymerase gene. LMV: Lamivudine; ADV: Adefovir; ALT: Alanine aminotransferase.

After lamivudine was switched to adefovir, HBV-DNA dropped by 2 logs with ALT normalization. Viral breakthrough (defined as a  $\geq 1 \log_{10}$  increase in HBV DNA from nadir after initial viral response) and rtN236T mutation developed after 30 mo of adefovir therapy. Lamivudine was restarted with discontinuation of adefovir. After lamivudine was restarted, the rtM204I mutation reemerged and rtN236T adefovir-resistant mutants disappeared 1 mo after the treatment. He showed persistently high levels of HBV-DNA, ALT and total bilirubin. Lamivudine was changed to adefovir and entecavir combination treatment as a rescue therapy. After the combination treatment, HBV-DNA and ALT dropped significantly.

Patient 2 (Figure 2), a 49-year-old man with cirrhosis, showed the similar response to lamivudine retreatment as shown in patient 1. Viral breakthrough with YMDD mutation developed after 15 mo of the

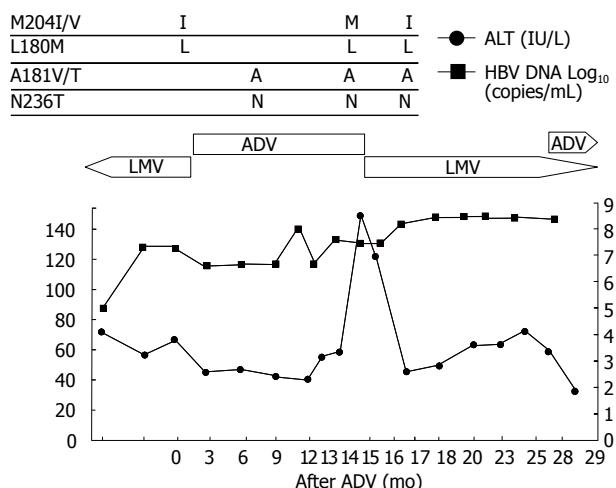


**Figure 3** Viral responses and clinical courses in a 50-year-old woman with decompensated cirrhosis (patient 3). The underline represents sequential evolution of lamivudine- or adefovir- resistant genotypic mutations in HBV polymerase gene. LMV: Lamivudine; ADV: Adefovir; ALT: Alanine aminotransferase.

initial lamivudine treatment. Lamivudine was switched to adefovir, however, there was no decrease in HBV-DNA level but ALT elevation. After reintroduction of lamivudine for adefovir resistance, ALT level decreased without a significant drop in HBV-DNA level initially. Adefovir-resistant mutant was replaced by wild type, however, reemergence of the rtM204I/V mutation followed by HBV-DNA rebound and ALT fluctuation were observed. Adefovir was added to the ongoing lamivudine therapy.

Patient 3 (Figure 3), a 50-year-old woman, had cirrhosis and received lamivudine as an initial treatment. After 16 mo of lamivudine therapy, she had elevated HBV-DNA followed by hepatic decompensation. Mutation profile showed rtM204I and rtL180M at the time of viral breakthrough. Lamivudine was switched to adefovir, and HBV-DNA decreased and her liver function was restored. Viral breakthrough occurred after 27 mo of adefovir treatment and rtN236T mutation was detected at this time. Lamivudine monotherapy was reintroduced consecutively. Rapid increase in HBV-DNA with the rtM204I mutants 2 mo after the lamivudine retreatment and ALT flare with jaundice and ascites occurred subsequently. The rtN236T mutation changed to wild type after the LMV treatment. Adefovir was added to lamivudine as a rescue therapy, and a rapid drop in HBV-DNA and ALT level was observed. The bilirubin decreased from the peak level of 18.7 to 1.6 mg/dL 6 mo after the combination treatment.

Patient 4 (Figure 4), a 50-year-old man with cirrhosis, received lamivudine for 14 mo. He had viral breakthrough with mild elevation of ALT, therefore lamivudine was changed to adefovir. However, he had no viral response though he showed a normal ALT level during the adefovir treatment. Adefovir was switched to lamivudine due to the high viral load. The ALT level was stable despite no decrease in HBV-DNA. After 2 mo



**Figure 4** Viral responses and clinical courses in 50-year-old man with compensated cirrhosis (patient 4). The underline represents sequential evolution of lamivudine- or adefovir- resistant genotypic mutations in HBV polymerase gene. LMV: Lamivudine; ADV: Adefovir; ALT: Alanine aminotransferase.

of lamivudine retreatment, HBV-DNA rebound with ALT flare developed. HBV mutation rtM204I was reappeared at this time. Adefovir was added to the ongoing lamivudine treatment.

All the patients were positive for HBeAg and had no seroconversion of HBeAg during the sequential antiviral treatment except for patient 3. Patient 3 had HBeAg loss after the lamivudine/adefovir combination therapy. No death occurred, however, two of them showed hepatic decompensation following the reemergence of YMDD mutations and viral breakthrough.

## DISCUSSION

These cases showed that reintroduction of lamivudine could induce rapid reemergence of lamivudine-resistant mutations in chronic hepatitis B patients with prior lamivudine resistance. Lamivudine is well tolerated and significantly reduces HBV-DNA level<sup>[10]</sup>. However, lamivudine resistance associated with mutations in the polymerase gene, particularly in rtM204I/V known as YMDD mutant, occurs at a rate of 14%-30% annually<sup>[3,4]</sup>. Development of lamivudine resistance is associated with high baseline HBV-DNA level, duration of lamivudine treatment, precore variant, insufficient serum HBV-DNA suppression and elevated serum ALT level during treatment<sup>[11-13]</sup>. All the cases had positive HBeAg and high viral load at the time of lamivudine resistance. The high viral replication status may predispose to develop antiviral resistant mutations and subsequent viral breakthrough.

Adefovir is an effective rescue therapy for lamivudine-resistant HBV and significantly improves biochemical, virological, and histological features of lamivudine-resistant patients<sup>[5,6]</sup>. Compared with lamivudine, adefovir is associated with a low rate of antiviral resistance<sup>[14]</sup>. However, adefovir resistance occurs more common in patients with preexisting lamivudine resistance<sup>[7]</sup>. Among our cases, rtN236T mutation was detected in two

patients at the time of viral breakthrough after adefovir rescue therapy. The other two showed no virologic response, and rtA181V was found in one patient.

YMDD mutations, mainly rtM204I in our cases, disappeared after the adefovir treatment, suggesting that cessation of antiviral therapy leads to disappearance of drug-resistant mutations. However, it was reported that lamivudine-resistant HBV mutants reappear rapidly after reintroduction of lamivudine<sup>[15]</sup>. Once antiviral-resistant HBV mutants have been selected, the mutants are archived even if treatment is stopped. In our cases, YMDD mutation reemerged within 3 mo after reintroduction of lamivudine, suggesting that re-treatment to which the virus has been previously resistant has a limited efficacy even after wild-type YMDD has restored. It could not be excluded that the adefovir-resistant mutations may affect the rapid emergence of lamivudine-resistant mutation.

The emergence of lamivudine resistance can result in viral breakthrough, flares of hepatitis and worsening of the initial histological improvement<sup>[1,2]</sup>. In patients with cirrhosis, severe exacerbation of hepatitis may result in hepatic failure<sup>[16]</sup>. It was reported that patients with YMDD mutations experience a higher rate of hepatitis flares than those without YMDD mutations<sup>[17]</sup>, which is possibly because the YMDD locus takes part in cellular immune response in some cases.

Serum HBV DNA level was higher than baseline in the cases after emergence of the YMDD mutant before exacerbation occurred. Two patients experienced a very rapid deterioration of hepatic function accompanying the reemerging YMDD mutants only a few months after lamivudine reintroduction. These findings clearly demonstrate that sequential monotherapy, particularly retreatment with the antiviral agent to which the HBV has been previously resistant, should be avoided.

Combination therapy with adefovir and lamivudine or other antiviral agents could be a better option to prevent the emergence of antiviral-resistant mutations in patients with lamivudine- or adefovir -resistance. Combination therapy is not likely to improve virologic response, but rather decrease the rate of viral resistance<sup>[18,19]</sup>. Unfortunately, combination therapy could not be commonly used in Korea so far because of the high cost of antiviral agents. Other antiviral agents, such as tenofovir or pegylated interferon, could be promising agents for multi-drug resistant HBV<sup>[20,21]</sup>. All the patients in this study received combination therapy with adefovir and lamivudine or entecavir as a rescue therapy for lamivudine resistance. After the combination treatment, the HBV DNA and ALT level dropped and hepatic function was restored. It was reported that entecavir has a potent antiviral efficacy against naïve HBV and lamivudine-refractory HBV<sup>[22]</sup>. Case 1 received adefovir and entecavir as the rescue therapy. These two agents have antiviral activity on wild type or YMDD mutants without cross resistance. The other three patients received adefovir and lamivudine combination treatment. Case 3 showed a rapid reduction in HBV DNA level and HBeAg seroconversion soon after the combination

therapy. Long-term data are not available from cases 2 and 4. However, they showed a significant decrease in HBV DNA level and improvement in hepatic function after combination therapy.

Taken together, lamivudine retreatment may induce rapid reemergence of the YMDD mutation and significant viral rebound and subsequent hepatic flare in patients with preexposed YMDD mutants. Sequential lamivudine treatment of those with a prior history of YMDD mutation should be abandoned.

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LETTERS TO THE EDITOR

## Nutritional therapy for active Crohn's disease

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### Abstract

Nutritional therapy for active Crohn's disease (CD) is an underutilized form of treatment in adult patients, though its use is common in the paediatric population. There is evidence that nutritional therapy can effectively induce remission of CD in adult patients. Enteral nutrition therapy is safe and generally well tolerated. Meta-analysis data suggest that corticosteroids are superior to nutritional treatment for induction of remission in active CD. However, the potential side effects of such pharmacotherapy must be taken into consideration. This review examines the evidence for the efficacy of elemental and polymeric diets, and the use of total parenteral nutrition in active CD.

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**Key words:** Crohn's disease; Nutrition; Dietary; Treatment; Steroids

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### TO THE EDITOR

Nutritional therapies studied for the induction of remission in active Crohn's disease (CD) include enteral

nutrition (EN), and total parenteral nutrition (TPN). EN by means of a polymeric diet can be given via the nasogastric or per oral route. Compliance tends to be greater with polymeric nutrition support than with an elemental diet, as the feed is considered more palatable. Polymeric diets provide nitrogen in the form of whole protein, and carbohydrates as hydrolysates of starch. Fat is most often provided as medium chain fatty acids. Fiber is commonly added to polymeric feeds though there is little evidence to suggest that it has a substantial positive or negative effect in hospitalized patients<sup>[1]</sup>. Elemental diets contain nutrients in simple forms (such as amino acids, simple carbohydrates, fats, vitamins and minerals) requiring little or no digestion to take place prior to absorption.

The theory behind the mechanism of action of an elemental diet is multi-factorial. Malnutrition can have effects on immune function and wound healing, as well as psychological and cognitive effects. Improvements in wound healing by ensuring a good nutritional status would theoretically lead to enhanced mucosal healing. Increased gut permeability has been implicated in the pathophysiology of CD. This is thought to relate to abnormalities in the tight junctions between enterocytes allowing an increase in luminal antigen uptake-potentially a factor contributing to increased inflammatory activity<sup>[2]</sup>. Treatment with an elemental diet has been shown to decrease intestinal permeability<sup>[3]</sup>. The incidence of CD dramatically increased in the twentieth century<sup>[4]</sup>. This coincides with many changes in our daily lives, including changes in our dietary intake. As elemental diets involve the ingestion of specific substances, it may be that pro-inflammatory antigens are avoided. The normal commensal bacterial population of the gut may play a role in the development of inflammation in CD, though the mechanism is unclear. In experimental animal models, inflammation does not develop in mice reared in a sterile environment<sup>[5]</sup>. An early study suggested the amount of bacteria per gram of faeces was reduced in patients on an elemental diet, though there is no consensus on this issue<sup>[6]</sup>. The constituents of an elemental diet are primarily absorbed in the proximal small bowel-proximal to the most commonly affected sites of inflammation in CD. The reduction in the workload of digestion and absorption, and a reduction in peristalsis and digestive tract secretions may also play a role<sup>[7]</sup>. In general, elemental diets contain a low proportion of fat compared to polymeric or normal diets. A recent Cochrane review concluded that there is a non-significant trend towards greater efficacy with

very low fat and long-chain triglyceride elemental diets compared to standard elemental diet regimes<sup>[8]</sup>.

High quality randomised controlled trials looking at the use of nutritional therapy for the management of acute CD are difficult to perform. There have been very few studies where the remission rate with nutritional therapy is as low as that seen in the placebo arm of drug trials in active CD. So, if we accept that nutritional treatments have an effect, then the question arises as to the magnitude of this effect. According to some studies, remission rates may be as high as 84% with the use of an elemental diet<sup>[9]</sup>. The Cochrane review (2007) of enteral feeding in active CD provides us with very useful meta-analysis data<sup>[8]</sup>. As found in previous meta-analyses, the Cochrane review concluded that steroids have superior efficacy to enteral nutrition in inducing remission<sup>[8]</sup>. The exact role of enteral nutrition (EN) in adults to treat active CD is therefore undefined.

## TRIALS COMPARING ELEMENTAL DIET TO CORTICOSTEROIDS

A number of studies have been conducted looking into the efficacy of elemental diets in CD patients. Riordan *et al*<sup>[9]</sup> studied 136 patients with active CD. An elemental diet was introduced and all other CD treatments discontinued. The intention was to give an elemental diet for 2 wk, though 31% of the patients did not tolerate the diet for more than 1 wk. Of the 78 remaining patients, 84% achieved disease remission after a 14-d treatment course. The group was then split into 38 patients receiving a tapered course of prednisolone and advice on healthy eating, and 40 patients receiving placebo instead of steroid—this ‘diet’ group was asked to introduce one new food each day and exclude foods that worsened symptoms. There was a median remission of 3.8 mo in the steroid group compared to 7.5 mo in the diet group.

Gorard *et al*<sup>[10]</sup> compared 22 patients given an elemental diet (4 wk treatment) to 20 patients receiving prednisolone (0.75 mg/kg daily for 2 wk followed by reducing doses). All participants were CD patients requiring hospitalisation for an acute flare of the disease. Nine of the twenty-two patients (41%) in the diet arm of the trial withdrew because of intolerance. Disease activity was measured using a simple disease activity index. The reduction in disease activity was similar between the diet (score of 4.8 reducing to 1.7) and prednisolone (score of 5.3 reducing to 1.9) groups. In addition to this, similar reductions in C-reactive protein, and increases in serum albumin concentration were found. The probability ratio of remaining patients in remission was, however, much lower in the diet group. At 6 mo, this probability was 0.67 after steroid compared to 0.28 after elemental diet.

## TRIALS COMPARING POLYMERIC DIET TO CORTICOSTEROIDS

Many trials involving polymeric and enteral diets in CD

have been conducted in children, due to the perceived need to avoid corticosteroids, to alleviate the additional risk of growth failure. Day *et al*<sup>[11]</sup> looked at 27 children with active CD (15 new diagnoses, 12 with known disease). They gave a polymeric feed as the exclusive source of nutrition for 6–8 wk either per oral or via a NG tube. No other medical therapy was used at that time. Twenty-four of the twenty-seven children completed the 6–8 wk course, while the other three did not tolerate enteral feeding. At the end of the treatment period, 80% of the newly diagnosed patients and 58% of the known-CD patients had entered remission. The CD remained inactive in all of the newly diagnosed patients with entered remission, over the mean 15.2-mo follow-up period.

Borrelli *et al*<sup>[12]</sup> conducted a trial comparing polymeric diet to corticosteroids in 37 children with active treatment in naïve CD patients. The study period was 10 wk, and after this time 15 of the 19 children (79%) receiving a polymeric diet had remission compared to 12 of the 18 patients in the corticosteroid group (67%). The differences were not statistically significant. An additional interesting aspect of this trial was that mucosal healing was assessed by endoscopy with histology at weeks 0 and 10. The proportion of children with mucosal healing was significantly higher in the polymeric diet group (74%) than in the corticosteroid group (33%).

The use of a polymeric diet in adult patients with CD has also been studied. Gonzalez-Huix *et al*<sup>[13]</sup> conducted a randomised controlled trial comparing adults with acute CD receiving 1 mg/kg per day prednisolone ( $n = 17$ ) followed by a reducing course, to those on a polymeric diet and no medication ( $n = 15$ ). The polymeric feed was given via a fine-bore NG tube and no other nutrition allowed. The polymeric diet patients went back to a normal diet after clinical remission was achieved. Of the seventeen patients in the steroid group, fifteen entered remission after a mean time of 2 wk. One patient had an intestinal perforation requiring surgery, and the others entered remission after being started on a polymeric diet. Of the 15 patients in the polymeric diet group, 12 entered remission after a mean time of 2.4 wk. Of the 3 treatment failures, one improved when steroids were given, and the other two were said to have failed as they did not enter remission after 4 wk on the polymeric feed. Patients from both arms of the trial were started on oral 5-ASA preparations prior to discharge. The cumulative probability of relapse during the follow-up period was higher after steroid treatment than after polymeric diet though this did not reach a statistical significance.

There is some evidence to suggest that the amount and type of fat in polymeric feeds may have an impact on its efficacy in CD patients. Gassull *et al*<sup>[14]</sup> hypothesised that a polymeric diet rich in monounsaturated fatty acids (MUFA) would be more effective in inducing remission in active CD patients than an identical diet but with polyunsaturated fatty acids (PUFA)-precursors of some inflammatory cytokines. They randomised 62 patients with active CD to either one of these diets for no longer

than 4 wk, or to 1 mg/kg per day prednisolone. The steroid group reacted as expected from previous studies with a 79% remission rate. However, the diet group did not fare as well. Only 20 % in the MUFA group entered remission, while 52% in the PUFA group achieved this target. These results were quite the opposite of those expected. Leiper *et al*<sup>[15]</sup> conducted a randomised trial in 54 patients with active CD. They received a polymeric diet with either high or low long-chain triglyceride (LCT) content. A staggering 39% of patients withdrew from the trial within 3 wk because of an inability to tolerate the diet, which was offered by either the oral or nasogastric route. Of those completing the trial, the response rate was 46% for the low LCT group and 45% for the high LCT group, respectively, thereby demonstrating no significant difference in efficacy with differing fat composition.

## COCHRANE COLLABORATION REVIEW OF ENTERAL NUTRITION THERAPY FOR THE INDUCTION OF REMISSION IN ACTIVE CD

There have been four meta-analyses looking at the use of enteral nutrition therapy in comparison to steroids to induce remission in active CD patients. Overall, each of these meta-analyses showed steroids to be more effective than enteral nutrition strategies. However, when two large trials were excluded because of the concomitant use of other medicines in the steroid arm, both enteral nutrition and steroids were seen to have an equal efficacy. A recent review from the Cochrane Collaboration studied the results from trials comparing different types of enteral nutrition (EN) to each other, and trials comparing the use of EN to steroids<sup>[8]</sup>. When looking at differences between diet formulations used to treat patients with acute CD, they performed a meta-analysis which included data from 188 adult patients treated by elemental diet and 146 patients given a polymeric diet. No significant difference was found in the results achieved between elemental and polymeric diets.

Sub-group analysis showed no difference between formulae with high fat *versus* low fat content. Differences in the amount of fat in the form of high *versus* low long-chain triglyceride were also shown not to be significant. Meta-analysis of trials comparing enteral feeding to corticosteroids compared data from 192 enteral nutrition patients *versus* 160 patients treated with steroid, which revealed a pooled odds ratio of 0.33 favouring steroid treatment.

## TOTAL PARENTERAL NUTRITION AS A TREATMENT FOR ACTIVE CD

Controlled trials of total parenteral nutrition (TPN) in CD patients are few and far between. Greenberg *et al*<sup>[16]</sup> conducted a trial in 51 patients with active CD. They were

randomised to either TPN and nil by mouth ( $n = 17$ ), partial parenteral nutrition (PPN) and supplementary nutrition with a liquid feed of a defined formula *via* a NG tube ( $n = 19$ ), or PPN and supplementary normal food ( $n = 15$ ). Remission occurred in 71% of patients on TPN, in 58% of patients on the PPN/defined formula diet, and in 60% of patients on PPN/normal diet. Of those achieving remission, the chance of successfully remaining in remission at one year was 42%, 55%, and 56%, respectively. The differences were found not to be significant. The total bowel rest achieved through TPN was therefore not thought to be of importance.

## DISCUSSION

There is a disappointing lack of quality studies on the use of TPN in active CD patients. It is difficult to find a place for TPN as a treatment for active CD. The efficacy of TPN does not seem to be greater than that suggested by other trials of EN or steroids. TPN is known to be associated with an increased risk of adverse events, such as sepsis, although perhaps it has a place in patients intolerant to both EN and steroids. The value of TPN in malnourished patients with intestinal failure due to CD is beyond doubt.

There would seem no logical reason to choose EN over steroids for the vast majority of our patients. The European Society for Parenteral and Enteral Nutrition published guidelines in 2006 on the use of enteral nutrition in gastroenterology<sup>[17]</sup>. They suggested that a role could be found for EN in active CD in the following circumstances: steroid intolerance, patient refusal of steroids, EN in combination with steroids in undernourished individuals, and in patients with an inflammatory stenosis of the small intestine.

EN plays a greater role in children with active CD. In this group of patients, EN has been shown to have an efficacy equal to steroids. Therefore, it would seem perfectly reasonable to prescribe EN instead of steroids in the hope of avoiding steroid side-effects, including deleterious effects upon growth and development of children. The use of corticosteroids increases the risk of permanent growth failure in children, and 20%-30% will become adults with an abnormally short stature whether or not they are exposed to prolonged courses of steroids<sup>[18]</sup>.

The nutritional status in those with acute CD is important. Differences are seen between patients in an active phase of disease and those in remission. Weight loss is found in up to 75% of patients hospitalized with an exacerbation of CD, with a negative nitrogen balance present in more than 50%, whereas the majority of patients in remission are of normal nutritional status<sup>[17]</sup>. The role of nutritional therapy in the maintenance of a good nutritional status in CD patients is important, especially as the condition itself will predispose to malnutrition.

When EN is to be used, the type of formula does not make any difference to the efficacy. Polymeric diets are less expensive and more palatable than elemental

diets, and therefore it would seem reasonable to suggest that there is no place for the elemental diet.

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## Meetings

### Events Calendar 2008-2009

FALK SYMPOSIA 2008  
January 24-25, Frankfurt, Germany  
Falk Workshop: Perspectives in Liver Transplantation

International Gastroenterological Congresses 2008  
February 14-16, Paris, France  
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies  
[www.easl.ch/hepatitis-conference](http://www.easl.ch/hepatitis-conference)

February 14-17, Berlin, Germany  
8<sup>th</sup> International Conference on New Trends in Immunosuppression and Immunotherapy  
[www.kenes.com/immuno](http://www.kenes.com/immuno)

February 28, Lyon, France  
3<sup>rd</sup> Congress of ECCO - the European Crohn's and Colitis Organisation Inflammatory Bowel Diseases 2008  
[www.ecco-ibd.eu](http://www.ecco-ibd.eu)

February 29, Québec, Canada  
Canadian Association of Gastroenterology  
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK  
British Society of Gastroenterology Annual Meeting  
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China  
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea  
Asian Pacific Association for the Study of the Liver  
18<sup>th</sup> Conference of APASL: New Horizons in Hepatology  
[www.apaslseoul2008.org](http://www.apaslseoul2008.org)

March 29-April 1, Shanghai, China  
Shanghai-Hong Kong International Liver Congress  
[www.livercongress.org](http://www.livercongress.org)

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco  
OESO 9<sup>th</sup> World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas  
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA  
SAGES 2008 Annual Meeting - part of Surgical Spring Week  
[www.sages.org/08program/html/](http://www.sages.org/08program/html/)

April 18-22, Buenos Aires, Argentina  
9<sup>th</sup> World Congress of the International Hepato-Pancreato Biliary Association  
Association for the Study of the Liver  
[www.ca-ihpba.com.ar](http://www.ca-ihpba.com.ar)

April 23-27, Milan, Italy  
43<sup>rd</sup> Annual Meeting of the European Association for the Study of the Liver  
[www.easl.ch](http://www.easl.ch)

May 2-3, Budapest, Hungary  
Falk Symposium 164: Intestinal

### Disorders

May 18-21, San Diego, California, USA  
Digestive Disease Week 2008

May 21-22, California, USA  
ASGE Annual Postgraduate Course Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion  
E-mail: education@sgo.org

June 4-7, Helsinki, Finland  
The 39<sup>th</sup> Nordic Meeting of Gastroenterology  
[www.congrex.com/ngc2008](http://www.congrex.com/ngc2008)

June 5-8, Sitges (Barcelona), Spain  
Semana de las Enfermedades Digestivas  
E-mail: sepd@sepdi.es

June 6-8, Prague, Czech Republic  
3<sup>rd</sup> Annual European Meeting: Perspectives in Inflammatory Bowel Diseases  
E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey  
ESGAR 2008 19<sup>th</sup> Annual Meeting and Postgraduate Course  
E-mail: fca@netvisao.pt

June 11-13, Stockholm, Sweden  
16<sup>th</sup> International Congress of the European Association for Endoscopic Surgery  
E-mail: info@aes-eur.org

June 13-14, Amsterdam, Netherlands  
Falk Symposium 165: XX International Bile Acid Meeting, Bile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic  
Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management  
E-mail: idca2008@guantar.cz

June 25-28, Barcelona, Spain  
10<sup>th</sup> World Congress on Gastrointestinal Cancer  
Imedex and ESMO  
E-mail: meetings@imedex.com

June 25-28, Lodz, Poland  
Joint Meeting of the European Pancreatic Club (EPC) and the International Association of Pancreatology (IAP)  
E-mail: office@epc-iap2008.org  
[www.e-p-c.org](http://www.e-p-c.org)  
[www.pancreatology.org](http://www.pancreatology.org)

June 26-28, Bratislava, Slovakia  
5<sup>th</sup> Central European Gastroenterology Meeting  
[www.ceurgem2008.cz](http://www.ceurgem2008.cz)

July 9-12, Paris, France  
ILTS 14<sup>th</sup> Annual International Congress  
[www.ilts.org](http://www.ilts.org)

September 10-13, Budapest, Hungary  
11<sup>th</sup> World Congress of the International Society for Diseases of the Esophagus  
E-mail: isde@isde.net

September 13-16, New Delhi, India  
Asia Pacific Digestive Week  
E-mail: apdw@apdw2008.net

APDW 2008  
September 13-16, New Delhi, Indian Organized: Indian Society of Gastroenterology

III FALK GASTRO-CONFERENCE

September 17, Mainz, Germany  
Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany  
Falk Symposium 166: GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic  
Prague Hepatology Meeting 2008  
[www.czech-hepatology.cz/phm2008](http://www.czech-hepatology.cz/phm2008)

September 20-21, Mainz, Germany  
Falk Symposium 167: Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France  
Third Annual Meeting  
European Society of Coloproctology  
[www.escp.eu.com](http://www.escp.eu.com)



October 8-11, Istanbul, Turkey  
18<sup>th</sup> World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists  
E-mail: orkun.sahin@serenas.com.tr

October 18-22, Vienna, Austria  
16<sup>th</sup> United European Gastroenterology Week  
[www.negf.org](http://www.negf.org)  
[www.acv.at](http://www.acv.at)

October 22-25, Minnesota, USA  
Anstralian Gastroenterology Week 2008  
E-mail: gesa@gesa.org.au

October 22-25, Brisbane, Australia  
71<sup>st</sup> Annual Colon and Rectal Surgery Conference  
E-mail: info@colonrectalcourse.org

October 31-November 4, Moscone West Convention Center, San Francisco, CA  
59<sup>th</sup> AASLD Annual Meeting and Postgraduate Course  
The Liver Meeting Information: [www.aasld.org](http://www.aasld.org)

November 6-9, Lucerne, Switzerland  
Neurogastroenterology & Motility Joint International Meeting 2008  
E-mail: ngm2008@mci-group.com  
[www.ngm2008.com](http://www.ngm2008.com)

November 12, Santiago de Chile, Chile  
Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

November 28-29, Cairo, Egypt  
1<sup>st</sup> Hepatology and Gastroenterology Post Graduate Course  
[www.egyptgastrohep.com](http://www.egyptgastrohep.com)

December 7-9, Seoul, Korea  
6<sup>th</sup> International Meeting Hepatocellular Carcinoma: Eastern and Western Experiences  
E-mail: sglee@amc.seoul.kr

INFORMATION FOR ALL  
FALK FOUNDATION e.V.  
E-mail: [symposia@falkfoundation.de](mailto:symposia@falkfoundation.de)  
[www.falkfoundation.de](http://www.falkfoundation.de)

Advanced Courses - European

Institute of Telesurgery EITS - 2008 Strasbourg, France  
January 18-19, March 28-29, June 6-7, October 3-4

N.O.T.E.S  
April 3-5, November 27-29  
Laparoscopic Digestive Surgery

June 27-28, November 7-8  
Laparoscopic Colorectal Surgery

July 3-5  
Interventional GI Endoscopy Techniques  
Contact address for all courses:  
E-mail: [info@eits.fr](mailto:info@eits.fr)

International Gastroenterological Congresses 2009  
March 23-26, Glasgow, Scotland  
Meeting of the British Society of Gastroenterology (BSG)  
E-mail: [bsg@mailbox.ulcc.ac.uk](mailto:bsg@mailbox.ulcc.ac.uk)

May 17-20, Denver, Colorado, USA  
Digestive Disease Week 2009

November 21-25, London, UK  
Gastro 2009 UEGW/World Congress of Gastroenterology  
[www.gastro2009.org](http://www.gastro2009.org)



### Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London, The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.



## Instructions to authors

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In addition to the open access nature, another key characteristic of *WJG* is its reading guidance for each article which includes background, research frontier, related reports, breakthroughs, applications, terminology, and comments of peer reviewers for the general readers.

*WJG* publishes articles on esophageal, gastrointestinal, hepatobiliary and pancreatic tumors, and other esophageal, gastrointestinal, hepatic-biliary and pancreatic diseases in relation to epidemiology, immunology, microbiology, motility & nerve-gut interaction, endocrinology, nutrition & obesity, endoscopy, imaging and advanced hi-technology.

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#### Published by

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#### Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

#### Text

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and case reports, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the body text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, should be found at: <http://www.wjgnet.com/wjg/help/instructions.jsp>.

### Illustrations

Figures should be numbered as 1, 2, 3, etc., and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: <http://www.wjgnet.com/1007-9327/13/4520.pdf>; <http://www.wjgnet.com/1007-9327/13/4554.pdf>; <http://www.wjgnet.com/1007-9327/13/4891.pdf>; <http://www.wjgnet.com/1007-9327/13/4986.pdf>; <http://www.wjgnet.com/1007-9327/13/4498.pdf>. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: ...etc. It is our principle to publish high resolution-figures for the printed and E-versions.

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Data that are not statistically significant should not be noted. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 should be noted (*P* > 0.05 should not be noted). If there are other series of *P* values, <sup>c</sup>*P* < 0.05 and <sup>d</sup>*P* < 0.01 are used. A third series of *P* values can be expressed as <sup>e</sup>*P* < 0.05 and <sup>f</sup>*P* < 0.01. Other notes in tables or under illustrations should be expressed as <sup>1</sup>F, <sup>2</sup>F, <sup>3</sup>F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, □, ▲, △, etc., in a certain sequence.

### Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscripts and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

## REFERENCES

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### Style for journal references

Authors: the name of the first author should be typed in bold-faced letters. The family name of all authors should be typed with the initial letter capitalized, followed by their abbreviated first and middle initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR). The title of the cited article and italicized journal title (journal title should be in its abbreviated form as shown in PubMed), publication date, volume number (in black), start page, and end page [PMID: 11819634 DOI: 10.3748/wjg.13.5396].

### Style for book references

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### Format

#### Journals

English journal article (list all authors and include the PMID where applicable)

- 1 Jung EM, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

- 2 Lin GZ, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixudiarrhoea. *Shijie Huaren Xiaohua Zaishi* 1999; **7**: 285-287

In press

- 3 Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

- 5 Vallancien G, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

Volume with supplement

- 7 Geraud G, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment

of migraine and in comparison with sumatriptan. *Headache* 2002; 42 Suppl 2: S93-99 [PMID: 12028325]

*Issue with no volume*

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (401): 230-238 [PMID: 12151900]

*No volume or issue*

- 9 Outreach: Bringing HIV-positive individuals into care. *HRS A Careaction* 2002; 1-6 [PMID: 12154804]

## Books

*Personal author(s)*

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

*Chapter in a book (list all authors)*

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

*Author(s) and editor(s)*

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

*Conference proceedings*

- 13 **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

*Conference paper*

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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**Patent** (list all authors)

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## Statistical expression

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## Units

Use SI units. For example: body mass,  $m$  (B) = 78 kg; blood pressure,  $p$  (B) = 16.2/12.3 kPa; incubation time,  $t$  (incubation) = 96 h, blood glucose concentration,  $c$  (glucose)  $6.4 \pm 2.1$  mmol/L; blood CEA mass concentration,  $p$  (CEA) = 8.6 24.5  $\mu$ g/L;  $CO_2$  volume fraction, 50 mL/L  $CO_2$ , not 5%  $CO_2$ ; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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## Italics

Quantities:  $t$  time or temperature,  $c$  concentration,  $A$  area,  $l$  length,  $m$  mass,  $V$  volume.

Genotypes:  $gyrA$ ,  $arg\ 1$ ,  $c\ myc$ ,  $c\ fos$ , etc.

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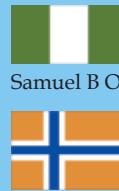
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[1]Passed away on October 20, 2007

[2]Passed away on June 11, 2007



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EDITORIAL

## New insights into calcium, dairy and colon cancer

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increasing dramatically in the rest of the world. In the United States, colorectal cancer is the second most common cause of cancer deaths, with an incidence of around 130 000 cases a year and a mortality rate of approximately 55-60 000<sup>[1]</sup>. The World Health Organization statistics for colorectal cancer incidences worldwide in 1996 showed about 875 000 cases with a mortality of over 500 000. The incidence of this tumor in the less well developed countries of the world is increasing dramatically so that the death rate for this tumor far exceeds the 7.2% of all cancer deaths reported by the WHO. Incidence and mortality rates differ markedly across countries with the highest rates reported from Australia and Northern Great Britain and the lowest rates in Southern Africa. This 30-fold difference in incidence underscores the importance of environmental factors in inducing this cancer.

Although the treatment of established colorectal cancer has improved remarkably over the last half century, mortality rates still are high, particularly in our aging population. Therefore, a major focus of the management of this tumor has been in the area of cancer prevention which is better termed "cancer risk reduction". There are three modalities of cancer prevention; tertiary prevention is when efforts are made to lower the risk of a second cancer once a primary tumor has been diagnosed. A good example of this is the use of tamoxifen for risk reduction of breast cancer in women who have had one breast cancer and the use of retinoid acting agents to lower the risk of second squamous cancers of the aero-digestive tract. Secondary prevention involves the abolition of pre-cancerous neoplastic lesions, thus lowering the risk of the later development of cancer. For the colorectum, secondary prevention by detection of neoplastic colorectal adenomas and adenoma polypectomy has become an established preventive technique and has been demonstrated to be effective in lowering the incidence of this tumor<sup>[2]</sup>. Primary prevention aims to reduce the development of a cancer before tissue pre-neoplastic changes occur. This is the approach where calcium and dairy products appear to have an important role in lowering the risk of colorectal cancer. It must be emphasized that in order to advocate a primary cancer prevention modality which is likely to be applied to large numbers of the population, it must have an excellent benefit to risk ratio. The term "chemoprevention" which is better called "risk reduction" has been applied to these approaches. Chemoprevention involves the use

### Abstract

This paper is to review recent information about the relationship of calcium and dairy foods to colon cancer. The review focuses on primary prevention, discusses the potential components in dairy foods that might be anti-neoplastic, reviews the epidemiologic information and describes intervention studies demonstrating efficacy of calcium and vitamin D in reducing colorectal polyp recurrence. Since vitamin D is important in cancer prevention, pertinent data is discussed and potential mechanisms of actions presented. Calcium and vitamin D are important agents for the primary prevention of colorectal neoplasia.

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**Key words:** Colorectal cancer; Dairy foods; Calcium; Vitamin D; Colorectal polyp recurrence; Colon cancer prevention

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Holt PR. New insights into calcium, dairy and colon cancer. *World J Gastroenterol* 2008; 14(28): 4429-4433 Available from: URL: <http://www.wjgnet.com/1007-9327/14/4429.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4429>

### INTRODUCTION

Colorectal cancer is a common and lethal disease in the Western World and the incidence and mortality is

of an agent to slow the progress, to reverse or inhibit the process of carcinogenesis. Such agents may act at different levels modulating cancer production at the level of the cell, at the molecular level, at the whole tissue level or potentially at the whole patient clinical level.

There are many components of dairy foods that have been shown experimentally to have protective effects against colon cancer. These components include calcium and vitamin D for which there is most evidence and (which will be discussed below), conjugated linoleic acid<sup>[5]</sup>, sphingolipids<sup>[4]</sup> and the potential of butyric acid formed by colonic lactobacilli from milk products. Clearly if one includes bacterial cultures, i.e. probiotics added to dairy products, there is an increasingly important literature that suggests that such agents may be beneficial in reducing the risk of colorectal neoplasia<sup>[5]</sup>.

## EPIDEMIOLOGY

There have been numerous epidemiologic studies that have suggested that calcium or dairy products may lower the risk of colorectal neoplasia. The data for many of these have been reviewed recently<sup>[6]</sup>. An important prospective epidemiologic study was performed in over 45 000 Swedish men, aged 45 to 79 years<sup>[7]</sup>. In this study, calcium intake was determined from food frequency questionnaires and there was a mean follow up of between 6 and 7 years. The data on colorectal cancer incidence, when analyzed for the highest versus the lowest quartile for calcium intake, showed a statistically significant reduction in colon cancer development with a mean odds ratio of 0.68 and for dairy intake a mean odds ratio of 0.46. Using multivariate analysis, the data from this study suggested that there might be a threshold effect at about 1200 mg to 1400 mg of calcium per day<sup>[7]</sup>.

## CALCIUM

At the present time, the gold standard for measuring risk reduction by an intervention in colorectal cancer uses determination of the incidence of recurrence of adenomatous polyps following removal of all colonic polyps by polypectomy. This approach was originally developed by Baron JA (Baron JA, Beach M, Mandel JS, van Stolk RU, Haile RW, Sandler RS, Rothstein R, Summers RW, Snover DC, Beck GJ, Bond JH, Greenberg ER. Calcium supplements for the prevention of colorectal adenomas. Calcium Polyp Prevention Study Group. *N Engl J Med* 1999; 340: 101-107) and his co-workers to analyze the beneficial effects of calcium for adenomatous polyp reduction<sup>[8]</sup>. Subsequent analyses have evaluated reduction of total adenomatous polyps and reduction of advanced polyps as defined by size and the presence of severe dysplasia. Calcium supplementation of 1200 mg per day reduced total adenomas by approximately 20%<sup>[8]</sup>, but advanced adenomas by about 45%<sup>[9]</sup>. If one translates these data into numbers of adenomas that would be reduced

in the United States by increased calcium intake this would total approximately 26 000 cases of adenomas with a more important impact on the advanced lesions. Subsequent analyses by Baron's group showed that most of the effect of calcium in lowering the incidence of recurrent adenomas occurred in individuals who had baseline levels of serum 25 hydroxy vitamin D above the median (about 29 ng per mL) with little effect in individuals with lower levels<sup>[10]</sup>. These data strongly suggest that it is the combination of calcium and vitamin D which is important in altering adenoma recurrence. A prospective US national study is ongoing to examine the relative effects of calcium, vitamin D or the combination of calcium plus vitamin D on colorectal adenoma recurrence. In a further recent publication, Baron JA and coworkers have followed calcium supplemented subjects for ten years after completing the ongoing study<sup>[11]</sup>. These data suggested that the beneficial effect of calcium upon adenoma recurrence persisted for five years even in subjects who were not taking supplemental calcium after stopping the formal study and showed 40% less adenoma recurrence when compared to control placebo treated subjects. After 5 years after no further beneficial effect of calcium administration was demonstrated<sup>[11]</sup>.

The classical hypothesis for the beneficial effects of calcium derived from an original physiochemical hypothesis by Newmark and colleagues in which they suggested that fatty acids and bile acids in the colon may be detrimental to the epithelium and important in the initial steps of colorectal carcinogenesis and that calcium could bring bile acids and fatty acids out of solution in the colonic lumen and, thus, reduce the cytotoxicity of these agents<sup>[12]</sup>. A number of studies by Van der Meer's group subsequently were consistent with this hypothesis even in *in-vivo* studies<sup>[13,14]</sup>.

However, calcium is known to have manifold cellular effects in colonic epithelial cells suggesting that these must be important in the action of this compound upon colorectal carcinogenesis. Two recent areas of research suggest that other mechanisms may well be crucial in the cellular effects of calcium. The human parathyroid calcium sensing receptor which senses minor changes in extra-cellular calcium concentrations is expressed in differentiated cells of the human colonic crypt. The receptor is partially or completely lost during colon carcinogenesis<sup>[15]</sup>. The calcium sensing receptor has two promoters with vitamin D response elements. *In vitro*, calcium and vitamin D stimulate the calcium sensing receptor promoter activity in colonic cells to reduce E-cadherin expression and inhibit TCF4. Thus, this receptor may function to regulate epithelial differentiation and be anticarcinogenic<sup>[15]</sup>. In addition, evidence suggests that the cardiac L-type calcium channel is present in colon tissue and may play a role in determining calcium influx into colonic epithelial cells<sup>[16]</sup>.

Vitamin D stores of the body derive from photolysis of 7 dehydrocholesterol in the skin to form pre-vitamin D<sub>3</sub>, which rapidly isomerizes at body temperature to form vitamin D<sub>3</sub> and then passes into the circulation.

Dietary vitamin D<sub>2</sub> and vitamin D<sub>3</sub> is absorbed from the intestinal lumen in micellar form and after transfer into intestinal lymph passes into the circulation where it is bound to a vitamin D binding protein. Vitamin D from both cutaneous and intestinal sources is taken up by the liver and converted to 25 hydroxy vitamin D (25 OHD<sub>3</sub>). The circulating levels of serum 25 OHD<sub>3</sub> reflect the body stores of this vitamin. 25 OHD<sub>3</sub> is transported to the periphery and converted to calcitriol, (1,25 dihydroxy vitamin D3 1,25 (OH)<sub>2</sub>D<sub>3</sub>), mainly in the kidney but also in many peripheral tissues by the action of the enzyme 1 alpha hydroxylase (CYP 27 B2). 1,25 (OH)<sub>2</sub>D<sub>3</sub> is bound to vitamin D receptors present in many tissues and has pleiomorphic actions in bone, the gastrointestinal tract and uterus, etc.

Epidemiologic studies of vitamin D effects upon human health have included evaluation of effects of sunlight (ultraviolet) exposure, analysis of dietary and supplemental vitamin D intake as well as measurement of serum 25 hydroxy vitamin D levels.

There is abundant epidemiologic data to show that exposure to sunlight results in a reduction in the incidence of many cancers, but most clearly reduced colorectal cancer. The original observations of Cedric Garland<sup>[17]</sup> which followed upon a forgotten report in 1941<sup>[18]</sup> showed a distinct North to South latitude difference in colorectal cancer development. More recently, several studies have shown not only a lowering of colorectal cancer incidence, but also that for breast, ovary and prostate<sup>[19]</sup> accompanied by the expected increased non-melanoma skin cancer formation. Other studies have also pointed to beneficial changes of sunlight in esophageal, renal and bladder cancer as well as non-Hodgkins lymphoma<sup>[20]</sup>.

Many investigators have analyzed the relationship of colorectal cancer prevalence with vitamin D body stores, i.e. measurement of circulating 25 OHD<sub>3</sub>. A metanalysis of vitamin D intake studies using dose response gradient analysis showed a reduction in colorectal cancer of approximately 20%<sup>[21]</sup>. In 2006, Garland analyzed positive and negative studies comparing serum 25 OHD levels and the development of colorectal, breast and prostate cancer. Six of seven studies of colorectal cancers showed a significant reduction of cancer and one was borderline, whereas for breast cancer only one was significant and one showed no effect with similar negative results for prostate cancer<sup>[22]</sup>. Further evidence for the beneficial effect of higher levels of 25 OHD were shown in the distal colon of older women (OR = 0.45)<sup>[23]</sup> and in black men<sup>[24]</sup>, because of lower action of sunlight in pigmented skin to form this vitamin. A prospective 7.75 year study of serum levels of vitamin D showed a 55% reduction in cancer development in the highest compared to the lowest quartile<sup>[25]</sup>.

## MECHANISMS OF ACTION OF VITAMIN D

The cellular activity of vitamin D is dependent on the

action of calcitriol principally through interaction with a high affinity binding protein (VDR). VDR is a member of the steroid receptor superfamily ligand dependent transcription factor and the binding of calcitriol to VDR induces a configurational change in the receptor. The receptor then heterodimerizes with the retinol receptor (RxR) and the VDR-RxR complex binds to vitamin D response elements in the nucleus. This interaction induces gene transcription which results in cell cycle arrest through the regulation of CDK2, p21, p27, p53, KI67 and E-cadherin. In addition there are effects on differentiation and apoptosis, the latter through changes in Bcl-2, Bcl-x<sub>L</sub>, Mcl-1, etc. Furthermore, there are non-receptor dependent actions of vitamin D upon the cell which include activation of calcium channels at least in the small intestine and colon<sup>[26]</sup>.

The clinical effects of vitamin D are also dependent on polymorphisms in the vitamin D receptor. Such polymorphisms can occur at the 3' end of the receptor and includes Bsm1, Taq1 and at the 5' end FoK1. It is known that such polymorphisms are functionally associated with differences in bone density and in serum calcitriol levels, but whether they affect the action of vitamin D upon the colon is unclear<sup>[27]</sup>. However, high dairy intake effects upon colon adenoma recurrence has been restricted to individuals with the Apal aA/AA genotype<sup>[28]</sup>.

One small, but unique study has described the effects of 6 mo supplemental calcium (1200 mg) plus vitamin D (400 IU) to subjects with adenomatous polyps which were transected with one half tattooed and left *in situ*. Calcium plus vitamin D reduced proliferation indices in the remaining polyps and flat mucosa, but also dramatically down-regulated polyp mucin 5AC (MUC5AC)<sup>[29]</sup>. These data suggest that the combination alters important cellular processes both in the adenoma and the flat colorectal mucosa.

Turning to other studies of dairy products upon colon neoplasia formation, Cho *et al* (Cho E, Smith-Warner SA, Spiegelman D, Beeson WL, van den Brandt PA, Colditz GA, Folsom AR, Fraser GE, Freudenheim JL, Giovannucci E, Goldbohm RA, Graham S, Miller AB, Pieninen P, Potter JD, Rohan TE, Terry P, Toniolo P, Virtanen MJ, Willett WC, Wolk A, Wu K, Yau SS, Zeleniuch-Jacquotte A, Hunter DJ. Dairy foods, calcium, and colorectal cancer: a pooled analysis of 10 cohort studies. *J Natl Cancer Inst* 2004; 96: 1015-1022) published pooled data of dairy product intake from 10 cohort studies and reported a 12% reduction in colon cancer risk with each 500 mL increase in milk intake. There also was a significant and 17% reduction in colorectal cancer incidence with the ingestion of ricotta cheese greater than 25 mg per day<sup>[30]</sup>. An important study that has received a large amount of attention was the Women's Health Initiative Dietary modification study (WHI study) in which women 50-79 years of age received supplemental 1100 mg calcium plus 400 IU vitamin D with meals. Some of these women also participated in a study of the effects of estrogen replacement therapy upon colon cancer development.

This prospective study had a mean follow up of  $7.0 \pm 1.4$  years and had colorectal cancer as an end point<sup>[31]</sup>. There was no significant difference between the development of colorectal cancer in women taking the calcium plus vitamin D when compared to controls (OR = 1.08) (0.86-1.34). There clearly were a number of issues related to this study which have resulted in some considerable criticism. Such issues included the fact that the mean age of the women in the study was 62 and the increase in colon cancer in women occurs only after age 60, they had a high basal dietary intake of calcium of approximately 1100 mg to 1200 mg per day and relatively adequate vitamin D intake approaching 400 IU per day. There was low compliance with the intervention with only 70% of subjects consuming more than 50% of the pills and in addition the subjects were permitted to continue to take calcium and/or vitamin D supplements separately from study drugs. It also was felt that the duration of the study was too short for a cancer end point and the amount of vitamin D provided in the intervention was relatively low. Importantly however, women who showed a low serum 25 hydroxy vitamin D level at base line demonstrated a 2.5 fold increased risk of colorectal cancer compared to the top quartile of serum 25 hydroxy vitamin D levels ( $P < 0.02$ )<sup>[31]</sup>.

## VITAMIN D AND CANCER MORTALITY

A unique study by Lappe prospectively evaluated the development of any cancer in approximately 1200 postmenopausal women who received calcium 1500 mg/d with or without vitamin D 1100 IU per day. The unadjusted relative risk for the development of any cancer with calcium administration was 0.53 and for vitamin D plus calcium 0.40. Serum 25 hydroxy vitamin D also was a significant predictor of decreased cancer development<sup>[32]</sup>. A recent meta-analysis of 18 studies of effects of vitamin D on overall mortality by Philippe Autier showed an 8% reduction in overall mortality in subjects who received vitamin D<sup>[33]</sup>.

## ANIMAL STUDIES

Over the past decade Martin Lipkin's group have been evaluating the effects of a Western style diet (WD) relatively high in fat content (20%) and relatively low in vitamin D and calcium upon carcinogenic changes in the colon of mice. This represented the first demonstration of diet-induced colorectal tumor formation in the absence of a carcinogen<sup>[34]</sup>. When folic acid also was reduced in the WD over a period of 18-24 mo, adenomas and carcinomas developed with increases in both the percent of mice developing tumors as well as in tumor frequency. The addition of calcium and vitamin D to the diet dramatically reduced or eliminated most of these tumors<sup>[35]</sup>. It also is of interest that this Western style diet in mice produced hyperproliferation in mammary duct epithelial cells<sup>[36]</sup>, pancreatic epithelial cells<sup>[37]</sup> and prostate cells<sup>[38]</sup> and that calcium and vitamin D suppressed such hyperproliferation. The molecular

underpinnings of these observations are presently under study.

## CONCLUSION

Epidemiologic intake and intervention studies have shown that calcium administration lowers colorectal adenomatous polyps as well as cancer rates and this effect may be prolonged. Most evidence suggests that the effect of calcium is dependent or partially related to simultaneous vitamin D intake. Vitamin D may also reduce colon cancer risk independent of the presence of increased amounts of calcium or dairy products in the diet. Few studies have been performed with dairy products alone, but the data generally supports the positive effects shown with calcium and vitamin supplementation as well. Understanding the cellular effects of calcium and vitamin D in humans' *in-vivo* is crucial to make further progress in this field.

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EDITORIAL

## Molecular basis of the potential of mesalazine to prevent colorectal cancer

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### INTRODUCTION

Patients with ulcerative colitis (UC) and Crohn's disease (CD), the major forms of inflammatory bowel disease (IBD) in humans, are at increased risk of developing colorectal cancer (CRC)<sup>[1,2]</sup>. Chronic inflammation is believed to be the driving force for neoplastic transformation, and clinical factors that increase the risk include disease duration > 10 years, extensive disease, severity of colitis, a positive family history of sporadic CRC, and the concomitant presence of primary sclerosing cholangitis<sup>[3-5]</sup>.

While in sporadic CRC, the dysplastic precursor is usually the adenomatous polyp, dysplasia in IBD patients can be both polypoid and flat, localized or diffuse. Detection of dysplasia during programmed screening and surveillance colonoscopy is the goal of the current strategy for CRC prevention in IBD patients. When dysplasia or CRC is identified, proctocolectomy is performed to remove the at-risk organ<sup>[6]</sup>. However, no evidence exists that screening for colonic dysplasia and cancer with surveillance colonoscopy prolongs survival in IBD patients. Indirect evidence also suggests that this is a cost-effective approach<sup>[7,8]</sup>. Therefore, gastroenterologists have recently shifted their attention towards alternative strategies of chemoprevention, and particular interest has been given to the possibility of reducing the risk of IBD-related CRC by using mesalazine or 5-aminosalicylic acid (5-ASA). Mesalazine is widely used in the maintenance of remission and in the treatment of mild flare-ups of IBD, and recent epidemiological studies have suggested that this drug is chemopreventive for CRC development in UC patients<sup>[9-11]</sup>, even though some studies have documented no benefit<sup>[12]</sup>. The mechanisms behind the antineoplastic effect of mesalazine are incompletely understood, but it is likely that they are mostly dependent on the ability of the drug to attenuate ongoing mucosal inflammation. Indeed, it is well known that mesalazine can modulate various inflammatory pathways (e.g. production of inflammatory cytokines, activity of inducible nitric oxide synthase, activation of nuclear factor- $\kappa$ B) that are

### Abstract

Patients with ulcerative colitis (UC) and Crohn's disease (CD) are at increased risk for developing colorectal cancer (CRC), and this is believed to be a result of chronic inflammation. Although conclusive evidence is still missing, both epidemiological and experimental observations suggest that certain drugs used to treat inflammation, such as mesalazine, can reduce the incidence of colitis-associated CRC. Therefore, in recent years, several studies have been conducted to dissect the mechanisms by which mesalazine interferes with CRC cell growth and survival. This review summarizes the current information on the molecular mechanisms that underlie the antineoplastic action of mesalazine.

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**Key words:** Chemoprevention; Colorectal cancer; Cyclooxygenase-2; Epidermal growth factor receptor; Inflammatory bowel disease; Mesalazine; Wnt/ $\beta$ -catenin

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relevant to CRC initiation and progression<sup>[13-16]</sup>. There is also evidence that mesalazine inhibits the formation of reactive oxygen species (ROS) from polymorphonuclear leukocytes<sup>[17]</sup>, which leads to a decrease or complete inhibition of DNA damage, a phenomenon that has been involved in colon carcinogenesis. More recently, it has also been shown that mesalazine can directly target CRC cells and interfere with biological pathways that control their growth and survival<sup>[18]</sup>. The object of this article is to summarize recent data that elucidate the basic mechanisms of the antineoplastic effect of mesalazine.

## MESALAZINE HAS DIRECT INHIBITORY EFFECTS ON CRC CELL GROWTH AND SURVIVAL

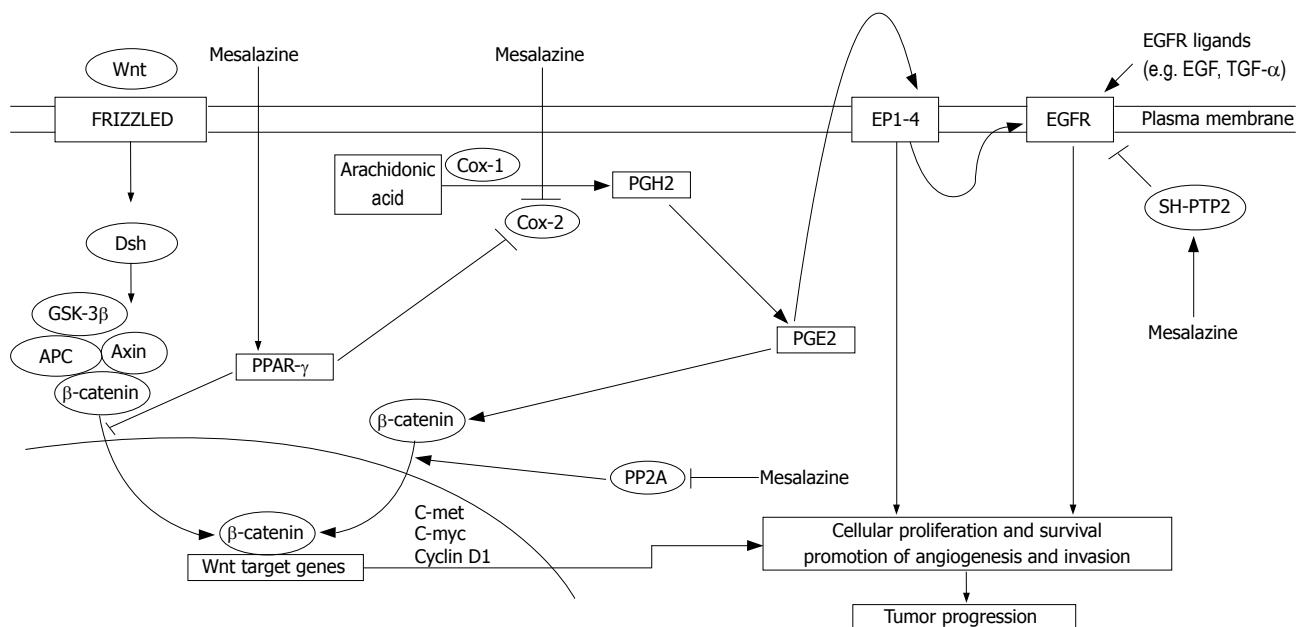
Carcinogenesis is a complex and multistage process that involves interactions between genes and environmental insults which ultimately affect cell proliferation and apoptosis. Apoptosis progressively decreases and proliferation increases in the sequential stages from normal colonic mucosa to dysplastic and CRC tissue. Therefore, strategies that inhibit cell proliferation and/or restore cell susceptibility to apoptosis have been shown to be effective in interfering with CRC initiation and/or progression.

Accumulating evidence indicates that mesalazine can block growth and promote apoptosis of CRC cells. This was initially suggested by *ex vivo* studies in patients with colonic adenoma. In particular, Reinacker-Schick *et al* have analyzed the effect of orally administered mesalazine on apoptosis and proliferation of colorectal mucosa in 21 patients with sporadic polyps. An increase in the apoptotic rate and decrease in cell proliferation were seen 1 and 3 d, respectively, after the initiation of treatment with mesalazine<sup>[19]</sup>. Bus *et al* have demonstrated that 2 wk treatment with 4 g/d mesalazine enema in patient with sporadic CRC resulted in enhanced apoptosis of tumor cells, while no change was seen in the normal mucosa that surrounded the tumor lesion. Moreover, the cellular proliferation rate as assessed by means of Ki-67 expression was unchanged in both the tumor and normal tissue<sup>[20]</sup>. Studies in rodent models of CRC showed that mesalazine inhibits tumor growth and reduces the number of aberrant cryptic foci<sup>[21,22]</sup>. Moreover, in a mouse model of colitis-associated CRC, Ikeda *et al* have shown that mesalazine, given in the remission stage of colitis, markedly suppresses the number and size of neoplasms. Notably, mesalazine treatment reduces the rate of proliferation of tumor cells, which leaves the proliferation of normal epithelial cells unaltered<sup>[23]</sup>. These observations have been reinforced by *in vitro* studies that show that mesalazine inhibits the growth and enhances apoptosis of several cultured CRC cell lines, in a time- and dose-dependent manner<sup>[18,24]</sup>. Altogether, these later findings

indicate that mesalazine has direct effects on CRC cells. This novel information has boosted new research aimed at dissecting the molecular mechanisms by which mesalazine interferes with CRC development/growth.

## EFFECTS OF MESALAZINE ON REPLICATION FIDELITY

Many of the molecular alterations that are believed to play a major role in the development of sporadic CRC are also seen in IBD-associated CRC tissue. For instance, both these types of CRC are characterized by a very similar frequency of the two main types of genomic instability, namely chromosomal instability (CIN, 85%) and microsatellite instability (MSI, 15%)<sup>[25]</sup>. CIN results in abnormal segregation of chromosomes and abnormal DNA content (aneuploidy). As a result, loss of chromosomal material often occurs, which contributes to the loss of function of important tumor suppressor genes [e.g. adenomatous polyposis coli (*APC*) and *p53*]. The MSI pathway involves the primary loss of function of genes that usually repair DNA base-pair mismatches that occur during the normal process of DNA replication in dividing cells. During this process, frameshift mutations, called microsatellites, tend to accumulate. Since microsatellites are mainly located in intronic DNA sequences, microsatellite mutations generally result in no gene function alteration. However, if microsatellites are located in exonic gene regions, the mutations can lead to a shift in the codon reading frame and changes in the amino acid sequence during mRNA translation. The introduction of an early stop codon can eventually cause protein truncation, and this is generally associated with a loss of protein function<sup>[26]</sup>. Recently, Gasche and colleagues, using an assay based on a stable transfection of a plasmid carrying a microsatellite sequence into HCT-116 cells, have shown that mesalazine improves replication fidelity in cultured CRC cell lines by reducing frameshift mutations at microsatellites. Since the effect of mesalazine is seen in mismatch repair-deficient CRC cells, it is highly likely that mesalazine acts on replication fidelity independently of post-replication mismatch repair. The molecular mechanism by which mesalazine inhibits the generation of frameshift mutations has not yet been characterized. However, studies by the same group have shown that mesalazine can interact with cellular machinery involved in cell-cycle progression<sup>[28]</sup>. In particular, it has been shown that mesalazine can slow down DNA replication and cell division, thus allowing cells to either repair DNA damage or undergo apoptosis. Analysis of cell-cycle phases has revealed that CRC cells are arrested in S-phase when treated with mesalazine for 24-48 h. Such results are, however, somewhat different from those published by Reinacher-Schick *et al* that have shown that CRC cells are arrested in G2-M phase when exposed to mesalazine<sup>[18]</sup>. The



**Figure 1** Some putative molecular mechanisms that underlie the antineoplastic activity of mesalazine. Mesalazine inhibits COX-2, thereby blocking synthesis of PGH2, an intermediate that is metabolized into various prostaglandins, including PGE2. PGE2 binds to its cell surface cognate receptors (EP1-4) and sustains various functions of tumor cells, including proliferation, survival, angiogenesis and invasion. These are mediated through the transactivation of EGFR or activation of other intracellular pathways. The antineoplastic effects of mesalazine rely also on its ability to inhibit Wnt/β-catenin, through inactivation of PP2A (and downstream down-regulation of β-catenin), and EGFR pathways. Moreover, mesalazine activates PPAR-γ, thus leading to inactivation of the Wnt/β-catenin pathway and down-regulation of COX-2.

reasons for these discrepancies remain unclear, but it is likely that they are due to differences in the culture systems used by these investigators.

### THE ANTI-MITOGENIC EFFECT OF MESALAZINE IS NOT STRICTLY DEPENDENT ON CYCLOOXYGENASE (COX)-2 INHIBITION

COX-2 is a major target of CRC chemopreventive programs, as it is highly expressed in both sporadic and familial CRC, and activation of COX-2 is known to trigger and/or amplify biological pathways that sustain CRC growth<sup>[29-32]</sup>. COX-2 is also over-expressed in IBD-related CRC tissue<sup>[33]</sup>. Since mesalazine inhibits COX-2 in inflammatory cells, it is hypothesized that the antineoplastic effect of this drug is strictly dependent on the inhibition of COX-2 in CRC cells. In this context, we have recently shown that mesalazine inhibits the growth of HT-115, a CRC cell line that expresses a functionally active COX-2, and that the anti-mitogenic effect of mesalazine is associated with a marked down-regulation of COX-2 at the protein and mRNA level<sup>[34]</sup>. Consistently, treatment of HT-115 cells with mesalazine causes a significant reduction in secretion of prostaglandin (PG) E2, a product of COX-2 activity that positively regulates CRC cell growth (Figure 1). However, exogenously added PGE2 does not abrogate the inhibitory effect of mesalazine on HT-115 cell growth. Moreover, mesalazine blocks the growth of DLD-1, a CRC cell line that does not express COX-2,

which suggests that the effect of this drug on CRC cell growth is partially independent of inhibition of the COX-2/PGE2 axis.

### MESALAZINE INHIBITS BOTH THE WNT/β-CATENIN AND EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) SIGNALING PATHWAYS IN CRC CELLS

An intriguing hypothesis that has emerged from the available experimental data is that mesalazine may act on one or more pathways that are both early and common in colorectal carcinogenesis. A potential candidate is the wingless and integration site growth factor (Wnt)/β-catenin pathway, since it is constitutively activated in the majority of CRC<sup>[35-37]</sup>. In this pathway, Wnt binds to the transmembrane Frizzled receptor, which leads to activation of the cytoplasmic disheveled (Dsh) protein. Dsh forms a complex with the β-catenin degradation complex, which consists of the APC gene product, glycogen synthase kinase-3β (GSK-3β), axin and β-catenin. The current model for the Wnt signaling pathway proposes that, in the absence of Wnt, GSK-3β phosphorylates β-catenin, thereby promoting ubiquitination and degradation of β-catenin<sup>[38]</sup>. In response to Wnt signals, β-catenin is no longer targeted for degradation and accumulates to high levels in the cytoplasm<sup>[39]</sup>. The accumulated β-catenin translocates to the nucleus, associates with the transcriptional enhancers of the lymphoid enhancer-binding factor/Tcf family, and stimulates the expression of genes, such as *Myc*, that

play important roles in tumor progression<sup>[40,41]</sup>.

Elegant studies by Bos *et al* have shown that mesalazine inhibits the Wnt/β-catenin pathway in APC-mutated CRC cells with intact β-catenin (Figure 1)<sup>[42]</sup>. Consistent with this, mesalazine treatment reduces expression of nuclear β-catenin and Wnt/β-catenin target genes (e.g. *cyclin D1*, *c-met* and *c-Myc*), and increased β-catenin phosphorylation. Mesalazine fails to inhibit the expression of Wnt/β-catenin target genes in β-catenin mutant CRC cell lines, in which the Wnt/β-catenin pathway is not regulated by β-catenin phosphorylation. These observations suggest that inhibition of the Wnt/β-catenin pathway by mesalazine is dependent on increased phosphorylation of β-catenin. In line with this, pre-incubation of CRC cells with okadaic acid, a specific phospho-serine/phospho-threonine phosphatase inhibitor, prevents mesalazine-induced β-catenin phosphorylation. The precise mechanism by which mesalazine enhances β-catenin phosphorylation remains to be ascertained, even though Bos *et al* have demonstrated that mesalazine reduces the activity of protein phosphatase 2A (PP2A), a known regulator of the β-catenin phosphorylation status and Wnt/β-catenin pathway in CRC cells.

Another target of mesalazine is EGFR (Figure 1). This receptor is highly activated in CRC cells, in which it is supposed to trigger mitogenic and pro-survival signals<sup>[43,44]</sup>. Consistent, EGFR inhibitors, such as monoclonal antibodies or small molecules that inhibit tyrosine kinase activity, have been shown to be effective in patients with advanced CRC<sup>[45]</sup>. A very high percentage of IBD-associated CRC displays immunohistochemical positivity for EGFR. Expression of EGFR is frequent in IBD-associated intestinal cancer<sup>[46]</sup>. Expression occurs at an early stage (i.e. premalignant lesions), as described in sporadic CRC<sup>[47]</sup>, which suggests that blocking EGFR activity is useful for reducing the occurrence of IBD-related CRC<sup>[48,49]</sup>. We have shown that exposure of CRC cell lines to mesalazine results in a marked suppression of EGFR phosphorylation/activation, and that mesalazine-induced EGFR dephosphorylation is dependent on neither CRC cell death induction nor shedding of the receptor<sup>[50]</sup>. Moreover, mesalazine suppresses EGFR activation induced by exogenous EGF or TGF-α, thus excluding the possibility that dephosphorylation of EGFR in mesalazine-treated cells is secondary to inhibition of EGFR ligand synthesis. EGFR phosphorylation is a tightly controlled phenomenon, which is the net result of the action of tyrosine kinase and phosphatases (PTPs). Therefore, we have examined whether mesalazine-mediated inhibition of EGFR activation reflects changes in the expression/activity of PTPs, which have been reported to control the extent of EGFR activation<sup>[51]</sup>. Notably, treatment of CRC cells with mesalazine causes a significant increase in the activity, but not expression, of phosphorylated-EGFR-targeting PTPs, and pre-incubation of cells with PTP inhibitors largely reduces the inhibitory effect of mesalazine on EGFR activation. Among these PTPs, both SH-PTP1 and SH-PTP2 interact with EGFR upon

mesalazine treatment. However, targeted silencing of SH-PTP2, but not SH-PTP1 prevents mesalazine-induced EGFR dephosphorylation. Consistent with these data, mesalazine also attenuates EGFR phosphorylation in *ex vivo* organ cultures of human sporadic CRC explants.

## MESALAZINE ACTIVATES PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR-γ (PPAR-γ) IN CRC CELLS

PPAR-γ is a nuclear receptor that is highly expressed in the colon, and plays a key role in bacteria-induced inflammation. Many factors can modulate the activity of PPAR-γ, but the most important activating factor in colon epithelial cells appears to be the luminal flora<sup>[52]</sup>. Activation of PPAR-γ also has anti-tumorigenic effects that are manifested as both anti-proliferative and pro-apoptotic activities<sup>[53, 54]</sup>, inhibition of the formation of aberrant cryptic foci<sup>[55]</sup>, and inhibition of CRC development<sup>[56]</sup>. It has also been shown that PPAR-γ suppresses tumor formation by interfering with the Wnt/β-catenin signaling pathway<sup>[57,58]</sup>. Recent *in vitro* and *in vivo* studies have shown that mesalazine can activate PPAR-γ (Figure 1). In particular, using HT-29 CRC cells, Rousseaux *et al* have shown that mesalazine enhances PPAR-γ expression, stimulates translocation of PPAR-γ to the nucleus, induces conformational changes in the PPAR-γ molecule, and increases the interaction between PPAR-γ and vitamin D3 receptor-interacting protein 205<sup>[59]</sup>. In competitive binding studies, mesalazine displaces rosiglitazone and the selective PPAR-γ ligand GW1929 from their binding sites on the PPAR-γ molecule<sup>[59]</sup>. In line with these *in vitro* findings, it has been shown that the antineoplastic effects of mesalazine are mediated by PPAR-γ in a model of CRC, in which SCID mice were engrafted with human CRC cells. In particular, in this model, locally administered mesalazine significantly reduced the growth of xenografts, and this effect was blocked by the selective PPAR-γ antagonist GW9662<sup>[60]</sup>.

## CONCLUSION

In recent years, there has been great interest in the possibility of chemoprevention of IBD-related CRC by mesalazine. Given the difficulty of performing double-blind, placebo-controlled, randomized clinical trials in patients, investigators have turned to experimental models of cancer, and indeed, the existing data suggest that mesalazine can reduce the risk of CRC by directly interfering with CRC cell biology, other than by simply controlling inflammation. However, definitive conclusions from experimental findings are not always completely correct, and therefore, future studies will be necessary to ascertain whether data generated from studies with cultured cells or animal models of CRC can be generalized to IBD-associated dysplasia or CRC.

Another important issue that needs further investigation regards the dosage/concentration of

mesalazine required to interfere with CRC cell growth and survival. *In vitro* studies have indicated that the antineoplastic effect of mesalazine is seen with relative high drug dosages (e.g. 10-50 mmol/L), which are not always reached within the colonic tissue under standard oral treatment. In this context, it is also relevant to take into consideration mesalazine metabolism, for instance, mesalazine oxidation and acetylation, which could differ considerably between *in vitro* and *in vivo* circumstances, and limit the amount of biologically active compound.

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EDITORIAL

## Thrombosis and inflammatory bowel disease—the role of genetic risk factors

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### Abstract

Thromboembolism is a significant cause of morbidity and mortality in patients with inflammatory bowel disease (IBD). Recent data suggest thromboembolism as a disease-specific extraintestinal manifestation of IBD, which is developed as the result of multiple interactions between acquired and genetic risk factors. There is evidence indicating an imbalance of procoagulant, anticoagulant and fibrinolytic factors predisposing in thrombosis in patients with IBD. The genetic factors that have been suggested to interfere in the thrombotic manifestations of IBD include factor V Leiden, factor II (prothrombin, G20210A), methylenetetrahydrofolate reductase gene mutation (MTHFR, 677T), plasminogen activator inhibitor type 1 (PAI-1) gene mutation and factor X III (val34leu). In this article we review the current data and future prospects on the role of genetic risk factors in the development of thromboembolism in IBD.

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### INTRODUCTION

Inflammatory bowel disease (IBD) is associated with an increased risk of vascular complications<sup>[1-4]</sup>. The most important of these complications are arterial and venous thromboembolisms, which represent a significant cause of morbidity and mortality in IBD patients. Thromboembolism is a disease-specific extraintestinal manifestation of IBD<sup>[5]</sup>, which is developed as the result of multiple interactions between acquired and genetic risk factors. Several studies have shown an imbalance of procoagulant, anticoagulant and fibrinolytic factors predisposing in thrombosis in patients with IBD<sup>[6-9]</sup>. The incidence of thromboembolism in IBD ranges between 1% and 7.7% in clinical studies<sup>[1,10,11]</sup>, rising to 39%-41% in postmortem studies<sup>[12]</sup>.

The most common thrombotic manifestations in IBD are deep vein thrombosis, usually in the leg, and pulmonary embolism. The latter may be fatal. Thrombotic events occur less frequently in other sites, such as the cerebrovascular system, portal vein, mesenteric veins, and retinal vein. Arterial thrombotic complications occur less frequently than venous thromboembolism in patients with IBD, and most occur after surgery. An increased atherosclerotic risk in patients with IBD has also been suggested<sup>[13]</sup>. This could be associated with the elevated levels of C-reactive protein and plasma sCD40L, which are features of the IBD<sup>[14,15]</sup>. Common carotid artery intima-media thickness has been found significantly higher in IBD patients compared with healthy controls<sup>[16]</sup>. Premature atherosclerosis with lower extremity arterial occlusions has been reported in young patients with Crohn's disease<sup>[17]</sup>. An increased risk of cardiovascular arterial thromboembolic diseases in both Crohn's disease and ulcerative colitis and an increase in cerebrovascular arterial thromboembolic diseases in Crohn's disease recently have been reported<sup>[18]</sup>. IBD patients have been found to develop thromboembolisms earlier in life than non-IBD thrombotic patients<sup>[19]</sup>. Furthermore, many IBD patients with thromboembolic disorders either have active disease or have undergone recent major abdominal surgery<sup>[2]</sup>. Conversely, thromboembolisms may also occur in quiescent IBD<sup>[1]</sup>. It has been suggested that the extent of colonic disease is correlated with thromboembolic risk. Extensive ulcerative colitis and colonic involvement of Crohn's disease was significantly associated with the development of thromboembolism<sup>[2]</sup>. The risk of recurrence of thromboembolism in IBD patients has been

reported to be 10%-13% despite medical therapy for the thromboembolic event<sup>[2]</sup>. The mortality from this complication in IBD has been reported to be 8%-25% during the acute episode<sup>[1,2]</sup>. The etiology of thrombosis in IBD is multifactorial. Thrombosis is a complex event in which several mechanisms and causal factors, inherited and acquired, are implicated, complicating the identification of its causes<sup>[20,21]</sup>. Several acquired prothrombotic risk factors are frequently observed, such as the inflammatory process per se, prolonged immobilization, use of corticosteroids, surgical treatment, fluid depletion, central venous catheters, hyperhomocysteinemia, vitamin deficiencies, smoking and use of oral contraceptives<sup>[4]</sup>. Importantly, approximately half of the patients with inflammatory bowel disease that develop a thromboembolic event have no identifiable risk factor<sup>[10]</sup>. Genetic factors may also play a role in thrombosis of patients with IBD. Based on the unraveling of the biochemistry and cell biology of the coagulation system, major advances in the understanding of genetics of thrombosis have been applied also in IBD complicating by thrombosis. The most common genetic variants that have been found to affect the risk of thrombosis are: factor V Leiden, factor II (prothrombin, G20210A), methylenetetrahydrofolate reductase gene mutation (MTHFR, 6777T), plasminogen activator inhibitor type 1 (PAI-1) gene mutation and factor XIII (val34leu). This review focuses on the role of genetic risk factors in the development of thromboembolism in IBD.

## EVALUATING GENETIC RISK OF THROMBOSIS IN INFLAMMATORY BOWEL DISEASE

Genetic risk factors have been suggested to predispose to thromboembolism in IBD. Several genetic markers located in coagulation genes have been examined in an attempt to document whether these markers are associated with increased thrombotic risk in IBD. Genetic studies of vascular complications of IBD pose enormous challenges, including resolution of immense genotypic and phenotypic heterogeneity, and gene-environment and gene-gene interactions. Past efforts to identify thrombosis-related genes in IBD have utilized population-based association methods, but the substantial progress that has been made recently with the strategy of using positional cloning of genes based on linkage studies is expected to apply to this field. These methods focus on measuring quantitative traits that are correlated with the risk of disease.

### **Factor V Leiden**

Factor V Leiden (FVL), an arginine to glutamine missense mutation in the factor V (FV) gene at position 506<sup>[22]</sup>, is the most prominent risk factor for venous thromboembolism<sup>[23,24]</sup>. The amino acid substitution in the activated protein C (APC) cleavage site of FV leads to increased thrombin generation due to decreased APC-mediated inactivation of FV, and due to decreased FV cofactor activity for FVIIIa inactivation<sup>[25]</sup>. Factor V Leiden is found in approximately 5% of Caucasians, and

increases the risk of thrombosis five- to eightfold for heterozygous carriers and 50- to 80-fold for homozygous carriers. The prevalence of FVL ranges from 20% to 30% in unselected patients with venous thrombosis. Genetic studies determining the prevalence of the FVL allele in IBD mostly have shown no difference in allele frequency between IBD patients and healthy controls<sup>[26-30]</sup>. The prevalence of FVL in thrombotic IBD patients was significantly higher than in IBD patients without thrombosis<sup>[31-33]</sup>. It is noteworthy that the FVL allele was associated mainly with venous thrombosis<sup>[33]</sup>. In addition, the prevalence of FVL in IBD patients with previous thromboembolism appears not to differ from that found in non-IBD patients with thromboembolism<sup>[33,34]</sup>. On the other hand, an Italian study showed a very low prevalence of FVL allele in thrombotic IBD patients<sup>[35]</sup>. The discrepancies between these results may be due to the different characteristics of the populations studied (genetic background, previous history of thrombosis, and small sample sizes). Furthermore, in a recent study of experimental colitis, the FVL allele had no effect in murine colitis and thus, the authors question the role of activated blood coagulation in IBD<sup>[36]</sup>. Although somewhat conflicting, these genetic studies suggest that the FVL as a risk factor for thrombosis in IBD patients matches that of the general population. Furthermore, FVL is not associated with IBD per se, but when present it increases the risk of thromboembolism<sup>[37]</sup>. Finally, it is important to realize that homozygous carriers of the FVL allele are rare and that all genetic studies are performed using heterozygotes, which have a milder thrombotic phenotype.

### **G20210A prothrombin gene mutation**

The G20210A mutation is a genetic variation of the prothrombin (Factor II) gene consisting of a single nucleotide change (guanine to adenine) at position 20210 of the 3'-untranslated region. The G20210A mutation is the second most frequent genetic prothrombotic mutation after FVL. It is present in approximately 2% of Caucasians, leads to greater prothrombin plasma levels (heterozygous carriers have about 30% higher PT levels than healthy controls), and increases the risk of venous thrombosis about threefold<sup>[38]</sup>. However, no definite association between this gene mutation and IBD has been detected in several studies<sup>[30,33-35,39]</sup>. Presence of this mutation is found at a similar prevalence in IBD patients as well as in IBD patients with thrombosis<sup>[27,32-33]</sup>. This mutation has only been studied in a limited number of thrombotic IBD patients so firm conclusions cannot be drawn.

### **Methylenetetrahydrofolate reductase C677T gene mutation**

Methylenetetrahydrofolate reductase is a critical enzyme involved in the remethylation pathway of homocysteine metabolism. A common mutation (C677T) has been identified in the MTHFR gene. This variant leads to 10%-20% increases in homocysteine plasma levels in homozygous carriers, which are found in around 10% of

the population. The effect of MTHFR 6777T carriership on the risk of thrombosis varies among studies, and a recent meta-analysis found a weak effect (10%-20% risk increase)<sup>[40-43]</sup>. Studies of the prevalence of C677T homozygosity in IBD have found discordant results<sup>[34]</sup>, probably because of regional and ethnic variations in the prevalence of polymorphism in the general population. In a recent population-based case-control study<sup>[50]</sup>, although some differences were observed among patients with IBD and healthy controls in the prevalence of MTHFR 6777T (decrease in mutant allele carriership in UC), these did not explain an excess risk of thrombosis. The prevalence of C677T homozygosity between IBD thrombotic patients and non-IBD thrombotic patients showed no significant difference<sup>[32,33]</sup>.

### **Factor X<sub>III</sub> gene mutation**

A common variant in subunit A of factor X<sub>III</sub>, usually indicated by the amino acid position and change (val34leu), is associated with a greater FXIII activation rate and leads to a 20%-40% reduction of the risk of venous thrombosis for homozygous carriers. These are found in approximately 10% of the population<sup>[44,45]</sup>. This variant, which is protective against thrombosis, has been evaluated in IBD patients<sup>[46]</sup>. Available data suggest that the prevalence of this polymorphism is similar in patients with IBD compared to the general population<sup>[47,48]</sup>. A slightly greater prevalence of factor X<sub>III</sub> mutation carriership in CD has been found in a recent population-based study<sup>[30]</sup>, but this could not explain the greater risk of venous thrombosis in CD. Finally, the prevalence of X<sub>III</sub> (val34leu) was similar in IBD patients with vascular complications and non-IBD thrombotic patients<sup>[33]</sup>.

### **Plasminogen activator inhibitor type 1 gene mutation**

Plasminogen activator inhibitor type 1 (PAI-1) is considered as inhibitor of fibrinolysis. The 4G/4G genotype is associated with an overexpression of PAI-1, which may cause a decreased fibrinolysis and, therefore, a hypercoagulability state contributing to the development of vascular complications. Several studies have demonstrated that the 4G/4G genotype is associated with an enhanced PAI-1 expression<sup>[49]</sup> and contributes as an additional risk factor to the development of myocardial infarction<sup>[50]</sup>, arterial thrombosis<sup>[51]</sup>, and deep venous thrombosis<sup>[52]</sup>. However, the evidence regarding the relationship between an elevated PAI-1 plasma level or PAI-1 genetic polymorphism and the risk of venous thromboembolism is rather conflicting. The allelic frequency of PAI-1 4G has been reported higher in IBD patients than in the reference population<sup>[53]</sup>. Moreover, a recent study showed a significantly higher allelic frequency of PAI-1 4G in IBD patients with vascular complications compared with IBD and healthy controls. However, the prevalence of this genotype does not differ in thrombotic IBD patients compared to non-IBD thrombotic patients<sup>[33]</sup>.

### **Janus kinase 2 gene mutation**

Janus kinase 2 (JAK2) mutations have been described in

several Philadelphia-negative myeloproliferative disorders (MPD)<sup>[54]</sup>. The point mutation in JAK2 encodes a valine to phenylalanine change at position 617 (JAK2 V617F) and confers constitutive tyrosine kinase activity<sup>[55]</sup>. It has been suggested that thrombosis in MPD may be due to JAK2 mutation. JAK2 is also important in vascular diseases, such as atherosclerosis, in which inflammation plays an important role. JAK2 V617F mutation has been found, in the absence of overt MPD, highly associated with splanchnic vein thrombosis and sporadically with cerebral thrombosis. A recent study investigated the role of JAK2 V617F mutation in 48 IBD patients with thrombotic complications, but no case with the JAK2 V617F mutation was found<sup>[56]</sup>. The small number of cases with splanchnic vein thrombosis in this series (but also in other IBD series) that are mainly associated JAK2 V617F mutation could be also an explanation of this finding.

### **Other genetic factors**

The role of the well recognized inherited thrombophilic states such as deficiencies of plasma antithrombin III, protein C, and protein S has been examined in several studies. Although deficiency of the proteins C and S in IBD patients have been proposed by some studies<sup>[20]</sup>, other studies failed to confirm these data<sup>[21]</sup>. These factors are rare (< 1% of the population) and are considered to play a less important role in the thrombosis, and therefore it is not surprising that their results in IBD are rather contradictory.

### **Future prospects**

We are entering a new era in genetic studies of venous thrombosis. It is believed that, combined, all the known mutations account for about the half of the genetic thrombosis risk. There is every reason to believe that additional genetic causes of thrombosis remain to be discovered. As a complex disease, thrombosis is considered to be the result of flexible combinations of variations of multiple genes that interact with lifestyle or other environmental factors to produce the disease. Future studies should investigate these interactions. Moreover, in IBD we need collection of large case-control series of patients complicating with venous thrombosis. This will provide the high quality clinical information related both to IBD and thrombosis that forms the basis of any genetic project. On the other hand, large-scale DNA analysis systems are now becoming available, which will allow us to study in the setting of IBD the genetics of venous thrombosis down to the single nucleotide level. As an example, we recently have reported a multigenetic analysis of polymorphisms of thrombophilic and vasoactive genes in a group of IBD patients with vascular complications compared with IBD patients without vascular complications and both thrombotic and healthy controls<sup>[33]</sup>. This approach, in a multicenter basis with a large number of patients, will give more insight into the genetic architecture of thrombotic risk in IBD. The final aim is personalized thrombosis prediction and appropriate management in a patient with IBD.

## CONCLUSION

Epidemiological data suggest that IBD is associated with an increased risk of thromboembolic complications. However, the cause for this strong association remains unclear. It can be speculated that gene mutations may underlie the greater risk for thromboembolic complications in IBD patients. Thus, several studies have investigated the role of genetic defects in the development of vascular complications in IBD. The most common genetic variants that affect the risk of thrombosis are factor V Leiden, factor II (prothrombin, G20210A), MTHFR (6777T) and factor XIII (val34leu). Furthermore, the role of other thrombophilic and vasoactive genes has been evaluated with the occurrence of thromboembolism in IBD. The available data in the literature have shown that genetic risk factors are generally not found more often in IBD patients than others. However, when they occur, those with IBD compared to healthy controls are more likely to suffer thromboembolic complications. The screening for genetic coagulation defects appears justified in all IBD patients with a history of thrombosis or a family history of venous thromboembolic events. Future multicentre studies with large number of cases and further investigation of the interaction between genetic and environmental factors might increase our understanding of the mechanisms of pathogenesis of thrombotic complications in IBD. Using the new large-scale DNA sequencing techniques that are becoming available and enable many genes to be studied in a single individual, we could expect an in-depth insight into how genetic risk factors are involved in thrombosis in IBD.

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TOPIC HIGHLIGHT

Harry HX Xia, PhD, MD, Series Editor

## Hepatocellular carcinoma: Defining the place of surgery in an era of organ shortage

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### INTRODUCTION

Liver resection (LR) has been the only potentially curative treatment available for hepatocellular carcinoma (HCC). With the advent of liver transplantation (LT) as a clinical modality patients with HCC were preferentially placed on the waiting list for transplantation<sup>[1]</sup>. To date, there have been no randomised clinical trials comparing LR to LT in patients with potentially resectable HCC (Child-Pugh A, wedged hepatic venous pressure < 10 mmHg)<sup>[2]</sup>. The decision of whether to resect or transplant is often determined by centre experience and the availability of LT. Unfortunately, the majority of patients have extensive tumours at presentation and are not candidates for either LT or LR. The use of neoadjuvant therapies to downstage the disease has recently been shown to be effective in a small number of selected cases. The results of newer tumour specific therapies that are currently being developed are awaited and may expand the number of potentially resectable cases. If livers for LT were freely available, a case could be made for transplanting all patients with HCC confined to the liver. However, there are insufficient numbers of grafts to transplant even good risk candidates. Determining which patients should be managed by resection or transplantation remains a subject of much debate.

### LIVER RESECTION

For the majority of patients with HCC, eligibility for LR is not only dependant upon anatomical location, but also on the extent of the underlying liver disease<sup>[3]</sup>. Consequently, only 15%-30% of patients with HCC are candidates for LR at the time of presentation<sup>[4]</sup>. Although the advantages of LR over LT are not clear-cut, patients with well-preserved liver function (Child-Pugh A) with small solitary (< 5 cm) HCCs should be considered for LR. The presence of hepatic decompensation (Child-Pugh C) is a contraindication to surgical LR, due to the high peri-operative mortality. The more difficult patients

### Abstract

Liver resection (LR) and transplantation offer the only potential chance of cure for patients with hepatocellular carcinoma (HCC). Historically, all patients were treated by hepatic resection. With the advent of liver transplantation (LT) patients with HCC were preferentially placed on the waiting list for LT. However, early experience with LT was associated with a high rate of tumour recurrence and poor long-term survival. The increasing scarcity of donor livers resulted in restrictions being placed on tumour size, and an improvement in patient survival. To date there have been no randomised clinical trials comparing LR to LT. We review the evidence supporting LR and/or LT for HCC and discuss the role of neoadjuvant therapy. The decision of whether to resect or transplant remains debatable and is often determined by centre experience, availability of LT and donor organs.

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**Key words:** Hepatocellular carcinoma; Liver transplantation; Liver resection; Adjuvant therapy; Salvage liver transplantation; Radiofrequency ablation; Transarterial chemoembolization

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**Table 1** Recurrence and survival rates following surgical treatment for hepatocellular carcinoma

Treatment	Author	n	Recurrence (%)	5-yr survival (%)
Liver resection				
	Ercolani (2003) <sup>[61]</sup>	224	54.4	42.0
	Sim (2003) <sup>[62]</sup>	81	NS	59.0 <sup>1</sup>
	Belghiti (2003) <sup>[63]</sup>	328	NS	37.0
	Bartlett (2007) <sup>[59]</sup>	53	47.0	42.6
	Nuzzo (2007) <sup>[64]</sup>	248	46.0	24.0
Deceased donor liver transplantation				
	Mazzaferro (1996) (Milan criteria) <sup>[33]</sup>	48	8.0	75.4 <sup>2</sup>
	Jonas (2001) (Milan criteria) <sup>[65]</sup>	120	16.0	71.0
	Figueras (2001) (5 cm, localised) <sup>[66]</sup>	307	21.0	63.0
	Yao (2001) (UCSF criteria) <sup>[34]</sup>	70	11.4	75.2
	Decaens (2006) <sup>[36]</sup>			
	Milan criteria	279	NS	60.0
	Beyond Milan, but within UCSF	44	NS	45.6
	Beyond UCSF and Milan criteria	145	NS	34.7
Living donor liver transplantation				
	Todo (2007) <sup>[67]</sup>			
	Milan criteria	137	1.4	79.4 <sup>1</sup>
	Extended criteria	172	22.2	60.0
	Hwang (2005) <sup>[68]</sup>			
	Milan criteria	173	NS	88.0
	Extended criteria	64	NS	60.0 <sup>1</sup>
	Jonas (2007) <sup>[69]</sup>			
	Milan criteria	8	NS	75.0
	Extended criteria	13	NS	62.0
	Kwon (2007) <sup>[70]</sup>			
	Extended criteria	139	NS	79.9
	Sugawara (2007) <sup>[71]</sup>			
	Extended criteria (5 nodules <5cm)	78	10.0	75.0
	Soejima (2007) <sup>[40]</sup>			
	Milan criteria	16	0.0	100
	Unlimited criteria	44	18.2	74.0 <sup>1</sup>
Salvage liver transplantation				
	Belghiti (2003) <sup>[55]</sup>	18	5.6	61.0
	Schwartz (2006) <sup>[19]</sup>	18	44.0	NS
	Hwang (2007) <sup>[54]</sup>	17	NS	NS

NS: Not stated; UCSF: University of California San Francisco. <sup>1</sup>3-year survival; <sup>2</sup>4-year survival.

are those with Child-Pugh B cirrhosis or those with large ( $> 5$  cm) or multiple tumours.

The natural history of HCC has been studied in small series 20–30 years ago. Those patients with a solitary small ( $< 3$  cm) tumour have good three-year survival irrespective of treatment modality. Of those that undergo LR approximately 70% will develop intrahepatic tumour recurrence within 5 years of resection<sup>[5]</sup>. This represents either tumour that was present, but not detected at the time of LR (synchronous), or a new tumour that has arisen in the diseased liver remnant (metachronous). The results of LR for HCC in some recent publications are summarised in Table 1.

A number of different clinico-pathological staging systems have been proposed to help predict survival outcomes for patients with HCC to assist clinicians in deciding whether patients are suitable for LR. The Barcelona Clinic Liver Cancer (BCLC) group stratifies patients with HCC into 4 categories (early, intermediate, advanced and terminal) and recommends different treatment options for each category<sup>[6]</sup>. Accordingly, LR is only indicated in patients with early stage HCC, that is; a single nodule  $\leq 5$  cm or up to 3 nodules

$\leq 3$  cm; Okuda stage 1 or 2<sup>[7]</sup>; Child-Pugh A or B<sup>[8]</sup>; Performance score of 0<sup>[9]</sup>; with no portal hypertension and a normal serum bilirubin level. The role of LR for BCLC group intermediate-stage HCC (single nodule  $> 5$  cm or multinodular tumours) in the presence of preserved liver function (Okuda stage 1 or 2; Child-Pugh A; Performance score 0–2) remains controversial. Patients with large ( $> 5$  cm) HCCs are not suitable for ablative therapies and are excluded from LT if the Milan criteria are used for patient selection. These patients are often deemed too high-risk to undergo LR due to the extent of the resection. The American Association for Study of Liver Diseases (AASLD)<sup>[10]</sup> and the European Association for Study of Liver (EASL)<sup>[11]</sup> guidelines state that LR is contraindicated for tumours  $> 5$  cm due to the high incidence of vascular invasion and the associated poor prognosis. However, a number of centres have reported acceptable outcomes for patients with resected HCC greater than 5 cm or even 10 cm. A multicentre study of 300 patients with HCC  $> 10$  cm reported a 5-year overall survival rate of 26.9%<sup>[12]</sup>. Poon *et al* reported a 5-year actual survival rate of 20.6% for 58 patients resected for tumours  $> 10$  cm<sup>[13]</sup>.

Much of the improvement in patient outcome following LR has been due to the adoption of a multidisciplinary approach to managing these patients with stringent preoperative evaluation of hepatic function and liver manipulation by selective portal vein or hepatic artery embolization. This has been borne out by nationally representative data which has shown inpatient mortality to be 40% less in high-volume hospitals compared to low-volume hospitals (odds ratio 0.60;  $P = 0.02$ )<sup>[14]</sup>. Supporting the concept that patients requiring hepatectomy, particularly in the presence of chronic liver disease, should be managed in high-volume centres.

Multi-focal HCC is associated with a poor outcome, due to the high rate of recurrence and is considered a relative contraindication to LR. A recent audit of LR in 380 patients with large and multifocal HCC and small ( $< 5$  cm) single nodules ( $n = 404$ ), revealed similar peri-operative morbidity and mortality rates between the two groups, but the three-year survival that was significantly better for small solitary HCC (76% vs 50%)<sup>[15]</sup>. Despite this the authors stated that survival of patients with multifocal HCC after LR was better than that achieved with trans-arterial chemoembolization (TACE), and suggested that if functional reserve was acceptable they should be considered for LR provided all identifiable tumour is able to be resected<sup>[15]</sup>.

Patients with early Child-Pugh B cirrhosis without evidence of significant portal hypertension should be considered for minor LRs. The difficulty is objectively assessing liver function and the extent of LR likely to be tolerated. The indocyanine green (ICG) clearance test<sup>[16,17]</sup> has been well validated and the Model of End Stage Liver Disease (MELD) also appears to predict peri-operative mortality following LR. A MELD score of  $\geq 9$  had a peri-operative mortality rate of 29%, whilst a score of  $< 9$  had no mortality<sup>[18]</sup>. However, no technique has been shown to be superior to that of the judgement of an experienced clinician.

The aim of LR is to achieve local control of the index tumour, accepting that new or unrecognised tumours may subsequently appear. Thirty-nine percent of patients with a solitary HCC  $< 5$  cm on preoperative cross sectional imaging are found after LT to have other lesions on histologic examination of the explanted liver<sup>[19]</sup>. Although preoperative imaging has improved, less than one third of HCCs  $< 1$  cm can be identified using either contrast enhanced computer tomography (CT) or magnetic resonance imaging (MRI)<sup>[19]</sup>. This suggests that progression of established disease present at the time of LR accounts for a significant proportion of tumour recurrences post resection.

Parenchymal preservation is important in preserving hepatic function and reducing the risk of small for size syndrome<sup>[3]</sup>. However, HCC has a propensity for vascular invasion resulting in intrahepatic metastases. Anatomical (segmental) LR, results in resection of a greater volume of liver parenchyma, leads to the *en bloc* resection of the primary tumour and all the potentially tumour-bearing portal tributaries. In support of this, anatomical resection for solitary HCC has been shown

to be associated with a lower rate of disease recurrence and improved overall survival<sup>[20-22]</sup>. In a retrospective study of 321 patients who underwent curative LR for solitary HCC  $< 5$  cm, patients with preserved synthetic function (Liver damage group A) that underwent anatomical LR had improved overall and recurrence free 5-year survival compared to those treated by non-anatomical LR (87% vs 76%,  $P = 0.02$  and 63% vs 35%,  $P < 0.01$ , respectively)<sup>[21]</sup>. Similarly, a recent retrospective analysis of 158 consecutive patients undergoing either anatomical ( $n = 95$ ) or non-anatomical ( $n = 63$ ) LR for HCC, demonstrated improved disease-free and long-term survival after anatomical LR, despite having larger tumours and higher prevalence of vascular invasion<sup>[22]</sup>. However, anatomical LR cannot always be performed due to limited hepatic reserve. The only option in these patients is a more limited (non-anatomical) LR or local ablative therapy. Patients with moderately impaired (Liver damage group B) synthetic function who underwent non-anatomical LR had significantly better 5-year overall and recurrence free survival compared to those treated by anatomical LR (72% vs 48%,  $P < 0.01$  and 43% vs 28%,  $P = 0.01$ , respectively)<sup>[21]</sup>. Although the reason for this dichotomy remains speculative, it is possible that non-anatomical LR is associated with less physiological stress, which is better tolerated by patients with limited hepatic reserve.

More recently ablative therapies, such as radiofrequency ablation (RFA) or percutaneous ethanol injection (PEI), have been shown to offer similar outcomes to LR for small tumours ( $< 4$  cm) without the associated operative morbidity. In a randomised controlled trial comparing PEI and RFA for HCCs  $< 3$  cm, 4-year local recurrence and survival rates were 1.7% and 74%, respectively<sup>[23]</sup>. This was achieved despite a 63% incidence of disease recurrence elsewhere within the liver. Outcome appears to be operator dependent, both in terms of patient selection and technique, with rates of complete ablation varying from 20% to 96%. In a trial comparing PEI with RFA in HCCs  $\leq 4$  cm, RFA achieved initial complete ablation in 96% of lesions<sup>[24]</sup>. Looking at explant pathology, Lu *et al* reported complete necrosis in 83% of HCCs  $< 3$  cm<sup>[25]</sup>. The higher failure rate of RFA compared to LR in larger lesions may be due to the presence of vascular invasion, which is present in 10%-15% and 46%-50% of 2 cm and 3-4 cm HCCs, respectively<sup>[26]</sup>. LR has the advantage of removing unrecognised regional metastases contained within the resected specimen and is supported by the finding of less intrahepatic recurrence after anatomical compared to non-anatomical LR<sup>[20]</sup>.

## INCREASING RESECTABILITY

Attempts to improve resectability include down staging the primary tumour and reducing the extent of surgery or increasing the size of the future liver remnant. Several techniques have been used to 'down-size' the tumour or to increase the size of the future liver remnant. Pre-operative portal vein embolization allows extensive resections to be performed by decreasing the likelihood

of post-operative liver insufficiency. This is achieved by embolizing the lobe of liver that is to be resected 6 wk prior to surgery, inducing hypertrophy in the future liver remnant. An increase of 40% to 60% in the size of the non-embolized liver is observed in non-cirrhotic livers.

TACE as a down-staging procedure in irresectable tumors has been shown to result in necrosis in 40%-100% of tumors with a three-year survival rate of 77%<sup>[26]</sup>. There is no clear evidence currently that chemoembolization is more effective than embolization alone<sup>[27]</sup>; however, combination of local ablative therapy with systemic chemotherapy or biological agents appears to increase resection rates in patients with compensated liver disease.

## ADJUVANT TREATMENT FOLLOWING LIVER RESECTION

Several strategies have been employed in an attempt to reduce tumour recurrence following LR for HCC, including systemic chemotherapy, regional chemotherapy and internal radiotherapy. Intra-arterial 131-iodine labelled lipiodol has been shown to increase disease-free and overall survival in randomised controlled trials<sup>[27]</sup>. These findings have subsequently been confirmed in a retrospective analysis, where the 3-year disease-free survival rates were 68.4% and 41.5% in those that did and did not receive 131-iodine labelled lipiodol, respectively<sup>[28]</sup>. A large randomised study is awaited to confirm these results. More recently, menatetrenone, a vitamin K2 analogue with known anti-proliferative effects against hepatoma cell lines, reduced tumour recurrence and improved patient survival in patients with HCC following LR or local ablative therapy<sup>[29]</sup>. Sorafenib (Nexavar, Bayer Pharmaceuticals Corporation), an oral multi-kinase inhibitor, has been shown in a phase III placebo-controlled randomised trial to improve overall survival by 44% in patients with stage IV HCC (Hazard Ratio = 0.69, P = 0.0006)<sup>[30]</sup>. Whether this will translate into an improvement in survival in patients following LR or ablation remains to be tested.

## LIVER TRANSPLANTATION

LT is theoretically the best option for treating HCC as it allows for both radical resection of the primary tumour and treatment of the underlying liver disease, thus eliminating the risk of developing new HCCs and progression to end-stage liver failure. For many patients LR is not feasible because of tumour size, anatomical location or poor liver function, and LT is the only surgical option.

Early experience with LT for HCC was associated with a high rate of tumour recurrence and poor long-term survival<sup>[31]</sup>. Improving results for LT and the increasing scarcity donor grafts resulted in restrictions on tumour size as a 20%-40% survival at 5-years was deemed unacceptable. Bismuth<sup>[32]</sup> proposed and Mazzaferro<sup>[33]</sup> popularised the Milan criteria (single HCC

$\leq 5$  cm or up to 3 nodules  $\leq 3$  cm in diameter) for LT in patients with HCC to restrict access and to improve long-term outcome<sup>[33]</sup>. In many centres this has become the 'gold-standard' in determining eligibility for LT; however, some consider these criteria as too restrictive. Yao *et al* analyzed the outcome of 70 patients with HCC undergoing LT and found that patients with a single lesion  $\leq 6.5$  cm, 2 to 3 nodules with the largest  $\leq 4.5$  cm or a total tumor diameter  $\leq 8$  cm had a 75% 5-year survival<sup>[34]</sup>. Patients exceeding these University of California at San Francisco (UCSF) criteria, however, had a 1-year survival rate of 50% following LT<sup>[34]</sup>. Onaca *et al*, in an analysis of 1206 patients that underwent LT, found that patients with 2-4 tumours  $\leq 5$  cm or a solitary HCC  $\leq 6$  cm had tumour free survival similar to those that were within the Milan criteria<sup>[35]</sup>. Despite these encouraging reports adopting expanded criteria, a recent a retrospective study found that patients meeting the Milan criteria pre-transplant had a 5-year survival rate of 60% compared to 45% for those exceeding the Milan criteria, but meeting the UCSF criteria<sup>[36]</sup>. Although the difference was not statistically different, such a clinical difference warrants further examination. The results from a multi-center audit being undertaken by the 'Mazzaferro' group looking at the preoperative number and size of tumours and outcome is awaited<sup>[37]</sup>.

Vascular invasion has been shown to be predictive of tumour recurrence and poor long-term survival in patients with HCC<sup>[38]</sup>. Vascular invasion is more common in large HCCs; however a significant proportion of tumours  $> 5$  cm do not have histological evidence of vascular invasion<sup>[38]</sup>. Using size as a surrogate marker of biological behaviour may, therefore, result in patients with large well-differentiated HCC, who would potentially benefit from LT being excluded, and include small poorly differentiated tumours that are at high risk of recurrence<sup>[34]</sup>. An alternative method of assessing the risk of tumour recurrence is undertaking preoperative percutaneous biopsies, which places the patient at potential risk of local and hematogenous recurrence. Studies have demonstrated that percutaneous biopsy impairs the chance of curative resection. A retrospective review of 85 HCCs resected over 12 years found that preoperative biopsy resulted in the 5-year disease-free survival rate falling from 52% to 24%<sup>[38]</sup>. A recent review estimated the risk of needle tract seeding as being less than 2%, but acknowledged that biopsy carries a risk of hematogenous dissemination, but considered the degree of risk as speculative<sup>[39]</sup>. Further studies are needed to evaluate using histological criteria from biopsy for patients with tumours exceeding standard criteria. It is likely histological and other molecular analyses of tissue samples will increasingly be used preoperatively to characterise the biological behaviour of HCC.

As a consequence of donor shortage, live donor LT (LDLT) has become increasingly utilized for patients with end-stage liver disease. Despite the initial enthusiasm, the number of LRLT performed in the United States has fallen since 2002 as a consequence of

the realisation of donor risk and the implementation of the MELD system for organ allocation, which gave greater priority to patients with HCC. Out of necessity the number of LDLT undertaken in Asian countries, where cadaveric donation is not routinely available, has increased dramatically over the last decade.

For patients with early HCC for whom a suitable donor is available, LDLT offers a number of benefits. It can be performed in a timely manner eliminating the risk of waiting list dropout due to disease progression. LDLT does not rely upon cadaveric donation that is dependent upon equitable allocation for all patients with end-stage liver disease. Consequently, there have been a number of studies looking at LDLT using extended criteria, where the size and number of lesions is not limited<sup>[40,41]</sup>. The largest study reviewed 125 patients that underwent LDLT, 55 of which had tumours that exceeded Milan criteria<sup>[41]</sup>. Patients that exceeded Milan criteria, but had ≤ 10 tumours, all of which were ≤ 5 cm in diameter, had a 5-year recurrence rate similar to those that were within the Milan criteria (7.3% and 9.7%, respectively;  $P = 0.89$ ). Multivariate analysis also demonstrated a preoperative des-gamma-carboxy prothrombin (PIVKA-II; protein induced by vitamin K antagonist-II) value of > 400 mAU/mL as strongly associated with disease recurrence, and a level of < 400 mAU/mL be included in the selection criteria<sup>[41]</sup>. Similarly, Soejima *et al* reported on 60 patients that underwent LDLT for HCC, and found that there were no recurrences in those that were within the Milan Criteria. Multivariate analysis identified only tumour diameter of > 5 cm and PIVKA-II of > 300 mAU/mL as strongly associated with disease recurrence<sup>[40]</sup>. Although these studies are small and have short follow-up, they suggest that expansion of the current tumour size and number with the use of preoperative PIVKA-II, may be associated with acceptable outcomes in patients undergoing LDLT.

## LOCAL ABLATIVE THERAPIES AS A BRIDGE TO LIVER TRANSPLANTATION

RFA and TACE have been used by many centres to downstage and/or prevent disease progression in patients with HCC. Currently there are no prospective randomised trials evaluating the effect of these therapies prior to LT.

RFA is operator dependent, both in terms of patient selection and technique, with rates of complete ablation varying from 20% to 96%<sup>[25]</sup>. Most studies have demonstrated a reduction in the dropout rate compared to historical controls. A study of 60 consecutive HCCs in 50 patients on the waiting list for LT treated by percutaneous and laparoscopic RFA demonstrated a 0% dropout rate and a 8% morbidity at a mean time to LT of 9.5 mo<sup>[42]</sup>. This compares favourably to an historical dropout rate of 10%-30% with waiting times of 6-12 mo<sup>[43]</sup>. More recently, a study of 52 patients treated by preoperative RFA reported a dropout rate of 5.7% at a mean of 12.7 mo with no evidence of tumour

recurrence post-transplant<sup>[44]</sup>. Although there remains a potential risk for needle track dissemination and its efficacy has not been demonstrated in large HCC, RFA should be considered in patients on the waiting list with small (< 3 cm) solitary tumours and reasonable synthetic function (Child-Pugh A, and selected B).

A number of cohort studies have evaluated the efficacy of TACE, alone or in combination with systemic chemotherapy prior to LT<sup>[45,46]</sup>. The results are conflicting, and a recent meta-analysis of TACE as a bridge to LT found that there was insufficient evidence to support the use of neoadjuvant TACE prior to LT as it did not improve long-term survival, allow for the expansion of selection criteria or reduce the dropout rates on the waiting list<sup>[47]</sup>. TACE has been proposed as a method of selecting patients with favourable tumour biology. In a study of 96 consecutive patients with HCC, 62 of whom exceeded Milan criteria, tumour recurrence was influenced by the response to pre-transplant TACE. Patients who had a sustained response to TACE pre-transplant ( $n = 39$ ) had a 5-year tumour free recurrence rate of 94.5%, whereas patients who had disease progression had a tumour free recurrence rate of 35.4% ( $P = 0.0017$ )<sup>[48]</sup>. Similarly, in a smaller study there were only 2 recurrences in 19 patients with tumours > 3 cm that had decreased the sum of two diameters by > 50% following pre-transplant TACE<sup>[49]</sup>.

The current practice guidelines from the American Association for the Study of Liver Diseases (AASLD) state that local ablation, RFA and TACE, are safe and effective in patients who are not suitable for LR, or as a bridge to LT if the waiting list time exceeds 6 mo<sup>[10]</sup>.

## SALVAGE LIVER TRANSPLANTATION

Salvage LT has been promoted as a way of managing patients with HCC in an era of organ shortage. LR is performed as the primary procedure, keeping LT in reserve for those who develop further intrahepatic tumours or decompensation. The strategy offers a number of potential benefits. With increasing waiting times for LT patients with HCC face the prospect of disease progression beyond transplant criteria whilst waiting for a suitable donor. Overall, 5 year survival decreases by 10%-20% (from 81%-58% to 62%-47%) for waiting times of 6-12 mo, and dropout rates range from 10%-30%<sup>[43]</sup>. Undertaking LR in the first instance allows one to observe the natural history of the disease and allow those patients with aggressive disease to declare extrahepatic disease, thus avoiding inappropriate LT and eliminating the risk of disease progression beyond transplant criteria while on the waiting list. In addition, the potential exists to reduce the number of patients requiring LT. In the medium term, disease-free survival for patients undergoing LR with early stage HCC has been shown to be comparable to that of primary LT. LR also allows for histological analysis of the tumour and those with poor prognostic criteria, such as macroscopic vascular invasion or poor differentiation, should be excluded from LT due to the high likelihood

of tumour recurrence, while resected patients who had solitary well-differentiated tumours without vascular invasion can be managed by surveillance and offered LT only if there is tumour recurrence or hepatic decompensation.

Salvage LT for HCC relies upon the principal that patients that have tumour recurrence following LR are still amenable to LT. Tanaka *et al* found that 8% of patients who underwent LR within the Milan criteria had tumour recurrence that exceeded Milan criteria<sup>[50]</sup>. Conversely, only 22% of patients undergoing LR for tumours outside the Milan criteria develop post-resection recurrence that is within Milan criteria<sup>[50]</sup>. Multivariate analysis identified size of the primary tumour and degree of differentiation as risk factors for recurrence exceeding Milan criteria<sup>[50]</sup>. Others have identified the presence of portal vein invasion in the resected liver specimen as the most important predictor of tumour recurrence<sup>[51]</sup>. A number of molecular indices have been examined to try to predict tumour recurrence. A high level of telomerase activity is reported as an independent predictor for tumour recurrence<sup>[52]</sup>. However, no marker has been confirmed to predict the risk of tumour recurrence reliably.

Salvage LT appears to have higher morbidity and mortality and an increased incidence of tumour recurrence compared to primary LT<sup>[53]</sup>. Of 18 patients that underwent salvage LT at Mount Sinai following LR, 2 died peri-operatively (11%), and 7 subsequently developed tumour recurrence (44%)<sup>[19]</sup>. Similarly, of 17 patients that underwent salvage LDLT, bleeding complications were more common, and the peri-operative mortality rate (5.9%) was significantly higher than after primary LT<sup>[54]</sup>. In contrast, Belghiti *et al* reported that LR prior to LT did not significantly increase the operative difficulty of the procedure<sup>[55]</sup>. Furthermore, they did not find any difference in disease-free or overall survival between primary and salvage LT. Patients who underwent salvage LT had a mean 20 mo disease-free interval before listing for LT<sup>[55]</sup>. The long-term outcome of these strategies is awaited.

## EFFECT OF IMMUNOSUPPRESSION ON TUMOUR RECURRENCE

Calcineurin inhibitors, cyclosporine and tacrolimus, are currently the mainstay of immunosuppression in LT recipients. Sirolimus, a novel immunosuppressive drug that inhibits the mammalian target of rapamycin has been shown *in vitro* to allow for the maintenance of tumour immunosurveillance, and may theoretically offer survival benefit in patients transplanted for HCC. In a study of 70 patients transplanted for HCC receiving *de novo* sirolimus and low dose calcineurin inhibitor for 6-12 mo and either a short course (3 mo) or no steroids, tumour free survival at a median of 49 mo was comparable to that achieved with conventional immunosuppression<sup>[56]</sup>. However, 50% of patients had at least one episode of rejection and 34% developed

an incisional hernia. A better understanding of tumour biology and particularly the role of immunosuppression and tumour growth will provide further improvement in the treatment of HCC.

## DIRECT COMPARISONS: RESECTION VERSUS TRANSPLANTATION

The oncological advantage of LT compared to LR has not been universally demonstrated. For large tumours LR is not often possible and LT is the only potential treatment modality. Numerous retrospective studies from the 1990s have demonstrated that the results of LT for large HCCs are poor in relative terms, with 5-year survival rates of < 20%-30%<sup>[31,57,58]</sup>. In contrast the best therapeutic modality for small tumours (< 5 cm) is debatable. A retrospective analysis of 102 patients treated by LT ( $n = 50$ ) and LR ( $n = 52$ ) showed no difference in 3-year survival or recurrence rate for tumours < 5 cm<sup>[57]</sup>. In contrast, Bismuth found that LT was superior to LR for small (< 3 cm) tumours<sup>[32]</sup>. The 3-year survival rate for patients with tumours < 3 cm with 1 to 2 nodules was 83% and 41% for LT and LR, respectively<sup>[32]</sup>. The difference could be attributed to lower peri-operative mortality and tumour recurrence in the LT recipients. The operative mortality for LR for HCC varies from 0.5% and 21.5% and reflects the incidence of hepatic insufficiency-associated with underlying liver disease<sup>[59]</sup>. In addition, the rate of 'recurrent' disease is significantly higher after LR compared to LT with a 3-year recurrence-free survival rate of 83% and 18%, respectively<sup>[32]</sup>. Taken together, it is apparent that in the presence of chronic liver disease, LT offers the greatest chance of long-term survival for patients with small (< 5 cm) tumours. In the present climate of donor organ scarcity it is difficult to justify LT for large and/or advanced HCC.

## CONCLUSION

Currently, in the absence of large randomised clinical trials, the treatment strategy for patients with HCC remains a matter of choice depending upon the interpretation of retrospective studies, anecdotal evidence, unit experience, and availability of therapeutic options.

To date, we have relied upon radiological criteria as a surrogate marker of tumour behaviour. What is needed is an accurate predictor of the biological behaviour of the tumour at the time of presentation. The molecular analysis of tumour biopsies has yet to deliver, and is associated with a risk of needle track recurrence. Less invasive markers that can accurately predict the risk of tumour recurrence are needed to help stratify patients for appropriate therapy.

One of the confounding factors in comparing the outcome of different treatment modalities for HCC is the lack of a uniform staging system. A large multicentre trial examining the commonly used staging systems, found that the American Joint Committee

on Cancer/Union Internationale Contre le Cancer AJCC/UICC (sixth edition) staging system provides the best stratification of prognosis following LR or LT<sup>[60]</sup>. Adoption of a uniform staging system by all centres would help to provide a better comparison of therapeutic modalities in the future.

Although there is no consensus as to the best treatment for patients with HCC, it is apparent that LR appears to be the most appropriate treatment for patients with small (< 5 cm) solitary HCC with well-preserved synthetic function (Child-Pugh A) and normal portal pressures (hepatic vein wedge pressure < 10 mmHg). On account of the high rate of complete ablation that can be achieved in small tumours (< 3 cm), with a similar rate of local control compared to LR, it is hard to justify LR for patients with HCCs < 3 cm, especially if they have significant co-morbidities. Given the scarcity of donor organs and the lack of prospective data demonstrating an acceptable outcome in extending the current criteria, LT should be reserved for early stage HCC (solitary < 5 cm; ≤ 3 lesions 3 cm) that cannot be treated by LR. Medical treatments currently have limited efficacy, and their role, as a surgical adjuvant to LR and LT is yet to be determined.

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CLINICAL RESEARCH

# Clinical significance of NOD2/CARD15 and Toll-like receptor 4 gene single nucleotide polymorphisms in inflammatory bowel disease

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nificant difference was found between UC patients and control group ( $P > 0.05$ ). In CD and UC patients, no significant association with G908R variant was found. L1007finsC SNP showed an association with CD (9.8%) compared with controls (2.9%,  $P = 0.002$ ) and UC patients (2.3%,  $P = 0.01$ ). Moreover, in CD patients, G908R and L1007finsC mutations were significantly associated with different phenotypes compared to CD wild-type patients. No association of IBD with the TLR4 SNPs was found in either cohort (allele frequencies: D299G-controls 3.9%, CD 3.7%, UC 3.4%,  $P > 0.05$ ; T399I-controls 2.9%, CD 3.0%, UC 3.4%,  $P > 0.05$ ). **CONCLUSION:** These findings confirm that, in our IBD patients selected from Southern Italy, the NOD2/CARD15, but not TLR4 SNPs, are associated with increased risk of CD.

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**Key words:** Crohn's disease; Ulcerative colitis; NOD2/CARD15 gene; Toll-like receptor 4 gene; Single nucleotide polymorphisms

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## Abstract

**AIM:** To evaluate the role of genetic factors in the pathogenesis of Crohn's disease (CD) and ulcerative colitis (UC), we investigated the single nucleotide polymorphisms (SNPs) of NOD2/CARD15 (R702W, G908R and L1007finsC), and Toll-like receptor 4 (TLR4) genes (D299G and T399I) in a selected inflammatory bowel disease (IBD) population coming from Southern Italy.

**METHODS:** Allele and genotype frequencies of NOD2/CARD15 (R702W, G908R and L1007finsC) and TLR4 (D299G and T399I) SNPs were examined in 133 CD patients, in 45 UC patients, and in 103 healthy controls. A genotype-phenotype correlation was performed.

**RESULTS:** NOD2/CARD15 R702W mutation was significantly more frequent in CD (9.8%) than in controls (2.4%,  $P = 0.001$ ) and in UC (2.3%,  $P = 0.03$ ). No sig-

## INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are

idiopathic chronic inflammatory bowel disease (IBD). The molecular basis of their pathogenesis is not completely clear, but contributing factors may include persistent bacterial infection, a defective mucosal barrier, and an imbalance in the regulation of the intestinal immune response<sup>[1]</sup>.

Animal models of IBD support the concept that genetic factors, environmental triggers, and immune dysregulation may have a potential role in developing uncontrolled intestinal inflammation that determines the typical endoscopic manifestations and mucosal lesions compatible with CD or UC<sup>[1-4]</sup>.

Over the last decade, multiple genome-wide linkage searches have delineated numerous genomic regions containing putative IBD risk factors. In studies performed on unselected populations, an average of 8%-10% of CD patients and 6%-8% of UC patients have at least one relative affected by some type of IBD. However, these values vary from study to study and percentages of CD familial aggregation of less than 4% and more than 20% have been reported<sup>[2]</sup>.

Moreover, studies on twins demonstrate a greater genetic influence for CD compared with UC; combined study concordance rates for monozygotic twins are 36% for CD and 16% for UC<sup>[5]</sup>.

Recently, an association between CD and mutations in the NOD2/CARD15 gene located on chromosome 16q12 (IBD1) has been reported. NOD2/CARD15 acts as an intracellular receptor in monocytes for bacterial components, triggering activation of NF $\kappa$ B and thus leading to subsequent activation of the inflammatory response. Within the NOD2/CARD15 gene, three mutations have been identified as being associated with CD: two missense mutations (Arg702Trp in exon 4 and Gly908Arg in exon 8) and an insertion mutation of a C in exon 11 (1007finsC), the latter resulting in a truncated NOD2/CARD15 protein. These NOD2/CARD15 variants alter the structure of either the leucine-rich repeat (LRR) domain of the protein or the adjacent region. The activating function of nuclear factor NF $\kappa$ B is regulated by the carboxy-terminal LRR domain, which has an inhibitory role and also acts as an intracellular receptor for components of microbial pathogens. These observations suggest that the NOD2/CARD15 gene can confer susceptibility to CD by altering the recognition of these components and/or by over-activating NF $\kappa$ B in monocytes<sup>[6-9]</sup>. The question arises as to how NOD2/CARD15 mutations and impaired NF $\kappa$ B activation can confer susceptibility to CD. It has been suggested that the answer most likely lies within the leucine-rich repeats (LRR) of the NOD2/CARD15 gene and the family of Toll-like receptors (TLRs). These receptors, a family composed of at least 10 mammalian homologs of Drosophila Toll, serve as pattern recognition receptors for various microbial products and can mediate production of proinflammatory cytokines<sup>[10]</sup>. Toll-like receptor 4 (TLR4) functions as the main receptor for lipopolysaccharide (LPS) of Gram-negative bacteria<sup>[11]</sup>. After the recognition of pathogen-associated molecular patterns, the TLRs activate signal transduction pathways

of the innate immune response genes including inflammatory cytokines and the NF $\kappa$ B signalling pathway<sup>[12]</sup>. TLR4 is expressed in macrophages, dendritic cells, endothelial cells, and, less abundantly, in intestinal epithelial cells, which are partly tolerant to LPS, thereby preventing an exaggerated immune response caused by the large number of bacteria in the intestinal lumen<sup>[13]</sup>.

Mutations of the TLR4 gene are known to abolish responses to endotoxin in mice, as shown for the mice strains C3H/HeJ and C57BL/10SeCr<sup>[14]</sup>. Therefore, the ability to recognize bacterial wall products and to activate proinflammatory mechanisms by TLRs may be of great importance for immune reactions in the intestinal mucosa. The recently characterised D299G and T399I single nucleotide polymorphisms (SNPs) of TLR4 gene are probably associated with impaired LPS signalling and increased susceptibility to Gram negative infections<sup>[15]</sup>.

In this study, we investigated the frequencies of the three NOD2/CARD15 gene mutations (Arg702Trp, Gly908Arg and 1007finsC) and of the TLR4 gene D299G and T399I SNPs in a group of 178 Italian adult patients affected by IBD: 133 patients with CD and 45 with UC. The allele frequencies of the NOD2/CARD15 and TLR4 genes were evaluated, and a detailed genotype-phenotype correlation was performed.

## MATERIALS AND METHODS

### **Study population**

The study population was comprised of 133 patients with CD (70 males, 63 females; mean age,  $43.5 \pm 10.7$  years), 45 with UC (27 males, 18 females; mean age,  $43.2 \pm 11.0$  years) and 103 healthy, unrelated controls (68 males, 35 females; mean age,  $46.6 \pm 9.8$  years). Patients were consecutively recruited from Department of Paediatrics and Department of Medicine, University Hospital of Messina, Italy. All patients were from Eastern Sicily and Calabria (Southern Italy). Informed consent was obtained from each participant.

Diagnosis of CD and UC was established according to accepted clinical, endoscopic radiological, and histological criteria<sup>[16]</sup>. A detailed clinical questionnaire concerning different features of the disease was employed. The Vienna classification was used for CD phenotypes<sup>[17]</sup>, while localization was defined based on the largest extent of the disease, according to X-ray, endoscopy, or surgical reports.

The following data of patients with CD and UC were collected: age, age at diagnosis, gender, familial or spontaneous disease (familial disease was considered if one first or second-degree relative had IBD), disease localization, disease behaviour, extraintestinal manifestations (arthritis, affections of eyes or skin, primary sclerosing cholangitis), type and site of surgery. Disease localization was defined as the maximum extent of digestive tract involvement at the latest follow-up.

Patients were eligible if IBD was confirmed, and they had undergone full colonoscopy with biopsy and/or surgical resection.

**Table 1** Primers sequences and restriction enzymes used for genotyping TLR4 and NOD2/CARD15

Gene locus	SNPs	Sequence	Restriction enzyme
NOD2/CARD15	R702W	For 5' TTCAGATCACAGCAGCCTTC 3'	<i>Msp</i> I
		Rev 5' CCCACACTGAAAATGTCAC 3'	
	G908R	For 5' AGCCACTGAAAATCTTGG 3'	<i>Hha</i> I
		Rev 5' TCITCACCTGATCTCCCAA 3'	
	L1007finsC	For 5' CCTGCAGTCTTTAACCTGG 3'	<i>Nla</i> IV
		Rev 5' CTTACCAGACTTCCAGGATG 3'	
TLR4	D299G	For 5' TTAGAAATGAAGGAAACTTGAAAAG 3'	<i>Bsa</i> BI
		Rev 5' TTTGTCAAACAATTAAATAAGTGATTAATA 3'	
	T399I	For 5' GGTTGCTGTTCTCAAAGTGATTTGGGAGAA 3'	<i>Hinf</i> I
		Rev 5' CCTGAAGACTGGAGAGTGAGTAAATGCT 3'	

A group of 103 healthy, unrelated subjects coming from the Sicily and Calabria regions (mainly students, blood donors and hospital employees) were selected as controls.

#### DNA extraction

Genomic DNA was isolated from 1 mL of peripheral blood anticoagulated with EDTA as previously described<sup>[18]</sup>. DNA samples of the patients and control subjects were analyzed for the variants of NOD2/CARD15 and TLR4 genes by melting curve analysis.

#### Genotyping of the NOD2/CARD15 mutations

To detect the R702W, G908R, and L1007finsC mutations, we performed a polymerase chain reaction (PCR) using 0.5 U of Taq polymerase (Eurotaq, Euroclone Life Sciences Division, UK), 400 μmol/L dNTPs, and 0.1 μmol/L of each primer in a total volume of 25 μL. After an initial denaturation for 5 min at 95°C, PCR was performed by 35 cycles of denaturing at 95°C for 30 s, annealing at 65°C for 40 s, primer extension at 72°C for 30 s. The final extension was performed at 72°C for 7 min. PCR reactions were carried out using a GeneAmp PCR system 2700 (Applied Biosystem, CA, USA).

Genotyping of each SNP was performed by enzymatic digestion at 37°C, overnight. After enzymatic digestion, the fragments were separated and visualized by gel electrophoresis (3% NuSieve® GTG agarose gel BMA, Rockland, ME, USA).

The specific primers PCR and the restriction enzymes (New England Biolabs, Ipswich, MA) for each SNP are given in Table 1.

Wild-type/mutant genotype was confirmed by automatic sequencing using the ABI-PRISM Big Dye™ Terminator v. 3.0 Cycle sequencing Ready Reaction Kit (Applied Biosystems, CA, USA). The sequencing products were purified using DyeEx Spin Kits (Qiagen) and visualized on an ABI-PRISM 310 Genetic Analyzer (Applied Biosystems, CA, USA).

#### Genotyping of the TLR4 polymorphisms

The two D299G and T399I SNPs of the TLR4 gene were determined by PCR-RFLP.

We performed PCR using 0.5 U of Taq polymerase (Eurotaq, Euroclone Life Sciences Division, UK), 400 μmol/L dNTPs, and 0.1 μmol/L of each primer in

a total volume of 25 μL.

For D299G SNP, cycle conditions were an initial denaturation for 5 min at 95°C, followed by 32 cycles of denaturing at 95°C for 30 s, annealing at 51°C for 30 s, primer extension at 72°C for 30 s, followed by a final extension at 72°C for 7 min. For T399I SNP, cycle conditions were an initial denaturation for 5 min at 95°C, followed by 35 cycles of denaturing at 95°C for 45 s, annealing at 55°C for 30 s, primer extension at 72°C for 45 s, followed by a final extension at 72°C for 7 min.

The specific primers PCR and the restriction enzymes for each SNP are given in Table 1.

The amplified samples of TLR4 gene D299G and T399I SNPs were digested at 37°C, overnight, with the *Bsa*BI and *Hinf*I restriction enzymes (New England Biolabs, Ipswich, MA, USA), respectively.

After enzymatic digestion, the fragments were separated and visualized by gel electrophoresis (3% NuSieve® GTG agarose gel BMA, Rockland, ME, USA).

As previously described here, the results of enzymatic digestion were confirmed by DNA sequence analysis of representative samples of each SNP.

#### Statistical analysis

Data are given as mean ± SD. Allele and genotypes frequencies in patients and in controls were compared by  $\chi^2$  test or Fisher exact test, when an expected value was < 0.5; *P* values were considered significant at a level of < 0.05. Odds ratio (OR) and *P* values were calculated using a standard package (StataCorp. Stata Statistical Software: Release 8.0 College Station. TX: Stata Corporation 2001).

Allele frequencies were tested for the Hardy-Weinberg equilibrium. Cases and controls were compared using Pearson's  $\chi^2$  test.

## RESULTS

#### Allele frequencies in IBD patients NOD2/CARD15 gene SNPs

In CD patients, the frequency of R702W mutation was significantly higher (9.8%) than in controls (2.4%, *P* = 0.001; OR, 4.09; 95% CI, 1.5-11.9) and in UC (2.3%, *P* = 0.03; OR, 4.49; 95% CI, 1.02-19.8; Table 2). No significant difference of the G908R mutation allele frequency was found

**Table 2** NOD2/CARD15 and TLR4 SNPs allele frequencies of CD patients vs control group and UC patients

Polymorphisms of NOD2/CARD15 and TLR4 genes	CD (n = 133)	Allele frequency (%)	Controls (n = 103)	Allele frequency (%)	<sup>1</sup> P	OR (95% CI)	UC (n = 45)	Allele frequency (%)	<sup>2</sup> P	OR (95% CI)
R702W										
Wild-type	107 (80.4%)	9.8	98 (95.1%)	2.4	0.001	4.09 (1.5-11.9)	43 (95.5%)	2.3	0.03	4.49 (1.02-19.8)
Heterozygous	26 (19.6%)		5 (4.9%)							
G908R										
Wild-type	120 (90.2%)	4.5	94 (91.2%)	4.3	NS	1.01 (0.3-3.5)	41 (91.1%)	4.4	NS	0.90 (0.28-2.92)
Heterozygous	13 (9.8%)		9 (8.8%)							
L1007finsC										
Wild-type	107 (80.4%)	9.8	97 (94.1%)	2.9	0.002	3.92 (1.55-9.95)	43 (95.5%)	2.3	0.01	5.22 (1.19-22.98)
Heterozygous	26 (19.6%)		6 (5.9%)							
D299G										
Wild-type	123 (92.5%)	3.7	95 (92.2%)	3.9%	NS	0.96 (0.37-2.54)	42 (93.3%)	3.4	NS	0.87 (0.23-3.35)
Heterozygous	10 (7.5%)		8 (7.8%)							
T399I										
Wild-type	125 (94%)	3.0	97 (94.1%)	2.9%	NS	1.15 (0.28-4.64)	42 (93.3%)	3.4	NS	1.11 (0.28-4.40)
Heterozygous	8 (6.0%)		6 (5.9%)							

No patients homozygous for NOD2/CARD15 gene R702W, G908R and L1007finsC SNPs were found in this study population; No patients homozygous for TLR4 gene D299G and T399I SNPs were found in this study population. <sup>1</sup>CD patients vs control group; <sup>2</sup>CD patients vs UC patients. NS: No significance.

between CD (4.5%) and the control group (4.3%; P > 0.05; OR, 1.01; 95% CI, 0.3-3.5), and between CD and UC patients (4.4%, 0.05; OR, 0.90; 95% CI, 0.28-2.92; Table 2).

The frequency of the frameshift mutation L1007finsC was significantly higher in CD patients (9.8%) compared with controls (2.9%, P = 0.002; OR, 3.92; 95% CI, 1.55-9.95) or patients with UC (2.3%, P = 0.01; OR, 5.22; 95% CI, 1.19-22.98; Table 2).

In UC patients, the allele frequencies of the R702W, G908R, and 1007finsC mutations were not significantly different from the control group (R702W: P > 0.05; OR, 0.91; 95% CI, 0.17-4.88 and G908R: P > 0.05; OR, 1.01; 95% CI, 0.3-3.5 and L1007finsC: P > 0.05; OR, 0.75; 95% CI, 0.15-3.88).

No homozygous carriers of the three NOD2/CARD15 mutations were found in the study and control populations.

The NOD2/CARD15 allele frequencies were in Hardy-Weinberg equilibrium in all patients and in control subjects.

### TLR4 gene SNPs

The results of the genotype analyses in 133 patients with CD, in 45 patients with UC and in 103 control individuals, with regard to the TLR4 D299G and T399I SNPs are shown in Table 2.

In CD patients, the frequency of the D299G SNP (3.7%) was not significantly different from the controls (3.9%, P > 0.05; OR, 0.96; 95% CI, 0.37-2.54) or from UC patients (3.4%, P > 0.05; OR, 0.87; 95% CI, 0.23-3.35; Table 2). The T399I SNP allele frequency was not significantly different between CD patients (3.0%) and control group (2.9%, P > 0.05; OR, 1.15; 95% CI, 0.28-4.64); or between CD (3.0%) and UC patients (3.4%, P > 0.05; OR 1.11, 95% CI 0.28-4.40; Table 2). No significant difference was found between UC patients and control group as regards the D299G SNP (P > 0.05;

OR, 0.84; 95% CI, 0.21-3.36) or the T399I SNP (P > 0.05; OR, 1.15; 95% CI, 0.28-4.64).

No homozygous carriers of the two SNPs were found in the study and control populations.

The TLR4 allele frequencies were in Hardy-Weinberg equilibrium in all patients and in the control group.

### Genotype-phenotype correlations

When the contribution of each SNP of the NOD2/CARD15 gene was investigated, the major support to the genotype-phenotype correlation could be ascribed to the G908R and the L1007finsC alleles (Table 3). In particular, in CD patients, the occurrence of one risk allele of G908R was associated with stenosing phenotype (P = 0.03) and resective surgery (P = 0.003).

An increased frequency of ileal localization (80.7%, P = 0.001) and resective surgery (53.9%, P = 0.01) was found in CD L1007finsC heterozygotes compared with CD patients with wild-type NOD2/CARD15 gene (ileum 36.8% and resective surgery 26.4%, respectively).

Moreover, the clinical features of all CD patients were analysed with respect to the presence of one or two risk alleles of each SNP (heterozygous or compound heterozygous) of any NOD2/CARD15 variants (Table 3).

By univariate analysis, the presence of one risk allele was significantly associated with ileal localization (P = 0.04) and resective surgery (P = 0.03). These significant associations increased in the compound heterozygotes (P = 0.03 and P < 0.0001, respectively). Moreover, the presence of two risk alleles was significantly associated with stenosing disease (P = 0.02, Table 3).

In CD patients, TLR4 D299G and T399I SNPs were not found to be associated with age at diagnosis, sex, localization, disease type, resective surgery and extraintestinal manifestations.

Similarly, in UC patients, these TLR4 gene SNPs were not associated with any studied clinicopathological parameter.

**Table 3** Genotype-phenotype correlations in CD patients

Total CD patients (n = 133)	CARD15 no risk alleles (n = 68, 51.1%)	R702W 1 risk allele (n = 26, 19.6%)	G908R 1 risk allele (n = 13, 9.7%)	L1007finsC 1 risk allele (n = 26, 19.6%)	CARD15 at least 1 risk allele (n = 65, 48.8%)	CARD15 compound heterozygous (n = 48, 36.1%)
Age (mean ± SD)	41.5 ± 11.2	41.2 ± 11.9	43.02 ± 10.9	42.0 ± 12.8	42.3 ± 12.1	42.7 ± 11.9
Sex (m/f, 70/63)	32/36	13/13	8/5	10/16	30/35	25/23
Localization (%)						
Ileum (n = 61)	25 (36.8)	11 (42.3)	4 (30.8)	21 (80.7)	38 (58.5)	30 (62.5)
Ileo-colon (n = 39)	22 (32.3)	10 (38.5)	4 (30.8)	3 (11.5)	15 (23.0)	10 (20.8)
Colon (n = 30)	18 (26.5)	5 (19.5)	5 (38.4)	2 (7.8)	12 (18.5)	8 (16.7)
Upper GI (n = 3)	3 (4.4%)				0	0
P		> 0.05 <sup>1</sup>	> 0.05 <sup>1</sup>	0.001 <sup>1</sup>	0.04 <sup>2</sup>	0.03 <sup>3</sup>
Disease type (%)						
Inflammatory (n = 37)	14 (20.6)	7 (27.0)	6 (46.0)	10 (38.5)	23 (35.4)	8 (16.6)
Stenosing (n = 56)	32 (47.0)	11 (42.3)	7 (54.0)	6 (23.0)	24 (37.0)	34 (70.8)
Fistulizing (n = 40)	22 (32.4)	8 (30.7)	0	10 (38.5)	18 (27.6)	6 (12.6)
P		> 0.05 <sup>1</sup>	0.03 <sup>1</sup>	> 0.05 <sup>1</sup>	> 0.05 <sup>2</sup>	0.02 <sup>3</sup>
Resective Surgery (%)	18 (26.4)	9 (34.6)	9 (69.2)	14 (53.9)	32 (49.2)	31 (64.6)
P		> 0.05 <sup>1</sup>	0.003 <sup>1</sup>	0.01 <sup>1</sup>	0.01 <sup>2</sup>	0.000 <sup>3</sup>
Extraintestinal manifestations (n = 18, %)	10 (55.5)	3 (11.5)	1 (7.7)	4 (15.3)	8 (13.3)	3 (6.2)

No patients homozygous for R702W, G908R and L1007finsC SNPs were found in this study population. <sup>1</sup>CD patients no risk allele vs CD patients with risk allele; <sup>2</sup>CARD15 at least 1 risk allele vs CD patients no risk allele; <sup>3</sup>CARD15 compound heterozygous vs CD patients no risk allele.

## DISCUSSION

In our study, we investigated the prevalence of NOD2/CARD15 and TLR4 genetic variants in CD and UC patients. Moreover, we compared the results with clinical phenotype characteristics of IBD of our patients to identify a possible genotype-phenotype association.

There are several controversial data about the role of the SNPs of the NOD2/CARD15 (R702W, G908R and L1007finsC), and TLR4 genes (D299G and T399I) in the pathogenesis of CD. Indeed, there are significant phenotypic differences that exist among populations.

The NOD2/CARD15 mutations are absent in Asian CD populations and controls<sup>[19-21]</sup>. In this case, the findings indicate that the NOD2/CARD15 is not a major contributor to CD susceptibility in the Japanese population. Similar data have been found in Turkish patients with IBD<sup>[22]</sup>.

The highest recorded frequencies are reported in a small study of 55 paediatric patients in Europe with two thirds of the patients having at least one NOD2/CARD15 mutation<sup>[23]</sup>. Within Europe, there is evidence of a north-south gradient with lower allele frequencies in the Celtic and Scandinavian countries compared to Southern Europe<sup>[23]</sup>.

To our knowledge, this is the first study in a large series of sporadic IBD patients coming from Eastern Sicily and Calabria. Indeed, previous reports regarded a Sicilian, small town population in which a familial study was performed<sup>[24]</sup>. Other studies have examined a group of sporadic Sicilian IBD patients, but the number of cases was smaller than in our study<sup>[25]</sup>.

In our study, the reported rates of 48.8 % of patients carrying at least one NOD2/CARD15 mutation in CD and 19.4% in controls are consistent with previously reported rates of 30%-50% in CD and 7%-20% in controls from other European regions<sup>[26-31]</sup>. Moreover, 36.1% had two mutations (compound heterozygotes). Recently,

Renda *et al* examined a group of 182 CD patients coming from Western Sicily and they found that 56 patients (30%) had at least one mutation of the NOD2/CARD15 gene<sup>[32]</sup>. This percentage was lower in respect to our data (48.8%). This difference may be ascribed to a different ethnic background. Indeed, the patients of our study coming both Eastern Sicily and Calabria. Today, populations genetically similar to that of the Northern Italy (as well as of the Northern Africa) are present in the Eastern Sicily. This heterogeneous population, during the middle age, might explain the genetic differences in the patient CD samples of the Eastern in respect to Western Sicily<sup>[33]</sup>.

The allele frequencies of the R702W (9.8%) and L1007finsC (9.8%) mutations were significantly higher in CD patients compared to UC patients and controls, whereas the frequency of the G908R (4.5%) mutation was similar in CD and UC patients, and in the control group. Collectively, our study confirmed previous studies, which reported increased mutation carrier frequencies of one of the three variant alleles in CD patients compared to UC patients or healthy controls<sup>[2,28,32,34-36]</sup>.

We also found that different risk alleles might be associated with different clinical features: in particular, the G908R allele seems to be associated with stenosing phenotype and need for surgery. The L1007finsC seems to correlate with ileal localization and resective surgery. These data suggest a more aggressive course of the disease in carriers of risk alleles. The strongest observed effect for ileal location is consistent with the proposed involvement of ileal Paneth cells in the pathophysiology of NOD2/CARD15-mediated disease susceptibility<sup>[30,36,37]</sup>. NOD2/CARD15 mutations may, thus, abrogate normal Paneth cell behaviour, explaining preferential involvement of the terminal ileum<sup>[26]</sup>.

Moreover, in our study, the risk of developing CD with a more aggressive course was increased in compound heterozygotes. In other populations, stronger associations

have been reported for homozygotes and compound heterozygotes than for simple heterozygotes. One copy of the risk alleles confers a 2-4-fold risk for developing CD, whereas double-dose carriage increases the risk by 20-40-fold<sup>[23]</sup>. Our study is in agreement with such a gene-dosage effect, although at lower levels.

In our IBD patients, we also examined the allele frequencies of the TLR4 D299G and T399I SNPs and the possible genotype-phenotype correlation.

With regard to the role of the TLR4 gene in the pathogenesis of IBD, several studies have been conducted leading to divergent results<sup>[23]</sup>. The allele frequency of the D299G mutation ranges between 8%-13% in CD, 0%-10% in UC and 3%-15% in healthy controls<sup>[38]</sup>. This TLR4 SNP was associated with CD and UC in a Belgian study<sup>[39]</sup>. This association was replicated in Dutch, German, Australian and Greek populations with CD, and an association with colonic disease has been described<sup>[8,34,35,40]</sup>. In one German cohort, an association was demonstrated between UC and the TLR4 T399I SNP<sup>[41]</sup>. However, there is substantial heterogeneity between populations, and no association was noted in Scottish CD patients<sup>[30]</sup>.

In our study, we found no difference in the prevalence of these mutations in our CD and UC patients, and controls. Recently, other studies have failed to find the association of the D299G and T399I SNPs of TLR4 gene<sup>[24,42-45]</sup>. In a retrospective German and Hungarian cohort study, patients with CD and UC were genotyped for the presence of the CD14 c.1-260C>T promoter variant and the TLR4 D299G variant. In this study, in German and Hungarian populations, IBD appears to be associated with the CD14 c.1-260C>T promoter variant, but not with the TLR4 D299G variant<sup>[45]</sup>. Recent data suggest that neither of these 2 variants is causal, but they may be in linkage disequilibrium with, as yet unidentified, causal variants<sup>[46-48]</sup>.

We examined also whether the TLR4 D299G and T399I SNPs could be related to particular CD or UC phenotypes. Detailed analysis did not show any association of the examined TLR4 gene variants with either CD or UC patient subgroups. In other studies, in CD patients, an association has been reported between D299G SNP and ileal localization and structuring phenotype<sup>[49]</sup>. Our data are similar to those previously reported<sup>[28]</sup>. These contrasting results can be ascribed to the different ethnic background of the various IBD populations studied.

Although several studies have been performed, further research is warranted to clarify the role of the genetic variants of NOD2/CARD15 and TLR4, and to investigate whether these genetic risk factors might be confirmed and considered clinically relevant. Indeed, an eventual goal in the genomic study of IBD is to identify these biologically relevant genotype-phenotype associations and to apply them to clinical practice.

## COMMENTS

### Background

Crohn's disease (CD) and ulcerative colitis (UC) are idiopathic inflammatory

bowel disease (IBD) with genetic risk factors. There is evidence that NOD2/CARD15 and Toll-like receptor 4 (TLR4) genes may be involved in their pathogenesis.

### Research frontiers

In our study, we found that some single nucleotide polymorphisms (SNPs) of the NOD2/CARD15 gene, but not of the TLR4 gene, were significantly more frequent in CD patients. Therefore, it is possible that the NOD2/CARD15 gene plays an important role in the pathogenesis of IBD.

### Innovations and breakthroughs

We evaluated the allele and genotype frequencies of the more frequent SNPs of NOD2/CARD15 and TLR4 genes in a selected IBD population coming from Eastern Sicily and Calabria (Southern Italy), a geographical area for which very few data exist.

### Applications

Genotyping of patients with CD could be an important diagnostic tool in clinical practice for identifying high-risk patients with specific diagnostics and therapeutic needs.

### Peer review

This study underlines the association of the NOD2/CARD15 genotype with the behaviour and location of CD also in patients coming from Eastern Sicily and Calabria (Italy). Moreover, the CD patients carrying at least one major variant of NOD2/CARD15 gene had an aggressive clinical course. Test strategies with NOD2/CARD15 variations to predict the clinical course of CD could lead to the development of new therapeutic paradigms.

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BASIC RESEARCH

## Metabolomic changes in fatty liver can be modified by dietary protein and calcium during energy restriction

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**Author contributions:** Pilvi TK designed and conducted the study, analysed the data and prepared the manuscript. Seppänen-Laakso T, Simolin H and Orešič M did the metabolomic analyses. Huotari A and Herzig KH conducted the metabolic performance and calorimetry tests. Korpela R and Mervaala EM were involved in designing the study, reviewing and interpreting the results and all authors were involved in drafting the manuscript.

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analyses were performed using the UPLC/MS based lipidomic platform and the HPLC/MS/MS based primary metabolite platform.

**RESULTS:** ER on both diets significantly reduced hepatic lipid accumulation and lipid droplet size, while only whey + Ca diet significantly decreased blood glucose ( $P < 0.001$ ) and serum insulin ( $P < 0.01$ ). In hepatic lipid species the biggest reduction was in the level of triacylglycerols and ceramides while the level of cholesterol esters was significantly increased during ER. Interestingly, diacylglycerol to phospholipid ratio, an indicator of relative amount of diabetogenic diglyceride species, was increased in the control ER group, but decreased in the whey + Ca ER group ( $P < 0.001$ , vs obese). ER on whey + Ca diet also totally reversed the obesity induced increase in the relative level of lipotoxic ceramides ( $P < 0.001$ , vs obese;  $P > 0.05$ , vs lean). These changes were accompanied with up-regulated TCA cycle and pentose phosphate pathway metabolites.

**CONCLUSION:** ER-induced changes on hepatic metabolomic profile can be significantly affected by dietary protein source. The therapeutic potential of whey protein and calcium should be further studied.

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**Key words:** Fatty liver; Metabolomics; Energy restriction; Whey protein; Dietary calcium

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### Abstract

**AIM:** To characterise the effect of energy restriction (ER) on liver lipid and primary metabolite profile by using metabolomic approach. We also investigated whether the effect of energy restriction can be further enhanced by modification of dietary protein source and calcium.

**METHODS:** Liver metabolomic profile of lean and obese C57BL/6J mice ( $n = 10/\text{group}$ ) were compared with two groups of weight-reduced mice. ER was performed on control diet and whey protein-based high-calcium diet (whey + Ca). The metabolomic

### INTRODUCTION

Obesity is closely associated with different components of metabolic syndrome<sup>[1]</sup>. However, not all obese

individuals develop metabolic syndrome and not all individuals with metabolic syndrome are obese. It has recently been suggested that fat accumulation in the liver is the key feature distinguishing those individuals who develop metabolic syndrome from those who do not<sup>[2]</sup>. The mechanisms leading to hepatic fat accumulation are not fully understood and, hence, the means of preventing and treating this condition are limited. Once fat has accumulated, the liver is insulin resistant and overproduces major cardiovascular risk factors, such as C-reactive protein, very low density lipoprotein and plasminogen activator inhibitor-1<sup>[3]</sup>. At the moment, improving insulin resistance through energy restriction and subsequent weight loss remains the cornerstone of therapy for non-alcoholic fatty liver disease<sup>[4]</sup>.

Lipids are a highly diverse class of molecules, which have important roles as signalling and structural molecules in addition to serving as energy storage. Therefore, it is crucial to identify the variety of lipid species accumulating in the liver in order to understand the complex process of hepatic insulin resistance. The level of triacylglycerides (TAG) and diacylglycerides (DAG) has been shown to be increased in non-alcoholic fatty liver disease in humans, while total amount of phosphatidylcholines is decreased<sup>[5]</sup>. Similar changes in liver lipids have been detected in ob/ob mice with up-regulation of TAG and DAG, diacylphosphoglycerols and specific ceramide species and down-regulation of sphingomyelins<sup>[6]</sup>. Interestingly, a recent human study revealed that a high level of liver fat is also associated with changes in the lipidomic profile of subcutaneous adipose tissue<sup>[7]</sup>. Increased adipose tissue ceramides, SM, ether phospholipids and long-chain TAG were associated with higher liver fat level. Hence, the accumulation of ceramides and TAG also in the subcutaneous adipose tissue seems to reflect the development of fatty liver. However, more studies are needed to support the findings of fatty liver lipidomics.

Even though weight loss is the main therapeutic way to reduce liver fat, the information on the effect of energy restriction on liver lipidomic profile is scarce. Also the beneficial effect of different dietary components on liver fat species is nearly an unexplored area<sup>[8]</sup>. High intake of dairy products is related to lower risk of insulin resistance<sup>[9]</sup>, type two diabetes<sup>[10,11]</sup> and metabolic syndrome<sup>[12-15]</sup>, but the mechanism of action has not been established. The increased intake of dairy products or calcium has also been shown to augment weight loss both in humans and mice<sup>[16-19]</sup>. Although part of the effect of dairy products on weight loss can be attributed to calcium, it has been repeatedly demonstrated that the anti-obesity effect of dairy is superior to that of calcium alone<sup>[18,20]</sup>. It has been suggested that the whey protein fraction of milk is a source of bioactive peptides or other compounds capable of regulating adipose tissue metabolism, energy expenditure or satiety signals<sup>[21]</sup>. In our previous study, we showed that whey protein in combination with calcium attenuates weight gain<sup>[22]</sup>, but the effects of whey protein during energy restriction

have not been reported. Also, the effect of whey protein containing high-calcium diet on hepatic lipid profile has not been previously described.

The aim of this study was to characterise the effect of high-fat diet-induced obesity and the subsequent ER on liver lipidomic and primary metabolite profile in C57Bl/6 mice, a widely studied model of diet induced obesity. In addition we investigated whether the effect of ER may be significantly improved by modulating the protein source and calcium content of the weight loss diet.

## MATERIALS AND METHODS

### Animals and diets

Eight-week old male C57Bl/6J mice ( $n = 40$ ) were purchased from Harlan (Horst, The Netherlands). The mice were housed five in a cage in a standard experimental animal laboratory, illuminated from 6:30-18:30, temperature  $22 \pm 1^\circ\text{C}$ . The protocols were approved by the Animal Experimentation Committee of the University of Helsinki, Finland and the principles of laboratory animal care (NIH publication no. 85-23, revised 1985) were followed. The mice had free access to feed and tap water during the experiment. After a one-week acclimatisation period on a normal chow diet (Harlan Tekland 2018, Harlan Holding, Inc, Wilmington, DE, USA) thirty mice ( $25.5 \text{ g} \pm 0.3 \text{ g}$ ) were put on a high-fat diet (60% of energy from fat, D05031101M, Research Diets Inc., New Brunswick, NJ, USA) for 14 wk. Ten remaining mice continued on normal chow diet (*ad libitum*) throughout the study and served as a lean control group. After the weight gain period on high-fat diet one group of mice (obese group,  $n = 10$ ) was sacrificed, and the remaining mice were put on a calorie restriction diet for 7 wk. During the calorie restriction period, the mice were given 70% of the energy they ate during the *ad libitum* feeding. In the beginning of the calorie restriction period, the body weight matched mice were divided into two groups: whey + Ca group and control group. Whey + Ca group received high-fat diet (D05031104M, Research Diets Inc., New Brunswick, NJ, USA) with 1.8%  $\text{CaCO}_3$  and all protein (18% of energy) from whey protein isolate (Alacen™ 895, NZMP, Auckland, New Zealand). The control group continued with the same high-fat diet (D05031101M) as during the weight gain period. The powdered diets were moistened with tap water (200 mL/kg in whey + Ca, 110 mL/kg in control and 700 mL/kg in normal chow diet) using industrial dough mixer, packed in one-day portions and stored at  $-20^\circ\text{C}$ .

The body weight was monitored weekly during the weight gain period, and twice per week during the calorie restriction period using a standard table scale (Ohaus Scout™ Pro, SP4001, Nänikon, Switzerland). The consumption of feed was monitored daily using the same table scale. The body fat content was analysed by dual-energy X-ray absorptiometry (DEXA, Lunar PIXImus, GE Healthcare, Chalfont St. Giles, UK) at the end of the weight gain and calorie restriction periods.

### **Calorimetry and metabolic performance**

The dietary protein-induced differences in metabolic performance, energy expenditure, physical activity and drinking and feeding behaviour were analysed by housing an additional group of animals ( $n = 4$ /whey group and  $n = 3$ /casein group) in a home cage-based monitoring system for laboratory animals (LabMaster®, Bad Homburg, Germany). The instrument consists of a combination of highly sensitive feeding and drinking sensors for automated online measurement. The calorimetry system is an open-circuit system that determines  $O_2$  consumption,  $CO_2$  production, and respiratory quotient ( $RQ = VCO_2/VO_2$ , where  $V$  is volume), respiratory exchange rate and heat. A photobeam-based activity monitoring system detects and records every ambulatory movement, including rearing and climbing movements in every cage. The sensors for detection of movement operate efficiently in both light and dark phases, allowing continuous recording. All of the parameters were measured continuously and simultaneously in all animals over the subsequent 7 d after 5 d of adaptation in identical training cages.

### **Fecal fat excretion**

For the collection of feces, the mice were housed in metabolic cages for 72 h at the end of the weight gain and weight reduction periods. The intake of feed and drink was monitored daily and feces collected at the end of the 72 h period. The feces were weighed and stored in -70°C until assayed. The fat content of the fecal samples was determined by SBR (Schmid-Bondzynski-Ratzlaff) method modified for fecal sample analysis<sup>[23]</sup>. The apparent fat absorption was calculated from the amount of feed consumed and the amount of fat excreted during the housing in metabolic cages. Apparent fat absorption (%) was determined as  $100 \times (\text{fat intake-fecal fat})/(\text{fat intake})$ . To estimate the effect of fat excretion on energy absorption during the whole study period, we calculated the apparent cumulative energy absorption from fat using the cumulative energy intake data (apparent fat absorption %  $\times$  cumulative energy intake from fat) as described previously<sup>[24]</sup>.

### **Blood glucose and serum insulin**

Blood glucose and was analysed from the blood samples taken at the termination of the animals. Blood glucose was determined by glucometer (Super Glucocard™ II, GT-1630, Arkray Factory Inc., Shiga, Japan). Serum insulin was analysed from frozen serum samples by ELISA kit for mouse insulin (Ultra sensitive Mouse Insulin ELISA kit 90080, Crystal Chem Inc., IL, USA).

### **The sample preparation**

At the end of the treatment period, the mice were rendered unconscious with  $CO_2/O_2$  (95%/5%; AGA, Riihimäki, Finland) and decapitated. The blood samples were taken into chilled plastic tubes, and the serum was separated by centrifugation at 4°C for 15 min. The livers and subcutaneous, epididymal, abdominal and perirenal

fat pads were removed, washed with saline, blotted dry and weighted. The tissue samples for lipidomic and primary metabolite analysis were snap-frozen in liquid nitrogen and stored at -80°C until assayed. The samples for oil red O-staining were frozen in isopentane (-38°C) and stored at -80°C until further processed. The samples for histology were fixed in 40 g/L formaldehyde and embedded in paraffin with routine techniques.

### **Liver histology and Oil Red O staining**

For histological evaluation of the liver samples 4  $\mu\text{m}$  sections of the paraffin embedded samples were cut with a microtome, stained with H&E and examined with a light microscopy. The severity of the observed lesions was graded as previously described<sup>[25]</sup>.

In order to determine the relative amount of lipids in the liver samples, frozen sections (4  $\mu\text{m}$ ) were stained with Oil Red O, mounted and photographed. From the obtained microscopic images, the amount of Oil Red O-positive staining was determined with AnalySIS Pro-software (Soft Imaging System, Münster, Germany).

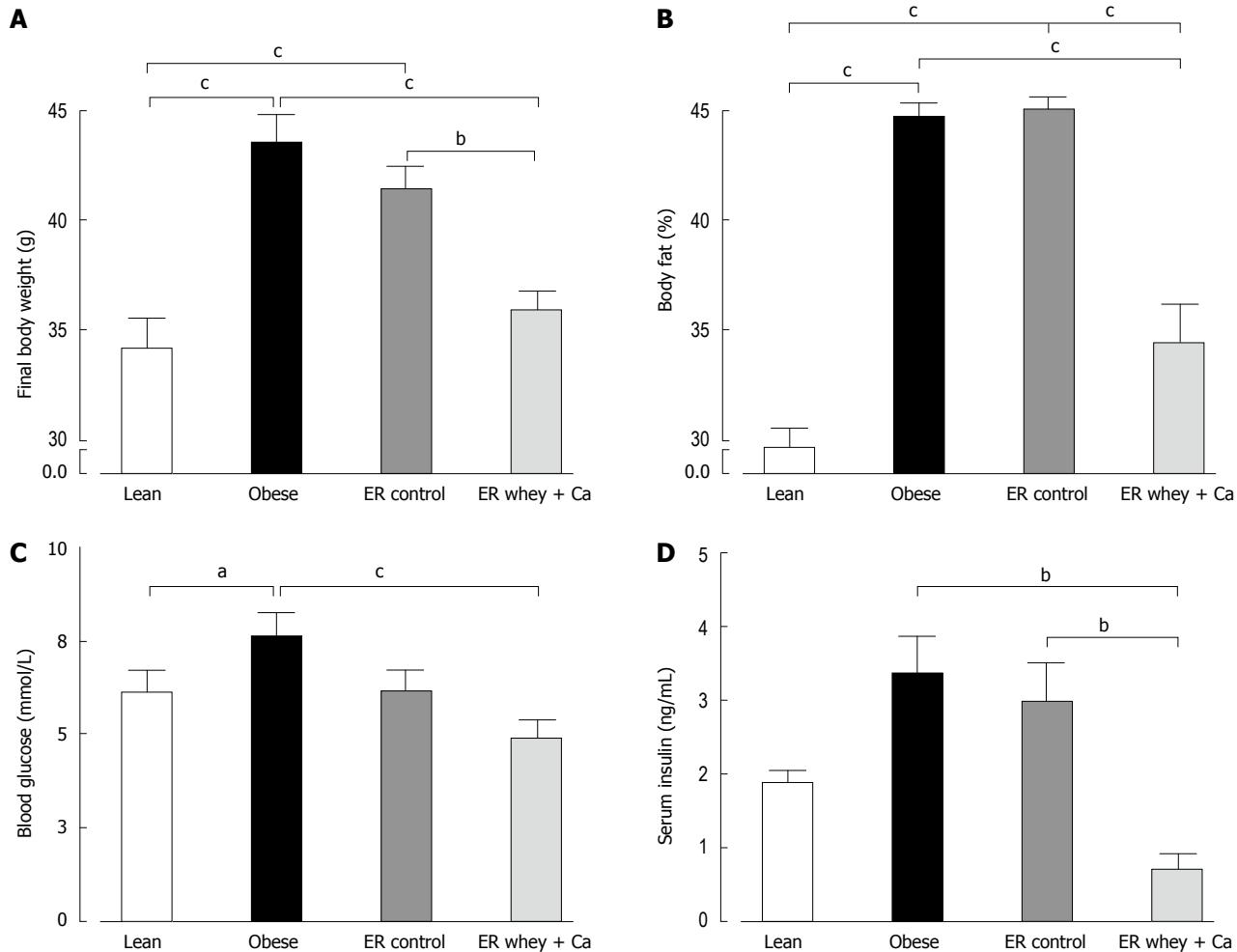
### **Lipidomics**

The lipidomic analysis of liver tissue samples ( $n = 10/\text{group}$ ) was performed as described previously described<sup>[26]</sup>. Liver tissue lipid extracts were examined by a Q-ToF Premier mass spectrometer by introducing the sample through an Acquity UPLCTM system equipped with an Acquity UPLCTM BEH C18 1 mm  $\times$  50 mm column with 1.7  $\mu\text{m}$  particles. The compounds were detected by using electrospray ionization in positive ion mode (ESI+). Data was collected at  $m/z$  300-1200 with a scan duration of 0.2 s. Data was processed using MZmine software version 0.60<sup>[27,28]</sup>, and metabolites were identified using internal spectral library or with tandem mass spectrometry as previously described<sup>[6,29]</sup>.

### **Primary metabolites**

Twenty mg of frozen liver tissue ( $n = 10/\text{group}$ ) was weighed into Eppendorf tubes and 200  $\mu\text{L}$  of methanol (-80°C) and 10  $\mu\text{L}$  of  $^{13}\text{C}$  labeled internal standard was added. Sample was homogenized with Micro Dismembrator S (Sartorius, Germany) by using glass beads (0.5-0.75 mm) and 3000 r/min for 3 min. Homogenized samples were boiled immediately in 80°C for 3 min and at 10000 r/min for 5 min. Supernatant was collected and evaporated to dryness under a stream of nitrogen. Samples were reconstituted in 100  $\mu\text{L}$  of ultra pure water.

The liver extracts were analyzed with HPLC-MS/MS method for quantitative analysis of phosphorous and TCA-cycle compounds. The system consisted of HT-Alliance HPLC (Waters, Milford, MA, USA) working at high pH. The analytes were resolved by anion exchange chromatography combined with post column ASRS Ultra II 2 mm ion suppressor (Dionex, Sunnyvale, CA) and detected with Quattro Micro triple quadrupole mass spectrometry (Waters, Milford, MA, USA) operating in electrospray negative ion mode. The analytical column



**Figure 1** A: The body weight of C57Bl/6J mice at the end of the study; B: The body fat content of C57Bl/6J mice measured by DEXA at the end of the study; C: The blood glucose of C57Bl/6J mice at the end of the study; D: The serum insulin of C57Bl/6J mice at the end of the study. Data is presented as mean  $\pm$  SE. The letters denote a significant difference between the groups (<sup>a</sup> $P < 0.05$ ; <sup>b</sup> $P < 0.01$ , <sup>c</sup> $P < 0.001$ ;  $n = 10/\text{group}$ ).

was IonPac AS11 (2 mm  $\times$  250 mm, Dionex, Sunnyvale, CA) and guard column IonPac AG11 (2 mm  $\times$  50 mm, Dionex, Dionex Sunnyvale, CA). Flow rate was 250  $\mu\text{L}/\text{min}$  and injection volume 5  $\mu\text{L}$ . The temperature of the column was 35°C and autosampler 10°C.

The compounds were detected in Multiple Reaction Monitoring mode for optimal sensitivity and selectivity. Mass spectrometric parameters, cone voltage and collision energy were optimized for each component. A small aliquot of <sup>13</sup>C-labelled metabolites from yeast-fed batch cultivation was used as an internal standard for both calibration standards and samples. Hexose phosphates (glucose-6-phosphate, fructose-6-phosphate, mannose-6-phosphate and 6-glucose-1-phosphate), pentose phosphates (ribose-5-phosphate and ribulose-5-phosphate), fructose bisphosphate, glycerate-2-phosphate and glycerate-3-phosphate, phosphoenolpyruvate, 6-phosphogluconate, succinate, malate,  $\alpha$ -ketoglutarate, oxaloacetate, citrate, iso-citrate, glyoxylate and pyruvate were quantitatively measured with this method. Data was processed with MassLynx 4.1 software and internal calibration curves were calculated based on response of <sup>12</sup>C analyte and <sup>13</sup>C labelled analogue.

### Statistical analysis

Data are presented as mean  $\pm$  SEM. Statistically significant difference in mean values were tested by ANOVA followed by Tukey's test. The data were analysed using GraphPad Prism, version 4.02 (GraphPad Software, Inc., San Diego, CA, USA). Statistical analyses of metabolomics data were performed using R statistical software ([www.r-project.org](http://www.r-project.org)).

## RESULTS

### Weight and fat loss and fat absorption during ER

The body weight of the high-fat fed mice increased significantly during the 14 wk ad libitum feeding. At the end of the weight gain period the high-fat fed mice weighed significantly more than the chow fed control mice (Figure 1A). The obese mice also had significantly more fat tissue than the lean controls (Figure 1B). The 7-week ER reduced the body weight in the whey + Ca group, to the level of lean controls, but the decrease in body weight was not statistically significant in the control group. Whey + Ca also reduced the fat pad weights more than the weight loss on control diet

**Table 1** Fat pad weights (g)

	Lean	Obese	ER		ANOVA P value
			Control	Whey + Ca	
Subcutaneous fat	0.4 ± 0.1 <sup>c</sup>	1.6 ± 0.1	1.4 ± 0.1 <sup>d,e</sup>	1.0 ± 0.1 <sup>c,d</sup>	< 0.0001
Epididymal fat	1.4 ± 0.1 <sup>a</sup>	1.9 ± 0.1	1.8 ± 0.1 <sup>f</sup>	1.3 ± 0.05 <sup>b</sup>	0.0006
Perirenal fat	0.7 ± 0.1 <sup>c</sup>	1.3 ± 0.1	1.4 ± 0.1 <sup>d,f</sup>	0.9 ± 0.1 <sup>b</sup>	< 0.0001

Data is presented as mean ± SE ( $n = 10$ /group). <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , <sup>c</sup> $P < 0.001$ , vs obese, respectively; <sup>d</sup> $P < 0.001$  vs lean; <sup>e</sup> $P < 0.05$ , <sup>f</sup> $P < 0.01$  vs whey + Ca, respectively.

**Table 2** Incidences of the observed histopathological lesions in liver samples

	Lean	Obese	ER	
			Control	Whey + Ca
Number of samples	8	10	10	10
No abnormalities detected	8	0	0	0
Macrovesicular fatty change (total)	0	10	10	10
Severe	0	1	0	0
Marked	0	1	0	0
Moderate	0	5	5	4
Slight	0	3	5	5
Minimal	0	0	0	1
Infiltration of inflammatory cells, minimal	0	1	1	3
Focal hepatocyte necrosis, total	0	2	0	2
Slight	0	1	0	0
Minimal	0	1	0	2

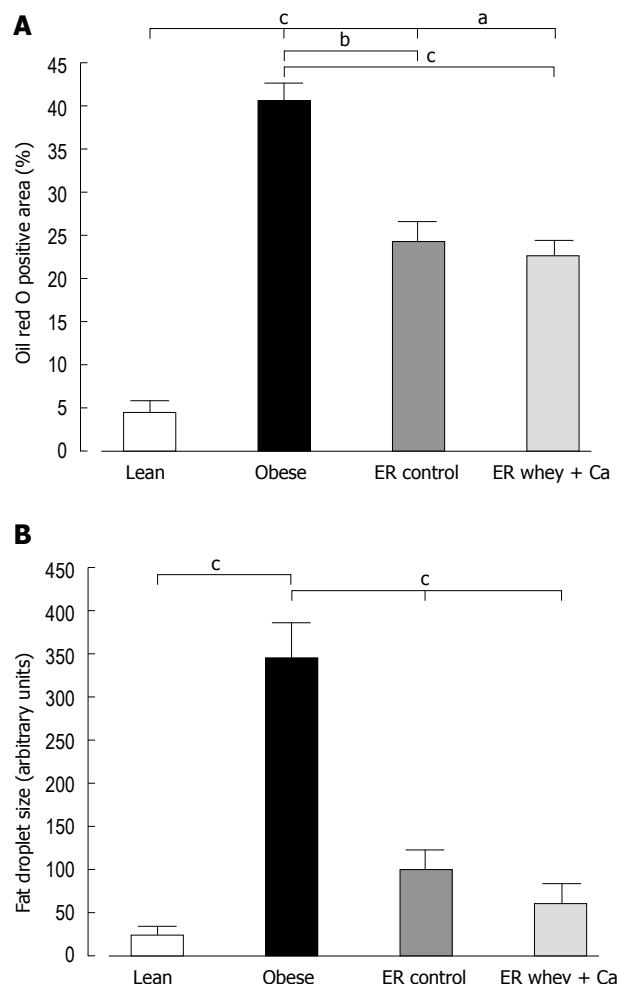
(Table 1). Apparent fat absorption was reduced in the whey + Ca group in comparison with the control diet ( $96.9\% \pm 0.3\%$  vs  $98.4\% \pm 0.1\%$  in whey + Ca and control diet, respectively;  $P = 0.0004$ ).

#### Blood glucose, serum insulin and liver histology

ER on both diets reduced the blood glucose to the level of lean controls, but the decrease was statistically significant only in the whey + Ca group (Figure 1C). Also the serum insulin was significantly decreased only in the whey + Ca group (Figure 1D). In the obese group, the liver histology showed an evident macrovesicular fatty change of diffuse pattern, with severity ranging from slight to severe (Table 2). In the ER groups, the observed fatty change was less severe. The fat droplets were smaller and mainly present in the perivenular regions. Minimal foci of inflammatory cells and necrotic hepatocytes were occasionally noted, but fibrosis was absent. Oil Red O-staining demonstrated that ER on control and whey + Ca diet significantly reduced hepatic lipid accumulation and lipid droplet size (Figure 2), but the amount of fat did not reach the level observed in the lean mice.

#### The effect of protein source on metabolic performance

In order to investigate whether the more pronounced weight loss effect in the whey protein fed mice was a result of differences in drinking and feeding behaviour, increased activity or changes in metabolic performance,

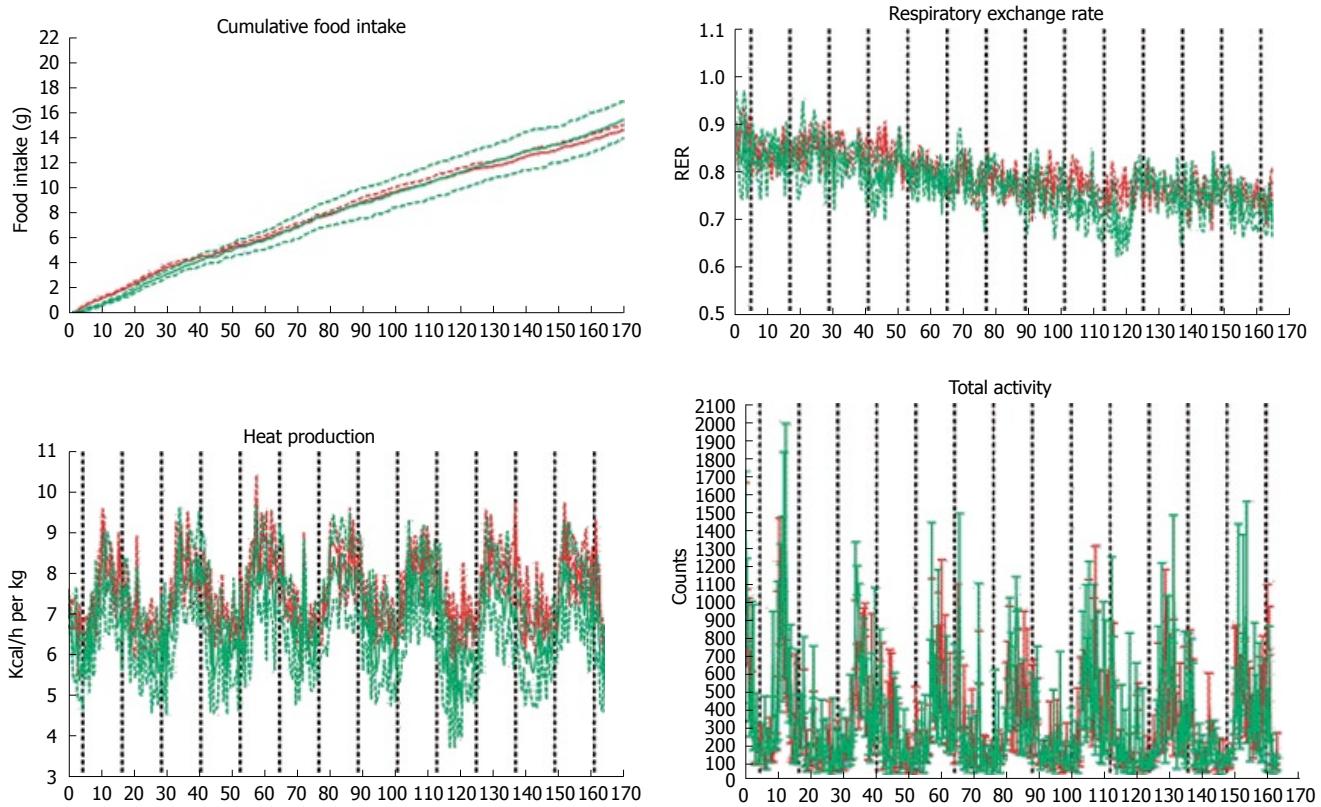


**Figure 2** **A:** The Oil Red O positive area of paraffin embedded liver samples of C57Bl/6J mice at the end of the study; **B:** The mean fat droplet area (arbitrary units) of paraffin embedded liver samples of C57Bl/6J mice at the end of the study. Data is presented as mean ± SE. The letters denote the significant difference between the groups (<sup>a</sup> $P < 0.05$ ; <sup>b</sup> $P < 0.01$ , <sup>c</sup> $P < 0.001$ ;  $n = 10$ /group).

an additional group of mice were housed in a home cage based monitoring system. A 7-day monitoring did not reveal differences in cumulative feed or water intake, respiratory exchange rate, heat production,  $O_2$  consumption,  $CO_2$  production, total or rearing activity or ambulatory movements during the observation period (Figure 3).

#### The effect of ER on liver lipid profile

Of the total 2498 hepatic lipid peaks detected, 391 major peaks were identified and included in further analysis. The reduction of lipids was mainly seen in the level of triacylglycerols (TAG) and ceramides and ER on whey + Ca diet even restored the level of ceramides to the level of lean mice (Figure 4). The amount of cholesterol esters was significantly increased in both ER groups. The TAG to phospholipid ratio, which reflects the relation of membrane lipids to storage lipids, was significantly reduced only in whey + Ca group, but it was still higher than in the lean controls (Figure 5A). Interestingly, diacylglycerol (DAG) to phospholipid ratio was increased in the control ER group, but decreased in



**Figure 3** A: Cumulative food intake over 7 d analyzed by LabMaster system. The cumulative total food intake was similar between the groups; B: LabMaster analysis of respiratory exchange rate (RER); C: Heat production measured by LabMaster; D: Total ambulatory movement did not differ between the groups. In all figures data is presented as mean  $\pm$  SE,  $n = 4$  in casein group (red) and  $n = 3$  in whey group (green).

the whey + Ca ER group (Figure 5B). ER on whey + Ca diet also totally reversed the obesity-induced increase in the ceramide to sphingomyelin ratio (Figure 5C). Phosphatidic acid (GPA) and phosphatidylglycerol (GPGro) peaks could not be uniquely distinguished by our method. The level of GPA/GPGro, phosphatidylcholines and lysophosphatidylethanolamines was not affected by obesity or ER.

The most significantly changed lipids are presented in Figure 6. Interestingly TAG (58:3) and TAG (58:2) were at higher level in the control weight loss group even though they were already increased as a result of obesity, and so was the level of TAG (50:8) in the whey + Ca group. Weight loss further increased the level of GPCho (32:2) even though its level was already over 10 times higher in the obese group than in the lean mice. On the other hand, the level of TAG (52:0) decreased during ER even though its level was already lower in obese than in the lean animals.

The most distinct features of whey + Ca ER group were the significant increases in the level of total phosphatidylserines, phosphatidylethanolamines and sphingomyelins (Figure 6). It is also of note that the level of certain phosphatidylcholine species was significantly decreased during ER on control diet whereas there was no change in whey + Ca group. Whey + Ca specifically affected certain ceramide species [Cer (d18:0/22:5), Cer (d18:0/22:6), Cer (d18:1/23:3), Cer (d18:1/23:5), Cer (d18:1/26:4)], whose level was reduced to the level of

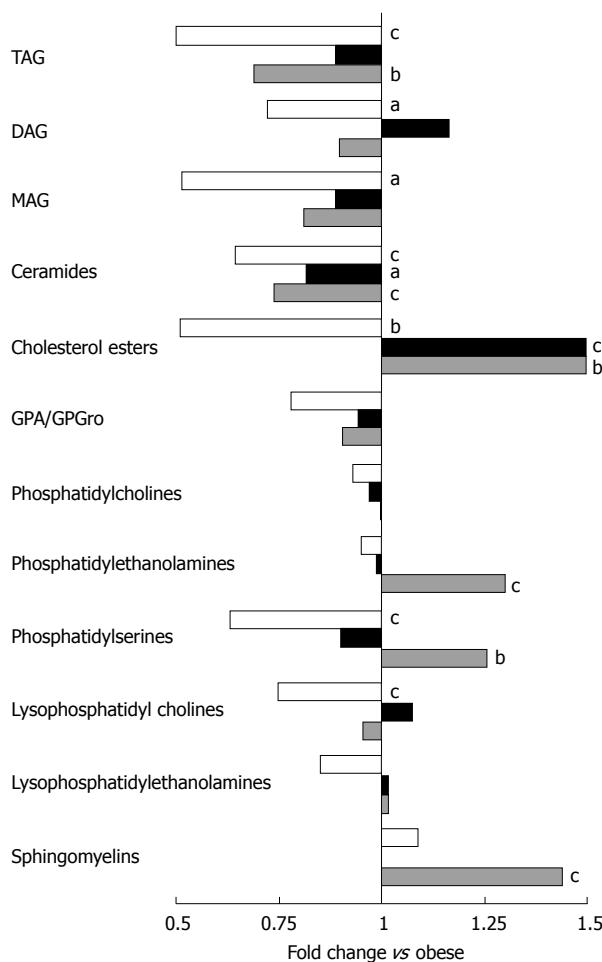
lean mice, whereas their level was unaffected by ER on control diet. Cer (d18:1/25:4) was even increased by ER on control diet while its level did not differ between lean, obese and whey + Ca group.

#### The effect of ER on primary metabolites

The primary metabolite analysis led to identification of 13 metabolites (glucose-6-phosphate, fructose-6-phosphate, mannose-6-phosphate, fructose bisphosphate, glycerate-3-phosphate, ribose-5-phosphate, succinate, malate, citrate, pyruvate, phosphoenolpyruvate, 6-phosphogluconate and fumarate). The high-fat diet feeding and subsequent obesity led to reduction of glycolytic metabolites, such as glucose-6-phosphate, fructose-6-phosphate and pyruvate (Table 3, Figure 7). ER with whey + Ca diet was associated with significant increases of succinate, which belongs to the TCA cycle and of ribose-5-phosphate, which is a product of pentose phosphate pathway. Whey + Ca diet also decreased the level of glycolytic metabolites glucose-6-phosphate, fructose-6-phosphate and fructose bisphosphate in contrast with ER on control diet, which did not affect the level of these metabolites.

#### DISCUSSION

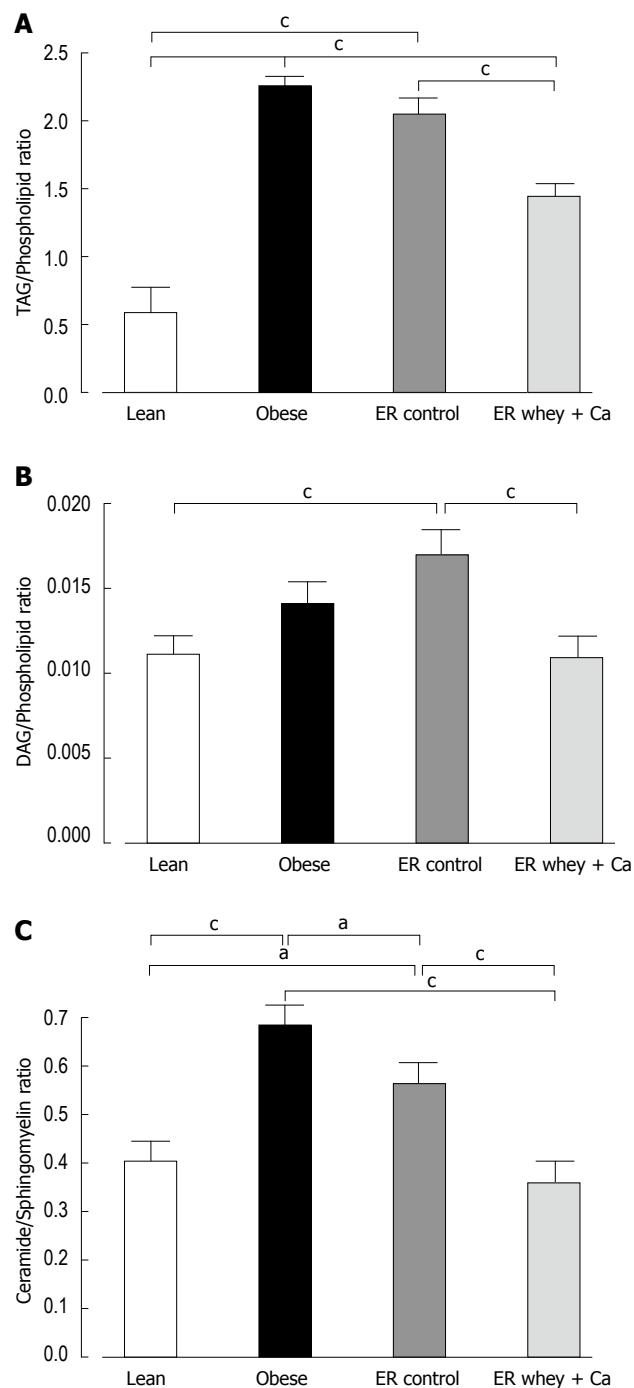
In this study, we showed that decreasing liver fat by ER significantly modulates the overall profile of liver



**Figure 4** Mean fold changes in lipid classes in lean (white), ER control (black) and ER whey + Ca (grey) groups in relation to the obese group ( $n = 10/\text{group}$ ). The letters denote a significant difference in comparison with the obese group ( $^{\circ}P < 0.05$ ,  $^{\circ}P < 0.01$ ,  $^{\circ}P < 0.001$ ).

lipid species. The main finding of the study was that the protein source and calcium content of the diet had a significant effect on the ER-induced hepatic lipid changes. Even though the histological analysis did not reveal significant differences in the amount of liver fat between the ER groups, the metabolomic data demonstrated that ER on whey + Ca diet was able to reduce the relative level of potentially diabetogenic ceramides and diacylglycerols to the level observed in lean animals. This finding is in accordance with the decreased level of serum insulin in this group. These changes were accompanied by a decrease in glycolytic metabolites while the metabolites from the pentose phosphate pathway and TCA cycle were increased together with a shift towards gluconeogenesis.

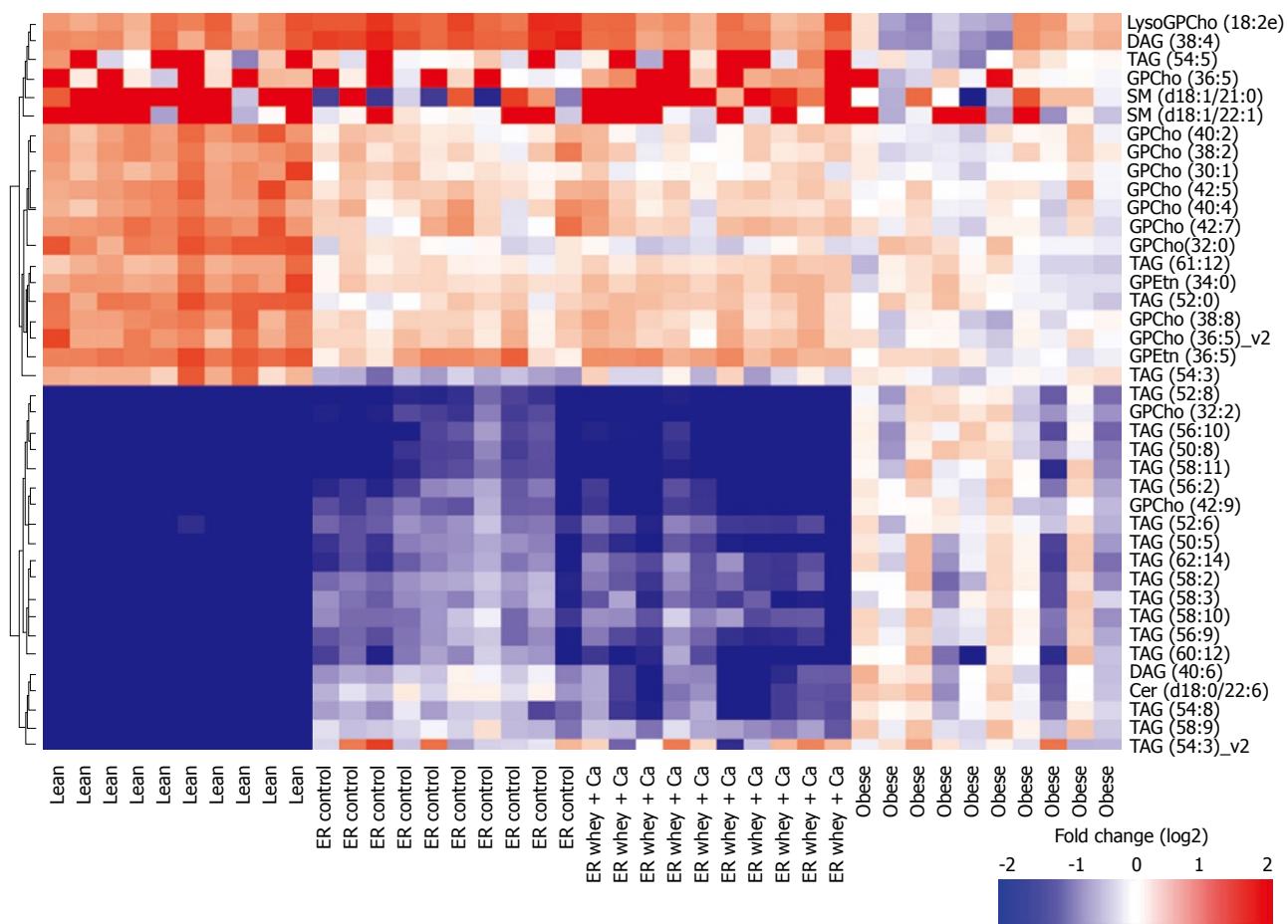
The UPLC/MS based lipidomics platform and the HPLC/MS/MS based primary metabolite platform techniques were used to characterise the hepatic lipid and primary metabolite changes in this study. These techniques provide an overview of key metabolites involved in energy metabolism, including a broad profile covering all major lipid classes present in liver as well as key metabolites of the central carbon metabolism. Traditional analyses of lipids have been generally limited



**Figure 5** A: The liver TAG/Phospholipid ratio; B: The liver DAG/Phospholipid ratio; C: The liver Ceramide/Sphingomyelin ratio. Lipids measured by UPLC/MS. Data is presented as mean  $\pm$  SE. The letters denote a significant difference between the groups ( $^{\circ}P < 0.05$ ;  $^{\circ}P < 0.01$ ;  $^{\circ}P < 0.001$ ;  $n = 10/\text{group}$ ).

to investigations of lipid class- or fatty acid-specific changes<sup>[30]</sup>. These new analytical methods combined with information technology provide extremely sensitive tools to measure the extended metabolome, and may help to explore the mechanisms of many complex diseases<sup>[31,32]</sup>. However, one evident shortcoming of the method is that a major part of the spectral peaks are still unidentified.

To our knowledge this is the first study to demonstrate the effect of ER on fatty liver lipidomic and primary metabolite profile in diet induced obese mice. In



**Figure 6** Twenty most significantly up- and down-regulated lipids between obese and lean group. Fold change for each individual mouse within each group as a log<sub>2</sub> ratio between the lipid concentration in individual sample and the median lipid concentration in the obese group. Hierarchical clustering using Ward linkage was applied.

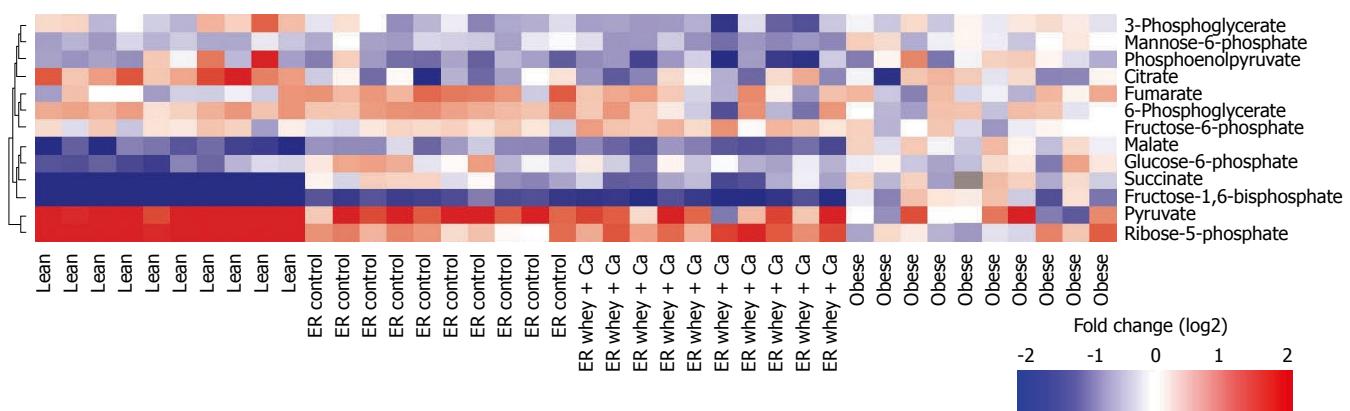
**Table 3** Concentrations of primary metabolites in liver samples ( $\mu\text{mol/g}$  tissue)

	Lean	Obese	ER		ANOVA P value
			Control	Whey + Ca	
Glucose-6-phosphate	28.6 ± 2.7 <sup>a</sup>	19.2 ± 3.5	22.7 ± 1.7 <sup>b</sup>	11.2 ± 3.2 <sup>f</sup>	< 0.0001
Fructose-6-phosphate	7.0 ± 0.7	4.9 ± 0.6	6.9 ± 0.7 <sup>b</sup>	3.2 ± 0.9 <sup>e</sup>	0.001
Mannose-6-phosphate	1.7 ± 0.2	1.7 ± 0.2	1.4 ± 0.1 <sup>g</sup>	0.8 ± 0.2 <sup>c,f</sup>	0.0003
Fructose bisphosphate	3.2 ± 0.6	8.7 ± 2.0	9.5 ± 2.4 <sup>d,h</sup>	0.5 ± 0.1 <sup>b</sup>	0.0005
Glycerate-3-phosphate	26.2 ± 3.7	21.0 ± 0.8	17.4 ± 1.2 <sup>d</sup>	13.1 ± 1.4 <sup>f</sup>	0.0008
Ribose-5-phosphate	0.3 ± 0.02 <sup>a</sup>	0.7 ± 0.1	0.7 ± 0.1 <sup>i,i</sup>	1.3 ± 0.1 <sup>c,f</sup>	< 0.0001
Succinate	24.5 ± 3.3 <sup>c</sup>	5.3 ± 1.1	6.7 ± 2.4 <sup>f,i</sup>	24.0 ± 4.0 <sup>c</sup>	< 0.0001
Malate	42.0 ± 5.2 <sup>a</sup>	54.5 ± 3.7	61.3 ± 4.7 <sup>d,g</sup>	40.5 ± 5.1 <sup>a</sup>	0.008
Citrate	4.9 ± 0.7 <sup>c</sup>	2.0 ± 0.3	1.6 ± 0.2 <sup>f</sup>	1.8 ± 0.3 <sup>f</sup>	< 0.0001
Pyruvate	5.6 ± 0.8 <sup>c</sup>	0.9 ± 0.2	2.6 ± 0.4 <sup>e</sup>	1.5 ± 0.3 <sup>f</sup>	< 0.0001
Phosphoenolpyruvate	2.8 ± 0.8	2.3 ± 0.3	1.4 ± 0.2	1.2 ± 0.2	0.0426
6-phosphogluconate	3.5 ± 0.2 <sup>a</sup>	2.4 ± 0.2	3.7 ± 0.2 <sup>e,g</sup>	2.6 ± 0.4 <sup>a</sup>	0.0023
Fumarate	9.6 ± 1.0 <sup>a</sup>	10.4 ± 1.1	17.8 ± 1.7 <sup>b,f,g</sup>	11.9 ± 1.3 <sup>a</sup>	0.0003

Data is presented as mean  $\pm$  SE ( $n = 10$ /group). <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , <sup>c</sup> $P < 0.001$  vs obese, respectively; <sup>d</sup> $P < 0.05$ , <sup>e</sup> $P < 0.01$ , <sup>f</sup> $P < 0.001$  vs lean, respectively; <sup>g</sup> $P < 0.05$ , <sup>h</sup> $P < 0.01$ , <sup>i</sup> $P < 0.001$  vs whey + Ca, respectively.

accordance with previous studies on lipidomic profile of non-alcoholic fatty liver disease, we also found increased levels of TAG, DAG and specific ceramide species and down-regulation of sphingomyelins in the obese group<sup>[6]</sup>. Interestingly, only ceramides were significantly decreased by ER on control diet, while the level of DAG increased non-significantly and sphingomyelins stayed un-changed.

However, ER on whey + Ca diet significantly increased the level of sphingomyelins and decreased the level of DAG changing the ceramide/sphingomyelin and DAG/phospholipid ratios to the level of lean animals. The accumulation of both ceramides and DAG in peripheral tissues contribute to insulin resistance<sup>[33-35]</sup> and, therefore, the decrease of these lipids can be considered



**Figure 7** Primary metabolite profiles for each individual mouse as a log<sub>2</sub> ratio between the metabolite concentration in individual samples and the median metabolite concentration in the obese group. Hierarchical clustering using Ward linkage was applied.

particularly beneficial.

Additionally an increase in liver cholesterol ester level, which was seen in both of the ER groups, has been demonstrated to occur also as a result of acute 24 h food deprivation<sup>[36]</sup>. One of the main functions of cholesterol is maintaining of membrane fluidity by interacting with other membrane lipid components such as phosphatidylcholines and sphingomyelin<sup>[37]</sup>. However, only cholesterol esters were found to be increased as a result of ER in the control group.

The level of primary metabolites was particularly affected in the whey + Ca group. Energy restriction in normal weight, healthy mice, is known to enhance hepatic gluconeogenesis<sup>[38,39]</sup> and suppress glycolysis<sup>[40]</sup>. This effect was particularly pronounced in the whey + Ca group as indicated by the striking decrease of fructose bisphosphate, the key regulator of gluconeogenesis, and significant decrease of glycolytic intermediates glucose-6-phosphate and fructose-6-phosphate. An increased level of ribose-5-phosphate in the whey + Ca group indicates enhanced flux through pentose phosphate pathway, which is known to be triggered by low concentrations of fructose-2,6-bisphosphate<sup>[41]</sup>. ER on whey + Ca diet also decreased the level of succinate to the level of lean animals, whereas the level of succinate did not change in the ER control group. The significance of the decrease of mannose-6-phosphate in whey + Ca groups remains to be elucidated.

One of the few dietary components which are known to influence the liver fat profile during ER is the type and amount of dietary fat<sup>[8]</sup>. In this study there were no differences in either the type or amount of dietary fat between the ER groups. However, the apparent fat absorption was decreased in the whey + Ca group. Calcium preferentially binds saturated fatty acids in the intestine<sup>[42]</sup>, and therefore, also the quality of the absorbed fat might have been influenced in the whey + Ca group.

Even though these findings may help to understand why increased dairy calcium intake may lower the risk of metabolic syndrome, the molecular mechanism by which whey protein and calcium modulate the liver lipid profile remain unanswered. Whey protein consists of several

small protein types, including alfa-lactalbumin, beta-lactoglobulin, bovine serum albumin, lactoferrin and other minor peptides<sup>[43]</sup>. In order to investigate the possible effects of whey protein on energy expenditure and food intake, we measured the metabolic performance of mice fed either casein or whey based diet in a calorimetry system, but did not see any differences between the proteins. The principal question regarding the mechanism is whether the beneficial effect is derived only from the amino acids or if bioactive peptides are formed during the digestion and absorption of the protein.

The present study demonstrates that ER-induced changes in fatty liver are significantly affected by dietary protein source and calcium. Reducing liver fat by ER is currently the main treatment for non-alcoholic fatty liver disease and therefore, it is crucial to understand which dietary factors have significant effects on the outcome of ER in liver. These results indicate that whey protein and calcium could be beneficial in the dietary treatment of fatty liver, and are likely contribute to the inverse relationship between dairy intake and the risk of insulin resistance. The therapeutic potential of whey protein and calcium in clinical setting, and the mechanism of action remain to be elucidated.

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## COMMENTS

### Background

Fatty liver is considered to be an important link between obesity and the development of metabolic syndrome and insulin resistance. Liver fat can be reduced by weight loss, but the effect of weight loss on the quality of hepatic lipid profile is currently not well established.

### Research frontiers

Some dietary factors, like the quantity and quality of fat during weight loss have been shown to have an effect on liver fat. However, the importance of dietary protein source and calcium content has not been investigated previously.

### Innovations and breakthroughs

This study characterises the changes in hepatic lipid profile during energy

restriction in a mouse model of diet induced obesity. The effect of protein source and calcium content of the weight loss diet is also studied. This study demonstrates for the first time that dietary protein source may beneficially modulate the lipid profile of fatty liver during energy restriction.

### Applications

Weight loss by life style changes is the main therapeutic approach to reduce liver fat. Therefore, it is crucial to identify dietary components which may improve the outcome of weight loss in the level of hepatic lipid profile. These results indicate that whey protein and calcium beneficially modulate the hepatic lipid profile targeting specifically the lipotoxic diacylglycerol and ceramide species.

### Peer review

The study is well conducted and has important information about the pathophysiology of fatty liver.

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# Lysophosphatidic acid induced nuclear translocation of nuclear factor- $\kappa$ B in Panc-1 cells by mobilizing cytosolic free calcium

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kinase C inhibitor, attenuated translocation of NF- $\kappa$ B induced by LPA.

**CONCLUSION:** These findings suggest that protein kinase C is activated endogenously in Panc-1, and protein kinase C is essential for activating NF- $\kappa$ B with cytosolic calcium and that LPA induces the nuclear translocation of NF- $\kappa$ B in Panc-1 by mobilizing cytosolic free calcium.

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**Key words:** Lysophosphatidic acid; Nuclear translocation; Nuclear factor- $\kappa$ B; Cytosolic free calcium; Panc-1

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## Abstract

**AIM:** To clarify whether Lysophosphatidic acid (LPA) activates the nuclear translocation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) in pancreatic cancer.

**METHODS:** Panc-1, a human pancreatic cancer cell line, was used throughout the study. The expression of LPA receptors was confirmed by reverse-transcript polymerase chain reaction (RT-PCR). Cytosolic free calcium was measured by fluorescent calcium indicator fura-2, and the localization of NF- $\kappa$ B was visualized by immunofluorescent method with or without various agents, which effect cell signaling.

**RESULTS:** Panc-1 expressed LPA receptors, LP<sub>A1</sub>, LP<sub>A2</sub> and LP<sub>A3</sub>. LPA caused the elevation of cytosolic free calcium dose-dependently. LPA also caused the nuclear translocation of NF- $\kappa$ B. Cytosolic free calcium was attenuated by pertussis toxin (PTX) and U73122, an inhibitor of phospholipase C. The translocation of NF- $\kappa$ B was similarly attenuated by PTX and U73122, but phorbol ester, an activator of protein kinase C, alone did not translocate NF- $\kappa$ B. Furthermore, the translocation of NF- $\kappa$ B was completely blocked by Ca<sup>2+</sup> chelator BAPTA-AM. Thapsigargin, an endoplasmic reticulum Ca<sup>2+</sup>-ATPase pump inhibitor, also promoted the translocation of NF- $\kappa$ B. Staurosporine, a protein

## INTRODUCTION

Lysophosphatidic acid (LPA) is the smallest and structurally simplest of all glycerophospholipids, and exists in serum at concentrations between 1-5  $\mu$ mol/L<sup>[1]</sup>. LPA is mainly released by activated platelets<sup>[2]</sup>, and immediately complexes with high affinity to serum albumin<sup>[3]</sup>. It is well known that LPA exhibits hormone- and growth factor-like activities<sup>[4,5]</sup>. LPA represents a major bioactive constituent of serum. LPA increases [<sup>3</sup>H]-thymidine incorporation, inositol phosphates, intracellular calcium, and protein kinase C activities<sup>[4,5]</sup>. Indeed, LPA acts on a large number of cells to achieve a broad range of immediate and long lasting effects. Specific responses to LPA include changes in cell shape and tension, chemotaxis, proliferation and differentiation<sup>[6,7]</sup>. LPA binds to putative G protein-coupled receptors found on nearly all cell types, including cancer cell lines. Numerous other cellular

and biochemical responses to LPA have also been documented<sup>[8]</sup>.

The molecular actions of LPA have been characterized best in rodent fibroblasts, where LPA stimulates phosphoinositide hydrolysis<sup>[4]</sup> and promotes the Rho-dependent formation of stress fibers and focal adhesions<sup>[9]</sup>. The stimulation of phosphoinositide hydrolysis is thought to occur through the GTP-binding regulatory protein (G protein) Go or Gq. The formation of stress fibers and focal adhesions is consistent with activation of Rho through G<sub>i0</sub> or G<sub>i1</sub><sup>[8]</sup>. Whether G proteins are sufficient for this action is unclear, but the sensitivity of the phenomenon to pertussis toxin (PTX) implies that G<sub>i0</sub> represents at least one necessary input. Receptors that recognize LPA have been identified that conform to the seven-transmembrane domain motif characteristic of G protein-coupled receptors, and identified as LP<sub>A1</sub>/Edg-2, LP<sub>A2</sub>/Edg-4, LP<sub>A3</sub>/Edg-7<sup>[10]</sup>.

On the other hand, nuclear factor-κB (NF-κB) is the prototype of a family of dimers whose constituents are members of the Rel family of transcription factors<sup>[11]</sup>. In most types of cells, NF-κB is present as a heterodimer comprising p50 (NF-κB1) and p65 (RelA). NF-κB is normally retained in the cytosol in an inactive form through interaction with I κB inhibitory proteins. Release of NF-κB for translocation to the nucleus and interaction with cognate DNA sequences is accomplished through a signal-induced phosphorylation and subsequent degradation of I κB. Originally described as a necessary element for expression of the immunoglobulin gene in mature B cells, NF-κB is now recognized to be an important transcriptional regulatory protein in a variety of cell types.

It is reported that LPA translocates NF-κB to the nucleus in fibroblasts<sup>[12]</sup>, lymphocytes<sup>[13]</sup>, breast cancer cells<sup>[14]</sup>, colon cancer cells<sup>[15]</sup>, prostate cancer cells<sup>[16]</sup>, and ovarian cancer cells<sup>[17]</sup>. However, until now it is not clear whether LPA translocates NF-κB in pancreatic cancer cells or not. In this study, we demonstrate for the first time that LPA induces translocation of NF-κB to nucleus in the pancreatic cancer cell line, Panc-1, by mobilizing cytosolic free calcium.

## MATERIALS AND METHODS

### Materials

The human pancreatic cancer cell line, Panc-1 (ATCC CRL 1469), was obtained from American Type Culture Collection (MD, USA). Media and supplements were from GIBCO BRL (New York, USA). Fetal bovine serum and fetal calf serum were from Hyclone (Utah, USA). Glass-bottomed chambers were from Costar (Massachusetts, USA). LPA and fatty-acid free albumin were from Sigma (Montana, USA). PTX, staurosporine, genistein and PD98059 were from Calbiochem (California, USA). U73122, U73343 and Fura-2/AM were from Seikagakukogyo (Tokyo, Japan). Rabbit anti-human NF-κB p65 polyclonal antibody was obtained from Upstate Biotechnology (New York, USA); and rhodamine-conjugated donkey anti-rabbit polyclonal

antibody and normal donkey serum from Chemicon International (California, USA), respectively.

### Methods

**Cell culture:** Panc-1 was maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with penicillin (50 units/mL), streptomycin (50 units/mL), and 10% fetal bovine serum (FBS). Panc-1 was maintained in DMEM without FBS for 48 h prior to use for the following experiments.

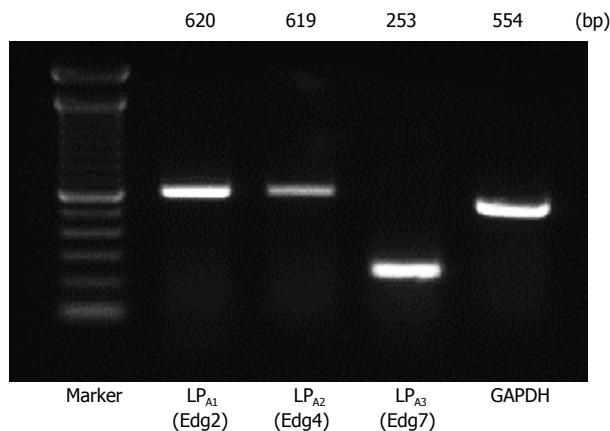
**Total RNA extraction and reverse-transcription polymerase chain reaction (RT-PCR) analysis for detection of LPA receptors:** Total RNA was extracted from Panc-1 using ISOGEN (Nippon Gene, Tokyo, Japan). The first strand cDNA synthesis was carried out using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen™ life technologies, California, USA). One hundred nanogram of the synthesized first strand cDNA was subjected to PCR using Platinum® Taq DNA Polymerase (Invitrogen™ Life Technologies, California, USA). The first strand cDNA was then amplified with specific primers; GAPDH (as an internal control), Edg-2, Edg-4, and Edg-7. The PCR primers and conditions are listed in Table 1. In all cases after the RT step the templates were heated at 94°C for 30 s, annealed for another 30 s, and finally subjected to a 72°C extension for 60 s. The PCR products were separated in 2% agarose gel and visualized under UV illumination.

**Measurement of cytosolic free calcium:** Cytosolic free calcium concentration was measured as previously described<sup>[18]</sup>. Confluent monolayers of Panc-1 cells grown in 175 cm<sup>2</sup> flasks were harvested by incubation in 0.9% (w/v) NaCl, 0.02% (w/v) EGTA and 10 mmol/L Hepes, pH 7.2. Cells were washed twice with PBS, resuspended to a density of 1 × 10<sup>6</sup> cells/mL, and then incubated with 6 μmol/L fura-2/AM for 1 h at room temperature. Aliquots of this suspension were washed twice with Krebs-Ringer's HEPES buffer in a stirred fluorimetry cuvette at 37°C. Fluorescence of fura-2 was measured with Shimazu RF-5000 luminescence spectrometer (alternate excitation wavelengths of 340 nm and 380 nm; emission wavelength, 505 nm). R<sub>max</sub> was determined by the addition of 0.1% Triton X-100, then R<sub>min</sub> was estimated using 4 mmol/L EGTA.

**Immunofluorescence:** Panc-1 cells were cultured on glass-bottomed culture dishes, and experiments were conducted as described above. Panc-1 cells were washed twice with ice-cold PBS and fixed with methanol for 5 min, permeabilized with 0.2% Triton X-100. Once the dishes had air-dried, the cells were incubated in 10% FCS for 2 h to block nonspecific antibody binding. The cells were then incubated with rabbit anti-human NF-κB p65 polyclonal antibody (1:50) in PBS containing 0.2% BSA for 6 h at room temperature. The dishes were then washed three times in PBS with 0.2% BSA for 5 min at room temperature. Cells were then incubated with rhodamine-conjugated donkey anti-rabbit polyclonal

**Table 1** The PCR primers and conditions for detection of LPA receptors

	Sense primer	Antisense primer	Temperature (°C)	Size (bp)
GAPDH	5'-AATGCATCCCTGCACCACCAACTGC-3'	5'-GGAGGCCATGTAGGCCATGAGGTC-3'	59	554
Edg-2	5'-TCCACACACGGATGAGAAC-3'	5'-GTGATCATTCGCTGTGAACCTCC-3'	62	620
Edg-4	5'-CCACCAGCCCATCTACTACCT-3'	5'-CTCACAGCTAAACCATCCAG -3'	62	619
Edg-7	5'-GCTGGAATTGCCTCTGCAAC -3'	5'-ACCACAAACGCCCTAAGAC -3'	62	253



**Figure 1** Expression of LPA receptors in Panc-1 cells. LPA-specific receptors LP<sub>A1</sub>/Edg-2, LP<sub>A2</sub>/Edg-4, and LP<sub>A3</sub>/Edg-7 are expressed in PANC-1 cells. Total mRNA of PANC-1 cells was extracted and used for RT and PCR using primers designed from the sequence of LP<sub>A1</sub>, LP<sub>A2</sub>, and LP<sub>A3</sub>, and GAPDH. PCR products were separated by electrophoresis in 2.0% agarose gel. The molecular size of the amplification products is inferred from the electrophoretic migration of the DNA markers.

antibody (1:100) in PBS containing 0.2% BSA for 1 h at room temperature. The dishes were then washed three times in PBS containing 0.2% BSA for 5 min at room temperature and mounted with glass coverslips using Vectashield mounting medium from Vector Laboratories (California, USA). Immunofluorescence was observed by using an LSM410 Confocal Laser Scanning Microscope from Carl Zeiss, Inc. (Oberkochen, Germany).

**Treatment of Panc-1 cells:** Panc-1 cells were treated with various agents before measuring cytosolic free calcium or detecting translocation of NF- $\kappa$ B. For PTX-pretreatment, monolayers of Panc-1 cells were cultured in DMEM with 100 ng/mL PTX overnight, and then washed twice with PBS. Panc-1 cells were treated with other agents; 10  $\mu$ mol/L U73122 for 10 min, 10  $\mu$ mol/L U73343 for 10 min, 10  $\mu$ mol/L BAPTA for 15 min, 10 nmol/L staurosporine for 10 min, 50  $\mu$ g/mL genistein for 1 h, and 10 nmol/L PD98059 for 1 h before stimulation of LPA.

#### Statistical analysis

The Student's *t*-test was used to determine significant differences between the groups.

## RESULTS

### Panc-1 cells express LP<sub>A1</sub>, LP<sub>A2</sub>, and LP<sub>A3</sub> mRNA

The expression of LP<sub>A1</sub>/Edg-2, LP<sub>A2</sub>/Edg-4, and LP<sub>A3</sub>/

Edg-7 receptors was determined by RT-PCR in Panc-1 cells. As shown in Figure 1, a significant expression of mRNA encoding LP<sub>A1</sub>/Edg-2, LP<sub>A2</sub>/Edg-4, and LP<sub>A3</sub>/Edg-7 was observed, as judged from the appearance of unique cDNA bands of the expected size.

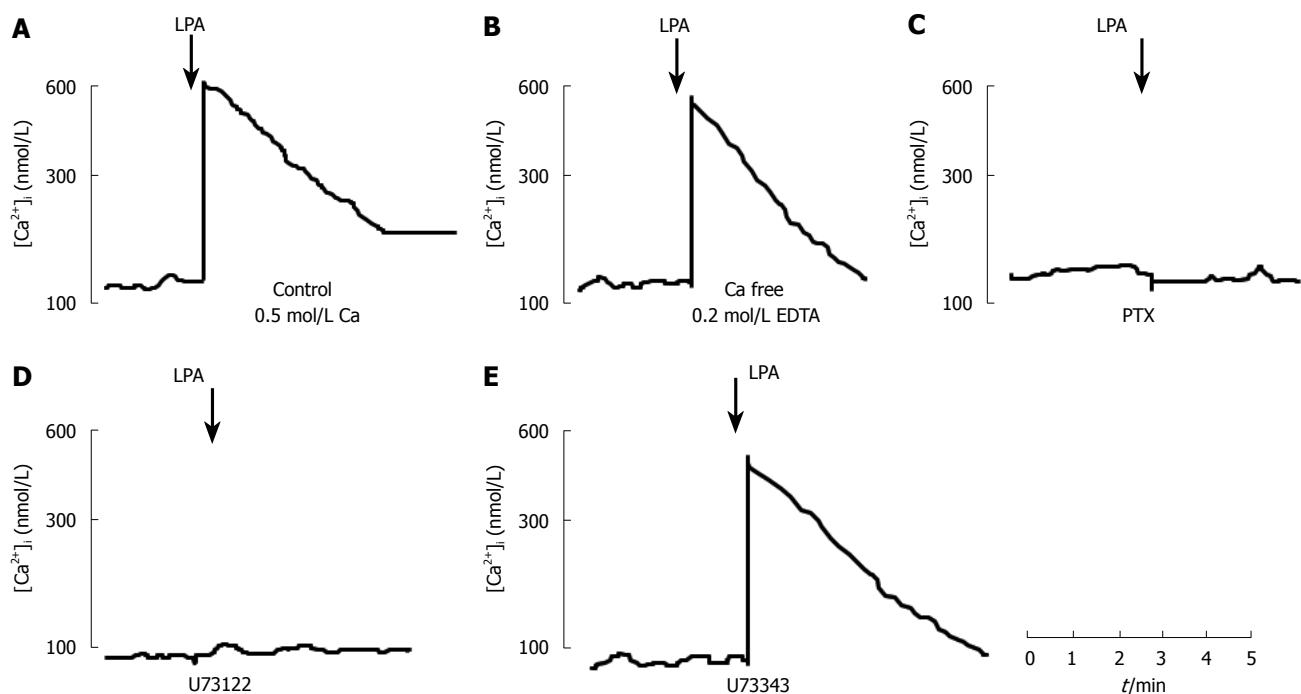
### LPA elevates [Ca<sup>2+</sup>]<sub>i</sub> by mobilizing Ca<sup>2+</sup> from intracellular stores

LPA, at concentration of 10  $\mu$ mol/L, elevated [Ca<sup>2+</sup>]<sub>i</sub> by  $600 \pm 45$  nmol/L ( $n = 5$ ) above a resting [Ca<sup>2+</sup>]<sub>i</sub> of  $108 \pm 30$  nmol/L within 20 s of addition to intact fura2-loaded Panc-1 cell suspension (Figure 2A). This increase in [Ca<sup>2+</sup>]<sub>i</sub> was followed by a return to near resting levels within 3 min. The elevation of [Ca<sup>2+</sup>]<sub>i</sub> by LPA in Panc-1 cells was concentration-dependent, with an EC<sub>50</sub> of 870 nmol/L (Figure 3). Responses to LPA were dependent upon the presence of fatty acid-free BSA (0.1% w/v), without which [Ca<sup>2+</sup>]<sub>i</sub> increases were a quarter-maximal. Fatty acid-free BSA itself did not cause a significant increase in [Ca<sup>2+</sup>]<sub>i</sub> for 30 min (data not shown), as other researchers previously reported<sup>[4]</sup>. LPA-induced elevation of [Ca<sup>2+</sup>]<sub>i</sub> resulted predominantly from mobilization of intracellular [Ca<sup>2+</sup>]<sub>i</sub> store. In the condition of Ca<sup>2+</sup>-free with 0.2 mmol/L EDTA, LPA increased [Ca<sup>2+</sup>]<sub>i</sub> with a similar potency and maximal effect to the condition with extracellular Ca<sup>2+</sup> (Figure 2B). After overnight treatment with PTX (100 ng/mL), LPA did not evoke [Ca<sup>2+</sup>]<sub>i</sub> in Panc-1 cells (Figures 2C and 3). Treatment of Panc-1 cells with a phospholipase C inhibitor, U73122, at a concentration of 10  $\mu$ mol/L for 10 min, abolished LPA-induced increase in [Ca<sup>2+</sup>]<sub>i</sub> (Figure 2D and 3). Pretreated with U73343, an inactive analogue of U73122, at a concentration of 10  $\mu$ mol/L for 10 min had no effect on LPA-induced elevation of [Ca<sup>2+</sup>]<sub>i</sub> in Panc-1 cells (Figure 2E). These findings suggest that LPA induces elevation of [Ca<sup>2+</sup>]<sub>i</sub> by mobilizing Ca<sup>2+</sup> from Ca<sup>2+</sup> store through PTX-sensitive G-protein (Gi or Go) and phospholipase C. Treatment of Panc-1 cells with thapsigargin, the endoplasmic-reticulum Ca<sup>2+</sup>-ATPase pump inhibitor, at a concentration of 500 nmol/L caused a slow increase in [Ca<sup>2+</sup>]<sub>i</sub> followed by a sustained plateau (data not shown).

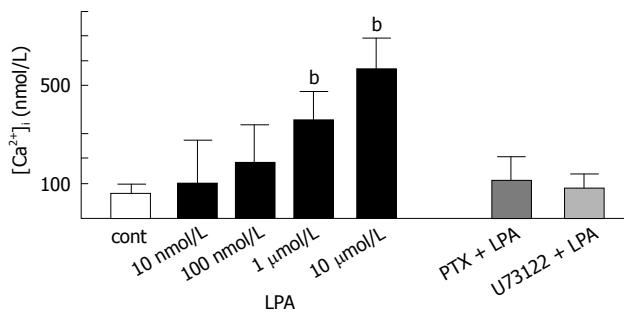
### Effect of LPA on translocation of NF- $\kappa$ B to nuclei in Panc-1 cells

It has been reported that LPA activates NF- $\kappa$ B in fibroblasts<sup>[19]</sup> and endothelial cells<sup>[20]</sup>. We, therefore, investigated the possibility that LPA activates the transcription factor NF- $\kappa$ B in Panc-1 cells.

NF- $\kappa$ B is normally retained in the cytosol in an inactive form in Panc-1 cells (Figure 4A). LPA, at a



**Figure 2** Effect of LPA on cytosolic free calcium concentration  $[Ca^{2+}]_i$  in Panc-1 cells. **A:** Representative  $Ca^{2+}$  signal was evoked by 10  $\mu\text{mol/L}$  LPA in Panc-1 cells; **B:** Effect of chelation of extracellular  $Ca^{2+}$  by addition of 0.2 mmol/L EDTA on LPA-induced increases in  $Ca^{2+}$ ; **C:** PTX-sensitive effect of LPA on cytosolic free calcium. Panc-1 cells were pretreated with 100 ng/ml of PTX for overnight and loaded with fura-2/AM; **D:** Effect of U73122, a phospholipase C inhibitor, on LPA-induced increases in cytosolic free calcium in Panc-1 cells. Panc-1 cells were treated with U73122 at a concentration of 10  $\mu\text{mol/L}$  for 10 min, and then stimulated by 10  $\mu\text{mol/L}$  LPA; **E:** Effect of U73343, an inactive analogue of U73122, on LPA-evoked increases in cytosolic free calcium in Panc-1 cells.



**Figure 3** Effect of LPA on cytosolic free calcium in Panc-1. LPA evoked cytosolic free calcium in concentration-dependent manner. PTX-treatment at a concentration of 100 ng/ml for overnight abolished 10  $\mu\text{mol/L}$  LPA-induced mobilization of  $[Ca^{2+}]_i$ . Pretreatment of U73122 (10  $\mu\text{mol/L}$ ) for 10 min also abolished LPA-induced mobilization of  $[Ca^{2+}]_i$ . <sup>b</sup> $P < 0.01$ .

concentration of 10  $\mu\text{mol/L}$ , translocated NF- $\kappa$ B to the nucleus within 30 min (Figure 4B). NF- $\kappa$ B was re-established in the cytosol after 3 h (data not shown).

After Panc-1 cells were preincubate with 100 ng/ml PTX, LPA did not translocate NF- $\kappa$ B to nuclei (Figure 5A). A phospholipase C inhibitor, U73122, also abolished the translocation induced by LPA (Figure 5B). Pretreatment of Panc-1 with BAPTA-AM, an intracellular calcium chelator, at a concentration of 10  $\mu\text{mol/L}$  for 15 min, completely abolished NF- $\kappa$ B translocation (Figure 5C). Thapsigargin is known to block a  $Ca^{2+}$ -ATP pump of calcium stores, and elevates cytosolic free calcium slowly. In the present study, thapsigargin alone caused NF- $\kappa$ B translocation in Panc-1 cells at a concentration of 500 nmol/L (Figure 5D). These data suggest that elevation of

cytosolic free calcium is necessary for activation of NF- $\kappa$ B in Panc-1 cells.

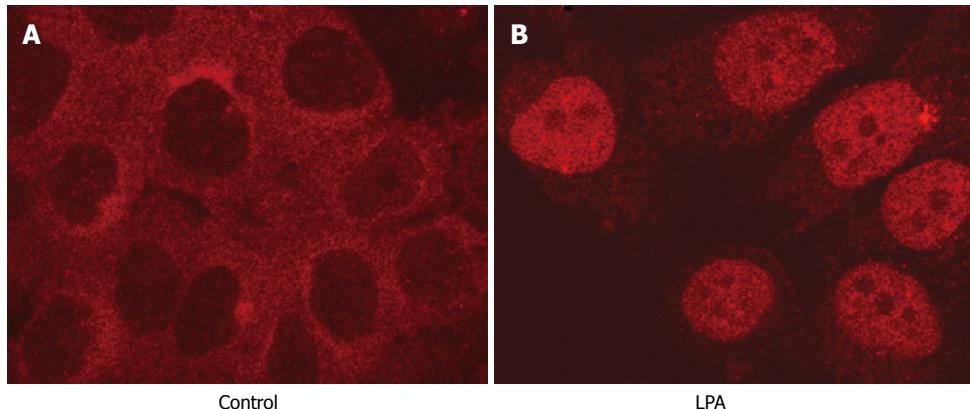
Phorbol myristate, which is well known to be a potent activator of protein kinase C, alone failed to induce the translocation at a concentration of 100 nmol/L (Figure 5E). On the other hand, staurosporine, a protein kinase C inhibitor, attenuated translocation of NF- $\kappa$ B induced by LPA (Figure 5F). These findings suggested that protein kinase C is activated endogenously in cancer cells, such as Panc-1, and that protein kinase C is essential for activating NF- $\kappa$ B with cytosolic calcium. It is reported that PMA could activate NF- $\kappa$ B in some types of cells including rat pancreatic acinar cells. However, the present data revealed that cytosolic calcium might have a more crucial role in activating NF- $\kappa$ B than protein kinase C in Panc-1 cells.

A tyrosine kinase inhibitor genistein or a MEK inhibitor PD98059 did not have significant effects on the translocation of NF- $\kappa$ B induced by LPA (data not shown).

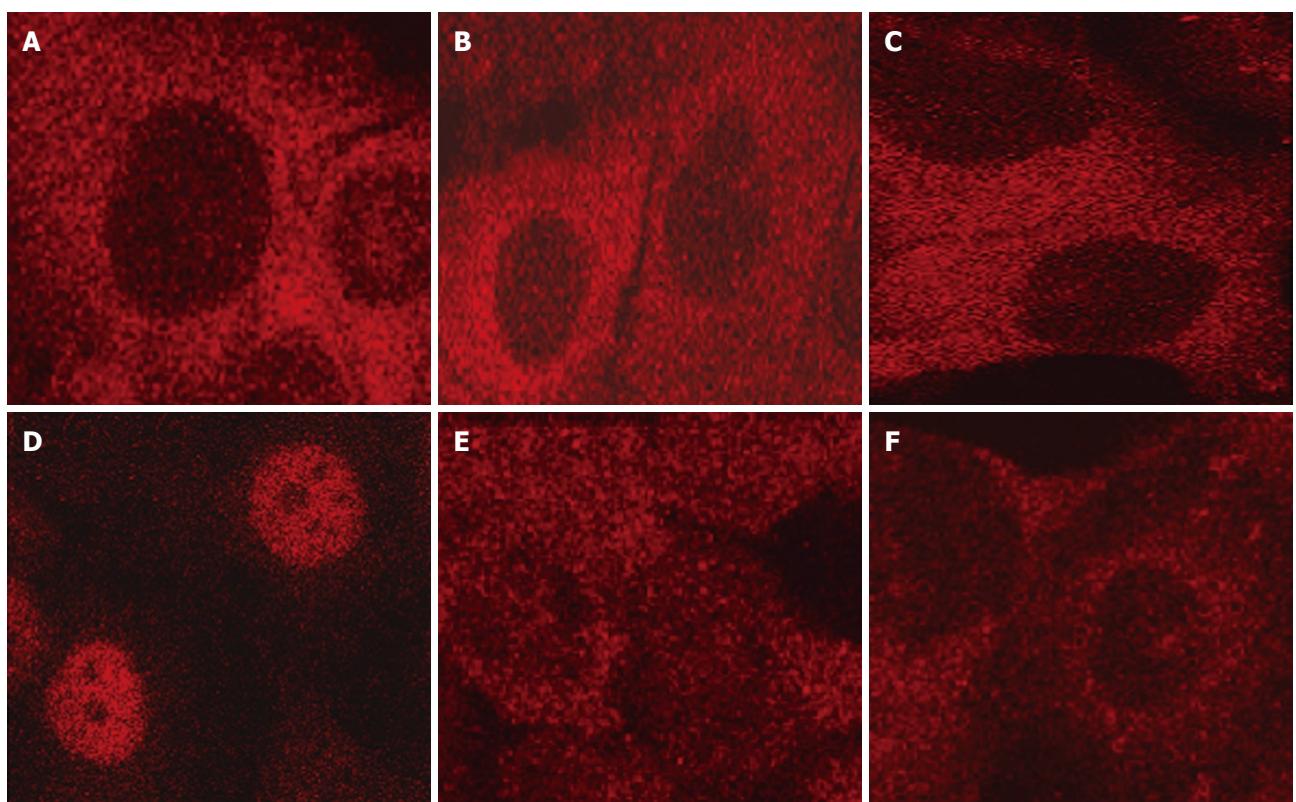
## DISCUSSION

In the present study, we first confirmed that Panc-1 cells express LPA receptors, and then showed that LPA induced  $Ca^{2+}$  mobilization and translocation of NF- $\kappa$ B into the nucleus.

LPA acts as an intercellular messenger molecule bound to serum albumin<sup>[3]</sup>. LPA exerts multiple biological functions through G protein-coupled receptors. It has been identified a new family of



**Figure 4** Effect of LPA on nuclear translocation of NF-κB. LPA-induced nuclear translocation of p65 in Panc-1 cells. Localization of p65 was visualized by indirect immunofluorescence staining using rabbit anti-p65 polyclonal antibodies (1:50) which only recognized NF-κB p65. Donkey anti-rabbit antibodies (1:100) conjugated to rhodamine was performed, and visualized under a confocal microscope. **A:** Cytosolic localization of p65 in an inactive form in unstimulated Panc-1 cells; **B:** Nuclear localization of p65 in Panc-1 cells after treatment of 10  $\mu$ mol/L LPA was observed at 60 min.



**Figure 5** Localization of NF-κB p65 in Panc-1 cells. **A:** PTX-treatment prevented nuclear translocation of NF-κB p65 induced by LPA in Panc-1 cells at 1 h. Panc-1 cells were preincubated with 100 ng/mL PTX, and then stimulated with 10  $\mu$ mol/L LPA; **B:** U73122-treatment prevented nuclear translocation of NF-κB p65 induced by LPA in Panc-1 cells at 1 h. Panc-1 cells were pretreated with 10  $\mu$ mol/L U73122 for 10 min before adding 10  $\mu$ mol/L LPA; **C:** Nuclear translocation of p65 induced by LPA was reduced by an intracellular calcium chelator, BPTA-AM at 1 h. Panc-1 cells were treated with 10  $\mu$ mol/L BAPTA-AM for 15 min and then stimulated with 10  $\mu$ mol/L LPA; **D:** Thapsigargin alone caused nuclear translocation of p65 at 1 h. Panc-1 cells were treated with 500 nmol/L thapsigargin; **E:** Phorbol myristate (PMA) alone failed to mobilize p65 into nuclei at 1 h. Panc-1 cells were treated with 100 nmol/L PMA; **F:** Staurosporine reduced nuclear localization of p65 induced by LPA at 1 h. Panc-1 cells were treated with 10 nmol/L staurosporine for 10 min and then stimulated with 10  $\mu$ mol/L LPA.

receptor genes for LPA. Members of this family include at least three G-protein-coupled receptors belonging to the endothelial differentiation gene (Edg) family, Edg-2/LP<sub>A1</sub>, Edg-4/LP<sub>A2</sub>, and Edg-7/LP<sub>A3</sub><sup>[10]</sup>. Recent investigation has revealed that Panc-1 cells also express functionally active LPA receptors (LP<sub>A1</sub>/Edg-2, LP<sub>A2</sub>/Edg-4, LP<sub>A3</sub>/Edg-7)<sup>[21]</sup>. LPA has a major

role in migration of Panc-1 cells via phosphorylation of ERK<sup>[21]</sup>, but was reported to not act as mitogen in pancreatic cancer cell lines, Panc-1 cells and Bx-PC cells<sup>[21]</sup>. LPA also mobilized cytosolic calcium in Panc-1 cells as neuropeptides including neuropeptidin, bombesin, cholecystokinin, and vasopressin<sup>[22]</sup>. However, it is still unclear of roles of calcium mobilization in Panc-1 cells.

Some researchers have tried to identify which subtype of LPA receptors is crucial for calcium mobilization. An investigation using human-recombinant G protein has suggested that LP<sub>A1</sub>/EDG-2 transduces Ca<sup>2+</sup> mobilization largely through PTX sensitive G<sub>i/o</sub> proteins<sup>[18]</sup>. LP<sub>A2</sub>/Edg-4 was supposed to be linked with G<sub>q</sub> and phospholipase C<sup>[18]</sup> and cooperated with other G proteins<sup>[19]</sup>. Another study, using a knock-out technique, revealed that nearly all PLC activation in response to LPA is dependent on endogenous expression of LP<sub>A1</sub> and LP<sub>A2</sub><sup>[20]</sup>.

The results presented in this paper show that PTX and U73122 attenuated LPA-induced calcium mobilization in Panc-1 cells, which suggests that LPA evokes cytosolic calcium via PTX-sensitive G protein and inositol phosphate production. We have not yet determined which subtypes of LPA receptors have a major role in mobilizing cytosolic calcium in Panc-1 cells. It is likely that LP<sub>A1</sub> or LP<sub>A2</sub> might have a major role in mobilization of cytosolic calcium in Panc-1 cells.

The present data suggests that LPA-induced cytosolic calcium mobilization is necessary for activation of NF-κB. PTX and U73122 attenuated both cytosolic calcium mobilization and nuclear translocation of NF-κB induced by LPA. Thapsigargin exerted more potent and long lasting actions than those achieved by LPA. PMA alone failed to stimulate the nuclear translocation of NF-κB. This result suggests that evoked cytosolic calcium concentration is crucial for this translocation, and that a resting level of cytosolic calcium might be insufficient for activation of NF-κB by phorbol ester-stimulated protein kinase C.

Activation of NF-κB by LPA may function as a counterpart to proliferative signaling. It is well known that activation of NF-κB is related to resistance to apoptosis. Recent studies have revealed that chemosensitivity of human pancreatic cancer cells, including Panc-1, is enhanced by I-κBα super-repressor<sup>[23]</sup> and that NF-κB has a major role in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) resistance of pancreatic cancer cell lines<sup>[24]</sup>.

LPA is supposed to utilize G proteins to achieve its activation of NF-κB. The fact that PTX attenuates activation of NF-κB would suggest a role for G<sub>i/o</sub>. NF-κB can be activated by oncogenic Ras<sup>[25]</sup>, and both Ras and ERKs are activated by LPA through pathways partly sensitive to PTX<sup>[20]</sup>. Cummings *et al* has shown that protein kinase C is also related with activation of NF-κB, which was coupled to G<sub>i/o</sub> and G<sub>12/13</sub> proteins<sup>[25]</sup>.

G<sub>i/o</sub> might have a major role in the activation of NF-κB<sup>[25]</sup>. It is conceivable that low concentrations of LPA activates G<sub>i/o</sub> and that higher concentrations activates G<sub>q</sub>. G<sub>q</sub> protein may be responsible for the activation of protein kinase C and mobilization of Ca<sup>2+</sup> of sufficient magnitude or duration to bring, together with signals from G<sub>i/o</sub>, activation of NF-κB<sup>[26]</sup>.

In conclusion, our results show for the first time that LPA induces nuclear translocation of NF-κB in Panc-1 cells by mobilizing cytosolic free calcium. This effect might be caused by activation of phospholipase C via PTX-sensitive G protein.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Lysophosphatidic acid (LPA) is a growth factor that exerts a number of biological actions on some cancer cell lines. However, the effect of LPA on pancreatic cancer cells has not been well estimated. In the present study, the authors investigate the effects of LPA on cytosolic calcium and translocation of NF-κB in Panc-1 cells.

### Research frontiers

The article focuses on the relationship of cytosolic calcium mobilization and NF-κB in Panc-1 cells induced by LPA.

### Innovations and breakthroughs

The present study shows that LPA-induced cytosolic calcium mobilization is necessary for activation of NF-κB in pancreatic cancer cells, and that a resting level of cytosolic calcium might be insufficient for activation of NF-κB by phorbol ester-stimulated protein kinase C. It is suggested that protein kinase C is activated endogenously in Panc-1, and protein kinase C is essential for activating NF-κB with cytosolic calcium and that LPA induces the nuclear translocation of NF-κB in Panc-1 by mobilizing cytosolic free calcium.

### Applications

It is known that LPA represents a major bioactive constituent of serum, and that activation of NF-κB is related to resistance to apoptosis. By knowing the mechanism of action of LPA, it can be used that target apoptosis in pancreatic cancer cells.

### Peer review

The authors investigated the effect of LPA on calcium mobilization, and localization of the transcription factor, NF-κB in the pancreatic cell line, Panc-1. This is an interesting paper. They demonstrated that LPA stimulates calcium mobilization from intracellular stores via PTX sensitive G proteins and PLC.

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RAPID COMMUNICATION

# Comparison of esophageal capsule endoscopy and esophagogastroduodenoscopy for diagnosis of esophageal varices

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**CONCLUSION:** We conclude that capsule endoscopy has a limited role in deciding which patients would benefit from EGD with banding or beta-blocker therapy. More data is needed to assess accuracy for staging esophageal varices, PHG, and the detection of gastric varices.

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**Key words:** Esophageal varices; Capsule endoscopy; Portal hypertension

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## Abstract

**AIM:** To investigate the utility of esophageal capsule endoscopy in the diagnosis and grading of esophageal varices.

**METHODS:** Cirrhotic patients who were undergoing esophagogastroduodenoscopy (EGD) for variceal screening or surveillance underwent capsule endoscopy. Two separate blinded investigators read each capsule endoscopy for the following results: variceal grade, need for treatment with variceal banding or prophylaxis with beta-blocker therapy, degree of portal hypertensive gastropathy, and gastric varices.

**RESULTS:** Fifty patients underwent both capsule and EGD. Forty-eight patients had both procedures on the same day, and 2 patients had capsule endoscopy within 72 h of EGD. The accuracy of capsule endoscopy to decide on the need for prophylaxis was 74%, with sensitivity of 63% and specificity of 82%. Inter-rater agreement was moderate ( $\kappa = 0.56$ ). Agreement between EGD and capsule endoscopy on grade of varices was 0.53 (moderate). Inter-rater reliability was good ( $\kappa = 0.77$ ). In diagnosis of portal hypertensive gastropathy, accuracy was 57%, with sensitivity of 96% and specificity of 17%. Two patients had gastric varices seen on EGD, one of which was seen on capsule endoscopy. There were no complications from capsule endoscopy.

## INTRODUCTION

Cirrhosis affects 3.6 out of every 1000 adults in North America. A major cause of cirrhosis-related morbidity and mortality is the development of variceal hemorrhage, a direct consequence of portal hypertension. The reported prevalence of esophageal varices in patients with chronic liver disease varies from 24% to 81%<sup>[1-3]</sup>. Variceal hemorrhage occurs in 25%-40% of patients with cirrhosis, and is associated with a mortality rate of up to 30%<sup>[1,2]</sup>. Accurate identification of patients with an increased risk of bleeding allows for primary prophylaxis to prevent variceal bleeding. Prophylactic use of beta-blockers has been shown to decrease the incidence of first variceal bleeding and death in patients with cirrhosis, and is currently the standard of care in patients who are at high risk for variceal hemorrhage<sup>[4,5]</sup>. Factors predictive of variceal hemorrhage include location of varices, size of varices, appearance of varices, clinical features of the patient, and variceal pressure<sup>[6]</sup>.

Esophagogastroduodenoscopy (EGD) is the standard of care for evaluation of varices. An EGD is currently recommended at diagnosis of cirrhosis, and

then yearly screening for patients with no varices on initial EGD for patients with progression of their liver disease or every two years for those who remain stable<sup>[5]</sup>. In patients with small varices, endoscopy should be performed every year to assess for a change in size<sup>[7]</sup>.

Currently, there is no universally accepted grading system for varices. Reliability of endoscopy is affected by inter-observer variability<sup>[8,9]</sup>. The subjective grading, invasiveness, risks of sedation, and cost of EGD has prompted a search for other alternatives. As of yet, no alternative had proven to be as accurate as EGD.

Several pilot studies have been published comparing capsule endoscopy (CE) to EGD for variceal screening. Eisen *et al* studied 32 patients, and found an overall concordance rate of 96.9% for the diagnosis of esophageal varices and 90.6% for the diagnosis of portal hypertensive gastropathy<sup>[10]</sup>. Lapalus *et al* performed unsedated EGD and capsule endoscopy in 21 patients, with an accuracy of 84.2% for the presence or absence of esophageal varices<sup>[11]</sup>.

Herein, we report the results of a study designed to assess the ability of capsule endoscopy to correctly identify the presence of esophageal varices and related features of portal hypertension in patients undergoing screening or surveillance endoscopy, and to determine the need for treatment or prophylaxis of esophageal varices.

## MATERIALS AND METHODS

All patients enrolled were from the patient population of Scripps Clinic, La Jolla, California. Patients were eligible if they were scheduled to undergo EGD for screening or surveillance of esophageal varices. Screening was performed in patients with either biopsy-proven cirrhosis, or biochemical and imaging studies consistent with cirrhosis. Surveillance was performed in patients who had previously been diagnosed with esophageal varices *via* EGD and were repeating the test to assess for progression of varices. Patients who had previously undergone banding of esophageal varices were included in the study if they were stable and had not had a variceal hemorrhage for  $\geq 6$  mo. Consecutive patients scheduled for EGD as screening or surveillance of esophageal varices were screened for eligibility to participate. All patients were age  $> 18$  years, able to give informed consent, and hemodynamically stable.

Exclusion criteria included dysphagia, known Zenker's diverticulum, the presence of cardiac pacemaker or other implantable electro-medical devices, pregnancy, or a scheduled MRI within 7 d after capsule ingestion. Patients also were excluded if they had a history of or risk for intestinal obstruction, including any prior abdominal surgery of the gastrointestinal tract other than uncomplicated cholecystectomy or appendectomy.

All patients who consented underwent capsule endoscopy and EGD on the same day or within 72 h. The endoscopies were performed under moderate sedation by three staff hepatologists at Scripps Clinic. The hepatologists were blinded to the results of the capsule endoscopy, but not to the patient's prior history or

previous endoscopy findings. Photographs were taken of any pertinent findings at endoscopy and grading of varices was agreed to by all three physicians after unblinding.

EGDs and CEs were both graded by the following scale: F0, no varices; F1, small straight varices; F2, tortuous varices and  $< 50\%$  of esophageal radius; F3, large and tortuous varices with or without red spots<sup>[6,12]</sup>. Presence or absence of high risk stigmata, defined as neovascularization or red or white spots was noted separately. Each observer decided whether or not treatment was indicated based on presence of F2 or F3 varices or the presence of high risk stigmata on any size varix. Portal hypertensive gastropathy (PHG) was graded on the following scale: none, mild (mucosal mosaic pattern), moderate (mosaic mucosal pattern with occasional red spots), or severe (mosaic mucosal pattern, extensive red or black spots, active oozing)<sup>[13,14]</sup>. Portal hypertensive gastropathy was diagnosed on capsule endoscopy *via* photographs of any area of the gastric mucosa as it was not possible to assess the location of the visualized area. The presence or absence of gastric varices was noted separately, as well as other findings unrelated to portal hypertension such as esophagitis, gastritis (defined as erythema or erosions of gastric lining), peptic ulcer disease, or duodenal lesions.

Capsule endoscopy was administered in the following manner in all patients. After imbibing 100 mL of water with 0.6 mL of simethicone, patients lay supine and then ingested the pill with 5 mL of water without raising their head. Any difficulty with ingestion was recorded by the administrator, and patients were instructed not to speak after pill ingestion. After 2 min supine, they were raised to a 30 degree incline. After another 2 min they were raised to 60 degrees, and after 1 min at 60 degrees the patient imbibed a sip of water. They then sat up completely and imbibed another sip of water, at which time they were placed in the left lateral decubitus position in order to improve visualization of the fundus. Three minutes after being placed on their left side the patients were instructed to sit up or walk around for the remaining 12 min of the examination.

Capsule endoscopies were read by two separate investigators, who were blinded to EGD findings, patient medical history, and reading of the other investigator. Both capsule readers had prior experience in endoscopic evaluation and diagnosis of esophageal varices. Prior to the study, both readers underwent training as recommended by the capsule manufacturer, consisting of review of a CD Rom and participation in an online course, which included review of 10 cases of capsule endoscopy. Each CE was read twice by each investigator on two separate occasions at least 60 d apart. Capsule images were evaluated for the presence and grade of esophageal varices, the presence and grade of PHG, the presence of gastric varices, and any other findings. Esophageal transit time and time spent reading each examination was recorded.

One week after capsule ingestion, each patient was contacted by telephone to assess for symptoms of capsule retention. At that time, patient satisfaction was

**Table 1 Demographics of 50 patients undergoing esophageal capsule endoscopy and EGD for diagnosis of esophageal varices (*n* = 50)**

	Patient population (%)
Male gender	34 (68)
Average age	58 (range, 25-74)
Average MELD <sup>1</sup>	9.48 (range, 6-23)
Average Child-Pugh	6.8 (range, 5-13)
Race	
Caucasian	40 (80)
Hispanic	6 (12)
African American	3 (6)
Middle Eastern	1 (2)
Etiology of cirrhosis	
Hepatitis C	24 (48)
Hepatitis C and alcohol	7 (14)
Alcohol	6 (12)
Nonalcoholic steatohepatitis	6 (12)
Other <sup>2</sup>	7 (14)

<sup>1</sup>MELD: Model for End Stage Liver Disease; <sup>2</sup>Primary biliary cirrhosis, sarcoidosis, cryptogenic cirrhosis, autoimmune hepatitis, Wilson's disease, idiopathic pulmonary hypertension.

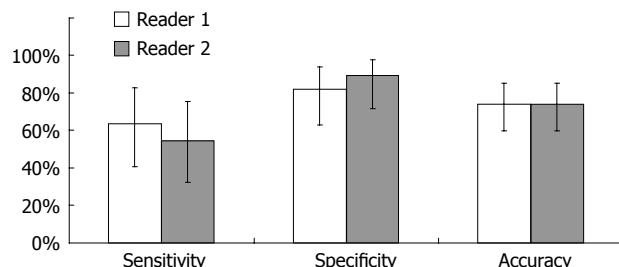
assessed. Patients were asked if they would be willing to undergo CE or EGD again, and which study they preferred.

Statistical analysis was performed to assess sensitivity, specificity, and accuracy of CE versus EGD in determining need for prophylaxis or treatment. A weighted kappa scale was used to determine agreement of variceal grade by CE compared to EGD, as well as inter- and intra-observer agreement<sup>[14-17]</sup>. Inter-observer agreement was defined as comparing results from Reader 1 to results from Reader 2. Intra-observer agreement measured results from the first read and results from the second read of each reader independent of the other reader. The sample size of 50 was chosen because with these numbers one typically will expect a standard deviation of 0.10 and coefficients of variation of 15% or less.

The study was approved by the local institutional review board.

## RESULTS

Fifty-five patients were screened to participate in the study. Five patients were not included: 2 patients refused, 1 patient had a history of an esophageal stricture, and 2 patients had history of surgery on the gastrointestinal tract. Fifty patients successfully underwent EGD and esophageal capsule endoscopy. In most cases, patients underwent CE on the same day as and just prior to EGD. There were two patients who underwent CE on a different day but within 72 h and two patients who underwent CE immediately after EGD. Median esophageal transit time was 249.5 s (range, 1-352 s). The esophageal transit times were as follows: 2 capsules 0-5 s, 15 capsules 5-60 s, and 33 capsules 60-352 s. Five patients (10%) had a mild amount of difficulty swallowing the capsule, and four patients (8%) had a moderate amount of difficulty, one of whom had to swallow it in a sitting position.



**Figure 1** Sensitivity, specificity and accuracy of esophageal capsule endoscopy compared to EGD for two separate blinded investigators. The error bars represent 95% confidence intervals.

Demographics of the patients can be seen in Table 1. Thirteen patients (26%) were undergoing surveillance of varices and had a history of previous variceal banding; the remainder were undergoing screening examinations. The patients who were undergoing surveillance had not been banded for at least 6 mo, and previously had been obliterated. All patients had undergone banding in the past for history of variceal bleeding. Based on EGD findings, prevalence of esophageal varices was 66%: 17 patients had no varices, 16 patients with F1 varices, 15 patients with F2 varices, and 2 patients with F3 varices. 5 patients underwent banding at the time of EGD.

In determining need for prophylaxis using EGD as the gold standard, sensitivity of CE was 63% (95% CI, 0.40-0.83; SD, 0.04), specificity was 82% (95% CI, 0.63-0.94; SD, 0.03), and accuracy was 74% (95% CI, 0.59-0.85; SD 0.04; Figure 1). The accuracy was not improved when patients with prior banding were excluded or when patients with difficulty swallowing the capsule were excluded. Positive predictive value in this population was 73% (95% CI, 0.48-0.91; SD, 0.04) and negative predictive value was 74% (95% CI, 0.55-0.88; SD, 0.04). There was no association between time of esophageal transit of the capsule and accuracy of the results, assessed by splitting the group at the median time of 249 s and comparing the two groups. Inter-rater reliability for need for prophylaxis was 0.56 (moderate agreement). Intra-rater reliability was 0.61 (good) for Reader 1 and 0.41 (moderate) for Reader 2. For grade of varices, agreement between EGD and CE was 0.53 (moderate). Inter-rater reliability for grade of varices was 0.77 (good), and intra-rater reliability was 0.76 (good) for Reader 1 and 0.69 for Reader 2.

Two patients (4%) had gastric varices. One of these patients had gastric varices suspected on CE by Reader 2, and neither of the patients had large esophageal varices requiring primary prophylaxis. It was not possible to gauge the location of the varices based on the capsule photographs.

Forty-five patients (90%) had portal hypertensive gastropathy: 28 patients with mild disease and 17 patients with moderate disease. In determining the presence or absence of PHG, sensitivity was 96% (95% CI, 0.78-0.99) and specificity was 17% (95% CI, 0.05-0.39). Accuracy was 57% (95% CI, 0.41-0.71). Inter-rater reliability for presence of PHG was 0.61 (good).

Seventeen patients (34%) had other findings seen on

EGD. Seven patients had gastritis seen on EGD, two of which were detected by CE. Two patients had Barrett's esophagus; one was detected by Reader 1 and one was detected by Reader 2. Two patients had esophagitis seen on EGD but not on CE. One patient had gastric polyps and one had duodenal polyps seen on EGD, and neither was detected on CE. One patient had an esophageal ring seen on EGD that was also detected on CE by Reader 2. One patient had scarring from prior banding that was seen on EGD but not CE. 11 patients underwent biopsy at time of EGD: 10 to rule out *H pylori* and one for diagnosis of Barrett's esophagus.

There were no complications from either CE or EGD. Thirty-six patients (72%) were satisfied equally with EGD and CE. Thirteen (26%) preferred CE to EGD, and one patient preferred EGD to CE. There were no instances of capsule retention.

## DISCUSSION

Complications of portal hypertension remain one of the major causes of morbidity and mortality in patients with cirrhosis. Up to 33% of cirrhotics will experience bleeding from varices, and 70% of these will be plagued with recurrent variceal bleeding<sup>[1,2,6]</sup>. In 1998, an AASLD single-topic symposium on portal hypertension devised the following current recommendations for variceal screening: EGD at time of diagnosis of cirrhosis, and if no varices were present, on a biyearly basis if liver function is stable, or yearly if liver function worsens, and yearly if small varices were present on initial screening<sup>[7]</sup>. Numerous studies have demonstrated the efficacy of beta-blocker therapy for reduction of risk of variceal bleeding and related mortality, decreasing the risk of variceal bleeding by 50%<sup>[18-20]</sup>. Recent data have suggested that variceal banding is also effective as primary prevention of variceal bleeding in patients with high risk varices<sup>[18-21]</sup>. Despite these recommendations, compliance with screening has been quite poor. Arguedas *et al* in 2001 reported that just 46% of cirrhotic patients underwent variceal screening by EGD prior to referral for liver transplantation, despite having a diagnosis of cirrhosis for a median duration of 3 years<sup>[22]</sup>. Results of a survey of practicing gastroenterologists suggested an even lower screening rate of 39%<sup>[23]</sup>.

Alternative methods to EGD have been studied for variceal screening, including transnasal endoluminal ultrasound<sup>[24]</sup>, platelet count/spleen diameter ratio<sup>[25]</sup>, multidetector computed tomography esophagography<sup>[26]</sup>, and esophageal capsule endoscopy<sup>[10,11,27]</sup>. To date, no method has proven accurate enough to replace EGD.

The results of our study are different from the two published pilot studies, showing a lower sensitivity, specificity, and accuracy for esophageal capsule endoscopy. Because there is known variability in grading of varices by EGD<sup>[8,9]</sup>, the accuracy of capsule endoscopy when measured against EGD may be wrong. We attempted to decrease this effect by verification of variceal grade diagnosed at endoscopy after unblinding by all physicians involved in the study, through

inspection of photographs. Other possible reasons that our study results may vary include the small size of prior studies compared to ours. Our trial size was still somewhat small, but we balanced that expectation with the recognition that a much larger trial would be needed for confirmation of this as a pilot trial. Other confounders for the data could include the absence of complete industry funding in our study as opposed to the prior ones, and our relative lack of expertise with capsule endoscopy or other technical difficulties.

Concern has been raised regarding the utility of capsule endoscopy in patients who have previously undergone banding of esophageal varices. Patients were included in our study if they had not undergone banding for at least 6 mo. We chose to include these patients because we felt that varices would still be able to be diagnosed at esophageal capsule endoscopy. When patients with previous banding were excluded from analysis, our accuracy did not improve significantly. A total of 5 patients out of 13 who were undergoing surveillance for esophageal varices required repeat banding at the time of EGD. This underscores a limitation of capsule endoscopy: that patients with varices seen at diagnosis may then have to undergo EGD for therapy.

There has been some concern about the mixed results of capsule endoscopy use for evaluation of esophageal pathology, such as varices, Barrett's esophagus, or esophagitis<sup>[27-29]</sup>. It is thought that the mixed results of capsule endoscopy may have to do with deviations from the standard ingestion procedure recommended by the manufacturer<sup>[30]</sup>. We note that in our study, all patients were able to successfully swallow the capsule, with only 9 patients having some difficulty, including two patients that needed to lift their heads from the supine position and one patient that had to ingest the pill in the sitting position. We feel that there is little chance these deviations influenced our results. When we looked at patient history of banding, time of esophageal transit, and reader experience/learning curve, none of these factors significantly changed the results of our study. We, therefore, feel that the accuracy reported here may be more reflective of what can be expected with capsule endoscopy use in community gastroenterology practice.

Esophageal capsule endoscopy has been designed specifically to look at the esophagus; there is no way to ensure that full inspection of the gastric mucosa and duodenum will occur, as it would with EGD. When screening for varices, this usually is not an issue. However, as in our study, there are patients who have gastric varices in the absence of significant esophageal varices that would require pharmacologic prophylaxis against bleeding. These patients may be missed if screening was done solely with capsule endoscopy. In addition, capsule endoscopy had poor accuracy for diagnosis of portal hypertensive gastropathy. Capsule endoscopy limits the patient to diagnosis only. In 11 of our patients, biopsies were performed for diagnosis of *H pylori* or Barrett's esophagus. Obviously, these biopsies would not have been able to be performed if capsule endoscopy was the only diagnostic method used.

Given our results for capsule endoscopy, we are uncertain if its routine use can replace EGD at this time as a screening tool. It may be useful for those patients who are unable or unwilling to undergo upper endoscopy, but clinicians need to be cognizant of the possibility of a false negative result. At this time, we would recommend use of esophageal capsule endoscopy only in the setting of a clinical trial.

In conclusion, we feel that capsule endoscopy has a limited role in deciding which patients would benefit from EGD with banding or beta-blocker therapy in early cirrhosis, as well as for determining the specific grade of esophageal varices, PHG, or gastric varices. More data is needed to assess accuracy for staging esophageal varices, PHG, and the detection of gastric varices. Clinicians who choose to employ capsule endoscopy as part of their routine clinical practice should be cognizant of the lower accuracy for esophageal variceal screening.

## COMMENTS

### Background

Esophageal varices are found in up to 81% of patient with cirrhosis, and results in significant gastrointestinal bleeding in up to half of patients. In order to prevent variceal bleeding, screening is recommended with upper endoscopy every 1-3 years, with prophylaxis given to those patients with large varices. Esophageal capsule endoscopy is a new device designed to image the esophagus in a noninvasive way. The utility of esophageal capsule endoscopy in the diagnosis of esophageal varices is not known.

### Research frontiers

To date, two pilot studies have been published regarding the use of esophageal capsule endoscopy for the diagnosis of esophageal varices. These initial studies were performed in 32 and 21 patients, respectively, and showed high concordance and accuracy for the diagnosis of esophageal varices with capsule endoscopy (96.9% and 84.2%, respectively).

### Innovations and breakthroughs

In this publication, 50 patients underwent upper endoscopy and esophageal capsule endoscopy. The capsule endoscopies were independently read by two blinded investigators. The accuracy of capsule endoscopy for diagnosis of esophageal varices was found to be 74% in determining the need for prophylaxis based on the presence of large varices. The sensitivity was 63% and the specificity was 82%. Inter-rater reliability was moderate for determining the need for prophylaxis. Intra-rater reliability was moderate for one reader and good for the other reader. 34% of patients studied had other findings seen at upper endoscopy that were not reliably diagnosed with capsule endoscopy, including gastric varices, gastric and duodenal polyps, esophagitis, and Barrett's esophagus. Accuracy for diagnosis of portal hypertensive gastropathy was poor at only 57%.

### Applications

Currently, the use of capsule endoscopy for variceal screening cannot be routinely recommended. Refinements to the capsule procedure may improve the accuracy in the future. Further studies are needed to verify these results.

### Peer review

This paper details the use of esophageal capsule endoscopy for the diagnosis of esophageal varices. Two pilots studies suggested that capsule endoscopy may be useful for detection of large varices. In this largest cohort to date, we found that capsule endoscopy has a poor sensitivity in detecting large varices requiring prophylactic therapy. In addition, there is also poor inter- and intra-observer agreement when using this method for grading esophageal varices. Finally, since three quarters of all patients do not prefer one method over the other, it appears that capsule endoscopy would have a limited role in diagnosis of esophageal varices.

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RAPID COMMUNICATION

## Association between *calcium sensing receptor* gene polymorphisms and chronic pancreatitis in a US population: Role of *serine protease inhibitor Kazal 1type* and alcohol

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alcohol are necessary co-factors in its etiology.

**METHODS:** Initially, 115 subjects with pancreatitis and 66 controls were evaluated, of whom 57 patients and 21 controls were predetermined to carry the high-risk *SPINK1* N34S polymorphism. We sequenced *CASR* gene exons 2, 3, 4, 5 and 7, areas containing the majority of reported polymorphisms and novel mutations. Based on the initial results, we added 223 patients and 239 controls to analyze three common nonsynonymous single nucleotide polymorphisms (SNPs) in exon 7 (A986S, R990G, and Q1011E).

**RESULTS:** The *CASR* exon 7 R990G polymorphism was significantly associated with CP (OR, 2.01; 95% CI, 1.12-3.59;  $P = 0.015$ ). The association between *CASR* R990G and CP was stronger in subjects who reported moderate or heavy alcohol consumption (OR, 3.12; 95% CI, 1.14-9.13;  $P = 0.018$ ). There was no association between the various *CASR* genotypes and *SPINK1* N34S in pancreatitis. None of the novel *CASR* polymorphisms reported from Germany and India was detected.

**CONCLUSION:** Our United States-based study confirmed an association of *CASR* and CP and for the first time demonstrated that *CASR* R990G is a significant risk factor for CP. We also conclude that the risk of CP with *CASR* R990G is increased in subjects with moderate to heavy alcohol consumption.

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**Key words:** Calcium sensing receptor; *Serine protease inhibitor Kazal 1type*; Chronic pancreatitis; Alcohol

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### Abstract

**AIM:** To test the hypothesis that calcium sensing receptor (*CASR*) polymorphisms are associated with chronic pancreatitis (CP), and to determine whether *serine protease inhibitor Kazal 1type* (*SPINK1*) N34S or

## INTRODUCTION

Chronic pancreatitis (CP) is a debilitating, inflammatory disease of the pancreas, characterized by progressive organ destruction and fibrosis. CP results in profound exocrine and endocrine insufficiency and, in many cases, intractable, chronic pain. As a complex disorder, CP can develop from a variety of etiologies with multiple pathological pathways<sup>[1]</sup>. For several years, alcohol abuse has been considered the most likely causative agent for CP in Western countries, although etiologies including toxic, metabolic (hypercalcemia, hyperlipidemia), genetic mutations, autoimmune, and duct obstruction, have also been implicated<sup>[2,3]</sup>.

Consistent experimental evidence links elevated acinar cell calcium levels with acute pancreatitis in association with premature trypsinogen activation to trypsin<sup>[4]</sup>. Recurrent acute pancreatitis (RAP), as illustrated in patients with hereditary and sporadic pancreatitis, can lead to CP<sup>[5-7]</sup>. Hypercalcemia itself has been associated with the development and complications of CP<sup>[2]</sup>. Recent studies from Germany and India have reported that novel *calcium sensing receptor* (*CASR*) gene mutations in combination with the presence of *serine protease inhibitor Kazal 1 type* (*SPINK1*) N34S increased the risk of CP<sup>[8-10]</sup>. The *SPINK1* N34S "high-risk haplotype" is strongly associated with CP, but only a limited portion of mutation carriers develop CP during their life time, suggesting that additional factors are necessary to develop this complex disorder<sup>[11,12]</sup>.

*CASR* is a member of the G-protein-coupled receptor (GPCR) superfamily<sup>[13]</sup>. *CASR* plays an important role in calcium homeostasis, as is reflected in its expression by cells of the parathyroid gland and renal tubules that are involved in calcium metabolism. *CASR* has been identified in both human pancreatic acinar and ductal cells, as well as in various non-exocrine cells<sup>[14]</sup>, although its functional significance in the pancreas has not been determined.

The human *CASR* gene is located on chromosome 3q 13.3-21<sup>[15,16]</sup>. *CASR* possesses a coding region of 3234 base pairs (bp) which is contained within 6 of the seven exons that make up the gene. One hundred and twelve functional mutations (40 activating and 72 inactivating) have been described in the *CASR* mutation database related to familial hypocalciuric hypercalcemia (FHH), neonatal severe primary hyperparathyroidism (NSHPT), and autosomal dominant hypocalcemia (ADH) families as well as in *de-novo* disease<sup>[17]</sup>. In addition, single activating or inactivating *CASR* mutations may cause hypercalcemic or hypocalcemic disorders<sup>[18,19]</sup>.

We hypothesized that *CASR* polymorphisms are associated with the development of CP and that *SPINK1* N34S mutations or alcohol may be important co-factors in its etiology. We tested this hypothesis by evaluating subjects with RAP, CP and healthy controls with known *SPINK1* genotypes and alcohol intake for common and novel *CASR* polymorphisms in exons 2, 3, 4, 5 and 7.

## MATERIALS AND METHODS

### Study population

Subjects were recruited from the North American

Pancreatic Study2 (NAPS2). The NAPS2 study is a multicenter, molecular epidemiology study designed to evaluate the genetic and environmental factors predisposing to recurrent acute pancreatitis (RAP) and CP. Detailed description of methods are presented elsewhere<sup>[20]</sup>. The subjects were stratified into alcohol categories based on self-reported average number of drinks consumed per week during the period of heaviest lifetime drinking. Alcohol categories were defined based on the drinking pattern as: (1) abstainers: no alcohol use or < 20 drinks in lifetime; (2) light drinkers: ≤ 3 drinks/week; (3) moderate drinkers: 4-7 drinks/week for females; 4-14 drinks/week for males; (4) heavy drinkers: 8-34 drinks/week for females; 15-34 drinks/week for males; (5) very heavy drinkers: ≥ 35 drinks/week for both males and females. For analysis, alcohol drinking categories were combined into 3 groups based on their risk for causing CP: (1) abstainers and light drinkers were considered very low risk, (2) moderate and heavy drinkers were considered moderate risk, and (3) very heavy drinkers were considered substantial risk<sup>[20]</sup>.

One hundred and fifteen affected individuals and 66 controls were selected initially from four sites of the NAPS2 cohort. These subjects were selected based on the presence or absence of *SPINK1* N34S mutations, of which 57 patients and 21 controls were determined by previous genetic analysis to carry the high-risk *SPINK1* mutation. From the twenty site NAPS2 consortium, 219 affected subjects and 239 controls were later screened for the three common nonsynonymous single nucleotide polymorphisms (SNPs) seen in the coding region of the intracellular *CASR* tail in exon 7 which appeared to be the region of interest. These were A986S (rs # 1801725), R990G (rs # 1042636), and Q1011E (rs # 1801726).

### DNA preparation and mutation analysis

Genomic DNA was extracted from whole blood as described<sup>[20]</sup>. PCR primers were designed for *CASR* gene exons 2, 3, 4, 5 and 7, which contains most of the commonly seen activating and inactivating mutations as well as the novel mutations found in Germany and India (Table 1). Exons 4 and 7 were lengthy and thus were divided into 2 and 4 fragments respectively.

PCR was performed in a total volume of 25 μL; 200 nmol of forward and reverse primer, 200 μmol of dNTP and 1 × PCR Buffer II (ABI, CA) with 10 ng of DNA. Amplification settings were 95°C for 12 min × 1 cycle, 95°C for 30 s, annealing temperature (Table 1) × 20 s and 72°C × 20 s for 35 cycles and 72°C for 2 min × 1 cycle. Annealing temperatures and magnesium concentrations for different primers are shown in Table 1. PCR amplification products were purified with exonuclease I (NEB, Beverly, MA) and shrimp alkaline phosphatase (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's recommendations. Cycle sequencing was performed using the ABI Prism Big Dye Terminator Sequencing Kit v3.1 diluted 1:8 (ABI, Foster City, CA) using the appropriate PCR primers. Products from the sequencing reaction were purified by ethanol EDTA precipitation. Sequence products were run on

**Table 1** Polymerase chain reaction primer pairs, magnesium concentration and annealing temperatures used for genetic analysis of the *CASR* gene

Scanning region	Forward and reverse primer sequences	MgCl <sub>2</sub> (mmol/L)	Annealing temperature (°C)
Exon 2	5'-ACCACCCACATTACAAGTC-3' 5'-GCTTTTCTCCAACCACATCAG-3'	2.5	55
Exon 3	5'-ATGAAGCCAGAGAGTAGTAAC-3' 5'-TAAACCGTATGGCTATGGG-3'	2.5	58
Exon 4a	5'-GCTTTTCTTACCCCTTCATTCATC-3' 5'-ATCACCTCTACCATGCTG-3'	2	58
Exon 4b	5'-CAGATCTTGAGCCCCATC-3' 5'-GCAGCCCAACTCTGCTTAT-3'	2	59
Exon 5	5'-TGGGGCTTGTACTCATTTCTT-3' 5'-CTGGTTTCTGATGGACAGC-3'	1.5	59
Exon 7a	5'-CACACAATAACTCACTCTCAC-3' 5'-CAGAGAAAACCAGCAGGAAC-3'	2.0	61
Exon 7b	5'-AAAACCAACCGTGTCTCCCTG-3' 5'-ATGGCAATCACCTCTACGGC-3'	1.0	53
Exon 7c	5'-GCTCATCTCTCATCGTCTGG-3' 5'-CGTATCGTGTCTTCTGGG-3'	1.0	58
Exon 7d	5'-CCCAGCAAGAGCAGCAG-3' 5'-ACAACTCTCAGGGTCTCC-3'	1.0	58

**Table 2** Participant characteristics

Demographic	CP (n = 219)	RAP (n = 115)	Controls (n = 305)
Age, mean (SD)	45.3 (18.1)	46.1 (16.2)	54.7 (14.5)
Race, % White	91	91	94
Sex (M/F)	125/94	49/66	121/184
Alcohol drinking pattern (%)			
Abstainers	45 (22.5)	30 (27)	72 (25)
Light	38 (19)	26 (23)	83 (28)
Moderate	34 (17)	22 (20)	56 (19)
Heavy	39 (19.5)	20 (18)	58 (20)
Very heavy	44 (22)	13 (12)	23 (8)

CP: Chronic pancreatitis; RAP: Recurrent acute pancreatitis; SD: Standard deviation. Abstainers: No alcohol use or < 20 drinks in lifetime; Light: < 3 drinks/week; Moderate: 4-7 drinks/week for females, 4-14 drinks/week for males; Heavy: 8-34 drinks/week for females, 15-34 drinks/week for males; Very heavy: > 35 drinks/week.

an ABI Prism 3730 Genetic Analyzer and sequence data were analyzed using Sequencher 4.7 (Gene Codes Corp., Ann Arbor, MI)<sup>[5,21]</sup>.

### Statistical analysis

Genotype frequencies were assessed for Hardy-Weinberg equilibrium. The frequencies of genotypes among cases and controls were compared using Chi square test or the Fisher's exact test when appropriate. Odds ratio (OR) and 95% confidence intervals (95% CI) for genotypes were calculated using an autosomal dominant model. For all statistical comparisons, *P* < 0.05 was considered significant.

## RESULTS

Subject demographics and alcohol drinking patterns are given in Table 2. The proportion of subjects reporting a moderate or heavy alcohol drinking pattern was similar between patients and controls. Of the 334 patients with pancreatitis, 219 (66%) had CP and 115 (34%) had RAP.

The initial study consisted of 115 patients (CP = 82

and RAP = 33) and 66 controls, of which 57 patients (CP = 47 and RAP = 10) and 21 controls carried the *SPINK1* N34S high risk haplotype. Of the 58 patients without *SPINK1* N34S, 35 were diagnosed with CP and 23 had RAP.

The genotype frequencies were found to be in Hardy-Weinberg equilibrium. The R990G polymorphism (AGG → GGG transition) in exon 7 of the *CASR* gene, the G allele was more common among CP patients (*n* = 35) than controls (*n* = 45), but only in subjects without *SPINK1* N34S. In comparing CP patients (*n* = 47) and controls (*n* = 21) with *SPINK1* N34S, there was a non-significant trend towards an increased occurrence of the G allele in patients (OR, 4.03; 95% CI, 0.48-190.8, *P* = 0.255). One limitation of this study was the small number of *SPINK1* N34S subjects for comparison; Therefore, caution must be exercised before this association is either accepted or rejected.

From the 112 mutations reported previously, the following three mutations--E191E, Y440C and A746A were each observed once in CP patients with *SPINK1* N34S. Another mutation, P748P, was identified in two CP patients without *SPINK1* N34S. Recently identified novel *CASR* mutations from Germany and India seen in exons 3 (P163R), 4 (L173P, F391F, I425S, D433H), 5 (V477A) and 7 (E870E, R896E)<sup>[8-10]</sup> were not observed in either patients or controls. Two intronic polymorphisms 493-94 C>T and 493-134 T>C included in exon 4 amplicon occurred with similar frequency in CP patients and controls, both with and without *SPINK1* N34S polymorphisms.

Secondarily, 219 patients (137 CP and 82 RAP) and 239 controls from the NAPS2 study who did not carry *SPINK1* N34S were analyzed to test the association of *CASR* A990G and CP. This ancillary analysis confirmed that the R990G was significantly associated with CP, as shown in Table 3 (OR, 2.01; 95% CI, 1.12-3.59; *P* = 0.015). The frequencies of R990G among RAP patients and controls, with and without *SPINK1* N34S were similar. There was

**Table 3** Genotype analysis of *CASR* R990G polymorphism in patients and controls

	Patients	Controls	P	OR (95% CI)
CP patients vs controls without <i>SPINK1</i> N34S (%)				
AA	140 (82)	255 (90)		
AG	31 (18)	28 (10)		
GG	1 (1)	1 (0.5)		
AA vs AG/GG			0.015	2.01 (1.12-3.59)
RAP patients vs controls without <i>SPINK1</i> N34S (%)				
AA	93 (89)	255 (90)		
AG	10 (9)	28 (10)		
GG	2 (2)	1 (0.5)		
AA vs AG/GG			0.712	1.28 (0.67-2.47)
<i>SPINK1</i> N34S positive CP patients vs controls (%)				
AA	39 (83)	20 (95)		
AG	8 (17)	1 (5)		
GG	0	0		
AA vs AG/GG			0.255	4.1 (0.48-35.14)

CP: Chronic pancreatitis; RAP: Recurrent acute pancreatitis. *SPINK1*: Serine protease Kazal type 1 gene. <sup>1</sup>Fisher exact test.

**Table 4** *CASR* genotype comparison for R990G polymorphism in CP patients and controls with similar alcohol drinking patterns

	Patients	Controls	P	OR (95% CI)
A/L Alcohol CP patients vs controls (%)				
AA	69 (83)	136 (88)		
AG	13 (16)	18 (11)		
GG	1 (1)	1 (1)		
AA vs AG/GG			0.332	1.45 (0.69-3.07)
M/H Alcohol CP patients vs controls (%)				
AA	59 (81)	106 (93)		
AG	14 (19)	8 (7)		
GG	0	0		
AA vs AG/GG			0.018	3.12 (1.14-9.13)
VH Alcohol CP patients vs controls (%)				
AA	37 (84)	21 (91)		
AG	7 (16)	2 (9)		
GG	0	0		
AA vs AG/GG			0.708	1.99 (0.38-0.45)

A: Abstainer; L: Light; M: Moderate; H: Heavy; VH: Very heavy. Alcohol categories: Abstainers: No alcohol use or < 20 drinks in lifetime; Light drinkers: < 3 drinks/week; Moderate drinkers: 4-7 drinks/week for females, 4-14 drinks/week for males; Heavy drinkers: 8-34 drinks/week for females, 15-34 drinks/week for males; Very heavy drinkers: > 35 drinks/week. <sup>1</sup>Fisher's exact test.

no difference in A986S and Q1011E polymorphisms among RAP and CP patients, and controls.

To determine if the risk was modified with alcohol use we compared *CASR* R990G genotypes in subjects with moderate and heavy alcohol drinking pattern. CP was strongly associated with the *CASR* R990G in moderate and heavy alcohol drinkers, as is demonstrated in Table 4 (OR, 3.12; 95% CI, 1.14-9.13; P = 0.018). No association was observed with this particular polymorphism in abstainers or in subjects with self-reported light or very heavy alcohol drinking patterns.

## DISCUSSION

In the past, CP was commonly attributed to heavy alcohol consumption. More recent studies, however, suggest there is also a strong genetic basis for this illness<sup>[22]</sup>. Growing knowledge of complex gene-environment interactions has

provided fundamental insight into the pathophysiological mechanisms that result in fibrotic destruction of the pancreas<sup>[11,23-25]</sup>. Studies from Germany and India have recently identified 8 novel *CASR* mutations that were associated with *SPINK1* N34S in idiopathic and tropical CP subjects. Our study did not detect these novel *CASR* mutations. However, we were able to demonstrate and verify that *CASR* R990G confers significant risk for developing of CP especially when linked to moderate and heavy alcohol consumption.

Three common nonsynonymous SNPs are located in the region coding the intracellular tail of *CASR*<sup>[26]</sup> and play an important role in cellular signal transduction that alters serum ionized calcium level<sup>[27,28]</sup>. Previously, it was reported that individuals carrying the 990 variant G allele may experience very mild decrease in serum ionized calcium levels from 4.92 mg/dL to 4.84 mg/dL<sup>[28]</sup>. Although serum ionized calcium levels

alter the cytosolic calcium ion concentrations in acinar cells in a concentration-dependent manner, and may alter the risk of acute pancreatitis<sup>[29,30]</sup>, the *CASR* R990G allele associated with increased risk of CP should slightly reduce the risk of acute pancreatitis. Furthermore, the magnitude of change in serum calcium levels due to *CASR* R990G alone is small, and it is difficult to imagine that this small change would, by itself, significantly alter the risk of acute pancreatitis. Indeed, our data suggests that *CASR* R990G is associated with CP rather than RAP. Our speculation is that *CASR* R990G might induce direct changes in the acinar and ductal cells that increase the risk for CP. However, the mechanism remains unknown.

Interestingly, while 55%-80% of pancreatitis cases may be attributed to alcohol abuse, less than 5% of heavy alcohol users develop pancreatitis<sup>[31]</sup>. Alcohol abuse may not be the sole risk for the development of CP<sup>[32]</sup>; rather alcoholic CP is likely the result of an interaction of several co-factors<sup>[2]</sup>. It has been demonstrated that chronic alcohol consumption accelerates fibrosis in response to cerulein-induced CP in rats<sup>[33]</sup>. Alcohol metabolites in pancreatic acinar cells induce persistent cytosolic Ca<sup>2+</sup> signals in a concentration-dependent manner and depolarize mitochondria.

The discovery and characterization of a genetic cause of hereditary pancreatitis generated renewed interest in a possible genetic predisposition to alcoholic CP<sup>[34]</sup>. Several CP-related gene mutations have been described previously with *CFTR*, *PRSS1*, *SPINK1* and others<sup>[35]</sup>. Our study also demonstrates the association of *CASR* R990G with CP, especially with moderate and heavy alcohol consumption. The presence of *CASR* R990G alone doubled the risk of developing CP, while in those individuals reporting moderate and heavy alcohol consumption, the risk was increased by 3-fold. Our hypothesis for testing *CASR* R990G in subjects with moderate and heavy drinking patterns is that this group represented a "threshold" alcohol pancreatitis risk group in which the addition of another risk factor would increase the overall risk of developing CP. The risk of CP in subjects with *CASR* R990G but with minimal or no alcohol consumption would be lower, while very heavy drinkers would be at high risk, regardless of the *CASR* genotype. Our experimental findings support this hypothesis.

The novel *CASR* gene mutations that were identified in German and Indian populations appeared to be closely associated with the *SPINK1* N34S haplotype. We did not detect these, or other novel *CASR* mutations, and our study was not powered to demonstrate an interaction between *SPINK1* N34S and *CASR* R990G. On the other hand, it was not clear whether or not the German and Indian studies tested for an effect of alcohol in a "threshold" dose range. However, both studies suggest that the overall effect of *CASR* polymorphisms are relatively small, and become clinically significant in the presence of additional risk factors in an additive or multiplicative way. This is consistent with current concepts that CP is a complex syndrome.

The present study confirmed the association of *CASR* genetic variants with CP. Our genotyping results

in a US population were different from those reported from Germany and India. *CASR* R990G significantly increased the risk of developing CP and this effect was enhanced in subjects who consumed alcohol in a moderate to heavy dose range. Certain polymorphisms in the *CASR* gene may be considered risk factors for the development of CP, especially within the context of alcohol consumption. The relationship with *SPINK1* mutations warrants further study.

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## COMMENTS

### Background

Chronic pancreatitis is a highly morbid, complex disease whose development depends on the combination of genetic and environmental factors. Elucidating the genetic links to this illness is critical in diagnosis, treatment and risk assessment.

### Research frontiers

This study adds another gene to the growing number of genetic and other factors that confers increased risk of chronic pancreatitis. As new factors continue to be identified and confirmed, the emphasis will turn to integrating these risks, using systems approaches, as described in reference #1.

### Innovations and breakthroughs

This study is one of the first to consider the complexity of gene-environment and gene-gene interactive paradigms by evaluating alcohol consumption and serine protease inhibitor Kazal 1type (*SPINK1*) N34S variants with calcium sensing receptor (*CASR*) polymorphisms. The confirmation of *CASR* genetic variants as risk factors for chronic pancreatitis strengthens the importance of dysfunctional calcium regulating genes in the etiology of pancreatitis.

### Application

With the inclusion of associated *CASR* polymorphisms in comprehensive evaluation of selected patients, we may improve the accuracy of overall pancreatitis risk prediction and may be able to provide a target for preventive approaches and possible treatment options.

### Peer review

Our peer reviewers noted this brief manuscript to be well-developed and well-written. They felt that the abstract was clear and the hypothesis being tested and methodology were sound and well presented.

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RAPID COMMUNICATION

## Folic acid supplementation inhibits recurrence of colorectal adenomas: A randomized chemoprevention trial

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### Abstract

**AIM:** To determine whether folic acid supplementation will reduce the recurrence of colorectal adenomas, the precursors of colorectal cancer, we performed a double-blind placebo-controlled trial in patients with adenomatous polyps.

**METHODS:** In the current double-blind, placebo-controlled trial at this VA Medical Center, patients with colorectal adenomas were randomly assigned to receive either a daily 5 mg dose of folic acid or a matched identical placebo for 3 years. All polyps were removed at baseline colonoscopy and each patient had a follow up colonoscopy at 3 years. The primary endpoint was a reduction in the number of recurrent adenomas at 3 years.

**RESULTS:** Of 137 subjects, who were eligible after confirmation of polyp histology and run-in period to conform compliance, 94 completed the study; 49 in folic acid group and 45 in placebo group. Recurrence of adenomas at 3-year was compared between the two groups. The mean number of recurrent polyps at 3-year was 0.36 (SD, 0.69) for folic acid treated patients compared to 0.82 (SD, 1.17) for placebo treated subjects, resulting in a 3-fold increase in polyp recurrence in the placebo group. Patients below 70 years of age and those with left-sided colonic

adenomas or advanced adenomas responded better to folic acid supplementation.

**CONCLUSION:** High dose folic acid supplementation is associated with a significant reduction in the recurrence of colonic adenomas suggesting that folic acid may be an effective chemopreventive agent for colorectal neoplasia.

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**Key words:** Folic acid; Adenoma; Colorectal cancer

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### INTRODUCTION

Colorectal cancer is the second most common cancer in the United States<sup>[1]</sup>. Although the etiology of this disease is related to genetic susceptibility, dietary factors such as vitamins and micronutrients are thought to influence carcinogenesis<sup>[2]</sup>. Considerable interest has recently been focused on the water soluble vitamin folic acid. Although the specific mechanism(s) by which folate deficiency enhances colorectal carcinogenesis have not been fully elucidated, it has been hypothesized that aberrations in DNA methylation may contribute to abnormalities in DNA synthesis and genomic instability<sup>[3]</sup>.

Several clinical trials have noted an inverse relationship between dietary folic acid and the development of colorectal cancer<sup>[4-7]</sup>. A folate deficient diet is thought to increase the risk of colonic neoplasia<sup>[8-11]</sup>, whereas supplementation of this nutrient may be chemopreventive<sup>[12-15]</sup>. However, the timing of folate supplementation may be particularly important since folate intervention after the establishment of microscopic neoplastic foci in the colorectal mucosa may promote

rather than suppress colorectal carcinogenesis<sup>[16]</sup>.

Accumulating data from murine studies have also supported a role for folic acid in the prevention of colon carcinogenesis. Folate deficient rats demonstrate an increased susceptibility to dimethylhydrazine induced colonic neoplasia as compared to folate replete animals<sup>[10]</sup>. In a similar model, folate supplementation protected against the development of colonic neoplastic lesions in a dose dependent manner<sup>[17]</sup>. We have previously demonstrated that folic acid supplementation can reduce the age-related susceptibility of murine colorectal mucosa to a colonic carcinogen<sup>[18]</sup>. In the azoxymethane-induced colon cancer rat model, supplemental folic acid has also been shown to decrease the formation of aberrant crypt foci, which are considered to be precursors of colorectal adenomas and carcinoma<sup>[19,20]</sup>. Additionally, *in vitro* studies have further demonstrated that supplemental folic acid greatly inhibits proliferation of colon cancer cells<sup>[21,22]</sup>. Although these studies suggest a chemopreventive role for folic acid in colorectal cancer, to the best of our knowledge, no conclusive long-term clinical trials have been performed to evaluate the efficacy of folic acid in preventing the recurrence of colorectal adenomas. The current 3-year placebo-controlled clinical trial was, therefore, undertaken to test the hypothesis that folic acid will inhibit the recurrence of colorectal adenomas.

## MATERIALS AND METHODS

### Objectives

The primary objective of this chemopreventive trial is to determine if supplementation of folic acid for 3 years will inhibit the recurrence of colorectal adenomas. The study was initiated in December, 1998 with a 2-year patient accrual followed by a 3-year treatment with folic acid (5 mg/d) or placebo. The study was completed in June, 2005. The study protocol was approved by the Human Investigation Committee of Wayne State University. All subjects provided written informed consent.

### Study subjects and treatment

Eligible subjects were male or female, from the age of 18-80 years. However, the youngest subject enrolled in this clinical trial was 44 years of age. All subjects underwent a colonoscopy for colon polyps noted on screening flexible sigmoidoscopy or as routine surveillance for a history of colon polyps at the Detroit VA Medical Center. Prior to colonoscopy, potential subjects agreed in advance to participate if they were found to have at least one adenoma (tubular, tubulovillous, villous) > 0.5 cm, and had no exclusionary factors including hyperplastic histology of the index polyp. The histology of all polyps was examined by a pathologist blinded to the sample coding.

At study entry, all patients completed a lifestyle questionnaire. Nutritional assessment was evaluated by a registered dietitian using a Block Dietary Data System for California, Berkley. Nutrient intakes were computed according to the composition values from the U.S. Department of Agriculture<sup>[23]</sup>, supplemented with other sources<sup>[24]</sup>.

Eligible participants underwent a complete colonoscopy and had all adenomas removed at colonoscopy (with at least one adenoma > 0.5 cm). They were then randomized in a double-blind trial to receive either a 5 mg folic acid tablet (Stanley Pharmaceutical, Toronto, Canada) or one identical placebo tablet (sucrose/fructose base) daily per oral with breakfast for 3 years. Compliance was monitored by both pill count and telephone contact. Patients were seen or contacted by telephone every 90 d by the study coordinator to obtain pill counts, assess adverse events and to renew a 90 d supply of study medication. Patients were required to take ≥ 90% of their prescribed study treatment. At the end of 3 years, a repeat colonoscopy was performed, and all identified polyps were removed endoscopically. Serum and RBC folate concentrations were monitored at baseline and every 6 mo. During the course of the trial all adverse events including deaths were reported to the Institutional Review Board (IRB).

### Choice of folic acid dose

A 5 mg dose of folic acid was chosen on the basis of the previous observations that diets high in folate protect against the development of colorectal neoplasia. Although lower doses of folic acid (0.4-1 mg) resulted in a reduced relative risk of neoplasia, the risk reduction did not achieve statistical significance<sup>[12,14]</sup>. Kim *et al* noted a significant increase in colonic mucosal and systemic folate concentrations in patients who were treated for 1 year with 5 mg folic acid<sup>[25]</sup>. Folate supplementation, even at a dose of 15 mg/d, has been rarely associated with gastrointestinal or CNS adverse effects<sup>[26]</sup>. In addition, the high prevalence of dietary supplementation of folic acid (up to 1 mg/d) in the general population would have been a confounding variable.

### Exclusion criteria

Subjects were excluded if they had any of the following criteria: severe co-morbid conditions, such as severe heart disease, cancer, or other diseases causing organ dysfunction or contraindications for colonoscopy and polypectomy. Subjects with gastrointestinal disorders that affect absorption or metabolism of folic acid, B12 deficiency, and hereditary predisposition to colorectal cancer were excluded. In addition, pregnant or nursing mothers were excluded. Sexually active females agreed to use an effective method of birth control. Patients who drank more than 2 alcoholic drinks daily or who were regularly ingesting or anticipating chronic therapy with vitamin, mineral or any other nutritional supplement, steroids and non-steroidal anti-inflammatory drugs (excluding cardiopreventive aspirin doses), antineoplastic agents or folate were also excluded. Patients were asked if they had a family history of familial colorectal cancer syndrome. This question was asked to exclude obvious known history of FAP or HNPCC.

### Placebo run-in

Subjects were supplied with a known number of placebo

**Table 1** Baseline characteristics of the subjects

Characteristics	Folate group (n = 80)	Placebo group (n = 97)	P
Age (yr)	60.36 ± 10.34	62.64 ± 9.59	NS
Sex (male, %)	93	92	NS
Race			
African American	48%	50%	NS
Caucasian	51%	49%	
Other	1%	1%	
BMI (kg/m <sup>2</sup> )	31.62 ± 4.68	29.84 ± 5.71	NS
Dietary intake			
Total calories	2069.58 ± 902.9	1823.53 ± 741.12	NS
Protein (g/d)	79.57 ± 30.07	74.31 ± 36.33	NS
Fat (g/d)	88.89 ± 52.04	75.2 ± 38.38	NS
Carbohydrate (g/d)	237.29 ± 129.32	206.48 ± 86.37	NS
Fiber (g/d)	7.28 ± 5.84	8.51 ± 7.93	NS
Folate (μg/d)	184.45 ± 231.7	162.64 ± 140.23	NS
Calcium (mg/d)	577.14 ± 433.68	569.69 ± 353.75	NS
Aspirin users (≤ 325 mg/d)	24%	24%	NS
Number with advanced polyp (%)	59	53	NS
Adenomas per patient	2.34 ± 1.46	2.06 ± 1.38	NS
Total polyps per patient including hyperplastic polyps	2.88 ± 1.73	2.87 ± 2.21	NS
Current smokers	16 (35%)	19 (39%)	NS
Serum folic acid (ng/mL)	14.53 ± 19.51	11.35 ± 6.65	NS
RBC folate (ng/mL)	446.57 ± 164.81	477.82 ± 148.76	NS
Serum vit B12 (pg/mL)	472.97 ± 456.10	393.02 ± 190.93	NS
Serum calcium (mg/mL)	9.31 ± 0.48	9.33 ± 0.37	NS

NS: Not significant. Advance adenoma: ≥ 2 adenomas, large (> 1 cm) or adenoma with villous component or high grade dysplasia. Number of patients in placebo and folate group represents those who completed the baseline colonoscopy and satisfied the criteria for enrollment. Ninety-four subjects completed the 3-year study.

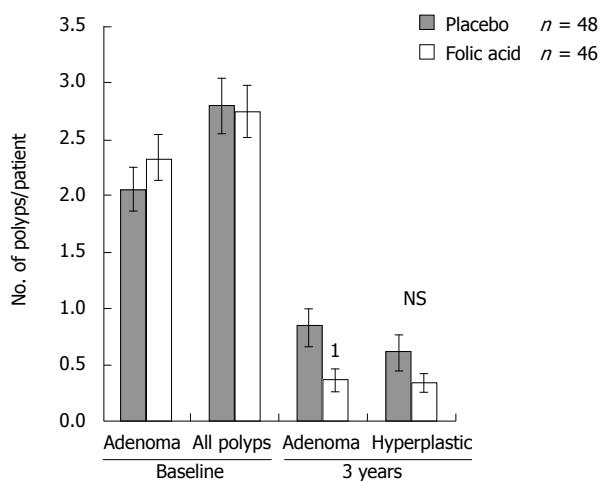
tablets to be taken daily during breakfast for 4 wk. Those who had taken ≥ 90% of their tablets were randomized.

### Randomization and stratification

Participants were randomized to the folic acid or placebo group using a stratified randomization block scheme. There were 3 stratification factors: number of adenomas (1, 2-5 and ≥ 6), size of the largest adenoma (≤ 1 cm, >1 cm) and history of polyps (no, yes). Block randomization was used in a block size of 8 to ensure that at no time during the study would there be a large imbalance between the intervention and control groups. Subject assignment was made in advance and recorded in sealed envelopes, numbered consecutively.

### Statistical analysis

The statistical analyses were all performed using the Statistical Package for Social Sciences (SPSS, version 8.0; 1997, Chicago, IL). All t-tests were two sided. Initially, the two treatment groups were compared across demographic information using independent t-tests for continuous data and Chi-Square analyses for categorical information. Treatment efficacy was assessed between intervention groups using independent t-tests across classifications of polyp morphology, lateralization, and age grouping. Logistic regression was utilized to assess the incidence of recurring polyps three years post-removal for individuals taking folic acid versus those



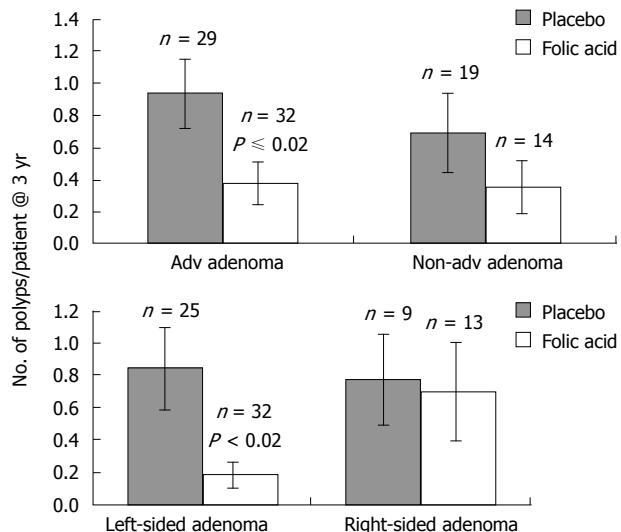
**Figure 1** Number of adenomas versus treatment. Histograms showing the number of adenomas or all types of polyps in folic acid and placebo-treated groups at baseline and 3 years after treatment.  $P = 0.02514$ , compared to the placebo-treated group. Each histogram represents the mean ± SD.

taking placebo. A contingency table was computed via Chi-Square analysis, and Odds Ratios were computed via logistic regression analysis.

## RESULTS

One hundred and thirty seven patients fulfilled the eligibility criteria. Ninety four completed the 3-year follow up colonoscopy and were included in this analysis. There were 43 subjects that dropped out from this study; of which 28 died from various causes unrelated to colon cancer and 15 subjects had geographic relocation precluding further participation. Of those who did not complete the study, there were no statistically significant differences (age, BMI, sex, NSAID/multivitamin, baseline adenoma, RBC folate, deaths) between those assigned to receive folic acid or placebo. Forty nine of the subjects who completed the 3-year follow-up received supplemental folic acid and 45 were given placebo tablets. At post-randomization, there was no statistical difference in the serum levels of folic acid between the two groups (Table 1). Demographic data and other baseline parameters were also comparable between these two groups (Table 1). At the 3-year follow-up colonoscopy, patients in the folic acid group showed a significantly lower number of adenomas per patient ( $0.36 \pm 0.69$ ) with a 64% lower risk ratio, compared to the placebo group ( $0.82 \pm 1.17$ ; odds ratio, 2.77;  $t = -2.26$ ,  $P = 0.02514$ , 95% CI, 0.06-0.84; Chi Square = 11.2,  $P = 0.00142$ ; Figure 1). The recurrence of adenoma at the 3-year follow-up was twice as high in the placebo group, compared to the folic acid group. There was no significant difference in the recurrence of hyperplastic polyps between the groups (folic acid:  $0.44 \pm 0.89$ , placebo:  $0.51 \pm 0.94$ ;  $P = 0.74$ ; 95% CI, 0.31-0.43).

Folic acid supplementation caused a significant reduction ( $P = 0.02335$ ) in the recurrence of adenomas in patients with advanced adenoma [large (> 1 cm)



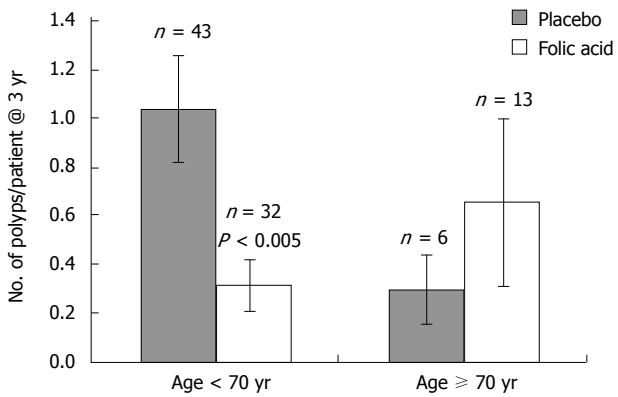
**Figure 2** Polyp characteristics and response to treatment. Recurrence of advanced [large ( $> 1$  cm) and polyps with villous component] or non-advanced adenomas (upper panel) as well as right or left-sided adenomas (lower panel) following 3 years of treatment with a high dose of folic acid. The numbers of subjects as well as the levels of significance between the two groups are shown.

adenoma or adenoma with villous component or high grade dysplasia], compared to the placebo-treated controls (Figure 2, upper panel). Those with non-advanced adenomas also showed a reduction in the recurrence of adenomas with folic acid, compared to placebo controls, but this was not statistically significant (Figure 2, upper panel). On further stratifications, it was noted that subjects with left-sided polyps had a significantly lower ( $P = 0.01964$ ) recurrence of adenomas than those with right-sided polyps in response to folic acid supplementation, when compared with the corresponding placebo-treated controls (Figure 2, lower panel).

Since colorectal cancer is an age-related disease, the data were analyzed to determine the age-related differences in responsiveness to folic acid. We observed that the younger subjects responded better than older subjects in that the recurrence of adenomas was significantly lower ( $P = 0.00496$ ) in younger patients, compared to older patients (Figure 3). This response was maintained until 70 years of age (Figure 3). However, patients older than 70 years of age failed to respond to folic acid supplementation demonstrating a higher recurrence rate of polyps as compared to the placebo group. This difference was not statistically significant (Figure 3). There were more deaths in the folic acid group, compared to the placebo-treated group, but this difference was not statistically significant (19 in folic acid vs 9 in placebo,  $P > 0.1$ ).

## DISCUSSION

Despite recent advances in medicine, the mortality from colorectal cancer, a leading cause of death in the USA and other Western countries, still remains unacceptably high. Therefore, the search for strategies to prevent the development and progression of colorectal cancer has



**Figure 3** The effect of age on the response to treatment. Recurrence of adenomas in response to 3 years of folic acid treatment in patients over or below 70 years of age is shown. The number of subjects in each group as well as the levels of significance is shown in the figure.

greatly intensified. Chemoprevention offers a viable option to block neoplastic inception or delay disease progression. Since colorectal cancer is an age-related disease, typically diagnosed after the age of 50, any delay in the onset and subsequent progression of this disease through the use of dietary agents is likely to have significant health benefits. Folic acid has recently emerged as a major contender in the repertoire of promising colorectal cancer prevention agents. A number of animal, as well as a few case controlled human studies, strongly support folic acid as a potentially efficacious chemopreventive agent with a negligible toxicity profile<sup>[3]</sup>. However, there have been no systematic conclusive studies to examine the effect of supplemental folic acid on recurrence of adenomas in the colon.

Our data, for the first time, show that the daily consumption of a high dose of folic acid over a period of 3 years prevents the recurrence of colorectal adenomas. This reduction could not be attributed to differences in diet or lifestyle. The patients completed a detailed lifestyle questionnaire and nutritional assessment with both study groups demonstrating statistically similar caloric, fiber, fat and protein intake as well as similar baseline BMI, folate, B12 and calcium status. Additionally, the groups were similar with regard to aspirin use and the number and type of adenoma at baseline. Most patients were male which is consistent with the Veterans Affairs based population. Interestingly, patients who had large adenomas or adenomas with a villous component (referred to as advanced adenomas) responded better to high dose folate supplementation, as evidenced by the significantly reduced number of recurrent adenomatous polyps. A similar phenomenon was also observed among patients with left-sided adenomas and those who were less than 70 years of age. Although the reasons for this are not fully understood, it is plausible that the increased responsiveness of these subjects could be a result of greater tissue accumulation of folic acid due to a better active folate transport system. The basis for this inference comes from the observations by Mennan *et al* which suggest that mucosal folate levels may be a determinant factor

in the development of adenomas<sup>[27]</sup>. They demonstrated that the levels of folate in adenoma, carcinoma as well as normal appearing adjacent mucosa are lower than the corresponding polyp-free controls<sup>[27]</sup>. Future studies analyzing folate levels in adjacent tissue near recurrent adenomas need to be completed.

Although several clinical trials have suggested a role for folic acid in the prevention of colorectal adenomas, there are no prospective controlled trials addressing this issue at the dose of 5 mg<sup>[5-8]</sup>. It has also been demonstrated that supplementation of a high dose of folic acid in animals with colonic neoplasia may accelerate the progression of carcinogenesis<sup>[16]</sup>. A more recent human study showed that supplemental folic acid may not reduce the incidence of colorectal adenomas and in some cases may actually increase the risk<sup>[28]</sup>. Although the reasons for these controversial issues are not fully understood, one possibility could be attributed to the dual modulatory effect of folic acid on carcinogenesis. It has been demonstrated that the timing and the dose of folate intervention has a promoting effect on the progression of established neoplasms, while it could have a chemopreventive effect if given in premalignant conditions. Data from our clinical trial clearly supports a chemopreventive role of folic acid since supplementation of this vitamin for 3 years inhibits the recurrence of colonic adenomas. More importantly, none of the patients in the folate treatment group were found to have histologically aggressive adenomas or carcinoma at final endoscopy.

The mechanisms by which folic acid exerts its chemopreventive role in colorectal carcinogenesis are becoming increasingly understood. Since folic acid plays a key role in DNA methylation and cellular homeostasis, folate deficiency may result in a variety of cellular consequences including misincorporation of uracil for thymidine during DNA synthesis resulting in an increased spontaneous mutation as well as chromosomal abnormalities and errors in DNA synthesis<sup>[29-33]</sup>. The restoration of DNA methylation status in patients with colorectal neoplasms treated with supraphysiological doses of folic acid lends further support to the hypothesis. In a recent study, we examined the changes in mutational status of APC, DCC and p53 genes in macroscopically normal appearing rectal mucosa at baseline and after 1 year of treatment with either folic acid or placebo<sup>[34]</sup>. We have observed that folate supplementation prevented the loss of heterozygosity (LOH) of the DCC gene in 5 out of 5 patients who demonstrated baseline heterozygosity, whereas 2 out of 4 placebo treated patients with baseline heterozygosity demonstrated complete allelic loss. Mucosal protein levels of DCC were also reduced in 70% of placebo treated patients compared to only 10% of folate treated patients<sup>[34]</sup>. Cell culture studies have further demonstrated that supplemental folic acid and its metabolite 5-methyltetrahydrofolate (5-MTF) inhibit EGF-receptor (EGFR) promoter activity in colon cancer HCT-116 cells by enhancing methylation<sup>[35]</sup>. Since EGFR is known to play a critical role in the development and progression of a wide variety of epithelial cancers,

including colorectal cancer<sup>[36,37]</sup>, the inhibition of basal as well as serum-stimulated EGFR promoter activity by folic acid and 5-MTF suggests that these changes may partly contribute to specific inhibition of growth-related processes in colorectal neoplasia. Supplemental folic acid may also attenuate the downstream events of EGFR signal transduction pathways that are critically involved in modulating growth-related processes. We have observed that in polypectomized patients, supplemental folic acid for 1 year leads to a decreased nuclear translocation of β-catenin<sup>[38]</sup>, which interacts with the T-cell factor 4 (TCF-4) transcription factor to induce expression of specific target genes, including cyclin D1, VEGF and c-myc, which promote cell growth and proliferation<sup>[39-42]</sup>.

The dose of folic acid supplementation may be important when considering the differing effects of supplementation. This has been more explored in the cardiovascular literature in attempting to modulate homocysteine levels where the VISP study showed greater efficacy at higher doses in lowering homocysteine levels<sup>[43]</sup>. A cogent example of this was the recently published large scale study interventional study of over 1000 men and women who were randomized to receive either 1mg folic acid or placebo. The endpoints were similar to our study, but the 3 year follow up data were very different in that no effect was seen for the dose used<sup>[28]</sup>. Of interest, there was no effect of gender in that study which may have important implications for our study in terms of applicability to the general population. The timing of supplementation may also be important<sup>[44]</sup>.

In summary, daily consumption of a high dose of folic acid over 3 years prevents the recurrence of colorectal adenomas. Patients below 70 years of age and those with left-sided colonic adenomas or advanced adenomas responded better to folic acid supplementation. We conclude that folic acid is an effective chemopreventive agent for colorectal adenomas, and more specifically for that category of adenomas which are believed to possess the highest risk of cancer progression.

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## COMMENTS

### Background

Colorectal cancer is one of the major causes of cancer related deaths. In the US and the other developed countries, 50% of the subjects diagnosed with colon cancer die. Therefore, there is a need to prevent the development and progression of colon cancer using chemopreventive agents. Water soluble vitamins, such as folic acid, have shown to have chemopreventive potential for colon cancer. Aim of this investigation was to determine whether folic acid supplementation will reduce the recurrence of colorectal polyps, the precursors of colorectal cancer, we performed a double-blind placebo-controlled trial in patients with polyps.

## Research frontiers

Several clinical trials have noted an inverse relationship between dietary folic acid and the development of colorectal cancer. A folate deficient diet is thought to increase the risk of colonic neoplasia, whereas supplementation of this nutrient may be chemopreventive. However, the timing of folate supplementation may be particularly important since folate intervention, after the establishment of microscopic neoplastic foci in the colorectal mucosa, may promote rather than suppress colorectal carcinogenesis. A similar approach using aspirin and similar non-steroidal anti-inflammatory agents have shown promising activity in prevention of colon cancer after resection of colon polyps.

## Innovations and breakthroughs

This is a large randomized, single institution, double-blind placebo controlled trial demonstrating the efficacy of folic acid in secondary chemoprevention of colorectal cancer. This is the only study examining high dose supplementation over a period of three years further establishing safety and efficacy of large dose of folic acid. It should also be noted that the present study is the only study of its kind specifically targeting the US veteran population.

## Applications

Daily consumption of a high dose of folic acid over 3 years prevents the recurrence of colorectal adenomas. Particularly, patients below 70 years of age and those with left-sided colonic adenomas or advanced adenomas responded better to folic acid supplementation. We conclude that folic acid is an effective chemopreventive agent for colorectal adenomas, and more specifically for that category of adenomas which are believed to possess the highest risk of cancer progression.

## Peer review

This is an important study which, for the first time, demonstrates that daily consumption of a high dose folic acid over a prolonged period of time leads to a significant reduction in the recurrence of colonic adenomas. The results suggest that folic acid may be an effective chemopreventive agent for colorectal neoplasia.

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RAPID COMMUNICATION

## CT colonography after incomplete colonoscopy in subjects with positive faecal occult blood test

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surgery for colonic masses of indeterminate nature. Four subjects refused further examinations. CTC correctly identified 2 colonic masses and 20 polyps. PPV for masses or polyps greater than 9 mm was of 87.5%. Per-lesion and per-segment PPV were, respectively, 83.3% and 83.3% for polyps greater or equal to 10 mm, and 77.8% and 85.7% for polyps of 6-9 mm.

**CONCLUSION:** In the context of a screening program for CRC based on FOBT, CTC shows high per-segment and per-lesion PPV for colonic masses and polyps greater than 9 mm. Therefore, CTC has the potential to become a useful technique for evaluation of the non visualized part of the colon after incomplete colonoscopy.

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**Key words:** Computed tomography colonography; Virtual colonoscopy; Incomplete colonoscopy; Positive faecal occult blood test; Colorectal cancer screening

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### Abstract

**AIM:** To report our experience with computed tomography colonography (CTC) systematically performed in subjects with positive faecal occult blood test (FOBT) and an incomplete colonoscopy in the setting of a population-based screening for colorectal cancer (CRC). **METHODS:** From April 2006 to April 2007, 43290 individuals (age range 50-70) who adhered to the regional screening program for the prevention of CRC underwent immunochemical FOBT. FOBT was positive in 1882 subjects (4.3%). 1463 (77.7%) of these subjects underwent colonoscopy, 903 performed in a single center. Of 903 colonoscopies 65 (7.2%) were incomplete. Forty-two of these subjects underwent CTC. CTC was performed with a 16-MDCT scanner after standard bowel prep (polyethylene glycole) in both supine and prone position. Subjects whose CTC showed polyps or masses were referred to the endoscopist for repeat colonoscopy under sedation or underwent surgery. Per-lesion and per-segment positive predictive values (PPV) were calculated.

**RESULTS:** Twenty-one (50%) of 42 CTCs showed polyps or masses. Fifty-five of these subjects underwent a repeat colonoscopy, whereas 2 subjects underwent

### INTRODUCTION

Randomized clinical trials have demonstrated that screening with faecal occult blood test (FOBT) reduces mortality for colorectal cancer (CRC)<sup>[1]</sup>. Accordingly, population-based screening with FOBT is currently recommended by the European community health associations and has been applied in many countries, including Italy<sup>[2]</sup>.

Subjects with a positive FOBT are usually examined with a total colonoscopy which allows removal of polyps and histological diagnosis of the lesions. However, colonoscopy can be incomplete due to several reasons, including intolerance to the procedure, adhesions from previous surgery, redundant colon and the presence of stenosis. The reported rates of incomplete colonoscopy from various studies carried out in U.S. and Europe over the past 15 years range from 4% to 25%<sup>[3,4]</sup>.

In order to complete evaluation of the colon, radiological examinations can be performed such as double contrast barium enema (DCBE)<sup>[5]</sup> and computed tomography colonography (CTC). In particular, several studies have shown that CTC is a valuable tool to evaluate the proximal colon after incomplete colonoscopy<sup>[6-10]</sup>, and the American Gastroenterologists Association (AGA) recognized that CTC is indicated for adults with failed colonoscopy<sup>[11]</sup>.

We report the results of CTC systematically performed in subjects with positive FOBT and incomplete colonoscopy in the context of a population-based screening programme for CRC with FOBT.

## MATERIALS AND METHODS

### Subjects

This prospective study was approved by our institutional review board; informed consent was obtained in all subjects. A population-based screening program for CRC has been active in the Tuscany Region, Italy, since 1998. The screening protocol is directed to all subjects aged 50-70 living in the regional area who are invited via mail every second year to perform immunochemical FOBT. Subjects with negative FOBT are notified of their result by mail and advised to repeat screening after two years. Subjects with positive test are invited to perform colonoscopy<sup>[12]</sup>.

From April 2006 to April 2007, 43 290 asymptomatic individuals aged 50-70 years attended the FOBT-based Florence District screening program and were tested. FOBT was positive in 1882 (4.3%) subjects. These subjects were invited to undergo colonoscopy assessment: 1463 (77.7%) subjects underwent colonoscopy and 419 refused. 903 colonoscopies were performed in a single center by two experienced endoscopists.

According to the screening protocol, colonoscopy was performed without sedation. 838 (92.8%) colonoscopies were complete (i.e. the caecum was reached) and 65 (7.2%) were incomplete. The levels at which colonoscopy was interrupted were the sigmoid colon in 33 subjects, the descending colon in 21, the transverse colon in 8 and the ascending colon in 3. According to the endoscopist's report, presumptive reasons for incomplete colonoscopy were dolichocolon (14.8%), diverticular disease (23.1%), adherences due to previous abdominal surgery (16.4%) or intolerance to the procedure (12.1%).

Forty-two of these 65 subjects (17 males, 25 females; mean age 60.7 years; age range 51-70) agreed to complete colonic examination with CTC and constitute of the base for this report.

CTC was performed within 6 wk after incomplete colonoscopy (mean interval 16 d). In those subjects in whom endoscopic polyp removal was performed during the incomplete colonoscopy, CTC was delayed for at least 1 mo after polypectomy.

### CTC technique

All subjects underwent a standard bowel preparation for CTC with 4 L of a polyethylene glycole solution (Isocoloran; Giuliani, Milan, Italy) administered the day before the procedure and a low residue diet for 3 d. All subjects received intravenously 30 mg of scopolamine butylbromide (Buscopan; Boehringer Ingelheim, Florence, Italy) before air insufflation, in order to improve colonic distension<sup>[13]</sup>.

The subjects were placed on the right lateral decubitus and a 24 Fr rubber catheter, Foley type, with a small retention balloon (10 mL) was inserted into the rectum. After catheter positioning the patient was turned in supine position and colonic distension was obtained with manual insufflation of room air. Air was administered from an enema bag connected to the rectal tube with a maximum capacity of 2 L. Insufflation was performed by gently squeezing the enema bag during 3 to 5 min up to subject tolerance.

Both supine and prone CT scans were obtained in all subjects. Colonic distension was evaluated with an anterior-posterior scout view in both supine and prone position, and additional air was inflated using a manual bulb if distension was unsatisfactory. In one subject, unable to stay prone because of abdominal pain, a right lateral decubitus acquisition was obtained instead of the prone scan. Intravenous contrast medium was not used.

CTC was performed with a 16-MDCT scanner (Sensation 16; Siemens, Erlangen, Germany) using a detector configuration of 16 mm × 0.75 mm, 120 kVp, 50 effective mAs, tube rotation time of 500 ms and a pitch of 1.25. Data were reconstructed using a slice thickness of 1 mm with a reconstruction increment of 0.7 mm (30% overlap). For each acquisition CTDI<sub>vol</sub> was 4.15 mGy with a calculated equivalent dose of 3.5 mSv for females and 2.7 mSv for males (CT Patient Dosimetry Calculator, ImPACT; measures executed on MonteCarlo Phantom).

### CTC evaluation

The images of each study were transferred to a workstation equipped with CTC dedicated software (Syngo; Siemens, Erlangen, Germany). The software provides axial, multi-planar reformatted (MPR), endoluminal surface-shaded images and double-contrast-like reconstructions of the colon. All studies were interpreted on the workstation by two readers, one experienced gastrointestinal radiologist and one radiology resident, by consensus.

Preliminarily, the degree of colonic distension was evaluated on axial images. The colon was divided into six segments: caecum, ascending, transverse, descending, sigmoid and rectum (the different segments were evaluated both in supine and prone acquisitions). Distension for each segment was graded on a scale from 0 to 3, in

which a grade of 0 indicated complete collapse and a grade of 3 optimal distension<sup>[13]</sup>. The least-distended section of any individual segment was used to assign the overall distention score for that segment. Colonic distension was deemed clinically adequate if all segments had a score of 2 or 3 at least in supine or prone acquisition.

Moreover, we assessed the adequacy of preparation by evaluating the proportion of colonic segments containing residual faecal matter or fluid for each subject (no specific attempt was made to rank the amount of fluid or stool).

CTCs were evaluated with a primary 3D approach, using 2D for problem solving. In all cases, endoluminal navigation was performed from rectum to caecum and backwards for both supine and prone acquisitions. Then axial images were examined with an abdominal window (level 40 HU, width 350 HU), in order to discover areas of colonic wall thickening, and to look for extra-colonic findings.

All lesions detected at CTC were localized according to their segmental location in the colon. Each lesion was measured taking account of its maximum diameter on 2D images viewed with a bone window (level 400 HU, width 2000 HU).

### **Subjects management**

All subjects with CTC showing polyps were referred to the endoscopist to repeat colonoscopy under sedation. Also subjects with colonic masses were referred to the endoscopist who evaluated in agreement with the subject the opportunity of a repeat colonoscopy or a surgical consult.

The results of repeat colonoscopy and/or the pathological findings on surgical specimens were used as a gold standard for CTC performance assessment. Lesions were measured by open biopsy forceps at endoscopy and with ruler for pathological specimens.

CTC findings were classified as true-positive or false-positive results. A true-positive lesion at CTC was defined as a lesion that was confirmed at repeat colonoscopy or at surgery, a lesion that was in the same or an adjacent colonic segment, and a lesion for which the size correlated within 50% of the diameter. A lesion was defined as false positive if the lesion reported at CTC was not detected at repeat colonoscopy, was not in the same or an adjacent colonic segment or there was more than a 50% discrepancy in the lesion diameter. Endoscopic evidence of polyps or masses at repeat colonoscopy not detected at CTC was assumed as a false negative result for CTC.

Descriptive statistics were used to calculate per-lesion and per-segment positive predictive values (PPV) for polyps equal or greater than 10 mm, for polyps of 6–9 mm and for smaller lesions (< 6 mm). In per-segment analysis the colon was divided into six segments (caecum, ascending, transverse, descending, sigmoid and rectum) and the segments examined by initial colonoscopies were excluded from the evaluation.

Subjects with a negative CTC did not undergo further examination and were scheduled for standard follow-up according to the screening protocol<sup>[12]</sup>.

**Table 1** CTC results for polyps

	True positive	False positive	False negative	Per-lesion PPV (%)
Polyps < 6 mm	8	4	2	66.7
Polyps 6–9 mm	7	2	0	77.8
Polyps ≥ 10 mm	5	1	0	83.3
Total	20	7	2	

## **RESULTS**

Complete colonic distension was obtained in 36 (85.7%) of 42 subjects. Considering both supine and prone acquisitions, the rectum in one patient and the sigmoid colon in 5 patients were not adequately distended. Incomplete distension was mainly due to advanced diverticular disease. The mean overall bowel distension scores were  $2.75 \pm 0.37$  for supine position and  $2.84 \pm 0.23$  for prone position. Either fluid and faecal residua, or inadequate distension precluded evaluation of 14 (5.6%) of 252 colonic segments [rectum ( $n = 1$ ), sigmoid colon ( $n = 10$ ), descending colon ( $n = 3$ )].

Twenty one (50%) of 42 CTCs showed polyps or colonic masses of indeterminate nature. Fifteen patients with polyps at CTC underwent repeat colonoscopy under sedation. Two patients with colonic masses of indeterminate nature were referred to surgical consult and underwent colectomy. Four patients with CTC findings of polyps smaller than 6 mm did not undergo repeat colonoscopy because of medical problems or refusal.

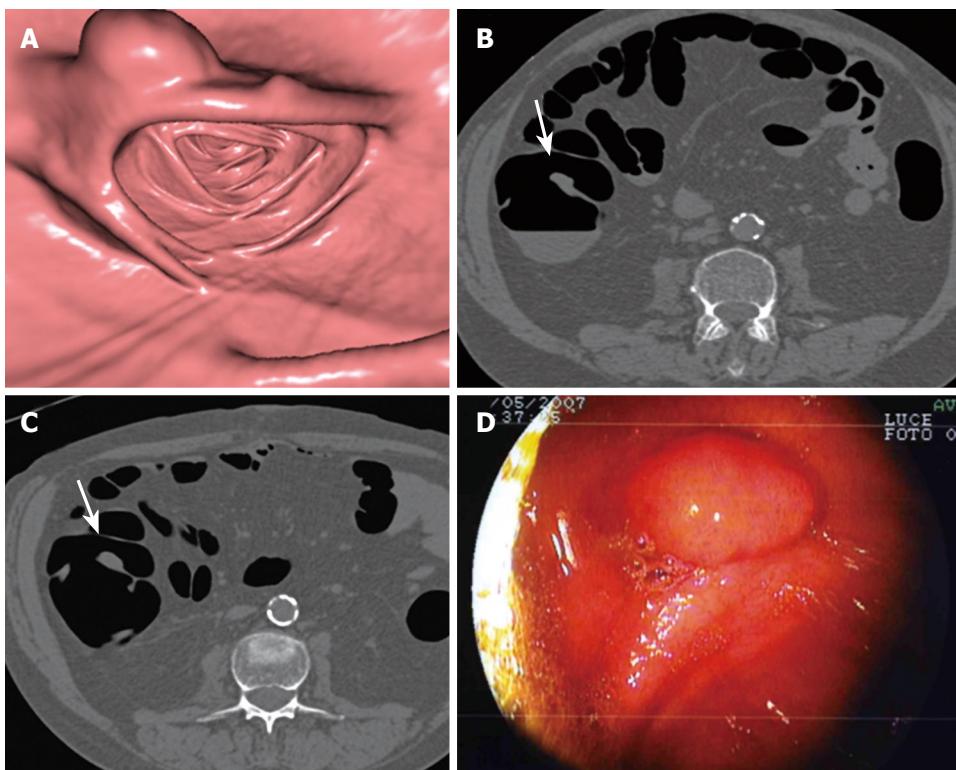
All repeat colonoscopic examinations were performed within a mean of 34 d after CTC (range 15 d to 6 mo) and were complete. No complications occurred after CTC or diagnostic and operative colonoscopy.

CTC correctly identified 20 polyps in segments not visualized at initial colonoscopy, and gave 7 false positive and 2 false negative results (Table 1). All polyps were endoscopically removed and histology was obtained (Figure 1). Of 20 polyps 11 were adenomas (2 tubulo-villous adenomas with high-grade dysplasia, 6 tubulo-villous adenomas, 3 tubular adenomas) and 9 hyperplastic or inflammatory polyps. No cancers were observed. The two false negative polyps not identified at CTC included one hyperplastic polyp and one tubular adenoma, both smaller than 6 mm. A total of 5 advanced adenomas were found at CTC and histologically proved.

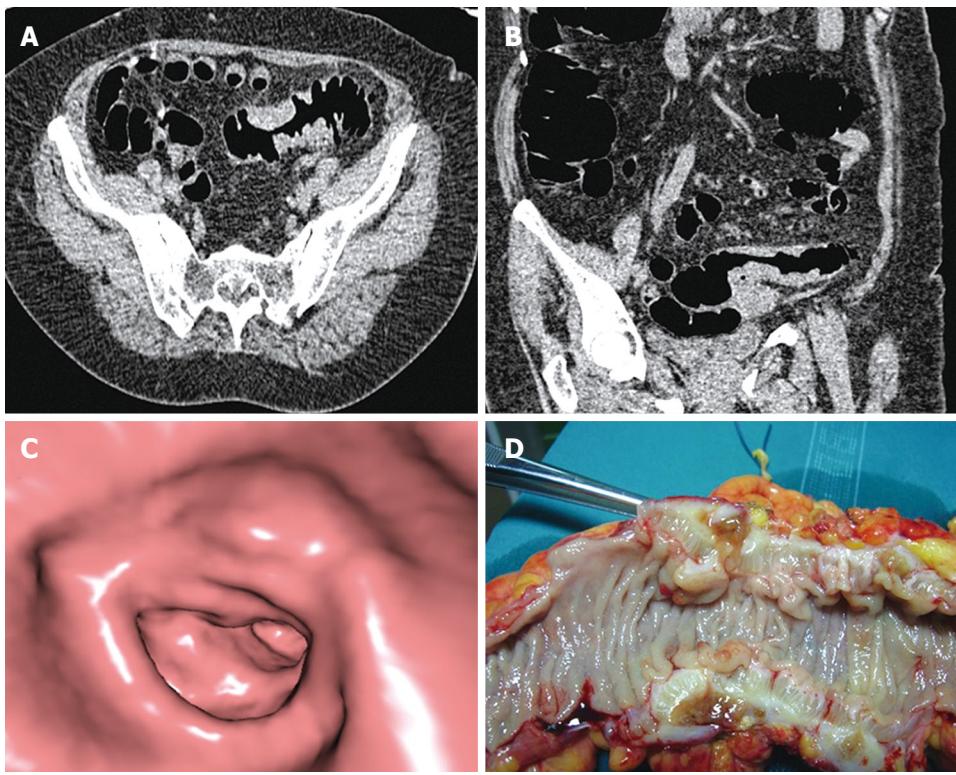
CTC correctly depicted two colonic masses of indeterminate nature at the level of the proximal sigmoid colon, which were found to be advanced diverticular disease complicated by stenosis at surgery (Figure 2). None of these patients had endoscopically visualized masses or adenomatous polyps at initial colonoscopy.

Per-segment analysis was performed on patients who completed repeat colonoscopy or underwent surgery ( $n = 17$ ). On a total of 102 colonic segments, 26 segments examined by initial colonoscopies were excluded, and the analysis was based on the remaining 76 segments.

CTC showed a PPV for masses or polyps greater than 9 mm of 87.5%. Per-lesion and per-segment PPV



**Figure 1** Adenomatous polyp of 13 mm of the ascending colon in a 61-year-old female with initial colonoscopy interrupted at the descending colon for severe discomfort. **A:** Endoluminal CT image of the ascending colon shows 13 mm sessile polyp lying on a fold; **B** and **C:** Axial CT images acquired in supine and prone position show the polypoid lesion (arrow) on a fold; **D:** Sessile polyp of 13 mm of the ascending colon found at repeat colonoscopy. Histology evaluation revealed adenomatous polyp.



**Figure 2** Stenosing mass of the proximal sigmoid colon in a 69-year-old female with initial colonoscopy interrupted at the distal sigmoid colon for diverticular disease. **A:** Axial CT image acquired in prone decubitus shows a stenosing lesion of the proximal sigmoid colon with CT findings suspicious for malignancy: eccentric wall thickening, "shoulder sign", absence of pericolonic fat stranding; **B:** Coronal oblique multiplanar reformation shows the lesion in the sigmoid colon; **C:** Endoluminal CT image shows the passage from the normal colonic wall to the stenosis; **D:** Surgical specimen from left hemicolectomy shows a stenosing lesion of about 5 cm in the proximal sigmoid colon with marked wall thickening due to advanced diverticular disease confirmed at histological evaluation.

were respectively 83.3% and 83.3% for polyps greater or equal to 10 mm, 77.8% and 85.7% for polyps of 6-9 mm, 66.7% and 50% for polyps smaller than 6 mm.

Diverticular disease was found in 20 subjects (47.6%) and 5 of these subjects (11.9%) showed signs of chronic diverticulitis such as diffuse wall thickening and pericolic fat stranding.

Major extra-colonic findings included aneurysm of the abdominal aorta ( $n = 1$ ), renal masses ( $n = 2$ ), he-

patic focal lesion other than cystic ( $n = 1$ ), splenomegaly ( $n = 1$ ) and pulmonary nodules ( $n = 2$ ).

## DISCUSSION

Due to its natural history CRC is an ideal candidate for screening<sup>[14]</sup>. In fact, most CRC originate from pre-existing adenomatous polyps that, in 10 to 15 years, undergo malignant transformation<sup>[15]</sup>. Likelihood for malignant

transformation is not the same for all adenomas. In particular adenomas equal or greater than 10 mm (advanced adenomas) tend to become malignant after an average of 5.5 years, whereas it is estimated that less than 1% of adenomas smaller than 10 mm contain a cancer<sup>[14]</sup>. Thus, advanced adenoma is a precancerous lesion and should be considered the main target of a screening test for CRC.

In the majority of screening programmes, subjects with positive FOBT are invited to undergo colonoscopy, which can be performed with or without sedation. Colonoscopies without sedation can be incomplete in up to 25% of the cases<sup>[4]</sup> and in our series incomplete colonoscopies were 7.2%. Before adoption of CTC in such cases, we previously performed DCBE.

Several studies showed that DCBE has a low accuracy in detecting colonic neoplasms, with sensitivity for adenomas greater than 9 mm in the range of 45%-50%<sup>[16]</sup>. CTC is more accurate in detecting colorectal neoplasms as shown in some meta-analyses in which the performance of CTC *versus* optical colonoscopy revealed sensitivity in the range of 85%-90% and specificity of about 95% for polyps greater than 9 mm<sup>[17,18]</sup>.

We evaluated the performance of CTC after incomplete colonoscopy in the setting of a large population based screening program with FOBT. In this context, CTC showed its potential for diagnostic assessment, identifying 2 colonic masses, 5 advanced adenomas and 6 smaller adenomas. CTC gave 7 false positive results which led to unnecessary repeat colonoscopy. One false positive was a polypoid lesion of 14 mm of the ascending colon that was visible only on the supine dataset. Four false positive results were for polyps smaller than 6 mm which should not be reported according to current recommendations<sup>[19]</sup>.

The possibility that diverticular disease simulates with colonic masses is well known, as is the fact that the differential diagnosis with cancer can be difficult with CT<sup>[20]</sup>. In our series, CTC detected two colonic masses of the proximal sigmoid colon which showed CT features suspicious for malignancy, but were demonstrated by pathology to be due to diverticular disease in absence of any malignancy. In both cases, initial colonoscopy was interrupted at distal sigmoid colon because of advanced diverticular disease. In the two cases, the endoscopist and the subject decided not to perform a repeat colonoscopy and the subjects were referred to the surgeon to undergo colectomy.

Colonic distension and cleansing were adequate for an accurate examination in the majority of our cases. In some cases, colonic collapse or repletion by fluid or faeces precluded evaluation of the rectum, sigmoid or descending colon. This limitation can be partially overcome by the fact that lower colonic segments are usually examined at initial colonoscopy. Indeed, segments examined by initial colonoscopies were excluded from per-segment analysis.

Almost 50% of 42 patients of our study had diverticular disease which represented an obstacle to complete conventional colonoscopy. Our series showed that

diverticular disease did not seriously compromise colonic distension and evaluation of the proximal colon at CTC.

Previous studies on CTC after incomplete colonoscopy have been conducted<sup>[6-10]</sup>. These studies were inhomogeneous regarding the patients' selection, because they included asymptomatic as well as symptomatic subjects. Our results in terms of PPV, acquired in a selected group of screening subjects, were comparable with those obtained in the largest study on CTC after incomplete colonoscopy conducted by Copel *et al*<sup>[10]</sup>. They reported per-lesion PPV of 91.7% for masses, of 70% for polyps of 10 mm or greater, and of 30.4% for polyps of 6-9 mm<sup>[10]</sup>. In our small group of subjects, we considered, altogether masses and polyps greater than 9 mm obtaining a similar result (PPV of 87.5%). Our better results for medium sized polyps (6-9 mm) with a per-lesion PPV value of 77.8% might be due to thinner collimation for CT scanning and double reading of the examinations we utilize.

We observed a significant number of false positive results which led to unnecessary repeat colonoscopy. The use of faecal tagging should reduce the number of false positive, enabling a better distinction between polyps and faecal residues, as showed in a series of CTC after incomplete colonoscopies<sup>[9]</sup>.

Our study had limitations. First, it was carried out on a small series of subjects. Second, repeat colonoscopy was conducted with segmental blinding, and this could have increased the number of false positive results of the CTC. Third, since subjects with negative CTC did not undergo further examinations, we could not evaluate sensitivity and specificity of CTC with respect to optical colonoscopy.

In conclusion, in the context of a population-based screening program for CRC based on FOBT, CTC showed a high per-segment and per-lesion PPV for colonic masses and polyps greater than 9 mm. Therefore, CTC has the potential to become a useful technique for evaluation of the non-visualized part of the colon after incomplete colonoscopy and should replace DCBE.

## ACKNOWLEDGMENTS

We thank Mr. Graziano Giannini, radiographer, for his collaboration in performing CTCs.

## COMMENTS

### Background

Colorectal cancer (CRC) is a relevant neoplastic disease for its high incidence and mortality. Due to its natural history CRC is suitable for screening. Screening with faecal occult blood test (FOBT) reduces mortality from CRC. Subjects with positive FOBT are usually examined by colonoscopy which can be incomplete.

### Research frontiers

Computed tomography colonography (CTC) is a non-invasive imaging technique with a high sensitivity and specificity in the diagnosis of colonic cancer and polyps equal or greater than 10 mm, which are the target for screening. Therefore, it might represent a second step examination before colonoscopy to examine subjects with positive FOBT.

### Innovations and breakthroughs

This study on CTC after incomplete colonoscopy was conducted in the frame of a population based screening program. Previous reports on this topic were car-

ried out in heterogeneous samples of symptomatic and asymptomatic subjects or patients with known colonic pathology.

### Application

In the context of a screening program with FOBT, CTC has high positive predictive value (PPV) for colonic masses or polyps equal or greater than 10 mm and should replace double contrast barium enema (DCBE) for evaluation of the non visualized part of the colon after incomplete colonoscopy.

### Terminology

CTC is a thin slice CT scan of the abdomen after adequate bowel preparation and colon insufflation in which data are reconstructed providing axial, multiplanar, and endoluminal views (virtual colonoscopy), in order to visualize colonic wall. Colonoscopy is the more accurate technique to evaluate colonic internal surface and it is performed passing a flexible tube with fiber optic through the anus. FOBT is a chemical test that can detect tiny traces of blood in the stool that may indicate the presence of CRC.

### Peer review

This paper shows the usefulness of CTC after insufficient colonoscopy in order to detect colorectal lesions. It was conducted as a part of population-based screening programme of CRC. It's an interesting study.

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RAPID COMMUNICATION

## Development of hepatorenal syndrome in bile duct ligated rats

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placed in metabolic cages and, at the end of the experiment, blood and urine samples were obtained. Histology and hydroxyproline content were analyzed in liver and renal tissue.

**RESULTS:** Rats with 2 wk of BDL increased free water clearance ( $P = 0.02$ ), reduced urinary osmolality ( $P = 0.03$ ) and serum creatinine ( $P = 0.01$ ) in comparison to the sham group. In contrast, rats at 6 wk of BDL showed features of HRS, including significant increase in serum creatinine and reductions in creatinine clearance, water excretion and urinary sodium concentration. Rats with 4 wk of BDL exhibited an intermediate stage of renal dysfunction. Progressive hepatic fibrosis according to post-procedure time was confirmed by histology. The increased levels of liver hydroxyproline contrasted with the absence of structural changes in the kidney, as assessed by histology and unchanged hydroxyproline content in renal tissue.

**CONCLUSION:** Our data show that BDL produced progressive renal dysfunction without structural changes in the kidney, characterizing HRS. The present model will be useful to understand the pathophysiology of HRS.

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**Key words:** Hepatorenal syndrome; Bile duct ligation; Renal function; Renin angiotensin system

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Pereira RM, dos Santos RAS, Oliveira EA, Leite VHR, Dias FLC, Rezende AS, Costa LP, Barcelos LS, Teixeira MM, Simões e Silva AC. Development of hepatorenal syndrome in bile duct ligated rats. *World J Gastroenterol* 2008; 14(28): 4505-4511 Available from: URL: <http://www.wjgnet.com/1007-9327/14/4505.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4505>

### Abstract

**AIM:** To evaluate in bile duct ligated rats whether there were progressive alterations of renal function without changes in histopathology.

**METHODS:** Male Wistar rats were submitted to sham-surgery or bile duct ligation (BDL) and divided according to the post-procedure time (2, 4 and 6-wk). To determine renal function parameters, rats were

### INTRODUCTION

Hepatorenal syndrome (HRS) has been defined as a progressive renal failure that occurs in patients with chronic liver disease and advanced hepatic failure in

the absence of any apparent clinical cause for renal insufficiency<sup>[1,2]</sup>. HRS represents the final stage of a process that gradually reduces the renal blood flow and the glomerular filtration rate (GFR) due to a marked renal vasoconstriction<sup>[1,4]</sup>. Despite the severity of renal failure, no significant histological abnormalities are found in the kidneys.

There are many experimental models to induce hepatic fibrosis<sup>[5]</sup>. However, none of them has been evaluated systematically as a model of hepatorenal syndrome. The two most frequently used experimental models of liver disease are the administration carbon tetrachloride, and the common bile duct ligation (BDL)<sup>[6]</sup>. The main advantage of BDL is to allow the study of renal function alterations in a short period of time with lower mortality rates than the administration of carbon tetrachloride<sup>[6]</sup>. In addition, this model mimics clinical conditions characterized by obstructive jaundice, such as biliary atresia and choledocal cysts<sup>[5,6]</sup>. In this study, we aimed to systematically evaluate renal function parameters, renal histology and tissue hydroxyproline content at different time-points of BDL.

## MATERIALS AND METHODS

### **Animals and experimental design**

Male Wistar rats weighing 220 to 300 g were maintained under temperature controlled conditions with an artificial 12-h light-dark cycle, and were allowed standard chow and water ad libitum. Hepatic fibrosis was induced by BDL. Briefly, the animals were anesthetized with intraperitoneal administration of 2.5% tribromoethanol (1 mL/100 g). A 1.5 cm midline incision was made and the common bile duct was located, double ligated with 4-0 silk and sectioned as previously described<sup>[7]</sup>. Our Ethics Committee approved all animal procedures.

### **Experimental protocol**

Animals were randomized into the following groups: sham-operated and those that underwent BDL. Sham-operated rats ( $n = 17$ ) underwent a midline incision and manipulation of the bile duct without ligation and were evaluated at various times following sham-surgery: 2-wk ( $n = 5$ ), 4-wk ( $n = 7$ ), and 6-wk ( $n = 5$ ). Bile duct ligated rats were also evaluated at the same post-procedure times: 2-wk ( $n = 8$ ), 4-wk ( $n = 7$ ) and 6-wk ( $n = 7$ ). Three days before blood sampling, all rats were placed in metabolic cages to measure urinary volume, water and food intake. At the end of the experiment, animals were weighed and blood samples were collected by decapitation to determine renal function parameters. Liver and renal tissue fragments were also obtained for histology and hydroxyproline determination.

### **Biochemical parameters**

Serum and urinary levels of creatinine (Jaffe method) were measured using Katal Kit and a semi-automatic analyzer BIO 2000. Urinary and serum osmolality were determined using a freezing point osmometer (Fiske

Osmometer, Fiske Ass. Inc., MA, USA). Serum and urinary levels of sodium and potassium were measured by flame photometry (Corning 400, Corning Inc., NY, USA).

### **Hydroxyproline determination**

Fragments (200 mg) of liver and renal tissue were removed for hydroxyproline determination as an indirect measure of tissue collagen content, as described by Reddy & Enwemeka<sup>[8]</sup>. Briefly, tissue fragments were homogenized in saline 0.9%, frozen and lyophilized. The assay was performed with 40 mg of the lyophilized tissue that was subjected to alkaline hydrolysis in 300  $\mu$ L plus 75  $\mu$ L NaOH 10 mol/L at 120°C for 20 min. An aliquot of 50  $\mu$ L of the hydrolysed tissue was added to 450  $\mu$ L of chloramine T oxidizing reagent (Chloramine T 0.056 mol/L, n-propanol 10% in acetate/citrate buffer pH 6.5) and allowed to react for 20 min. A hydroxyproline standard curve with the highest concentration of 400  $\mu$ g was prepared likewise. Colour was developed by the addition of 500  $\mu$ L of the Ehrlich reagent (p-dimethylaminobenzaldehyde, 1 mol/L) diluted in n-propanol/perchloric acid, 2:1 v/v). The samples then were centrifuged for 1500 g for 10 min at 4°C. An aliquot of 200  $\mu$ L of the supernatant was transferred to 96-well plates and the absorbance was read at 550 nm.

Five-micrometer sections of formalin-fixed and paraffin-embedded right liver lobes and kidney slices were processed routinely with hematoxylin-eosin, Masson's trichrome and ammoniac silver of Gomori. A single pathologist, blinded to experimental protocol, analyzed all liver and kidney fragments using light microscopy. The degree of liver fibrosis was measured based on the semi-quantitative scoring described by Ishak *et al*<sup>[9]</sup>.

### **Statistical analysis**

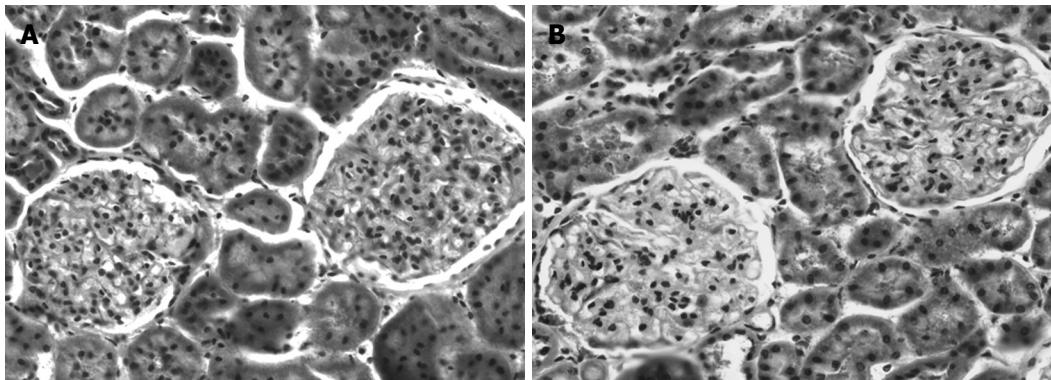
The data are expressed as mean  $\pm$  SD. Analysis of variance followed by Student Newman Keuls test was used to compare the differences between groups. Values of  $P < 0.05$  were considered significant.

## RESULTS

### **Morphological studies**

Because there did not appear to be any difference in the many variables studied in the sham group at two, four and 6 wk after sham operation, results from all the sham-operated groups were pooled for ease of presentation.

Hepatic fibrosis progressed over the time after BDL. Based on Ishak's score, the following score values for each group of BDL rats were obtained: sham operated rats scored 0 (normal hepatic architecture), 2-wk rats scored 3 (fibrous expansion of portal tracts with occasional portal-to-portal bridging), 4-wk rats scored 4 (fibrous expansion of portal tracts with marked portal-to-portal and portal-to-central bridging) and, at 6-wk, definite cirrhosis occurred (Score 6). In sharp contrast, as shown on Figure 1, no alterations in renal histology were observed in any bile duct ligated rats when compared to sham-operated animals.



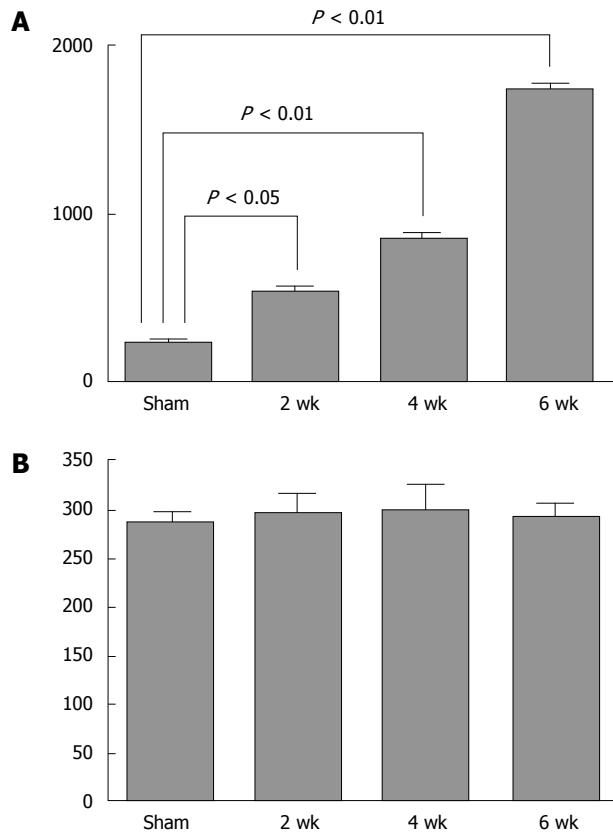
**Figure 1** Representative micrographs of the renal slices from bile duct ligated (BDL) and sham operated rats (HE,  $\times 100$ ). **A:** Sham operated rat with normal kidney; **B:** BDL rat at 6-wk also showing the absence of kidney histological alterations.

### Hydroxyproline determination

The progression of collagen deposition in liver tissue was also confirmed by the measurement of tissue hydroxyproline content at different time-points after bile duct ligation. Sham-operated rats represented the basal values of hydroxyproline content from a normal liver ( $235 \pm 45 \mu\text{g}/\text{mg}$  of liver tissue). As expected, according to the time after BDL, hydroxyproline content progressively increased in liver tissue, reaching values significantly higher than the control group in all time-points (2 wk:  $540 \pm 60 \mu\text{g}/\text{mg}$ ; 4 wk:  $863 \pm 57 \mu\text{g}/\text{mg}$ ; 6 wk:  $1735 \pm 73 \mu\text{g}/\text{mg}$ ;  $P = 0.0001$  for all comparisons, Figure 2A). The highest amount of liver hydroxyproline was detected in animals at 6 wk of BDL, indicating the significant degree of liver fibrosis (Figure 2A). Hydroxyproline content in renal tissue remained unchanged in sham-operated animals ( $288 \pm 31 \mu\text{g}/\text{mg}$  of kidney tissue) as well as in all groups of bile duct ligated rats (2 wk:  $298 \pm 55 \mu\text{g}/\text{mg}$ ; 4 wk:  $300 \pm 73 \mu\text{g}/\text{mg}$ ; 6 wk:  $294 \pm 39 \mu\text{g}/\text{mg}$ ;  $P > 0.05$  for all comparisons, Figure 2B). The results obtained with histological analysis and tissue hydroxyproline determinations showed the absence of structural changes in renal tissue during the development of liver fibrosis.

### Renal function parameters

Despite the well-preserved renal structure, important changes in renal function were clearly evidenced in bile duct ligated rats, as shown in Table 1 and Figure 3. As shown in Table 1, the 24-h urinary volume was significantly higher in animals with 4 and 6 wk of BDL compared to sham group. On the other hand, the 24-h urinary volume of 2 wk animals did not differ from sham group. Despite having the same urinary volume as sham-operated animals, rats with 2 wk of BDL exhibited an attempt to compensate the hydroelectrolyte imbalance produced by hepatic dysfunction. These animals significantly increased water excretion (Figure 3A,  $P = 0.02$ ), and reduced the urinary osmolality ( $P = 0.03$ ) and serum creatinine levels ( $P = 0.01$ ) in comparison to sham-operated rats (Table 1). An elevation in potassium excretion was also observed. However, the fractional excretion of this ion was unchanged in comparison with the sham group. Rats with 4 wk of BDL presented a progression in renal dysfunction as shown by a significant increase in serum creatinine ( $P = 0.01$ ) and a reduc-



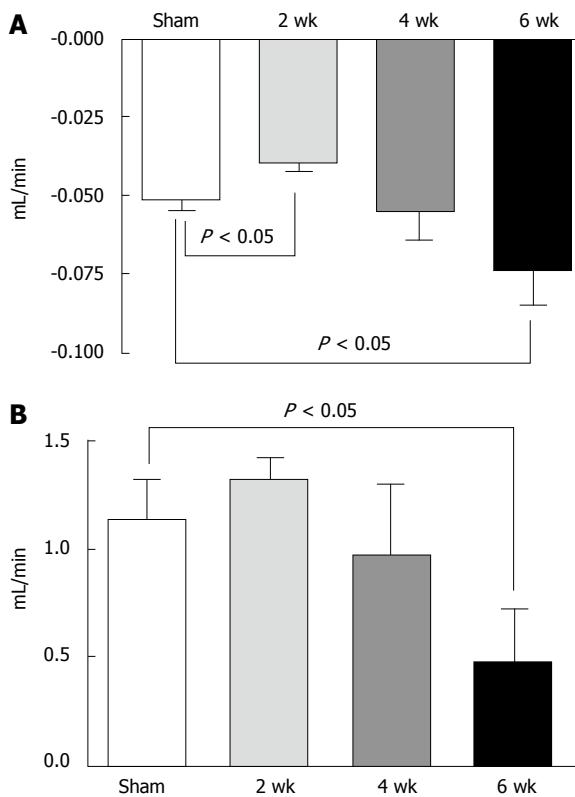
**Figure 2** Hydroxyproline determinations in the liver and renal tissue from bile duct ligated and sham operated rats. **A:** Hydroxyproline content in the liver tissue of sham operated rats (sham), and animals with 2-wk, 4-wk and 6-wk of bile duct ligation; **B:** Hydroxyproline content in renal tissue of sham operated rats (sham), and animals with 2 wk, 4 wk and 6 wk of bile duct ligation.

tion in urinary sodium concentration ( $P = 0.02$ ) when compared to sham-operated animals (Table 1). Rats with 6 wk of BDL clearly developed hepatorenal syndrome as revealed by a complete deterioration in renal compensatory mechanisms. These animals presented high levels of serum creatinine, a pronounced decrease in creatinine clearance (Figure 3B,  $P = 0.01$ ), and an important impairment in water excretion (Figure 3A,  $P = 0.02$ ) when compared to sham operated and 2 wk of BDL animals ( $P < 0.05$  for all comparisons, Table 1). Rats with 4 and 6 wk of BDL also presented dilutional hyponatremia and an elevation of fractional excretion of potassium when compared to sham group and animals with 2-wk of BDL ( $P < 0.05$  for both comparisons, Table 1). It

**Table 1** Renal function parameters in sham-operated (Sham) and bile duct ligated rats at 2-wk, 4-wk and 6-wk (mean  $\pm$  SD)

	Sham ( $n = 17$ )	2-wk ( $n = 8$ )	4-wk ( $n = 7$ )	6-wk ( $n = 7$ )
Urinary volume (mL/24 h)	12 $\pm$ 0.5	14.2 $\pm$ 1.0	19.4 $\pm$ 1.7 <sup>a</sup>	20.4 $\pm$ 2.8 <sup>a</sup>
Serum creatinine (mg/dL)	0.60 $\pm$ 0.10	0.28 $\pm$ 0.05 <sup>a</sup>	1.21 $\pm$ 0.25 <sup>a</sup>	2.50 $\pm$ 0.40 <sup>a</sup>
Creatinine clearance (mL/min)	1.14 $\pm$ 0.19	1.31 $\pm$ 0.11	0.97 $\pm$ 0.43	0.47 $\pm$ 0.25 <sup>a</sup>
Serum osmolality (mOsm/kg)	292 $\pm$ 2	289 $\pm$ 4	280 $\pm$ 14 <sup>a</sup>	282 $\pm$ 3 <sup>a</sup>
Urinary osmolality (mOsm/kg)	2147 $\pm$ 115	1578 $\pm$ 76 <sup>a</sup>	1499 $\pm$ 117 <sup>a</sup>	1745 $\pm$ 73 <sup>a</sup>
Osmolal clearance (mL/min)	0.061 $\pm$ 0.003	0.049 $\pm$ 0.002	0.071 $\pm$ 0.009	0.088 $\pm$ 0.013
Free water clearance (mL/min)	-0.052 $\pm$ 0.003	-0.040 $\pm$ 0.002 <sup>a</sup>	-0.056 $\pm$ 0.008	-0.074 $\pm$ 0.011 <sup>a</sup>
Serum [Na <sup>+</sup> ] (mEq/L)	137 $\pm$ 1	138 $\pm$ 3	125 $\pm$ 3 <sup>a</sup>	126 $\pm$ 2 <sup>a</sup>
Urinary [Na <sup>+</sup> ] (mEq/L)	111 $\pm$ 13	102 $\pm$ 10	57 $\pm$ 15 <sup>a</sup>	50 $\pm$ 15 <sup>a</sup>
Na <sup>+</sup> excreted (mEq)	1.47 $\pm$ 0.15	1.59 $\pm$ 0.19	1.20 $\pm$ 0.33	1.09 $\pm$ 0.34
Fractional Na <sup>+</sup> excreted (%)	0.65 $\pm$ 0.17	0.63 $\pm$ 0.13	0.91 $\pm$ 0.40	1.72 $\pm$ 0.61
Serum [K <sup>+</sup> ] (mEq/L)	4.5 $\pm$ 0.3	5.1 $\pm$ 0.4	4.0 $\pm$ 0.5	4.3 $\pm$ 0.1
Urinary [K <sup>+</sup> ] (mEq/L)	281 $\pm$ 12	269 $\pm$ 5	199 $\pm$ 21 <sup>a</sup>	180 $\pm$ 27 <sup>a</sup>
K <sup>+</sup> excreted (mEq)	3.35 $\pm$ 0.17	4.12 $\pm$ 0.31 <sup>a</sup>	3.65 $\pm$ 0.56	3.38 $\pm$ 0.39
Fractional K <sup>+</sup> excreted (%)	38 $\pm$ 6	43 $\pm$ 8	> 100 <sup>a</sup>	> 100 <sup>a</sup>

[Na<sup>+</sup>], sodium concentration; [K<sup>+</sup>], potassium concentration. <sup>a</sup>P < 0.05 vs sham group.



**Figure 3** Free water and creatinine clearance in bile duct ligated (BDL) and sham operated rats. **A:** Free water clearance of sham operated rats (sham), and animals with 2-wk, 4-wk and 6 of bile duct ligation; **B:** Creatinine clearance of sham operated rats (sham), and animals with 2 wk, 4 wk and 6 wk of bile duct ligation.

should be pointed out that body weights were similar in all groups at the beginning of the experimental protocol and no differences were observed in water and food intake (data not shown). No ascites was observed in the rats at 2-wk after BDL. In contrast, animals at 4 wk and 6 wk clearly exhibited ascites, also indicating the presence of water retention.

## DISCUSSION

This study supports the concept that the progression of hepatic damage promotes the manifestation of HRS. Indeed, the duration of BDL was positively correlated with renal function disarrangement without alterations in renal histology.

Animals at 2 wk of BDL seemed to be in a compensated state of hepatic injury, without ascites and alterations in water balance. These rats exhibited well-preserved renal function, suggesting that the homeostatic compensatory mechanisms remained intact at this moment of hepatic damage. Of note, serum creatinine was reduced in this group even when compared to sham operated animals. The creatinine clearance was slightly higher than in sham group, but significantly increased when compared to rats at 6 wk of BDL. These animals were also able to excrete water by increasing free water clearance. For this reason, serum osmolality remained at normal range and urinary osmolality was reduced when compared to sham. It has been reported that, at early stages of hepatic injury, as observed in animals with 2 wk of BDL, renal compensatory mechanisms against fluid retention still remain operating<sup>[1,3,4]</sup>. However, the progression of the process culminates in a non-compensated state by compromising the negative feedback loops of different regulatory systems<sup>[1,3,4]</sup>. Consistent with this, 4 wk after BDL, the rats already presented ascites, changes in water balance and an initial disturbance in renal function, revealed by sodium retention and an increase of the serum creatinine levels. After 6 wk of BDL, the hepatic damage evolved into a non-compensated stage with features of HRS<sup>[1,3,4]</sup>, including reduction in creatinine clearance and an evident fluid retention associated with significant reductions of the serum osmolality and of the free water clearance.

Clinical studies have attempted to delineate the natural history of cirrhotic patients with ascites with

respect to the development of HRS. Factors predictive for the development of HRS include intense urinary sodium retention, dilutional hyponatremia, and increased activity of systemic vasoconstrictors<sup>[10]</sup>. These features were clearly evidenced in our bile duct ligated rats, mostly at 6 wk. However, some characteristics of bile duct ligated rats are not typically observed in HRS seen in clinical medicine. While patients with HRS are usually oliguric<sup>[1,3]</sup>, our bile duct ligated animals at 4 wk and 6 wk increased the urinary volume when compared to the sham-operated group. It should be mentioned that, despite the elevation in the urinary volume, these animals still presented water and sodium retention, according to the observed reductions in free water clearance and in urinary sodium concentration. The so-called polyuria was not enough to excrete the whole amount of water and sodium retained by rats with 4 wk and 6 wk of BDL. Indeed, most investigators have used the BDL model to study pathological sodium retention<sup>[6,11]</sup>, which occurs in liver disease. Another possible explanation for this apparently elevated urinary volume is the well-known effect of circulating bile acids in kidney function during obstructive jaundice<sup>[12,13]</sup>. Acute cholaemia may cause volume depletion by increasing urinary salt loss, which, in turn, may aggravate the direct nephrotoxicity of circulating bile compounds<sup>[12]</sup>. *In vitro* addition of bile acids or bilirubin at concentrations comparable to those found in the plasma of BDL rats, to a mixture of reactive enzymes strongly inhibited most, particularly mitochondrial oxidative phosphorylation<sup>[13]</sup>. Thus, high concentrations of these substances in the blood may explain the development of renal failure during liver disease, and its reversibility when liver function returns to normal<sup>[13]</sup>. It also should be noted that the normal kidney histology and the unchanged levels of renal hydroxyproline content also favors the existence of HRS, a syndrome characterized by functional rather than structural disarrangement of the kidneys in presence of progressive liver disease<sup>[1,4]</sup>. Despite the differences in renal parameters in HRS in our system and in HRS observed in humans, this model seems to be very useful to evaluate the progression of renal dysfunction in hepatic diseases, since BDL rats are normally able to maintain a residual diuresis, probably allowing their long-lasting survival<sup>[6]</sup>.

The pathophysiology of HRS is still poorly understood. Hypoperfusion of the kidney due to active renal vasoconstrictors has been considered the hallmark of HRS<sup>[1-4]</sup>. In this context, Ozdogan and co-workers<sup>[14]</sup> conducted an elegant study that evidenced the role of endothelin-1, a potent vasoconstrictor, in an experimental model of HRS, which was induced by endotoxin administration to carbon tetrachloride-treated rats. In addition, the renin-angiotensin system (RAS) and the sympathetic nervous system, some of the major systems with a vasoconstrictor effect in the renal circulation, have been suggested as potential mediators of renal vasoconstriction in HRS<sup>[4,11,15]</sup>. During hepatic damage, systemic vasodilation and hyperdynamic circulation have been observed<sup>[16]</sup>, which

in turn promote an increase in sympathetic nervous activity, plasma renin activity (PRA), angiotensin II and aldosterone levels, especially in the presence of HRS<sup>[3,4]</sup>. It is well known that angiotensin II is one of the most powerful regulators of sodium excretion, operating through extrarenal as well as intrarenal mechanisms<sup>[17-19]</sup>. Some authors believe that, at the early stages of hepatic injury, the renal effects of angiotensin II represent a compensatory mechanism against the drop in organ perfusion pressure<sup>[3,4]</sup>. However, the development of renal impairment leading to HRS would occur as a result of an uncontrolled activation of systemic vasoconstrictor factors such as angiotensin II, sympathetic nervous system, endothelin and others that could not be counteracted by vasodilators such as nitric oxide, prostaglandins, bradykinin and maybe angiotensin-(1-7)<sup>[3,4]</sup>.

In this regard, we recently have shown that bile duct ligated rats presented different profiles of circulating RAS expression according to the progress of hepatic damage<sup>[20]</sup>. At early stages (1 wk and 2 wk of BDL), animals exhibited an elevation of angiotensin II and angiotensin-(1-7) levels, without concomitant changes in PRA and angiotensin I. With the progression of liver fibrosis (4 wk and 6 wk of BDL), RAS profile changed toward an overall enhancement of the PRA and the circulating levels of angiotensin I, angiotensin II and angiotensin-(1-7)<sup>[20]</sup>. According to these data<sup>[20]</sup>, we hypothesize that not only angiotensin II, but also angiotensin-(1-7) may possibly participate in the regulation of renal blood flow, glomerular filtration, and tubular transport in liver diseases. However, we still do not know how angiotensin-(1-7) could affect renal function in BDL rats. It has been clearly demonstrated that angiotensin-(1-7) also exerts complex renal actions<sup>[19,21,22]</sup>. Our group and others detected *in vivo* and *in vitro* antidiuretic effects of angiotensin-(1-7) by increasing fluid reabsorption<sup>[23-26]</sup>. These renal actions could contribute to sodium and water retention observed in bile duct ligated rats. In contrast, other studies showed that angiotensin-(1-7) has natriuretic and diuretic effects by inhibiting sodium reabsorption<sup>[27,28]</sup>. In addition, angiotensin-(1-7) seems to be involved in renal hemodynamic regulation by opposing the vasoconstrictive effects of angiotensin II in glomerular vessels<sup>[29,30]</sup>. However, it is difficult to know if the changes in the components of the RAS preceded or were caused by the decline in renal function. The liver, or maybe also the kidney, could produce angiotensin peptides, which, in turn, act either as systemic hormones or as locally generated factors. Accordingly, Paizis *et al*<sup>[31]</sup> detected an up-regulation of angiotensin converting enzyme 2 (ACE2), the main enzyme responsible for angiotensin-(1-7) synthesis<sup>[22]</sup>, in liver tissue from cirrhotic patients and bile duct ligated rats. Herath *et al*<sup>[32]</sup> showed increased expression of angiotensin-(1-7) receptor, the Mas receptor<sup>[33]</sup>, in experimental biliary fibrosis, suggesting a role for ACE2-angiotensin-(1-7)-Mas axis in liver injury.

Finally, the overall state of sodium and water

balance and the effect of many circulating and/or local regulators may influence the direction of the observed renal actions in bile duct ligated rats. Further studies are necessary to clarify the mechanisms involved in the development of HRS in experimental cirrhosis. However, our data indicate that BDL emerges as a good model for the study of HRS.

## COMMENTS

### Background

Hepatorenal syndrome (HRS) has been defined as a progressive renal failure that occurs in patients with chronic liver disease and advanced hepatic failure in the absence of any apparent clinical cause for renal insufficiency. HRS corresponds to a functional alteration without histological changes in kidney tissue. There are many experimental models to induce hepatic fibrosis. However, none of them has been systematically evaluated as a model of hepatorenal syndrome.

### Research frontiers

In this study, we aimed to systematically evaluate in bile duct ligated rats at different post-procedure time-points, whether there were alterations of renal function without changes in histopathology.

### Innovations and breakthroughs

Renal dysfunction without histological changes occurred according to the duration of bile duct ligation (BDL) in the absence of any additional treatment. Animals at 2 wk of BDL exhibited a well-preserved renal function, suggesting that the renal homeostatic compensatory mechanisms remained intact at this moment of hepatic damage. However, the progression of the process culminates in a non-compensated state, as already shown by rats at 4 wk of BDL with ascites, changes in water balance, sodium retention and increased serum creatinine levels. After 6 wk of BDL, features of hepatorenal syndrome (HRS) became evident, including reduction in creatinine clearance and fluid retention without alterations in renal histology and renal tissue collagen content. Our data showed that BDL seems to be a helpful model for the study of HRS, since it mimics clinical conditions characterized by obstructive jaundice, such as biliary atresia and choledochal cysts.

### Applications

The mechanisms for the renal changes observed in BDL animals remain unclear; however, this study indicates that BDL emerges as a good model for further studies of HRS and its treatment.

### Peer review

This study is a well-designed experimental work, which tries to define an experimental model for hepatorenal syndrome.

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RAPID COMMUNICATION

## Effects of microalgae chlorella species crude extracts on intestinal adaptation in experimental short bowel syndrome

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citrulline levels in blood were studied.

**RESULTS:** In rats receiving CCE, villus lengthening, crypt depth, mucosal DNA and protein levels, intestinal proliferation, and serum citrulline, protein and albumin levels were found to be significantly higher than those in control group. Apoptosis in CCE treated rats was significantly reduced when compared to EN group rats.

**CONCLUSION:** CCE has beneficial effects on intestinal adaptation in experimental SBS.

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**Key words:** Short-bowel syndrome; Intestinal adaptation; Chlorella; Nutrition; Microalgae

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Kerem M, Salman B, Pasaoglu H, Bedirli A, Alper M, Katircioglu H, Atici T, Perçin EF, Ofluoglu E. Effects of microalgae chlorella species crude extracts on intestinal adaptation in experimental short bowel syndrome. *World J Gastroenterol* 2008; 14(28): 4512-4517 Available from: URL: <http://www.wjgnet.com/1007-9327/14/4512.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4512>

## INTRODUCTION

Short bowel syndrome (SBS) is a clinical condition characterized by diarrhea, dehydration, electrolyte imbalance, malabsorption, and progressive malnutrition related to a wide resection of the small intestine<sup>[1-3]</sup>. In the pediatric population, necrotizing enterocolitis, gastroschisis, omphalocele, intestinal atresia and Hirschsprung disease, and in the adults mesenteric vascular occlusion, inflammatory bowel disease (IBD) and malignancies, are the most common reasons for performing extensive resection of the intestine<sup>[2]</sup>. Retaining intestinal autonomy depends on the length of remaining intestine and the adaptive capacity of the intestinal remnant. Some compensatory changes occur after massive intestinal resection in order to maintain adequate digestion. Restoration of the absorptive surface area and functional capacity result in morphologic and functional improvement. Structural adaptation after

## Abstract

**AIM:** To evaluate the effects of chlorella crude extract (CCE) on intestinal adaptation in rats subjected to short bowel syndrome (SBS).

**METHODS:** Wistar rats weighing 230-260 g were used in the study. After anesthesia a 75% small bowel resection was performed. Rats were randomized and divided into groups. Control group ( $n = 10$ ): where 5% dextrose was given through a gastrostomy tube, Enteral nutrition (EN) group ( $n = 10$ ): Isocaloric and isonitrogen EN (Altraq, Abbott, USA), study group ( $n = 10$ ): CCE was administrated through a gastrostomy tube. Rats were sacrificed on the fifteenth postoperative day and blood and tissue samples were taken. Histopathologic evaluation, intestinal mucosal protein and DNA levels, intestinal proliferation and apoptosis were determined in intestinal tissues, and total protein, albumin and

intestinal resection involves intestinal dilatation and elongation, villus lengthening, and increasing crypt cell proliferation. These changes result in a marked increase in the intestinal absorptive surface area<sup>[4]</sup>. The functional adaptation mechanism in the remnant intestine is still not entirely understood. Regulation of intestinal adaptation is an extremely complicated process influenced by many factors<sup>[5]</sup>. Some of these factors are nutrients, gastrointestinal secretions, hormones, and a variety of polypeptides stimulating growth capability<sup>[6]</sup>. The most important therapeutic objectives in the management of SBS are maintenance of the patient's calorie intake and nutritional status. Optimal intestinal rehabilitation should enhance the intestinal adaptation and shorten the period of intestinal recovery<sup>[7,8]</sup>; Yet, today no such optimal therapy exists. However, some enteral nutrition (EN) products are used for energy supports in order to reduce demand for total parenteral nutrition (TPN). The prospective, randomised and double blind clinical study of Byrne and colleagues<sup>[9]</sup> showed that glutamine, growth factor and optimum diet could reduce the length of TPN support. O'Dwyer and colleagues<sup>[10]</sup> emphasized that glutamine enriched TPN significantly improved the mucosal recovery and adaptation. New treatment alternatives to the current ones are still under research in experimental and clinical studies.

Chlorella is a species of green algae that grows in fresh water. The name Chlorella is taken from the Greek word meaning "small, fresh green", it contains the highest level of chlorophyll in the world when compared with all other nutrients. It has been consumed as a food source for centuries mainly in Japan and other Far East countries, and suggestion of its healing properties has enhanced consumption<sup>[11,12]</sup>. Biotechnological processing for single cell protein production is the most emphasized area of chlorella studies. Because of high protein ingredients, chlorella was considered to be a protein source in the beginning, but later it was seen as a "functional nutrient" first in Japan then Europe and America, and today it is accepted that chlorella is rich in nutritional ingredients<sup>[11,12]</sup>. Active ingredients of chlorella are: 61.6% protein, 12.5% fat, 13.7% carbohydrate, trace elements (Al, Ze, P, Ca, Mg, Mn, Ni, Se), vitamins (carotene, beta-carotene, thiamine, B<sub>1</sub>, B<sub>2</sub>, B<sub>6</sub>, C, D, E, K), nucleic acids (RNA and DNA), and various enzymes<sup>[12,13]</sup>. In our previous research, we showed that feeding with chlorella crude extract (CCE) has beneficial effects on malnourished rats which had undergone colon anastomosis<sup>[13]</sup>.

In the current study, we aimed to evaluate the efficacy of chlorella extract which is rich in amino acids, beta carotene, and trace elements on intestinal adaptation in SBS.

## MATERIALS AND METHODS

All procedures were conducted according to recommendations of the Animal Research Committee at Gazi University in Ankara, Turkey. Wistar rats weighing 230-260 g were used in the study. The rats were

maintained at 23°C in a 12 h light dark cycle, with free access to water and standard rat chow for a week. Eight hours prior to the start of experiments, rats were deprived of food while drinking water was available ad libitum. Animals were anesthetized by an intramuscular injection of 40 mg/kg ketamine (Ketalar®, Parke Davis, Eczacibasi, Istanbul, Turkey) and 5 mg/kg xylazine (Rompum®, Bayer AG, Leverkusen, Germany). Rats underwent central venous catheterization by inserting the catheter into the right external jugular vein, and were operated on under sterile conditions. A gastrostomy tube was placed for enteral feeding before the 75% small bowel resection. A 3 cm midline laparotomy was performed and intestinal transections were done 15 cm above the ileocecal junction and 5 cm from the duodenojejunal transition. Interrupted sutures of 7-0 PDS (Ethicon®, USA) were used for end to end bowel anastomosis. The venous catheter and the gastrostomy tube were tunneled subcutaneously through the dorsal cervical area, and attached with special apparatus (Swivel 56-1308; Harvard Apparatus, USA) which has a port and equipped with a spring system just beneath the skin. Before the closure of the abdominal cavity, 3 mL saline was administered intraperitoneally for the fluid resuscitation.

During the postoperative 3 d, rats received 60 kcal non-protein energy and 0.414 g nitrogen total parenteral nutrition (TPN). After postoperative day 4, rats were randomized and divided into the groups below: (1) Sham group: Laparotomy was performed; (2) Control group: 5% dextrose 12 mL/24 h was given to the rats by the gastrostomy tube with the infusion pump; (3) EN group: isocaloric (60 kcal/d) and isonitrogen (0.686 g/d) Alitraq (Abbott, USA) given to the rats through the gastrostomy tube with the infusion pump. Since, Alitraq is the EN product which has the highest amount of glutamine, and research clearly shows that glutamine has beneficial effect in SBS, Alitraq was used for enteral feeding in this study; (4) Study group: rats received CCE (60 kcal/d) through the gastrostomy tube by the infusion pump. In the sham, both EN and study group rats were not allowed to eat solid food, but were free to drink water.

Rats were anesthetized by intraperitoneal injection of 50 mg/kg sodium pentothal before laparotomy. The length of the small bowel was measured from the Treitz to the caecum. After withdrawal of blood samples from vena cava, rats were sacrificed by bleeding. Intestinal resection was quickly performed, and specimens were washed with cold saline. Histopathologic samples were taken from both the jejunal and ileal side of the anastomosis, and the rest of it was weighed.

### **Preparation of the algae extract, its ingredients and its use**

**Culturing and growth conditions:** Collection and isolation of microalgae were made in compliance with Rippka *et al*<sup>[14]</sup>. Microalgae were obtained from GUMACC (Gazi University Microalgae Culture Collection) *Chlorella* sp. C1 were expanded in number by culture in BG11 nutrition medium (blue-green medium 11) at less than 3000 lux light intensity, under illumination for 16 h

**Table 1** Total biochemical, histopathology and DNA results

	<b>Sham</b>	<b>Control</b>	<b>EN</b>	<b>CCE</b>	<b>ANOVA</b>	<b>Sheffe's</b>
Weight loss (g)	0 ± 0	52 ± 8	34 ± 6	24 ± 5	< 0.001	a, c, e
Width of jejunum (cm)	0.84 ± 0.1	0.48 ± 0.4	0.62 ± 0.3	0.78 ± 0.1	< 0.05	c, e, g
Length of jejunum villi (mm)	0.74 ± 0.22	0.38 ± 0.18	0.54 ± 0.12	0.76 ± 0.11	< 0.05	c, e, g
Depth of jejunum crypt (mm)	0.51 ± 0.13	0.32 ± 0.13	0.52 ± 0.10	0.68 ± 0.08	< 0.05	c, e, g
Number of jejunal mitosis (n)	11.2 ± 2.1	4.2 ± 2.1	13.4 ± 4.3	22.8 ± 5.2	< 0.001	c, e, g
Width of ileum (cm)	0.84 ± 0.1	0.48 ± 0.4	0.62 ± 0.3	0.78 ± 0.1	< 0.05	c, e, g
Length of ileum villi (mm)	0.74 ± 0.22	0.38 ± 0.18	0.54 ± 0.12	0.56 ± 0.11	< 0.05	a, c
Depth of ileum crypt (mm)	0.61 ± 0.10	0.36 ± 0.13	0.58 ± 0.19	0.64 ± 0.08	< 0.05	c, e, g
Number of ileum mitosis (n)	11.2 ± 2.1	4.2 ± 2.1	13.4 ± 4.3	22.8 ± 5.2	< 0.001	c, e, g
Total protein (mg/dL)	6.8 ± 1.8	4.3 ± 1.3	6.1 ± 1.4	6.6 ± 2.0	< 0.05	c, g
Albumin (mg /dL)	2.2 ± 0.2	1.1 ± 0.3	1.9 ± 0.1	2.0 ± 0.3	< 0.05	c, g
Serum citrulline (micromol/L)	72.2 ± 11.2	34.2 ± 6.2	52.3 ± 7.9	68.8 ± 9.8	< 0.001	c, g
Mucosal DNA (ng/µL)	622 ± 48	318 ± 32	716 ± 61	898 ± 182	< 0.001	c, g
Mucosal protein (mg/mL)	16.2 ± 3.8	6.2 ± 2.6	12.9 ± 3.8	15.3 ± 4.5	< 0.001	c, g
Cell proliferation index Jejunum	310 ± 27	550 ± 40	710 ± 50	850 ± 55	< 0.001	a, c, g
(BrdU (+) cells/ 10 crypts) Ileum	280 ± 32	510 ± 35	730 ± 45	970 ± 65	< 0.001	a, c, g
Apoptosis index Jejunum	12 ± 2.1	25 ± 3.2	18 ± 3.1	14 ± 2.1	< 0.001	a, c, g
(Apoptotic cell/1000 cell) Ileum	13 ± 2.3	29 ± 2.5	21 ± 4.1	16 ± 3.7	< 0.001	a, c, g

<sup>a</sup>P < 0.05, Sham group vs other groups; <sup>c</sup>P < 0.05, CCE and EN and control group; <sup>e</sup>P < 0.05, CCE vs EN group; <sup>g</sup>P < 0.05, control vs other groups.

and under darkness for 8 h. Algae were harvested after approximately a 15-d production period.

**Preparation of the extracts:** Algal mass from an axenic exponential culture of the microalgae strains grown in BG11 were separated from the culture medium by centrifugation and pellets were dried at 60°C for 24 h. Methanol extracts were prepared according to the methods of Khan *et al*<sup>[15]</sup> and Vlachos *et al*<sup>[16]</sup>, from dry algal mass (ratio 1:15 g/mL) extracted throughout 24 h. After separating the extraction phase, all of the extracts were preserved at 4°C. The *chlorella sp.* extract was resuspended at 1 g/mL in 0.9% sterile saline.

**Ingredients of algae extract and its use:** The dose of algae extract used was 50 g/kg BW/d<sup>[13,15,16]</sup>. The suspension was given *via* oral gavage three times a day in equal doses (each dose was less than 5 mL).

### Biochemical analysis

Serum protein, albumin and blood sugar levels were analyzed from blood samples. The mucosal layers of the intestinal samples were brushed with slides and then weighed. After a homogenization process, mucosal DNA and protein levels were evaluated by the techniques of Chomczynski as described previously<sup>[17]</sup>.

### Serum citrulline levels

Serum citrulline levels were measured by the tandem mass spectrophotometric technique with isotope embedded amino acid standards. The results were expressed as mmol/L.

### Histopathology

Jejunal and ileal tissue samples were fixed in a 10% solution of formaldehyde, and embedded in paraffin wax from which 3-µm-thick sections were mounted on slides. The sections were stained with hematoxylin-eosin

(HE), and mucosal widening, villus length and crypt depth were evaluated.

### Enterocyte proliferation and apoptosis

Crypt cell proliferation was determined using 5-bromodeoxyuridine (BrdU). Twelve hours before sacrifice, 100 mg/kg BrdU was given intraperitoneally to the rats. Sections were stained with anti-BrdU antibodies. Every 10 crypts stained with positive BrdU were calculated as a proliferation index. The TUNEL technique was used for the determination of apoptotic cells.

### Statistical analysis

All values were expressed as mean ± SE and the results were compared by analysis of variance (one-way ANOVA) and Scheffe's post hoc analysis. P < 0.05 was considered statistically significant. Statistical evaluation was carried out using SPSS 11.5 software (SPSS, Chicago, IL, USA).

## RESULTS

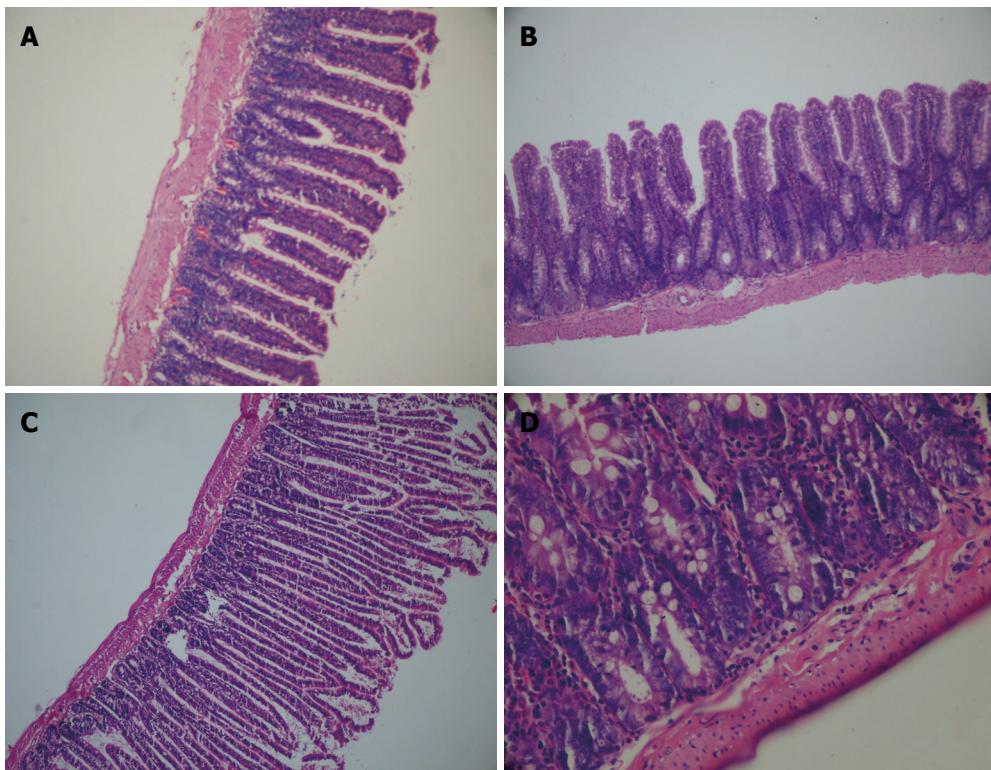
Three rats died during the creation of the SBS, and they were replaced with new ones.

### Weight lost

All rats subjected to SBS lost significant weight when compared with their original weight before the experiment. In the EN and study (CCE) groups, rats lost less weight than the control group (P < 0.05). When they were compared with each other, it was observed that in the CRE group rats lost significantly less weight than the EN group (P < 0.001, Table 1).

### Histopathologic evaluation

In the control group, mucosal widening, villous length, crypt depth and amount of mitosis markedly decreased



**Figure 1** Villi and crypts in the ileum of rats which underwent SBS (HE, x 100): Control group (**A**) and EN group (**B**); **C**: Significant elongation of villi in CCE group (ileum, HE, x 100); **D**: Significant mitotic figures in the crypts in the CCE group (ileum, HE, x 400).

when compared to sham, EN and CCE groups ( $P < 0.05$ ). Jejunal and ileal mitosis number in the EN and CCE groups were significantly higher than those at the control group ( $P < 0.05$ ). The amount of mitosis in both segments of intestine in the CCE group was markedly higher than those in the EN group. Villus length and mucosal widening were not significantly different between the EN and CCE groups, whereas crypt depth was remarkably increased in the CCE group over the others ( $P < 0.05$ , Table 1, Figure 1).

#### Mucosal DNA levels

Mucosal DNA levels significantly decreased in the control group when compared to the other groups. The mucosal DNA levels for EN and CCE groups were found to be remarkably higher than the sham and control groups. Moreover, the same parameters significantly increased in the CCE group when compared to the EN group ( $P < 0.05$ , Table 1).

#### Mucosal protein levels

Ileal mucosal protein levels were significantly higher in the CCE group than all other groups ( $P < 0.05$ ). The mucosal protein levels of the control group were markedly reduced when compared to the other groups ( $P < 0.05$ ). There was no difference between the EN and CCE groups ( $P < 0.05$ , Table 1).

#### Cell proliferation index

The jejunal and ileal cell proliferation indexes were significantly higher in the CRE group than all other

groups ( $P < 0.05$ ). The cell proliferation indexes in the control group were increased when compared to the sham group while they were significantly lower than the EN and CCE groups ( $P < 0.05$ , Table 1).

#### Apoptotic index

Apoptosis in the control group was markedly increased compared to other groups. The apoptotic index in subjects fed with CCE was significantly lower than those in the EN and control groups ( $P < 0.05$ ), and it was found to be insignificantly higher than the apoptotic indexes of the sham group ( $P < 0.05$ , Table 1).

## DISCUSSION

Loss of small bowel function caused by extended intestinal resection results in malabsorption and fluid and electrolyte imbalances<sup>[1]</sup>. In the early postoperative period the first priority of SBS treatment is adequate resuscitation of volume and electrolyte disturbances. When these parameters are stabilized, parenteral nutrition can be started<sup>[1-3]</sup>. Although parenteral nutrition causes a significant decrease in the mortality rates of SBS, the time required for optimal TPN therapy is too long and has many disadvantages and severe complications<sup>[4-7]</sup>. Searching for new treatment methods for increasing bowel adaptation mechanisms in order to reduce complications of SBS is an area which many recent studies concentrated on. The hormones; bombesin, growth factors, insulin like growth factors, ghrelin, leptin, and EN products; glutamine, fish oil (omega-3 fatty acids), immune

nutrients and fibers, have been investigated, and found to have beneficial effects on bowel adaptation mechanism in SBS<sup>[8-10,18]</sup>. We designed our research on the use of species of chlorella algae for healing effects because of its high protein, nucleic acid, antioxidant and fiber content<sup>[11,12]</sup>. In this study, it was observed that enteral feeding with CCE has beneficial effects on intestinal adaptation in rats with SBS. Lengthening of the intestinal villus, increasing crypt depth, intestinal proliferation, mucosal protein, DNA, serum citrulline, protein and albumin levels, and decrease of apoptosis were found in the study. This is the first study to demonstrate the healing effects of chlorella on SBS.

When we look at the current literature, we can find few trials of different algae species<sup>[19,20]</sup>. Tokida ameliorated murine chronic colitis through down-regulation of interleukin-6 production on colonic epithelial cells. Sakai *et al*<sup>[21]</sup> have also described that *Sargassum horneri*, a marine brown algae, increases Cl<sup>-</sup> absorption in isolated rat colon by activation of leukotrienes. In another study, it was shown that algae extract can reduce inflammation and ultrastructural changes in rats colitis induced by acetic acid<sup>[20]</sup>. Gonzalez *et al*<sup>[22]</sup>, demonstrated that intestinal myeloperoxidase enzyme levels remarkably decrease in response to algae extract. Dvir and colleagues<sup>[23]</sup> suggested that red algae can regulate intestinal physiology and lipid metabolism, it can also be used as a functional nutrient. The same investigators claimed that algae-derived polysaccharides can increase jejunal muscular hypertrophy, and algae fiber can lengthen both colon and small bowel and increase colonic transit time by 44% compared to control group. Significant increases in mucosal villous lengthening and crypt depth due to the effects of micro-algae were observed in the same study. Although it is very rare, we can see the use of algae for SBS in the literature.

To our knowledge, if fluid resuscitation is not subsequently provided following the surgery, mortality can be significantly high in animal models of SBS<sup>[1]</sup>. For this reason, we inserted an intravenous line in rats, before starting TPN, and we resuscitated them with iv fluid for a short period of time. After the third postoperative day, rats received dextrose in addition to EN and CCE. They were weighed daily until the end of the experiment. All rats subjected to SBS lost significant weight. However, weight lost in rats fed with EN and CCE was markedly less than the control group. Though both EN and CCE resulted in weight gain in rats, the CCE group rats gained more weight than the EN group. Both nutritional solutions have almost the same energy distribution, but CCE has higher nucleotide content, and better absorptive and adaptive capacity than the EN, so rats gained more weight in the CCE group than those in other groups. In our previous study, we observed that CCE increases weight gain in malnourished rats.

Recent articles feature the amino acid citrulline, the best marker of intestinal absorptive capacity in SBS due to massive intestinal resection<sup>[3-7]</sup>. The clinical study of Rhoads *et al*<sup>[24]</sup>, found that there was a correlation between serum citrulline levels and enteral tolerance, and

levels of serum citrulline could be used as a predictive test. In our study, fifteenth day serum citrulline levels were significantly lower than those in other groups. However, the CCE group serum citrulline levels were markedly higher than those in the control and EN groups. Another result of absorptive and adaptive responses was the significant increase in serum protein and albumin levels in CCE fed rats when compared to control rats. High amino acid and nucleotide levels in CCE could be responsible for these effects.

Mucosal DNA and BrdU proliferation index for evaluating intestinal proliferation rate were found significantly reduced in SBS rats when compared to control group rats. Mucosal DNA and intestinal proliferation index were markedly higher in CCE and EN groups than those in control group. These parameters also increased in the CCE group compared to the EN group. Excessive amount of nucleic acid, protein, vitamin and other substances in CCE may be responsible for these outcomes.

In conclusion, enteral administration of CCE increases intestinal adaptation and proliferation in experimental SBS. The current study provides preliminary data for future research. More studies are needed to investigate the use of algae species growing in water as a clinical nutrition product.

## COMMENTS

### Background

Chlorella is a species of green algae that grows in fresh water. The name chlorella is taken from the Greek word meaning "small, fresh green", it contains the highest level of chlorophyll in the world when compared with all other nutrients. It has been consumed as a food source for centuries mainly in Japan and other Far East countries, and suggestion of its healing properties has enhanced consumption. However, there is little data about the use of chlorella in disease, which is investigated in this experimental study.

### Research frontiers

Short bowel syndrome (SBS) which affects both children and adults is a generally seen disease. The basis of treatment for this disease is enteral and intravenous nutrition. Several enteral nutrition (EN) products have been used for SBS. The aim of this study is to evaluate the effect of chlorella in SBS in rats.

### Innovations and breakthroughs

In this study, positive effects of orally given chlorella were seen. This is the first study on this subject. It was seen that chlorella increased intestinal adaptation in SBS.

### Applications

This experimental study will guide new experimental and clinical studies. Chlorella is an algae which is widely found in both salt and fresh water. As a usage for EN, it can be used for acute pancreatitis, inflammatory bowel diseases, colitis studies and clinical studies.

### Peer review

In this experimental study about SBS, orally given CCE showed a positive effect on parameters of intestinal adaptation. Since it is the first study that shows positive effects of algae in SBS, this is an interesting study.

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RAPID COMMUNICATION

## Risk factors of thrombosis in abdominal veins

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### Abstract

**AIM:** To estimate the prevalence of inherited and acquired thrombophilic risk factors in patients with abdominal venous thrombosis and to compare the risk factor profiles between Budd-Chiari syndromes (BCS) and splanchnic vein thrombosis (SVT).

**METHODS:** In this retrospective study, 36 patients with abdominal venous thrombosis were studied. The patients were divided into Budd-Chiari group (hepatic vein, IVC thrombosis) and splanchnic venous thrombosis group (portal, splenic, superior mesenteric veins) based on the veins involved. Hereditary and acquired thrombophilic risk factors were evaluated in all patients.

**RESULTS:** Twenty patients had SVT, 14 had BCS, and 2 had mixed venous thrombosis. Ten patients (28%) had hereditary and 10 patients (28%) acquired thrombophilic risk factors. The acquired risk factors were significantly more common in the SVT group (SVT vs BCS: 45% vs 7%,  $\chi^2 = 5.7$ ,  $P = 0.02$ ) while hereditary risk factors did not show significant differences between the two groups (SVT vs BCS: 25% vs 36%,  $\chi^2 = 0.46$ ,  $P = 0.7$ ). Multiple risk factors were present in one (7%) patient with BCS and in 3 patients (15%) with SVT. No risk factors were identified in 57% of patients with BCS and in 45% of patients with SVT.

**CONCLUSION:** Hereditary and acquired risk factors play an important role in the etiopathogenesis of abdominal venous thrombosis. Acquired risk factors

are significantly more common in SVT patients while hereditary factors are similar in both groups.

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**Key words:** Budd Chiari syndrome; Splanchnic vein thrombosis; Risk factors; Hereditary; Risk comparison

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### INTRODUCTION

Abdominal venous thrombosis may present as Budd-Chiari Syndrome (BCS) (thrombosis of inferior vena cava and/or hepatic veins) or splanchnic venous thrombosis (SVT) (occlusion of portal, splenic, superior or inferior mesenteric veins). Hereditary and acquired risk factors have been implicated in the etiopathogenesis of abdominal venous thrombosis<sup>[1,2]</sup>. Hereditary risk factors for thrombophilia include Factor V Leiden gene mutation, prothrombin gene mutation, homozygous methyl tetrahydrofolate reductase (MTHFR) gene mutation, and deficiencies of coagulation inhibitor protein C, protein S and antithrombin III (AT III)<sup>[3-7]</sup>. Causes of acquired thrombophilia are myeloproliferative disorders, malignancy, surgery, antiphospholipid syndrome, pregnancy, oral contraceptives, and infection<sup>[8-11]</sup>. Identification of these risk factors may help in evaluation, planning therapy, or screening family members to evaluate an individual risk.

There are few studies from South Asian regions which have comprehensively evaluated prothrombotic risk factors in BCS and portal venous thrombosis (PVT)<sup>[12,13]</sup>. These studies did not assess risk factors in patients with mesenteric venous thrombosis. Other studies have evaluated individual risk factors or multiple risk factors in single venous thrombosis<sup>[14-17]</sup>. The aim

of the study was to analyse prothrombotic etiological profiles (hereditary and acquired) in patients with abdominal venous thrombosis and to compare the profiles of the BCS and SVT groups.

## MATERIALS AND METHODS

Patients admitted with abdominal venous thrombosis that had complete etiological work up during the period July 1997 to June 2006 were included in the study. Patients with incomplete evaluation (acute thrombosis or on anticoagulants) were excluded. Diagnosis of thrombosis was based on Doppler sonography, abdominal computed tomography (CT), or venography. For all selected patients, clinical information and laboratory data were collected by a standardized review of medical charts using uniform structured data forms. Details of acquired prothrombotic risk factors like abdominal surgery, oral contraceptives, pregnancy, liver cirrhosis, antiphospholipid syndrome, infection, or others were also obtained.

Genetic tests for mutation in Factor V Leiden gene (1691, G-A), *MTHFR* gene (677 C-T), and prothrombin gene (20210, G-A) were done in all the patients by PCR amplification of the respective gene segments<sup>[18-20]</sup>. The amplified products were subjected to restriction digestion fragment length polymorphism (RFLP) analysis. Protein C and AT III were assessed using chromogenic assays, done on the coagulation analyzer (Dade Behring's Sysmex CA 1500). Free protein S was estimated by an immunoassay (Chromogenix Coamatic Protein S Free, IL) done on the ACL Advance (Instrumentation Laboratory). The assays for protein C, protein S, and AT III were run concurrently with normal control and abnormal control (substrate present in low level simulating deficiency states) samples for validation as well as comparison with normal. The normal reference ranges of various tests were protein C: 50%-150% of normal; protein S: 50%-150% of normal; AT III: 80%-120% of normal. Patients were considered to have protein C, protein S, or AT III deficiency only if liver dysfunction was ruled out.

### Statistical analysis

Comparison between the BCS and the SVT group was done by Fischer's exact test for categorical variables and Mann Whitney U test for continuous variables. A *P* value of < 0.05 was considered significant. All analysis was performed in SPSS for Windows Version 11.

## RESULTS

Thirty-six patients with thrombosis of abdominal veins were studied. The mean age of the patients was 36.7 years (range: 3-69 years). There were 24 males (67%) and 12 females (33%). Abdominal pain, the commonest symptom, was seen in 16 (44%), hepatomegaly in 4 (11%), splenomegaly in 10 (28%), and ascites in 13 (36%) patients. Acute presentation was more common in SVT (40%) than in BCS (21%). Diagnosis of abdominal venous thrombosis was made by Doppler sonography in 21 patients (58%), CECT abdomen in 10 (28%), and

venography in 5 (14%) patients. Twenty patients had thrombosis of splanchnic veins (SVT), 14 had thrombosis of inferior vena cava and/or hepatic vein (BCS) and 2 had thrombosis in both splanchnic and IVC/hepatic veins.

The site of thrombosis along with details of hereditary and acquired risk factors in all patients studied is shown in Table 1. Hereditary risk factors were present in 10 (28%) patients and acquired risk factors in 10 (28%) patients. The most common hereditary risk factors were Factor V Leiden gene mutation (11%) and AT III deficiency (11%) followed by protein C deficiency (8%). None of the patients had a prothrombin gene mutation, protein S deficiency, or was homozygous for *MTHFR* gene mutation. *MTHFR* mutation (heterozygous) was seen in 22% patients, which is not considered a risk factor for thrombosis. In the BCS group (14 patients): IVC obstruction alone was present in 5 patients, hepatic vein (HV) obstruction alone in 6 patients, and IVC + HV obstruction in 3 patients. Hereditary risk factors were present in 5 (36%) patients and acquired risk factor in one (7%) patient. In SVT group (20 patients): portal vein (PV) obstruction alone was present in 4 patients, splenic vein (SV) obstruction alone in 2 patients, superior mesenteric vein (SMV) obstruction alone in 1 patient, PV + SMV obstruction in 3 patients, and PV + SV + SMV obstruction in 10 patients. Hereditary risk factors were present in 5 (25%) patients and acquired risk factors in 9 (45%) patients.

Comparison of risk factor profiles between the BCS and the SVT group is shown in Table 2. Hereditary risk factors were higher in the BCS group (BCS vs SVT: 36% vs 25%, *P* = 0.7), but this difference did not reach statistical significance. Acquired risk factors were significantly higher in the SVT group (SVT vs BCS: 45% vs 7%, *P* = 0.02). The prevalence of multiple risk factors in the BCS and the SVT group are shown in Table 3. More than one risk factor was seen in 1 (7%) patient in the BCS group and in 4 (20%) patients in the SVT group. No risk factor was identified in 57% of patients in the BCS group and in 45% of patients in the SVT group.

## DISCUSSION

This study evaluated hereditary and acquired risk factors in 36 patients with abdominal venous thrombosis. Hereditary risk factors were identified in 36% of patients with BCS and in 25% of patients with SVT. Acquired risk factors were detected in 7% of patients with BCS and in 45% of patients with SVT.

Prevalence of Factor V Leiden mutation (FVLM), the most common cause of inherited thrombophilia, is variable in different populations<sup>[21]</sup>. Risk of venous thrombosis is 5- to 8-fold in heterozygotes and 50- to 80-fold in mutation homozygotes<sup>[3]</sup>. Janssen *et al* showed that prevalence of FVLM in BCS (26%) and PVT (8%) was higher than in controls (3%) suggesting that FVLM is an important risk factor for BCS (OR 11.3) and PVT (OR 2.7)<sup>[22]</sup>. Mohanty *et al* also found FVLM to be an important risk factor in BCS (26%; OR 14.5) and in PVT (6%; OR 2.3)<sup>[12]</sup>. Bhattacharyya *et al* demonstrated FVLM mutation in 17% of BCS and in 3% of patients with

**Table 1 Site of thrombosis and presence of risk factors in individual patients**

Patient No.	Group	Site	Age(yr)	Sex	Hereditary risk factors					Acquired risk factors
					FVL	PT	MTHFR <sup>1</sup>	PrC	PrS	
1	IVC and/or Hepatic vein	IV	12	M	-/-	-/-	-/-	N	N	N
2		IV	43	M	-/-	-/-	-/-	N	N	N
3	thrombosis	IV	45	M	-/-	-/-	-/-	N	N	N
4	(BCS)	H	20	F	+,-	-/-	-/-	N	N	N
5		IV + H	39	M	-/-	-/-	-/-	N	N	N
6		H	42	F	-/-	-/-	-/-	N	N	N
7		H	20	M	-/-	-/-	+,-	N	N	N
8		IV	49	M	-/-	-/-	-/-	N	N	N
9		H	4	M	-/-	-/-	-/-	N	N	N
10		IV	46	M	-/-	-/-	+,-	N	N	Y
11		IV + H	54	M	+,-	-/-	+,-	N	N	N
12		IV + H	5	M	-/-	-/-	+,-	Y	N	Y
13		H	40	F	-/-	-/-	-/-	N	N	N
14		H	28	M	-/-	-/-	+,-	N	N	Y
15	Splanchnic vein	P + SP + SM	55	F	-/-	-/-	-/-	Y	N	N
16		P + SP + SM	49	F	-/-	-/-	-/-	N	N	N
17	thrombosis	P + SP + SM	44	F	-/-	-/-	-/-	N	N	N
18	(SVT)	SP	35	M	-/-	-/-	-/-	N	N	Abdominal surgery
19		P	3	M	-/-	-/-	+,-	Y	N	Abdominal surgery
20		P	22	F	-/-	-/-	-/-	N	N	Cirrhosis
21		P + SP + SM	51	F	-/-	-/-	-/-	N	N	Past peripheral DVT
22		P + SM + IM	47	F	-/-	-/-	-/-	N	N	N
23		P	30	M	-/-	-/-	-/-	N	N	N
24		P + SM	37	M	-/-	-/-	-/-	N	N	Cirrhosis
25		P + SM	43	M	-/-	-/-	-/-	N	N	N
26		SM	31	M	+,-	-/-	-/-	N	N	APLA
27		P	30	M	-/-	-/-	-/-	N	N	Y
28		P + SP + SM	28	M	-/-	-/-	-/-	N	N	N
29		SP	20	M	-/-	-/-	-/-	N	N	Acute pancreatitis
30		P + SP + SM	62	M	-/-	-/-	-/-	N	N	N
31		P + SP + SM	69	M	-/-	-/-	-/-	N	N	Cirrhosis, HCC
32		P + SM	49	F	-/-	-/-	-/-	N	N	N
33		P + SP + SM	53	M	+,-	-/-	-/-	N	N	N
34		P + SP + SM	42	F	-/-	-/-	+,-	N	N	Polycythemia, APLA
35	BCS + SVT	IV + H + P	50	F	-/-	-/-	+,-	N	N	N
36		H + P	25	M	-/-	-/-	-/-	N	N	N

IV: Inferior vena cava; H: Hepatic vein; P: Portal vein; SP: Splenic vein; SM: Superior mesenteric vein; IM: Inferior mesenteric vein; FVL: Factor V Leiden gene; PT: Prothrombin gene; MTHFR: Methyl tetrahydolate reductase gene; PrC: Protein C deficiency; PrS: Protein S deficiency; AT III: Antithrombin III deficiency; Y: Yes; N: No; APLA: Antiphospholipid antibody. -/-: Wild type; +,-: Heterozygous mutation. <sup>1</sup>Heterozygous MTHFR gene mutation is not considered a risk factor of thrombosis.

**Table 2 Characteristics and risk factors of patients with BCS and SVT**

	BCS (n = 14)	SVT (n = 20)	P <sup>1</sup>
Age: Median (IQR)	39.5 (27.25) yr	42.5 (20.5) yr	0.18
Female	21.4%	40%	0.30
Acute presentation	21.4%	40%	0.30
Hereditary risk factors	35.7%	25%	0.70
Factor V Leyden mutation	14.3%	10%	0.55
Prothrombin gene mutation	0%	0%	-
Homozygous MTHFR gene mutation	0%	0%	-
Protein C deficiency	7.1%	10%	1.0
Protein S deficiency	0%	0%	-
AT III deficiency	21.4%	5%	0.28
Acquired risk factors	7.1%	45%	0.02
No risk factor	57.1%	45%	0.70

<sup>1</sup>Fisher's exact test for categorical variables and Mann Whitney's U test for continuous variable.

PVT<sup>[13]</sup>. Koshy *et al* showed that the prevalence of FVLM was similar in patients with PVT (3%) and controls (1%) and suggested that FVLM is not associated with PVT in

**Table 3 Prevalence of multiple risk factors (inherited and acquired) among patients with BCS and SVT n (%)**

Number of risk factors	BCS (n = 14)	SVT (n = 20)	Total (n = 34)
0	8 (57)	9 (45)	17 (50)
1	5 (36)	7 (35)	12 (35)
2	1 (7)	3 (15)	4 (12)
3	-	1 (5)	1 (3)

India<sup>[14]</sup>. Similar observations were made by Sharma *et al* who demonstrated FVLM in 1.6% of patients with PVT and in 4% of controls<sup>[15]</sup>. In the present study, 14% of patients with BCS and 10% with SVT were heterozygotes, both higher than control data (1%-4%) reported earlier from India<sup>[12,14,15]</sup>. Though the numbers of patients in the study are small, results suggest that FVLM may be a risk factor in BCS and SVT.

Prothrombin gene mutation, a risk factor for venous thrombosis (homozygote: 10-fold; heterozygote: 2- to 4-fold) is rare in African and Asian populations compared to Caucasians<sup>[23]</sup>. None of the five Indian

studies have shown this gene mutation in cases or controls<sup>[12,13,15-17]</sup>. We also did not detect this mutation in any of our patients. Prevalence of heterozygote *MTHFR* gene mutation in patients with venous thrombosis is similar to healthy controls suggesting that this mutation is not an important prothrombotic factor<sup>[24]</sup>. It has been shown that homozygote *MTHFR* mutation, one of the causes of hyperhomocystinaemia (risk factor for vascular disease), is a risk factor for venous thrombosis<sup>[19,24]</sup>. None of the patients in the present study were homozygous for the *MTHFR* mutation. Three patients had heterozygote *MTHFR* mutation as the only abnormality. They were presumed to have idiopathic abdominal venous thrombosis as heterozygote *MTHFR* mutation alone is not considered a significant prothrombotic risk factor. Five heterozygous patients had additional hereditary or acquired risk factors. In a study from Northern India, Bhattacharyya *et al* investigated 57 BCS and 48 PVT patients, and reported none were homozygous for *MTHFR* gene mutation. Heterozygous mutations were seen in 24% of BCS and 21% of PVT patients<sup>[13]</sup>.

Indian and Western studies have shown that protein C deficiency is the second most common cause of inherited thrombophilia in patients with BCS and PVT<sup>[12,13,22]</sup>. Amarapurkar *et al* showed that protein C deficiency was the commonest hereditary risk factor (26%) in a study on 28 patients with mesenteric venous thrombosis<sup>[25]</sup>. Protein C was also the commonest risk factor (38% patients) in a series of 16 patients with mesenteric venous thrombosis reported by Harward *et al*<sup>[26]</sup>. In the present study, protein C deficiency was demonstrated in 7% of patients with BCS and in 10% of patients with SVT. Prevalence of protein S, and AT III deficiency as risk factors for inherited thrombophilia in patients with BCS and PVT were low in Indian and Western studies<sup>[12,13,22]</sup>. Protein S deficiency was not detected in any of our patients. AT III deficiency was higher in patients with BCS (21%) as compared to those with SVT (5%). Diagnosis of inherited deficiencies of protein C, protein S, and AT III, as a cause of abdominal venous thrombosis is difficult, because acquired deficiencies develop in liver failure, acute thrombosis, and during anticoagulant therapy<sup>[27]</sup>. None of the patients in the present study with protein C and AT III deficiency had liver failure or were on anticoagulant therapy.

Comparison of prothrombotic risk factor profiles between BCS and SVT showed a trend for hereditary risk factors to be more frequent in BCS (BCS *vs* SVT: 35.7% *vs* 25%; *P* = 0.7); two other Indian studies have made similar observations of hereditary factors being more frequent in BCS group compared to PVT group<sup>[12,13]</sup>. Studies on prevalence of acquired risk factors in abdominal venous thrombosis have shown variable results. Denninger *et al* and Janssen *et al* have shown that acquired risk factors are more frequent in PVT than in BCS<sup>[22,28]</sup>. Mohanty *et al* found the frequency of acquired risk factors to be similar in BCS and PVT<sup>[12]</sup>. In our study, acquired risk factors were significantly more common in the SVT group (BCS *vs* SVT: 7% *vs* 45%; *P* = 0.02) suggesting that SVT is a

heterogeneous disease where hereditary and local risk factors play important roles.

No risk factor was identified in 57% of BCS and 45% of patients with SVT. One possible reason may be the low prevalence of myeloproliferative disorders in our series (one patient). Myeloproliferative disorders (overt or latent) have been shown as an important risk factor in previous studies on abdominal vein thrombosis<sup>[28-31]</sup>. Tests for detecting latent myeloproliferative disorders (formation of "spontaneous" erythroid colonies in cultures of bone marrow progenitor cells in erythropoietin-poor medium<sup>[32,33]</sup>) were not performed on our patients. In a study from Western India, an etiological factor could be found in 59% of the BCS and 30% of the PVT patients<sup>[12]</sup>. Interestingly, in this study also, none had a myeloproliferative disorder.

Previous studies have suggested that venous thrombosis results from coexistence of several risk factors<sup>[28]</sup>. In the present study, ≥ 2 risk factors were detected in 7% of BCS and in 20% of patients with SVT.

Hereditary and acquired risk factors play an important role in etiopathogenesis of abdominal venous thrombosis. Acquired risk factors are significantly more common in patients with SVT while hereditary risk factors are similar in patients with BCS and SVT. Recognition and evaluation of these risk factors may help in therapy and prevention of disease progression. As a significant number of patients lack obvious etiology further research is required to identify as yet unrecognized risk factors.

## COMMENTS

### Background

Abdominal venous thrombosis may present as Budd-Chiari Syndrome (BCS) or splanchnic venous thrombosis (SVT). Hereditary and acquired risk factors are implicated in the etiopathogenesis of abdominal venous thrombosis. There are few systematic studies that have comprehensively evaluated both hereditary and acquired factors in BCS and SVT. Most studies have evaluated either a single prothrombotic risk factor or multiple risk factors in a single vein.

### Research frontiers

Concept of multifactorial theory of thrombogenesis suggests that thrombosis occurs by activation of a trigger factor (acquired) in a thrombophilic milieu (hereditary). The prevalence of inherited risk factors is variable between populations throughout the world. Possible reasons are small numbers of patients studied, non-standardized evaluation of parameters tested, and genetic differences between patient populations. Data need to be generated by good studies from different geographical areas in the world. Etiological factors for abdominal venous thrombosis were identified in 70%-80% of patients in Western studies. In Indian studies, no risk factor was identified in half the patients suggesting that other unknown hereditary/risk factors may be operating in these patients.

### Innovations and breakthroughs

The present study suggests that acquired risk factors which are preventable are important in etiopathogenesis of SVT. As no risk factors were identified in about half the patients, research needs to be ongoing to identify unknown hereditary/acquired risk factors operating in these patients.

### Applications

Abdominal venous thrombosis is a life threatening condition caused by single or multiple, hereditary or acquired prothrombotic risk factors. Prevention and therapy with non-invasive techniques and new anticoagulant drugs are now possible. Complete thrombophilia screening is, therefore, important for risk assessment, and therapy in patients with abdominal venous thrombosis. With continuing search for hereditary risk factors (genetic molecular defects), true

idiopathic thrombotic disease will become uncommon.

#### Peer review

This paper investigates genetic and acquired risk factors in patients with thromboembolism in abdominal veins. The authors make a difference between hereditary and acquired risk. It's a nice study.

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RAPID COMMUNICATION

# Laryngopharyngeal reflux in patients with reflux esophagitis

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## INTRODUCTION

Gastroesophageal reflux disease (GERD) has been known to be a common medical condition affecting approximately 35%-40% of the adult population in the Western world<sup>[1,2]</sup>. Forty-four of all Americans suffer from heartburn at least once per month, 20% at least once per week<sup>[3]</sup>. The role of GERD in causing extra-esophageal symptoms is also increasingly being recognized<sup>[4]</sup>. Chronic laryngeal signs and symptoms associated with GERD are often referred to as reflux laryngitis or laryngopharyngeal reflux (LPR)<sup>[5]</sup>. But, not all episodes of GERD are associated with LPR and not all patients with LPR have typical features of GERD<sup>[6]</sup>. Classic reflux symptoms (heartburn and regurgitation), which are referred to as "typical GERD," may be absent in more than half the patients presenting with extra-esophageal manifestations<sup>[7,8]</sup>. The "silent reflux" contributes to the difficulty in making a definite and correct diagnosis. The extra-esophageal manifestations of GERD provide the most challenging areas to perform good research<sup>[9]</sup>.

Furthermore, the prevalence of LPR in the patients with GERD has not been studied well in the past. The kind of patients with GERD who are more associated with LPR is still unknown. The cause-and-effect relationship between GERD and LPR remains elusive.

In patients with esophageal syndromes of GERD, reflux esophagitis (RE) is more easily diagnosed definitely by endoscopy than others. The objectives of our current study were to determine the prevalence of LPR in the patients with RE and to find out the factors that contribute to the development of LPR.

## Abstract

**AIM:** To assess the prevalence of laryngopharyngeal reflux (LPR) in patients with reflux esophagitis and disclose factors contributing to the development of LPR.

**METHODS:** A total of 167 patients who proved to have reflux esophagitis by endoscopy were enrolled. They received laryngoscopy to grade the reflux findings for the diagnosis of LPR. We used validated questionnaires to identify the presence of laryngopharyngeal symptoms, and stringent criteria of inclusion to increase the specificity of laryngoscopic findings. The data of patients were analyzed statistically to find out factors related to LPR.

**RESULTS:** The prevalence rate of LPR in studied subjects with reflux esophagitis was 23.9%. Age, hoarseness and hiatus hernia were factors significantly associated with LPR. In 23 patients with a hiatus hernia, the group with LPR was found to have a lower trend of esophagitis grading.

**CONCLUSION:** Laryngopharyngeal reflux is present in patients with reflux esophagitis, and three predicting factors were identified. However, the development of LPR might be different from that of reflux esophagitis. The importance of hiatus hernia deserves further study.

## MATERIALS AND METHODS

### Recruitment of patients

Consecutive patients who were diagnosed to have RE by gastroendoscopic examination due to various symptoms, such as epigastric pain, acid regurgitation, heartburn, nausea, abdominal fullness sensation, and so on, at the gastrointestinal clinic of Cathay General Hospital from September 2006 to October 2007 were enrolled. These qualified patients then were referred to the otorhinolaryngologic clinic for the further work-up of a laryngoscopic examination. Written, informed consent had to be provided by the participants before the endoscopic examination.

To improve the specificity of our study, the inclusion criteria of the patients were very strict. Patients were excluded from the study if they had a history of respiratory or gastrointestinal malignancy; radiation therapy to the head and neck, lung, or gastrointestinal tract; gastroesophageal surgery; use of H<sub>2</sub>-receptor antagonists or proton pump inhibitors in previous 1 mo; past or present smoker; excessive alcohol consumption; chronic cough attributable to known chronic pulmonary or tracheobronchial disease; professional voice users (e.g. singer, teacher); excessive voice use; exposure to occupational or environmental pollutants; history of seasonal allergic rhinitis; pharyngolaryngeal infection in the previous 3 mo; tracheal intubation in the previous 12 mo and use of inhaled corticosteroids<sup>[10,11]</sup>.

### Gastroendoscopic examination

All subjects received conventional endoscopic imaging, as well as imaging with the narrow-band imaging (NBI) system by using a video endoscope (GIF-H 260; Olympus Optical Co, Ltd, Tokyo, Japan). A group of experienced endoscopists performed the endoscopic examination. NBI is a novel, non-invasive optical technique that adjusts reflected light to enhance the contrast between the esophageal mucosa and the gastric mucosa<sup>[12]</sup>. The Los Angeles classification was used to grade esophagitis. A hiatus hernia was diagnosed if the hernia sac was more than 2 cm in length. We did not include patients suspected to have Barrett's esophagus due to diagnostic complexity.

### Questionnaire

The qualified participants needed to complete a questionnaire by answering "yes" or "no" with the aid of research nurses, right after the gastroendoscopic examination. It was used to identify the presence of any throat or reflux symptoms (cough, hoarseness, throat clearing, sore throat, thick drainage, globus sensation, bad taste in the mouth, swallowing problems, chest pain). Subjects were also asked to score the severity of each symptom based on a graded scale of 1 to 4 [1 = rare (once a month or less), 2 = occasional (2-3 times a month), 3 = frequent (several times a week); 4 = all the time (several times daily)]. The graded scales of more than 2 were considered significant, and the symptoms with such a scale could be included into the study<sup>[13]</sup>.

### Laryngoscopic examination

Each patient at the otorhinolaryngologic clinic underwent an endoscopic examination (Hopkins 70°C Telescope, Model 8706 CA, Karl Storz, Germany) of the larynx by two well-trained otolaryngologists, both with experience of over 10 years and good consensus, to grade the laryngoscopic findings. The otolaryngologists were not aware of the results of the questionnaire before the laryngoscopic examination. A reflux finding score (RFS) was obtained based on the laryngeal examination scoring system by Belafsky *et al*<sup>[14]</sup>. A RFS of > 7 was considered abnormal and to have LPR. Laryngeal signs suspected to be reflux-related were determined based on an agreement of the two experts.

### Statistical analysis

Statistical analyses were performed using the Stata 8.0 for Window (STATA Corp, College Station, TX). Patient characteristics were compared using the Student's *t* test and Pearson's  $\chi^2$  test for proportions. A logistic regression model was used to adjust for confounding covariates including, age, sex, BMI, disease (presence or absence), a hiatus hernia (presence or absence), and the grading of LA classification (A, B, C) *et al*. A two-tailed *P* value of less than 0.05 was taken to indicate statistical significance.

## RESULTS

Two hundred twenty-two patients with endoscopically proven RE initially were included in the study. However, 13 patients did not visit otorhinolaryngeal clinic due to personal reasons and 42 patients were excluded because they did not meet the strict inclusion criteria. Therefore, a total of 167 patients (80 males and 87 females) were enrolled in this study. The demographic characteristics of the studied subjects are listed in Table 1. 96.4% of the patients belonged to the groups of esophagitis grade A and B; only 3.6% were of grade C. A hiatus hernia was found in 13.8% of the patients.

Table 2 shows comparisons of demographic characteristics of patients with and without LPR. Among the 167 patients, 23.9% (40 cases) were diagnosed to have LPR. The difference in age between the patients with and without LPR was significant (45.2 *vs* 49.9 years, *P* = 0.04). The patients with LPR were younger than the ones without LPR. The presence of hoarseness symptom was significantly higher in the group with LPR (55.0% *vs* 26.8%, *P* = 0.001). In addition, a hiatus hernia was found more frequently in the group with LPR (27.5% *vs* 9.5%, *P* = 0.004).

If we combined the symptom of hoarseness and presence of a hiatus hernia together, the prediction of LPR was much higher (odds ratio increased up to 12.3, Table 3).

We also made a detailed analysis in the patients with a hiatus hernia. The distribution of esophagitis grading between the groups with and without LPR were compared. Of interest, in 23 patients with a hiatus hernia, the group with LPR (11 patients) had a relatively lower trend of esophagitis grading (LA grade A/B/C:

**Table 1 Demographic characteristics of 167 patients**

<b>Demographic characteristics</b>	<b>n</b>
Gender (male/female)	80/87
Age (yr)	
mean ± SD	48.8 ± 12.8
Range	21- 81
BMI (kg/m <sup>2</sup> )	
mean ± SD	23.4 ± 3.2
Range	16.1-36.3
LPR symptoms (%)	
Hoarseness	56 (33.5)
Globus	56 (33.5)
Cough	46 (27.5)
Throat clearing	59 (35.3)
LA grade (%)	
A	118 (70.7)
B	43 (25.7)
C	6 (3.6)
Hiatus hernia (%)	23 (13.8)

BMI: Body mass index; LPR: Laryngopharyngeal reflux; LA grade: The grade of Los Angeles classification of esophagitis.

**Table 2 Comparisons of demographic characteristics of patients with and without LPR**

	<b>LPR (n = 40)</b>	<b>Non-LPR (n = 127)</b>	<b>P</b>
Gender (%)			0.67
Male	18 (45.0)	62 (48.8)	
Female	22 (55.0)	65 (51.2)	
Age (yr)	45.2 ± 11.9	49.9 ± 12.9	0.04
BMI (kg/m <sup>2</sup> )	23.3 ± 3.2	23.4 ± 3.2	0.88
LPR symptoms (%)			
Hoarseness	22 (55.0)	34 (26.8)	0.001
Globus	14 (35.0)	42 (33.1)	0.82
Cough	12 (30.0)	34 (26.8)	0.69
Throat clearing	16 (40.0)	43 (33.9)	0.47
LA grade (%)			0.68
A	29 (72.5)	89 (70.1)	
B	9 (22.5)	34 (26.8)	
C	2 (5.0)	4 (3.1)	
Hiatus hernia (n, %)	11 (27.5)	12 (9.5)	0.004

BMI: Body mass index; LPR: Laryngopharyngeal reflux; LA grade: The grade of Los Angeles classification of esophagitis.

9, 81.8%/2, 18.2%/0, 0%), whereas the group without LPR (12 patients) had a higher trend of grading (LA grade A/B/C: 4, 33.3%/6, 50.0%/2, 16.7%). The difference was statistically significant ( $P = 0.04$ ).

## DISCUSSION

The association between LPR and GERD has not been firmly established yet<sup>[6]</sup>. Not all patients with GERD will develop LPR. On the other hand, it is estimated that 50%-60% of chronic laryngitis and difficult-to-treat sore throat may be related to GERD<sup>[8]</sup>. The causal association between acid reflux and laryngitis is highly plausible considering the close anatomical relationship. The vagally mediated reflexes (bronchospasm, laryngospasm and cough) stimulated by esophageal acid is also implicated in the pathogenesis of GERD-related extra-esophageal disorder<sup>[11,15]</sup>.

**Table 3 Logistic regression analyses on predictors of LPR**

	<b>Odds ratio</b>	
	<b>Model 1</b>	<b>Model 2</b>
Gender		
Female	-	-
Male	0.72	0.82
Age	0.96 <sup>1</sup>	0.96 <sup>1</sup>
BMI	0.98	0.99
LPR symptoms		
Hoarseness	4.12 <sup>1</sup>	-
Globus	1.77	1.21
Cough	0.91	1.23
Throat clearing	0.82	1.24
LA grade		
A	-	-
B	0.81	0.76
C	2.35	2.21
Hiatus hernia	4.78 <sup>1</sup>	-
Hiatus hernia and Hoarseness	-	12.3 <sup>1</sup>

BMI: Body mass index; LPR: Laryngopharyngeal reflux; LA grade: The grade of Los Angeles classification of esophagitis. <sup>1</sup>P value is significant at the 0.05 level.

Currently, there is no “gold-standard” for the diagnosis of LPR. Ambulatory 24-h dual or triple probe pH-metry was once considered the best method for reflux testing<sup>[16]</sup> but the position of the probes makes the measurement not easy to interpret, and there is no consensus about the pathological reflux at the level of laryngopharynx<sup>[6]</sup>. Moreover, extra-esophageal reflux is also intermittent. A negative pH study does not rule out extra-esophageal reflux<sup>[17]</sup>. The empiric therapy with aggressive acid suppression, usually BID dosing of proton-pump inhibitors (PPIs), is currently recommended as the most practical and cost effective approach for the patients suspected with extra-esophageal presentations of GERD<sup>[16]</sup>. Nevertheless, this therapeutic trial for the diagnosis of LPR could not provide direct evidence of pathologic imaging changes of patients, about which most clinicians want to learn.

Laryngeal examination with special emphasis on the posterior location of tissue injury can be helpful for the diagnosis of LPR<sup>[18]</sup>. The severity of mucosal injury may be graded according to the RFS by Belafsky 2001<sup>[14]</sup>. The RFS is an 8-item clinical severity scale based on findings during fiberoptic laryngoscopy. However, this RFS system has been criticized to have high inter- or intra-observer variability and low specificity for reflux laryngitis<sup>[6,10,19]</sup>. Therefore, it is very important to exclude meticulously other potential etiologies that can lead to laryngeal irritation. In our study, we did a very stringent selection of the patients to avoid the secondary causes of chronic laryngitis, such as smoking, alcohol, excessive voice use, allergies, or asthma.

The NBI system we used on gastroendoscopic examinations could offer a better image of capillary patterns and, thus, enhance the contrast between the esophageal and gastric mucosa and facilitate the endoscopic evaluation of esophagitis<sup>[20,21]</sup>. The better depictions of small erosive foci improves the intra- and inter-observer reproducibility in the grading of esophagitis, especially

in the grading of class A or B esophagitis<sup>[12]</sup>, which was very helpful in our study.

In our study, LPR was present in 23.9% of the studied subjects with RE. In the past, Koufman described posterior laryngitis in 74% and laryngeal edema with erythema in 60% of all patients with GERD<sup>[16]</sup>. Tauber *et al* also reported 85% of GERD-positive patients had posterior laryngitis and 69% had laryngitis with an interarytenoid erythema and edema<sup>[22]</sup>. Our prevalence rate of LPR is much lower than theirs; the different sample size of patients and method of enrollment in our research must have influenced the results. Because we used very stringent criteria to enroll the patients, it was possible that we missed some cases and underestimated the prevalence rate of LPR. In fact, this kind of report is quite rare in the literature. Most papers dealt only with the prevalence rate of GERD (ranging 20%-50%) in patients with LPR<sup>[4,21,23,24]</sup>.

Our results indicated that age, hoarseness and a hiatus hernia could be the predicting factors of LPR in the patients with RE. However, gender, body mass index, and the severity of esophagitis were not associated. A large cohort study performed by Jaspersen *et al* reported female gender, higher age, severe esophagitis, longer duration of GERD and smoking were significantly related to the extra-esophageal disorder<sup>[25]</sup>. Their risk factors were not the same as ours, which might be caused by the recruitment method they used. Though the case number of their study was large, they did not exclude the patients strictly and included patients who smoked. The patients they studied did not receive a laryngoscopic examination, and solely relied on the "symptom questionnaire" for the diagnosis of extra-esophageal disorders, which could be another factor that would induce diverse outcomes.

Increased GERD severity due to degradation of the gastroesophageal junction and impaired esophageal clearance was found in the elderly<sup>[26]</sup>. Yet, age as a factor contributing to LPR seldom has been mentioned before. In the present study, the RE patients with LPR were of a younger age than the patients without LPR. This finding is contradictory to the result of Jaspersen's study, in which they noted higher age was a risk factor for the occurrence of extra-esophageal disorder<sup>[25]</sup>. The opposite results again might be attributed to the different recruitment and research methods. However, the drawback of our study was that we had fewer patients. According to our findings, higher age, which implies the probable longer duration of GERD, is not essential for the development of LPR. In addition, our study also indicated the severity of RE had nothing to do with the occurrence of LPR. Therefore, the existence of LPR seems to be not associated with the duration or severity of RE.

LPR may have several clinical symptoms. Among them, throat-clearing, persistent cough, globus and hoarseness are the most common complaints<sup>[24]</sup>. In our study, the prevalence of hoarseness in all the patients was 33.5%. When we made comparison between the patients with and without LPR, the rate of hoarseness became 55.0% vs 26.8%, which was statistically significant. Our result indicated that more than 50% of the RE

patients with LPR had the symptom of hoarseness. As for the other symptoms (globus, throat discomfort and persistent cough), we did not find significant differences between the two groups.

Hoarseness is a common complaint of the patients at the otorhinolaryngologic clinic. Underlying causes include malignancy, vocal cord palsy, polyps and nodules of the vocal cords, laryngitis and functional disorders. Acute laryngitis is usually infective, whereas chronic laryngitis may result from a spectrum of insults including cigarette smoking, dehydration, acid reflux and muscular imbalance<sup>[6]</sup>. Hoarseness is not specific for LPR. Therefore, we must exclude several other possible causes before we can make sure the laryngitis-related hoarseness is induced solely by acid reflux. In our patients with RE, an additional symptom of hoarseness might reflect that the acid reflux has gone beyond the upper esophageal sphincter and injured the vocal cord. Extra-esophageal manifestation of GERD, thus, might be incurred.

Hiatus hernias have a higher detection rate in Western populations, ranging between 14.5% and 22%<sup>[27]</sup>. In the Far East, the prevalence rate is much lower, 7% of 464 subjects in Taiwan<sup>[28]</sup>, 2.9% of 11943 subjects in Singapore<sup>[29]</sup>, and 17.5% of 6010 individuals in Japan were reported<sup>[30]</sup>. In a recent series in Taiwan, hiatus hernia was found in 18.8% of patients with erosive esophagitis<sup>[31]</sup>. In our research, it was 13.8% of the studied subjects, which was also higher than that of the normal population here.

A hiatus hernia can disrupt both the anatomy and physiology of the normal anti-reflux mechanism. It is associated with decreased esophageal peristalsis; it also increases the cross-sectional area of the esophago-gastric junction and acts as a reservoir allowing reflux from the hernia sac into the esophagus during swallowing. The presence of a hiatus hernia is associated with symptoms of gastroesophageal reflux, and increased prevalence and severity of RE<sup>[27]</sup>. Because the presence of a hiatus hernia would increase esophageal acid exposure, it is emerging as an important factor in the pathogenesis of GERD<sup>[27]</sup>.

In our study, a confirmed hiatus hernia was found to be a risk factor contributing to LPR in the patients with RE. Considering the possible mechanism of reflux-related extra-esophageal disorders, it will not be surprising to disclose the importance of a hiatus hernia in these patients. With the existence of a hiatus hernia, the acid reflux could be potentiated and would result in more mucosal injury up to larynx. Animal studies have shown that even minute amounts of gastric acid and pepsin on laryngeal mucosa can induce significant inflammation and edema<sup>[32,33]</sup>. Further work is still needed to understand how a hiatus hernia influences the progression of GERD and its complications. At present, a hiatus hernia is known to be a marker of severe GERD<sup>[27]</sup> and must have a contributing effect in the pathogenesis of LPR.

In our patients with a hiatus hernia, we also analyzed their grade of esophagitis. Between the groups with and without LPR, the result was quite interesting and surprising. In this category, the patients with LPR had

a milder form of esophagitis than the ones without LPR. This finding again supports the concept that the development of LPR is not related to the severity of RE. To the contrary, LPR can be seen more frequently in the patients with mild RE when a confirmed hiatus hernia is present. Of interest, Li *et al* just reported that a hiatus hernia was found to be associated with more severe esophagitis in patients with RE<sup>[34]</sup>. Therefore, the development of LPR must be different from that of simple RE without LPR. Moreover, our patients who coexisted with a hiatus hernia and hoarseness had a very high odds ratio for LPR. Combining these two factors clinically, we could predict the presence of LPR more accurately in the patients with RE.

Regrettably, we had only 6 patients (3.6%) with LA grade C esophagitis and no patient with grade D. Thus, in our research, several factors could not be viewed and studied with the entire esophagitis spectrum from grade A to D. Another drawback of our study is that we did not include patients suspected to have Barrett's esophagus or endoscopically suspected esophageal metaplasia, which could be another intriguing field to see the relationship between GERD and extra-esophageal syndromes.

In conclusion, our study revealed that age, hoarseness and a confirmed hiatus hernia were the factors related to LPR in the patients with RE. LPR could be associated with RE, but the definite cause-and-effect relationship is still unknown. Our research was only a hospital-based study; more case numbers and convincing data are necessary in the future. Based on the aforementioned findings, the development of LPR seems to be different from that of RE. The importance of a confirmed hiatus hernia in LPR deserves further study.

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## COMMENTS

### Background

The role of gastroesophageal reflux disease (GERD) in causing laryngopharyngeal reflux (LPR) is being increasingly recognized, but the cause-and-effect relationship between them remains elusive.

### Research frontiers

This research is to assess the prevalence of LPR in the patients with reflux esophagitis (RE), and also to identify the factors contributing to the development of LPR.

### Innovations and breakthroughs

The prevalence rate of LPR in our studied subjects with RE was 23.9%. Age, hoarseness, and hiatus hernia were the factors significantly associated with LPR. In addition, the patients who coexisted with a hiatus hernia and hoarseness had a very high odds ratio (12.3) for LPR. Another interesting finding was that in 23 patients with a hiatus hernia, the group with LPR was incidentally revealed to have lower trend of esophagitis grading.

### Applications

LPR is present in patients with RE and three predicting factors could be identified. Combining the two factors of hoarseness and hiatus hernia together, we could predict the presence of LPR more accurately in the patients with RE. However, the development of LPR seems to be different from that of RE, based

on the findings of this research. The importance of hiatus hernia in LPR deserves further study.

### Peer review

In this study, the authors ascertained the association of LPR with GERD and analyzed the factors related to the development of LPR. The results could be very important because the readers could learn the newest knowledge and understand the future perspectives in this field.

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RAPID COMMUNICATION

## Macro-regenerative nodules in biliary atresia: CT/MRI findings and their pathological relations

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nodule does not imply that LT is withheld solely on the basis of presumed malignancy by imaging studies. Liver biopsy may be required in aid of diagnostic imaging to exclude malignancy.

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**Key words:** Biliary atresia; Macro-regenerative nodule; Liver neoplasm; Liver transplantation; Computed tomography; Magnetic resonance imaging

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### Abstract

**AIM:** To describe the radiological findings of a macro-regenerative nodule (MRN) in the liver of pre-transplantation biliary atresia (BA) patients and to correlate it with histological findings.

**METHODS:** Between August 1990 and November 2007, 144 BA patients underwent liver transplantation (LT) at our institution. The pre-transplantation computer tomography (CT) and magnetic resonance imaging (MRI) findings were reviewed and correlated with the post-transplantation pathological findings.

**RESULTS:** Nine tumor lesions in 7 patients were diagnosed in explanted livers. The post-transplantation pathological findings showed that all the lesions were MRNs without malignant features. No small nodule was detected by either MRI or CT. Of the 8 detectable lesions, 6 (75%) were in the central part of the liver, 5 (63%) were larger than 5 cm, 5 (63%) had intra-tumor tubular structures, 3 (38%) showed enhancing fibrous septa, 3 (38%) had arterial enhancement in CT, one (13%) showed enhancement in MRI, and one (13%) had internal calcifications.

**CONCLUSION:** Although varied in radiological appearance, MRN can be differentiated from hepatocellular carcinoma (HCC) in most of BA patients awaiting LT. The presence of an arterial-enhancing

### INTRODUCTION

Biliary atresia (BA), the congenital absence or destruction of the intra- or extra-hepatic biliary system<sup>[1]</sup>, affects about 5-10/100 000 live births<sup>[2]</sup>. Porto-enterostomy is typically performed as soon as possible in these children<sup>[3,4]</sup>. However, liver cirrhosis and its complications may develop in some patients even after a successful porto-enterostomy. Liver transplantation (LT) is, thus, beneficial to BA, and is the leading reason for LT in children<sup>[5]</sup>.

Macro-regenerative nodule (MRN), defined as a regenerating liver nodule > 0.5 cm in size, is occasionally encountered in cirrhotic livers<sup>[6]</sup> and may mimic a hepatocellular carcinoma (HCC)<sup>[7,8]</sup>. Differentiating this benign entity from HCC may be challenging, but is very important when considering patients for LT. Although the clinical importance of MRN in BA patients have been discussed<sup>[9]</sup>, further details of its computer tomography (CT) and magnetic resonance imaging (MRI) radiological appearances remain to be elucidated. The objective of this study was to describe the CT and MRI appearances of MRN in BA patients, and their radiological importance.

## MATERIALS AND METHODS

### **Patients**

We reviewed the images, medical records, and pathological reports of 144 BA patients who underwent LT from August 1990 to November 2007 in Chang Gung Memorial Hospital-Kaohsiung Medical Center, Taiwan, China. The diagnosis of BA was proven by surgical and pathological findings after porto-enterostomy in all patients. Of the 144 patients, routine liver CT angiography and MRI were not performed in 33 patients before September 2000 because no standard procedure was available. However, there were no liver masses described in the pathological reports in these 33 patients. A total of 111 patients were included in this study.

### **CT**

Preoperative imaging evaluation was performed in the 111 patients using Somaton plus 4 spiral CT scanner (Siemens, Erlangen, Germany). Sedation using intravenous propofol (0.5-1 mg/kg body weight) without tracheal intubation was given in uncooperative patients. The scanning protocol was 5-mm collimation and a 1:1.5 pitch. The images were subsequently reconstructed at a 4- or 5-mm interval with scanning range from lung base to liver edge. Non-contrast enhanced scanning was performed followed by contrast enhanced scans utilizing an intravenous contrast medium (1.5-2 mL/kg body weight) injected at 1.5 mL/s with an automated power injector via a 22- or 24-gauge intravenous catheter. The arterial phase acquisition started at 20-25 s, porto-venous phase at 60-70 s and equilibrium phase at 3-5 min after intravenous administration of a contrast medium.

### **MRI**

Seventy-nine of the 111 patients underwent MRI examination. We used a 1.5-T superconducting imager (Gyroscan Intera, Philips Medical system, Netherland B.V.) equipped with a phase-array body-coil. The liver was imaged in the axial planes with the following sequences: T1WI (GRE), T2WI (SENSE) and contrast-enhanced T1WI (GR). T1WI (GRE) was conducted using the following parameter: a repetition time/ echo time of 10/4.6 milliseconds. The T2WI (SENSE) was imaged using the following parameter: a repetition time/ echo time of 600/80 milliseconds.

For all the pulse sequences, a 5-8 mm thick slice was used with a 2 mm gap, 256 × 256 matrix size, echo train length of 1, number of average of 1 and a 35-40 field of view, depending on the size of the individual patient's liver.

A contrast medium was administrated using gadolinium-DTPA (0.1 mmole/kg body weight, Magnevist, Schering, Berlin, Germany) followed by a 20-mL saline flush. The delay for image acquisition timing was determined with a bolus tracking technique. Image reconstruction with 5-8 mm thickness was performed with source images at a MRI workstation.

### **Image interpretation**

Two radiologists experienced in reading abdominal

radiography retrospectively reviewed the images from the picture archiving and communicating system (PACS, GE medics) or from the patient's file storage (before 2002). Arterial, porto-venous and equilibrium phase images were interpreted conjointly. Preoperative assessment of the liver nodules included the number, location, size (the largest diameter in 3D orthogonal view), morphology, enhancing pattern, and signal intensity in MRI.

### **Histopathologic review**

All explanted livers were serially sliced at 0.5 cm intervals and carefully inspected to detect the focal lesions seen during preoperative imaging. The size and location of all visible nodules were recorded at gross inspection. All macroscopic nodules were examined microscopically for histological identification and differentiation. Representative sections of the liver were also examined.

### **Serum alpha-fetoprotein (AFP)**

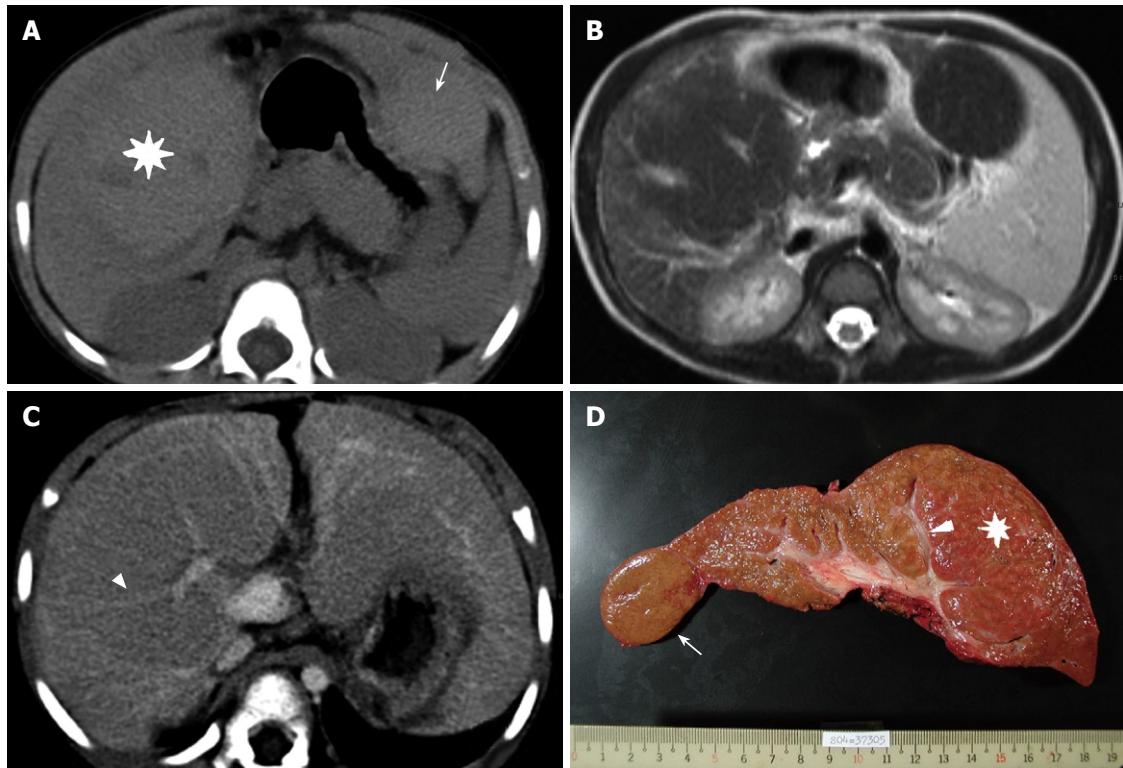
Serum AFP values were determined in all patients at the time of imaging or before transplantation.

## RESULTS

Nine MRN were detected in the explanted liver of 7 (4.8%) out of the 111 patients. The mean nodule diameter was 5.9 cm (range 1.6-9 cm). The MRN were located in the medial aspect (hepatic segments 4, 5, and 8) of 6 (67%) patients and in the lateral part of the liver (hepatic segments 2, and 6) of 3 (33%). The margin of the nodules was well-defined in all specimens. Of these 9 MRN, 7 were detected by CT and 7 by MRI. One was not detected by either MRI or CT, and one was found by MRI only. One patient with MRN did not undergo MRI. The CT and MRI findings of the 8 detectable MRN are listed in Table 1. At CT, the MRN were hyperdense compared with the surrounding liver parenchyma before contrast in 6 (75%) nodules (Figure 1A), the other 3 (38%) nodules were isodense (Figure 2A). After contrast medium enhancement, one nodule (13%) showed prominent enhancement both in arterial phase and in porto-venous phase, two nodules (25%) showed early enhancement and early wash-out pattern (Figure 3A), and four (50%) nodules showed no enhancement.

At MRI, the nodule was isointense to hyperintense on T1WI sequences and hypointense in T2WI in 5 (63%) nodules (Figure 1B) with one nodule showing T2WI central hyperintensity. Two nodules (25%) were hypointense on T1WI and hyperintense in T2WI (Figure 2B). After contrast enhancement, only one nodule showed enhancement. The other characteristic radiological findings in CT and MRI included stretching of intratumor tubular structures (5 lesions) (Figures 1C and 3B), fibrous septa in the periphery of the nodules (2 lesions), and internal calcifications (1 lesion). The septa were hypointense both before and after enhancement in T1WI.

Histopathology displayed that all lesions were MRN (Figures 1D and 3C), which were described as well-circumscribed liver cell nodules showing proliferating uniform liver cells bearing uniform round nuclei and



**Figure 1** A 3-year-old BA girl with segments 5-8 (asterisk) and segment 2 (arrow) MRN. **A:** The density of the MRN is slightly higher than that in the surrounding liver parenchyma during pre-enhanced phase of the CT; **B:** FSE/T2WI MRI shows a lower signal intensity in the MRN than in the surrounding liver; **C:** During portovenous phase of the CT, the tubular structure and splaying portal veins can be seen in the MRN (arrowhead); **D:** The explanted liver and intra-tumoral portal tract can be seen (arrowhead).

**Table 1 Summary of CT and MRI characteristics**

	<b>Case 1</b>	<b>Case 2</b>	<b>Case 3</b>	<b>Case 4</b>	<b>Case 5</b>	<b>Case 6</b>	<b>Case 7</b>
Size (cm)	9; 5; 1.6 <sup>1</sup>	4.5	2.1	3.5	7	7.5	13
Location (segment)	S5-8/S2	S6	S8	S5	S4-5-8	S4-5-8	S5-6-7-8
Density in CT (-) relative to liver parenchyma	Hyperdense; Hyperdense	Hyperdense	Isodense	Isodense	Hyperdense	Hyperdense	Hyperdense
Enhancement in CT	No  Early enhancement and early wash-out	Delayed portovenous enhancement	No	Arterial and portovenous enhancement	No	Early patchy enhancement early wash-out	
MRI T1WI/T2WI/C+	Hyperintense/ Hypointense	Hyperintense/ Hypointense	Hypointense/ Hyperintense	Hypointense/ Hyperintense	Not done	Hyperintense/ Hypointense	Isointense/ Hypointense
Septa T1WI/T2WI/C+	Not discernible	Hypointense/ Hypointense/ Enhanced	Not discernible	Not discernible	Not done	Hypointense/ Hypointense/ Enhanced	Not discernible
Presence of internal tubular structure (portal tract)	Yes; yes	No	No	No	Yes	Yes	Yes
Calcifications	No	No	No	Yes	No	No	No

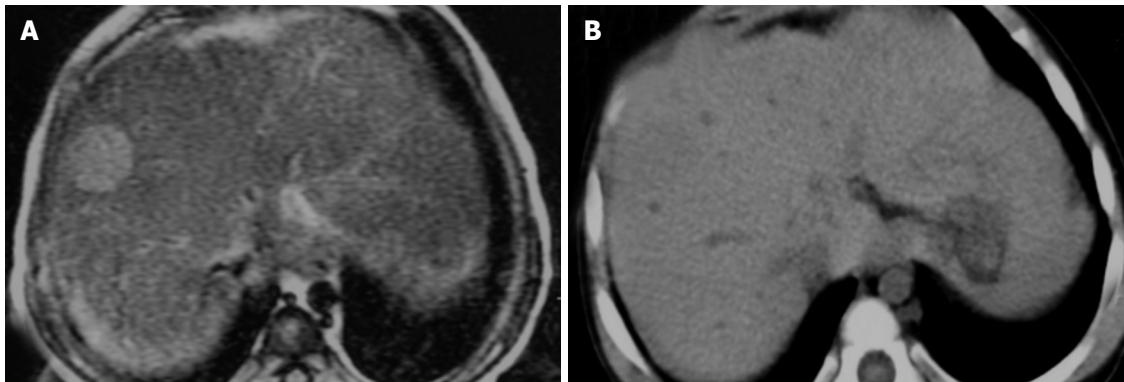
<sup>1</sup>This small nodule is not detected by either CT or MRI.

eosinophilic cytoplasm. No cellular atypia was found. The liver cells were arranged in one- to two-cell thick plates with intervening sinusoids (Figure 3D). No apparent sinusoidal capillarization was seen in the tumor lesion and no malignant foci were identified. Abortive portal tract formation was also noted. In all the seven MRN patients, the AFP level was < 3 ng/mL before LT.

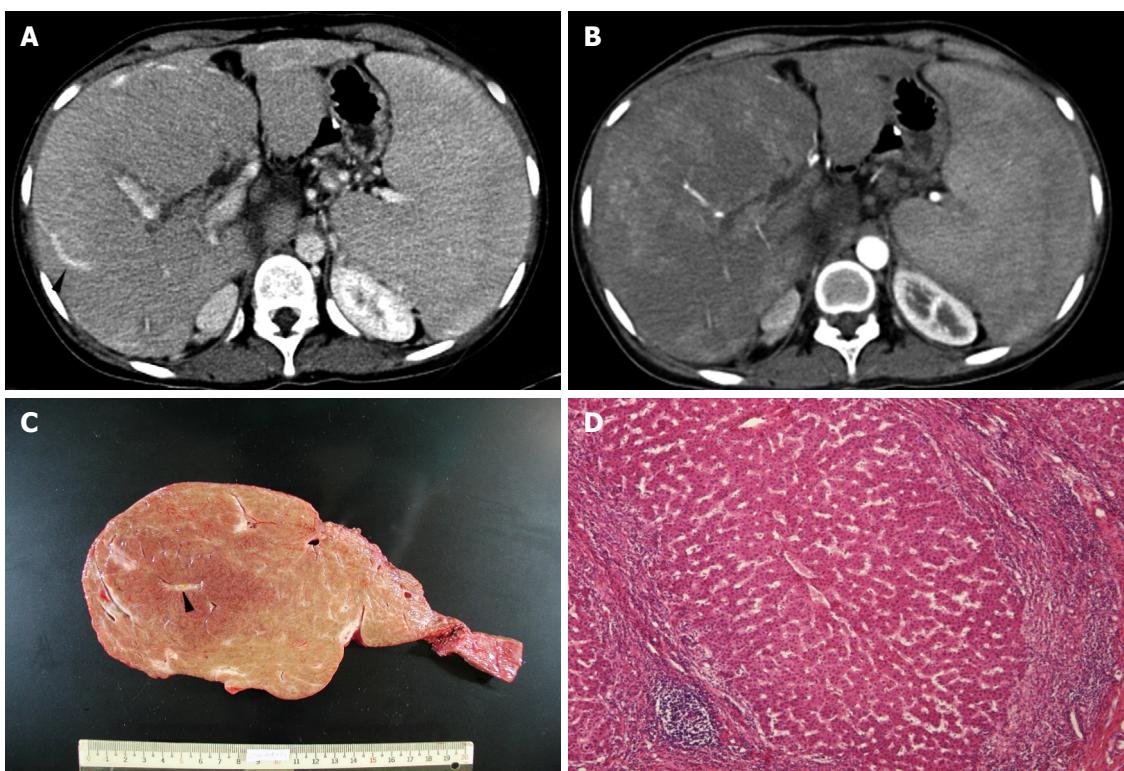
## DISCUSSION

Multi-acinar MRN, first described by Edmondson in

1976<sup>[10]</sup>, are sometimes seen in the cirrhotic liver. In 1996, an International Working Party defined MRN as “at least 5 mm regenerative nodules containing more than one portal tract”<sup>[10]</sup>. The reported prevalence in autopsy and explanted series varies from 14.2% in nodules > 1 cm in diameter to 37% in nodules > 0.5 cm in diameter<sup>[6,11,12]</sup>. It has been proposed that nodules > 2 cm in diameter in a background of cirrhosis are almost always dysplastic. However, these data were derived mainly from viral- or alcoholic-related cirrhotic livers. The smallest lesion found at gross pathology in our series was 1.6 cm in



**Figure 2** A 16-month-old BA girl with a 2.1 cm MRN in segment 8. **A:** During pre-enhanced phase of the CT, no nodule can be seen; **B:** The signal intensity is hyperintense on T2WI MRI.



**Figure 3** Patchy arterial enhancement within the mass in arterial phase of the CT (**A**), tubular structure (arrowhead) within the mass during portovenous phase of the CT (**B**), the explanted liver and intra-tumoral portal tract (arrowhead) (**C**), and microscopic examination (HE) showing uniform benign-looking liver cells arranged in one- to two-cell thick plates with intervening sinusoids and surrounding fibrous septa infiltrated with lymphocyte (**D**) in a 3-year-old BA girl with 13 cm MRN in right liver.

diameter and 56% (5/9) of the nodules were  $> 5$  cm in diameter (Figure 1A). In the literature, lesions  $> 10$  cm in diameter have been reported<sup>[13,14]</sup>.

MRN can be divided into siderotic and non-siderotic types<sup>[15]</sup> based on the presence of iron deposition within the mass. The presence of iron results in hypointense signal especially with longer TE MRI pulse sequences<sup>[16]</sup>. In this study, 63% (5/8) of the regenerating nodules fit this pattern (Figure 1B)<sup>[17,18]</sup>. In non-siderotic regenerating nodules (2/8), the patterns included T1 hypointensity and T2 hyperintensity (Figure 2B). These non-siderotic MRN are difficult to see on pre-enhanced CT due to their isodensity with the surrounding liver parenchyma (Figure 2A). Because of the retrospective

design of the current study, our methodology did not include histopathologic proof that the decreased signal intensity seen was caused by hepatic iron deposition. However, the same imaging techniques have been used to detect hepatic iron overload and siderotic regenerative nodules, and the results were confirmed with quantitative histopathologic measurements<sup>[19-21]</sup>.

In CT hepatic angiography, MRN have been characterized as non-enhancing nodules surrounded by enhancing fibrous septa<sup>[22]</sup>. However, in our study, this structural pattern was not easily seen. Only 25% (2/8) in our cases had broad vascular septa partially discernible by MRI. These septa were hypointense on T1W images, perhaps due to the fibrous component of the septum

itself. Enhancing septa were seen only in two nodules in this series after gadolinium-DTPA administration.

Fifty-three percent (5/8) of the MRN in our series had characteristic internal enhancing tubular structures during porto-venous phase. This was particularly true in the larger masses (Figures 1C and 3B). The enhancing tubular structures correlated with a distorted portal vein accompanying a bile duct on histopathology (Figures 1D and 3C). MRN virtually always contain some normal-appearing abortive portal tracts with complete portal veins, hepatic arteries and bile ducts<sup>[10]</sup>. Importantly, as a regenerative nodule progresses to become a dysplastic nodule or early HCC, one may notice the loss of visualization of these portal tracts and development of new arterial vessels, termed non-triadal arteries. These features are often used to differentiate MRN from adenoma or carcinoma pathologically. Therefore, the imaging appearance of these characteristic findings may be useful in diagnosing MRN during the pretransplant survey among BA patients awaiting LT.

Other useful imaging features used to differentiate MRN from HCC have been described elsewhere. First, a MRN is usually hyperdense to liver parenchyma in pre-enhanced study and often (but not always) does not enhance post contrast. A typical HCC is hypodense or isodense to liver parenchyma in pre-enhanced study and shows early arterial enhancement and early washout in porto-venous phase in dynamic-enhanced CT<sup>[23]</sup>. Second, the signal intensity of MRN in T2WI is often hypointense while malignant tumors are often hyperintense<sup>[23,24]</sup>. Third, MRN, especially larger ones, show splaying of intratumor portal veins while malignant tumors usually demonstrate displaced or obliterated portal veins. However, there are some overlapping features between MRN and HCC<sup>[25]</sup>. In a cirrhotic liver, early reports suggested that virtually all arterial-enhancing lesions are HCC. However, arterial enhancement may be seen in a regenerating nodule, non-tumor arterio-portal shunt, and aberrant venous drainage in a cirrhotic liver<sup>[8,26,27]</sup>. Our series demonstrated that arterial enhancement also occurred in MRN of BA patients.

A MRN has been presumed to be a precancerous lesion in virus-related or alcoholic-related liver cirrhosis because malignant foci are occasionally found in MRN<sup>[28,29]</sup>. Although rare, one case report has described a small HCC in a BA-related cirrhotic liver<sup>[30]</sup>. The possibility of early HCC could not be excluded in some of our pre-transplant imaging surveys of BA patients. According to the non-invasive diagnostic criteria for HCC proposed by the European Association for the Study of the Liver<sup>[31,32]</sup>, a diagnosis of HCC is established by the concomitant positive findings in two imaging techniques, or by a positive findings in one imaging technique with an AFP > 400 µg/L. The radiological interpretations for three arterial-enhancing tumor lesions (patient 2, 5 and 7) in this series were HCC initially (Figure 3A). Using the Barcelona Clinic Liver Cancer staging classification and treatment schedule, LT is considered for patients with three nodules < 3 cm in

diameter or with one tumor < 5 cm in diameter and liver function impairment<sup>[33]</sup>. LT may be precluded in patients 5 and 7 if the HCC diagnosis was made based solely on imaging. Furthermore, the AFP values for all cases were < 3 ng/mL. In such a situation, liver biopsy is necessary to confirm the nature of an arterial enhancing mass in order to exclude hepatic malignancy.

In conclusion, MRN are seen in about 5% of patients with BA awaiting LT. CT and MRI imaging features of MRN as described in this review can be useful in differentiating MRN from HCC in most of BA patients awaiting LT. The presence of an arterial-enhancing nodule should not imply that LT should be withheld solely on the basis of presumed malignancy by imaging studies, especially if the AFP value is incongruent with radiographic findings. Liver biopsy may be required in aid of diagnostic imaging to exclude malignancy in these cases.

## COMMENTS

### Background

End stage liver cirrhosis develops in some biliary atresia (BA) patients later in life. Liver transplantation (LT) is beneficial to such patients. Macro-regenerative nodules (MRNs) in cirrhotic liver are occasionally encountered in computer tomography (CT) / magnetic resonance imaging (MRI) and may be confused with hepatocellular carcinoma (HCC).

### Research frontiers

The authors described typical and atypical CT/MRI appearance of the MRN in BA patients, and the criteria for differentiation of MRNs from HCC. The strategy of managing atypical MRNs is also discussed.

### Innovations and breakthroughs

This study showed that the majority of MRNs can be easily differentiated from hepatocellular carcinoma by CT/MRI and unnecessary liver biopsy can be avoided.

### Applications

Using the radiographic features presented in this study will help manage BA patients awaiting LT properly.

### Terminology

MRN are defined as regenerative nodules larger than 0.5 cm in diameter in a cirrhotic liver. BA is a congenital absence or destruction of the intra- or extra-hepatic biliary system. It affects about 5-10/100 000 live births and is the leading cause for liver transplant in children especially in the oriental population.

### Peer review

The authors described the radiological findings of MRN in the liver of pre-transplantation BA patients and correlated it with histological findings. This is an interesting paper, which is informative to readers.

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RAPID COMMUNICATION

## Celecoxib-related gastroduodenal ulcer and cardiovascular events in a randomized trial for gastric cancer prevention

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**Author contributions:** Feng GS, Ma JL, Zhang L, Pan KF, and You WC contributed to the concept and designed the study on adverse events of celecoxib use; Ma JL and Liu WD collected the data; Feng GS and Ma JL wrote the manuscript; Feng GS was responsible for the statistical analysis; Shen L, Zhang XD, Li J, and Li JY contributed to the medical supervision in the trial; Xia HHX, Lam SK, and Wong BCY revised the manuscript; You WC supervised the whole study and polished the manuscript.

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**METHODS:** From 2004 to 2006, a total of 1024 Chinese patients (aged 35 to 64 years) with severe chronic atrophic gastritis, intestinal metaplasia or dysplasia were randomly assigned to receive 200 mg of celecoxib twice daily or placebo in Linqu County (Shandong Province, China), a high-risk area of gastric cancer. All gastroduodenal ulcer and cardiovascular events occurred were recorded and the patients were followed up for 1.5 years after treatment. At the end of the trial, a systematic interview survey about other adverse events was conducted.

**RESULTS:** Gastroduodenal ulcer was detected in 19 of 463 (3.72%) patients who received celecoxib and 17 of 473 (3.31%) patients who received placebo, respectively (odds ratio = 1.13, 95% CI = 0.58-2.19). Cardiovascular (CV) events occurred in 4 patients who received celecoxib and in 5 patients who received placebo, respectively. Compared with those who received placebo, patients who received celecoxib had no significant increase in occurrence of CV events (hazard ratio = 0.84, 95% CI = 0.23-3.15). Among the adverse events acquired by interview survey, only the frequency of bloating was significantly higher in patients treated with celecoxib than in those treated with placebo.

**CONCLUSION:** Treatment of gastric cancer with celecoxib is not associated with increased risk of gastroduodenal ulcer and cardiovascular events.

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**Key words:** Celecoxib; Gastroduodenal ulcer; Cardiovascular diseases; Adverse effects; Epidemiology; Randomized controlled trial

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Feng GS, Ma JL, Wong BCY, Zhang L, Liu WD, Pan KF, Shen L, Zhang XD, Li J, Xia HHX, Li JY, Lam SK, You WC. Celecoxib-related gastroduodenal ulcer and cardiovascular events in a randomized trial for gastric cancer prevention. *World J Gastroenterol* 2008; 14(28): 4535-4539 Available from: URL: <http://www.wjgnet.com/1007-9327/14/4535.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4535>

### Abstract

**AIM:** To evaluate the long-term risk of gastroduodenal ulcer and cardiovascular events induced by celecoxib in a population-based, randomized, double-blind, placebo-controlled study.

## INTRODUCTION

Celecoxib, approved by the US Food and Drug Administration (FDA) in 1998 for osteoarthritis and rheumatoid arthritis, is a cyclooxygenase-2 (COX-2) inhibitor. Owing to the selective inhibition of COX-2, this drug provides similar anti-inflammatory effects and a reduced risk of gastrointestinal complications in osteoarthritis and rheumatoid arthritis patients compared with nonsteroidal anti-inflammatory drugs (NSAIDs)<sup>[1,2]</sup>, which inhibit both COX-1 and COX-2. In addition, celecoxib, a selective inhibitor of COX-2, can block tumor growth by its antiangiogenic and proapoptotic effects, suggesting that it can be used in the prevention and treatment of cancers<sup>[3-5]</sup>.

However, it was reported that rofecoxib, also a COX-2 inhibitor, is associated with gastrointestinal toxic effects and cardiovascular (CV) events<sup>[6,7]</sup>; But, it has no gastrointestinal toxicity<sup>[8]</sup>. The conflicting results have raised the concern about the safety of celecoxib<sup>[9,10]</sup>. In 2005, the FDA Advisory Committee concluded that the adverse events of celecoxib are less than those of rofecoxib<sup>[11]</sup>. Therefore, we studied the safety issue of celecoxib. Gastroduodenal ulcer and CV events induced by celecoxib are reported in this paper.

## MATERIALS AND METHODS

### **Study population**

In 2004, a total of 1024 subjects, randomly selected from 12 villages of Linqu County (Shandong Province, China), participated in this study. Their age was 35-64 years. All subjects received a brief physical examination and their medical history was recorded. Subjects were ineligible if they had a history of stroke within two years, angina or congestive heart failure or myocardial infarction within one year, neoplastic diseases in the previous 10 years, esophageal or gastric surgery, inflammatory bowel disease, or bleeding diathesis, paracetamol allergy or hypersensitivity to aspirin, or other life-threatening illness. The remainders received <sup>13</sup>C-urea breath test (<sup>13</sup>C-UBT) and gastroscopic examination with biopsies from 5 standard sites of the stomach. Only those who had *Helicobacter pylori* (*H pylori*) infection and a histological diagnosis of severe chronic atrophic gastritis (CAG), intestinal metaplasia (IM) or dysplasia (DYS) were enrolled in the intervention trial. A written informed consent was obtained from each participant and the trial was approved by the Institutional Review Board (IRB) of Peking University School of Oncology (PUSO).

### **Study design and randomization**

Subjects were randomly assigned to received antibiotics and/or celecoxib or their placebo in a 2 × 2 factorial design. Finally, the subjects were divided into four groups. Group 1 received anti-*H pylori* treatment in the first week followed by 200 mg celecoxib twice daily for 24 mo, group 2 received anti-*H pylori* treatment in the first week followed by a look-alike celecoxib placebo for 24 mo, group 3 received a look-alike anti-*H pylori*

placebo in the first week followed by celecoxib twice daily for 24 mo, group 4 received a look-alike anti-*H pylori* placebo in the first week followed by a look-alike celecoxib placebo for 24 mo. We only observed and evaluated the risk of cardiovascular and other adverse events in the celecoxib and placebo groups (Figure 1). Both the participants and investigators were blinded to the treatment. Randomization of treatment assignments was generated at Westat Inc. in the US after eligibility was determined.

From March 16 to 30, 2004, the eligible participants were given a triple therapy with 20 mg omeprazole, 1 g amoxicillin and 500 mg clarithromycin or placebo twice daily for 7 d to eradicate their *H pylori* infection. Then 200 mg of celecoxib or placebo twice daily was given orally from April 8, 2004 to May 6, 2006, except for April 2005 because of the interim gastroscopic examination.

### **Follow-up**

During the period of study, labeled pill bottles of celecoxib or placebo were distributed to participants in each village by PUSO staff and trained field staff each month. The field staff visited each participant twice a month to monitor treatment-related events and to promote pill compliance in the entire duration of the study. The staff counted and recorded the number of pills remaining in each bottle before the new pill bottles were distributed each month. If a subject was not at home during the staff visit, an evening visit was scheduled. A subject was considered compliant if the pill bottle was empty at the end of that month. If a subject was unable to be contacted at the time of counting pills, he or she was considered non-compliant.

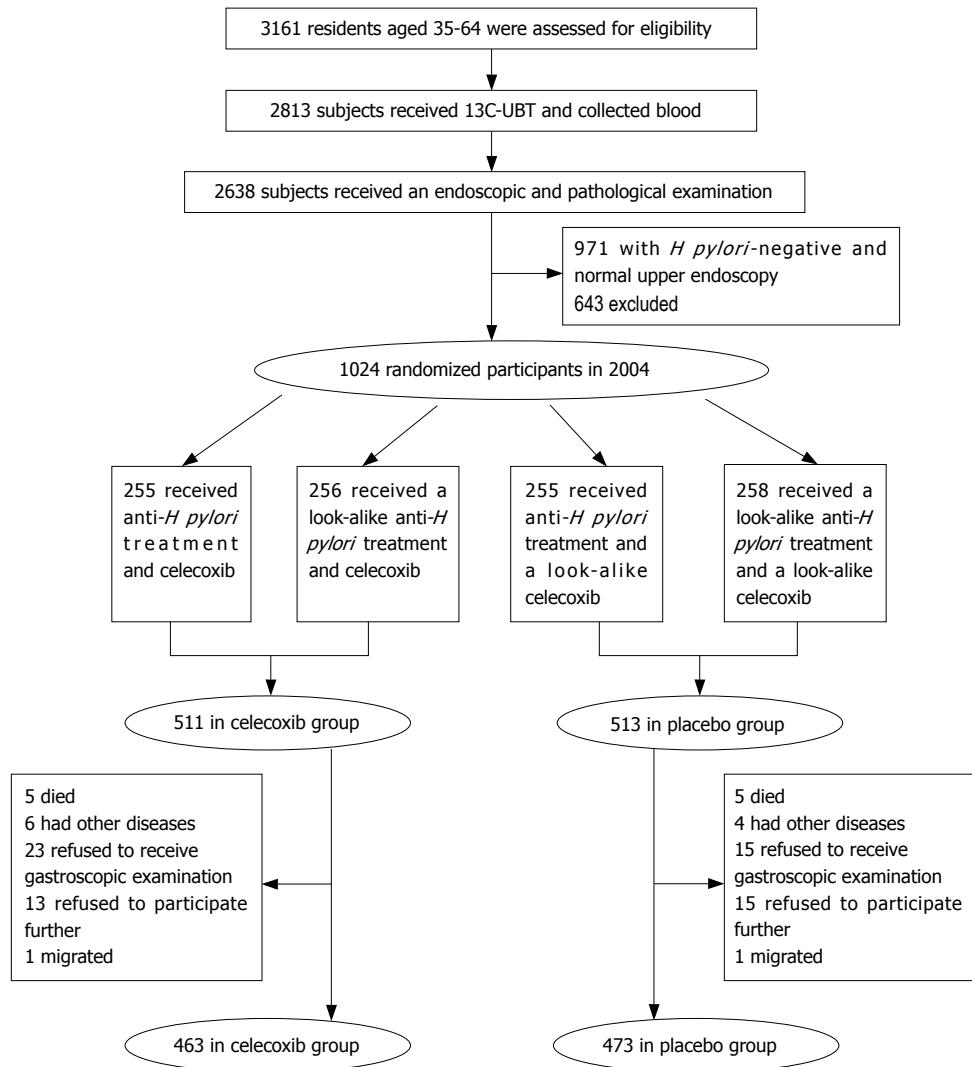
### **Adverse events**

Gastroduodenal ulcer was detected in 2005 and 2006 by the same group of PUSO physicians and gastroenterologists. Gastroscopic procedures, including biopsy samples taken from seven standard sites of stomach and histopathologic criteria, have been described elsewhere<sup>[12]</sup>. The gastroenterologist and pathologist were blinded to the subjects' intervention.

The CV events were defined as fatal or nonfatal myocardial infarction, ischemic and hemorrhagic stroke as previously described<sup>[13]</sup>. When visiting the participants, investigators recorded the CV events and other complaints of the participants. While investigators were absent, participants-reported symptoms were recorded by doctors in village clinics. All the CV events were diagnosed in local hospitals.

Other non-adjudicated adverse events were acquired by an interview among all the subjects at the end of the trial in May 2006. All the subjects' symptoms in the past two years were inquired and recorded by the trained interviewers, checked and categorized by two physicians in a blinded fashion after completion of the survey.

If the symptoms were related to treatment, PUSO physicians and field staff paid a close attention to the



**Figure 1** Trial profile.

subjects for at least 2 mo and these subjects received continuous treatment if the symptoms were aggravated.

### Statistical analysis

This study was designed to achieve a significant level of 95% ( $< 0.05$ ) and a power of 90% to detect a 20% regression of pre-malignant lesions, based on the background of 80% prevalence of gastric atrophy. At least 120 subjects were required in each group in order to detect a significant difference between the different treatment groups.

All data analyses were performed in a blinded fashion. The relative risks (with 95% confidence intervals) of gastroduodenal ulcer were analyzed using logistic regression by adjusting gender, age, smoking and drinking. The rate of CV events was determined and multivariate hazard ratio (HR) was calculated using the Cox proportional-hazard model. All  $P$  values were two-sided, and  $P < 0.05$  was considered statistically significant. All analyses were performed with SAS software, version 8.2.

## RESULTS

The 1024 participants were divided into celecoxib treatment group ( $n = 511$ ) and placebo treatment group ( $n = 513$ ). The baseline characteristics were balanced between the two groups (Table 1). During the two-year period of treatment, 88 participants who were relatively evenly distributed between the two groups withdrew from the study (Figure 1). The compliance rate was 90.61% in the celecoxib treatment group and 92.20% in the placebo treatment group, respectively.

From April 2004 to May 2006, gastroduodenal ulcer was detected in 19 of 463 (3.72%) participants of the celecoxib treatment group and in 17 of 473 (3.31%) participants of the placebo treatment group, respectively. The odds ratio (OR) was 1.13 (95% CI = 0.58-2.19, Table 2).

During the entire period of follow-up, CV events occurred in 4 participants of the celecoxib treatment group and in 5 participants of the placebo treatment group, respectively (Table 2). Compared with the placebo treatment group, the celecoxib treatment had no

**Table 1** Baseline characteristics of 1024 participants

	Celecoxib, n (%)	Placebo, n (%)	P
Male	238 (46.58)	235 (45.81)	0.81
Age (yr, means $\pm$ SD)	52.94 $\pm$ 6.51	52.93 $\pm$ 6.48	0.97
Smoking	146 (28.57)	142 (27.68)	0.75
Drinking	172 (33.66)	175 (34.11)	0.88
Hypertension	155 (30.33)	160 (31.19)	0.77

**Table 2** Incidence and risk of side effects in two groups

	Celecoxib, n (%)	Placebo, n (%)	OR (95% CI)
Gastroduodenal ulcer	19 (3.72)	17 (3.31)	1.13 (0.58-2.22)
CV events	4 (0.86)	5 (1.06)	0.84 (0.23-3.15)
Main nonadjudicated side effects			
Abdominal pain	8 (1.73)	13 (2.75)	0.62 (0.26-1.52)
Bloating	19 (4.10)	7 (1.48)	2.85 (1.19-6.84)
Constipation	9 (1.94)	15 (3.17)	0.61 (0.26-1.40)
Diarrhea	24 (5.18)	24 (5.07)	1.02 (0.57-1.83)
Dizziness	25 (5.40)	36 (7.61)	0.69 (0.41-1.17)
Gastric spasmus	15 (3.24)	15 (3.17)	1.02 (0.49-2.12)
Headache	26 (5.62)	23 (4.86)	1.16 (0.65-2.07)
Heartburn	29 (6.26)	23 (4.86)	1.31 (0.75-2.30)
Loss of appetite	25 (5.40)	16 (3.38)	1.63 (0.86-3.10)
Muscle pain	55 (11.88)	70 (14.80)	0.78 (0.53-1.13)
Nausea	14 (3.02)	17 (3.59)	0.84 (0.41-1.72)
Pain in the chest	16 (3.46)	17 (3.59)	0.96 (0.48-1.92)
Palpitations	22 (4.75)	16 (3.38)	1.43 (0.74-2.75)

significant increase in occurrence of CV events (HR = 0.84, 95% CI = 0.23-3.15).

The main nonadjudicated side effects are listed in Table 2. Except for bloating (OR = 2.85, 95% CI = 1.19-6.84), there were no significant differences in the frequency of other nonadjudicated adverse events between the two groups.

## DISCUSSION

In this study, gastroduodenal ulcer and CV events occurred in the subjects who took 200 mg celecoxib twice daily.

Two previous trials addressed the possibility that celecoxib has a lower rate of gastrointestinal complications than NSAIDs<sup>[14,15]</sup>. It was reported that the annual incidence rate of upper gastrointestinal complications and symptomatic ulcers is significantly lower in the celecoxib treatment group than in the combined diclofenac and ibuprofen treatment group (2.08% vs 3.54%;  $P = 0.02$ ) after 6 mo of treatment<sup>[14]</sup>. It has been shown that the incidence rate of gastric ulcer in the celecoxib treatment group and diclofenac treatment group is 18% and 34%, respectively ( $P < 0.001$ ), and the incidence rate of duodenal ulcer is 5% and 11%, in the celecoxib treatment group and diclofenac treatment group, respectively ( $P < 0.009$ )<sup>[15]</sup>.

Although the distinct role of celecoxib in ulcer is still unclear<sup>[16]</sup>, most studies suggested that celecoxib is not associated with gastric or duodenal ulcer<sup>[17-19]</sup>. Our trial compared the effects of celecoxib and placebo on

gastroduodenal ulcer, and the risk of gastroduodenal ulcer was not increased after treatment with 200 mg celecoxib daily compared with placebo.

The association between celecoxib and CV events was still debatable in our study. It was reported that a single dose of 400 mg celecoxib daily and placebo does not induce excess CV risk<sup>[20]</sup>. However, it was reported that 800 mg celecoxib increases the risk of death due to cardiovascular disease, myocardial infarction, stroke, or heart failure<sup>[21]</sup>.

The mechanism underlying the potential cardiovascular risk of COX-2 inhibitors is not fully understood. Although the imbalance caused by COX-2 inhibitors suppressing the COX-2 dependent prostacyclin production in endothelial cells without affecting the synthesis of platelet-derived thromboxane A<sub>2</sub>, may promote thrombosis and increase the risk of CV events<sup>[22-24]</sup>, the extent of instability to serum thromboxane and platelet function can be influenced by many factors, such as different doses of COX-2 inhibitors, variability among patients<sup>[16]</sup>.

In this study, different dose-effects of celecoxib on cardiovascular risk were observed. The dose of 800 mg celecoxib daily could increase the CV risk. However, 400 mg celecoxib daily did not increase the CV risk, suggesting that it can be used in the treatment of gastric ulcer.

In the present study, the frequency of bloating was higher in the celecoxib treatment group than in the placebo treatment group. However, the CV events were mild and tolerable, and none of the participants withdrew from this trial.

In conclusion, increases in gastroduodenal ulcer and CV events do not occur in subjects who take 200 mg celecoxib twice daily for two years. Celecoxib can be used in prevention and treatment of gastric cancer.

## ACKNOWLEDGMENTS

The authors thank the residents, field staff, and governments of Linqu County for supporting this long-term trial.

## COMMENTS

### Background

Celecoxib, a cyclooxygenase-2 inhibitor, is widely used as an analgesic and anti-inflammatory agent. In addition, it can prevent cancer. However, it is necessary to evaluate the risk of gastroduodenal ulcer and cardiovascular events, particularly in population-based studies.

### Research frontiers

No increase in gastroduodenal ulcer and cardiovascular (CV) events were found in the subjects who took 200 mg celecoxib twice daily for two years.

### Innovations and breakthroughs

This paper firstly reports an assessment of celecoxib-related gastroduodenal ulcer and cardiovascular events in Chinese population.

### Applications

Celecoxib (200 mg twice daily for two years) can prevent and treat gastric cancer in Chinese population.

### Peer review

The authors documented the absence of adverse effects of prolonged celecoxib administration at gastroduodenal and cardiovascular level in Chinese patients. The study was well designed and the results are reliable.

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RAPID COMMUNICATION

## Radiofrequency ablation as a treatment for hilar cholangiocarcinoma

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Fan WJ, Wu PH, Zhang L, Huang JH, Zhang FJ, Gu YK, Zhao M, Huang XL, Guo CY. Radiofrequency ablation as a treatment for hilar cholangiocarcinoma. *World J Gastroenterol* 2008; 14(28): 4540-4545 Available from: URL: <http://www.wjgnet.com/1007-9327/14/4540.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4540>

### Abstract

**AIM:** To explore the role of radio-frequency ablation (RFA) as a treatment for hilar cholangiocarcinoma.

**METHODS:** Eleven patients with obstructive cholestasis underwent Computed Tomography (CT) examination, occupying lesions were observed in the hepatic hilar region in each patient. All lesions were confirmed as cholangioadenocarcinoma by biopsy and were classified as type III or IV by percutaneous transhepatic cholangiography. Patients were treated with multiple electrodes RFA combined with other adjuvant therapy. The survival rate, change of CT attenuation coefficient of the tumor and tumor size were studied in these patients after RFA.

**RESULTS:** In a follow-up CT scan one month after RFA, a size reduction of about 30% was observed in six masses, and two masses were reduced by about 20% in size, three of the eleven masses remained unchanged. In a follow-up CT scan 6 mo after RFA, all the masses were reduced in size (overall 35%), in which the most significant size reduction was 60%. The survival follow-up among these eleven cases was 18 mo in average. Ongoing follow-up showed that the longest survival case was 30 mo and the shortest case was 10 mo.

**CONCLUSION:** RFA is a microinvasive and effective treatment for hilar cholangiocarcinoma.

### INTRODUCTION

Hilar cholangiocarcinoma (also known as Klatskin Tumor) was first reported by Gerald Klatskin in 1965. Since then, many therapeutic methods have been established to treat this type of tumor. For those patients presenting with type I and type II tumors, surgical resection is good and has a high 5-year survival rate<sup>[1-5]</sup>. However, for those tumors classified as type III and IV tumors, the surgical prognosis is poor even when combined with local tumor resection and left or right hemi-lobectomy of the liver, which can itself lead to further complications<sup>[6-8]</sup>. Thus, finding a surgical approach for the treatment of type III and IV Klatskin tumors is problematic. Percutaneous image-guided radiofrequency ablation (RFA) has received increasing attention as a promising technique for the treatment of liver tumors. This technique permits the destruction of tumors without necessitating their removal, and in many cases, can be used in place of more invasive and expensive surgical treatment. Initial attempts at tissue ablation with radiofrequency have been limited to the 1.6-cm diameter coagulation necrosis obtained from a single conventional electrode<sup>[9]</sup>. In order to achieve larger thermal necrosis, internally cooled, single or

clustered electrode technique, as well as expandable needle techniques has been introduced. Hence, from May, 2003 to December, 2005, we applied RFA therapy to a group of 11 patients with pathologically confirmed, type III and IV cholangiocarcinoma after percutaneous transhepatic cholangic drainage (PTCD) in order to determine its safety, efficacy, and outcome.

## MATERIALS AND METHODS

### Patient demographics

Eleven patients enrolled in this study were all male, ranging in age from 42 to 74 years, with a mean of 52 years. All patients presented with jaundice, and underwent CT examination. Occupying lesions were observed in hepatic hilar region in all cases. All lesions were confirmed as cholangioadenocarcinoma by biopsy and were classified as type III a ( $n = 4$ ), type III b ( $n = 2$ ), type IV ( $n = 5$ ) tumors by percutaneous transhepatic cholangiography. The average dimensions of the tumor masses were 3.4–4.5 cm.

### Instruments

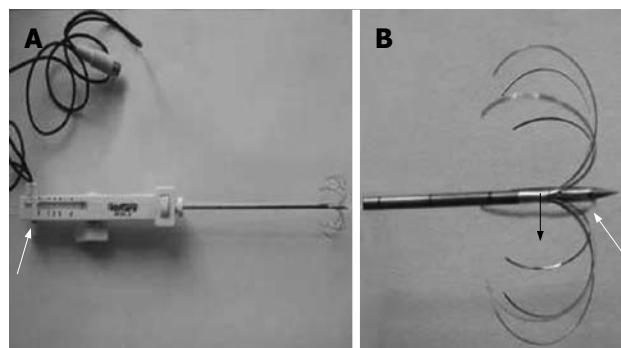
The Marconi CT-Twin flash, with the following parameters: volumetric scan with 5–10 mm thickness and pitch 1, WE7568 multiple electrode tumor RF ablator, 200W pulse output and 290 KHz pulse frequency, was applied (made by Beijing Welfare Electronic Co.). A temperature sensor was installed in the electrode to monitor the temperature and the sensor deviation was  $\pm 0.50^{\circ}\text{C}$ . A WHK-4 multiple electrode tumor RFA electrode with side holes ablation needle was applied. These systems deploy an array of multiple curved stiff wires in the shape of an umbrella from a single 14-gauge or 16-gauge canula (Figure 1). This array can produce zones of coagulation necrosis of up to 4–5 cm compared to the bipolar electrode. Furthermore, there are tiny side holes in the distal end of the central electrode, which make it possible for saline infusion during RFA in order to avoid charring and improve the ablation effect.

### Therapeutic strategy

All patients were hospitalized with proper blood analyses and preparations before synthetic interventional therapy. PTCD was performed for biliary drainage for 2 wk prior to RFA in order to preserve or improve hepatic function. The tumor size determined the number of sessions and duration of RFA.

### PTCD (percutaneous transhepatic cholangic drainage)

By using a 22 gauge Chiba needle, PTC was performed from the right middle axillary line to the most dilated intrahepatic biliary duct according to a prior CT scan. When the needle punctured the biliary duct, we fixed the needle and injected contrast media to perform cholangiography. The contrast media allowed better depiction of the stenotic segment and determined whether the left and right hepatic ducts were involved, allowing the tumor to be staged further. A unilateral



**Figure 1** **A:** The white arrow indicates the injection hole; **B:** The white arrow indicates the side hole, and the black arrow indicates hooked array radiofrequency needles

drainage catheter was applied for type III a and type III b. Bilateral kissing catheters were applied for type IV. The percutaneous drainage catheter was fixed onto the skin with stitches and connected with a drainage bag for daily observation of external bile drainage.

### RFA

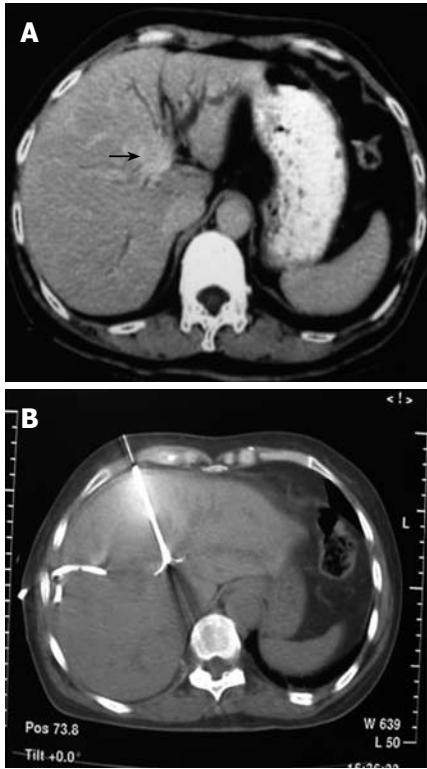
After 2 wk of biliary drainage, depending on the patient's general condition and improvement of the hepatic function, RFA was applied by percutaneous puncture into the tumor mass with an ablation electrode needle under CT guiding (a density survey of the tumor mass was performed before the procedure). After optimal tumor puncture, we delivered the electrodes in appreciable diameter. The electrodes were distanced from the drainage catheter during the RFA procedure, thereby protecting the catheters from mechanical puncture and thermal damage. The duration of ablation was dependant on the size of the tumor mass, with 10 min for those  $\leq 3$  cm, and 10–15 min for those around 3.1–4.0 cm. We prolonged the procedure time during which the temperature slowly increased. Injection of 1 mL of 10% saline from the side aperture of the needle every minute enhanced the excitement of ion vibrations, increasing the capability of the device. A CT scan was performed after delivering the electrodes to ensure that the entire lesion was fully covered within the region of RFA. This would provide complete tumor necrosis and eliminate the possible residual tumor infiltration in the adjacent area (Figures 2 and 3).

### Follow up

After RFA, regular monthly abdominal CT scans including a plain scan, double-phase scan and delayed image were performed with biochemical blood analysis in the first 6 mo. For the next 6 mo, follow-up work was every 2 mo, and 1 year after RFA, follow-up was every 3 mo.

### Evaluation of the therapeutic effect

One month after the RFA synthetic therapy, CT scans were compared to the baseline data. The treatment was counted as effective, in case where there was significant density attenuation of the tumor mass with no contrast



**Figure 2** A case with type IIIb Klatskin tumor. **A:** Plain CT scan reveals that the tumor is in the main trunk of left hepatic duct with dilatation of branches; **B:** The patient underwent RFA under CT guidance after internal and external drainage for two weeks.

enhancement, regardless of whether the tumor size was reduced or remained unchanged. Conversely, cases were counted as non-effective, if the tumor mass was enlarged and there was no significant density attenuation and inhomogeneous enhancement by IV contrast.

## RESULTS

### Quantification of tumor density change

The average density of the 11 tumor masses was 44.23 Hu before RFA therapy. One month after the therapy, it decreased to 21.6 Hu with the most marked case being a decrease of about 40 Hu.

### Quantification of tumor size

After RFA treatment, six masses had diminished in size by approximately 30%, two masses had been reduced by approximately 20% and no significant change in size was noted in three masses. These results were confirmed 1 mo later by a second CT scan. After 6 mo, the average size reduction in all the masses was approximately 35%. The most significant size reduction was 60%.

### Quantification of biochemical blood analysis

One month after PTCD, the direct and indirect bilirubin levels returned to their normal range (direct bilirubin  $\leq 8 \mu\text{mol/L}$  and indirect bilirubin  $\leq 15 \mu\text{mol/L}$ ) in nine cases. By 6 mo after RFA therapy, the bilirubin levels of all cases had returned to their normal range.

### Quantification of complications

No severe complications occurred during or after each procedure with the exception of fever post RFA.

### Survival analysis

The 11 patients were all alive at the end of the study. The survival period among these 11 cases was 18 mo on average. Ongoing follow-up showed that the longest survival case was 30 mo and the shortest case was 10 mo.

## DISCUSSION

Gerald Klatskin<sup>[10]</sup> first described the specific entities of adenocarcinoma as confined below, at or above the confluence of the common hepatic duct in 1965. Afterwards, Bismuth and Corlette<sup>[11]</sup> classified the tumor into four types: Type I: the lesion is confined to the common hepatic duct with no involvement of the left and right common ducts or confluence; Type II: confluence of the hepatic ducts is involved, but the lesion does not extend to the left and right intrahepatic biliary ducts; Type III a: the lesion spreads to the right hepatic duct; Type III b: the lesion spreads to the left hepatic duct; Type IV: involvement of the left and right hepatic ducts and confluence<sup>[12-18]</sup>.

For Type I and II tumors, surgical resection is the most effective form of therapy. Furthermore, a surgical approach is still possible in cases of local tumor recurrence<sup>[19,20]</sup>. However, for Type III tumors, radical resection is an aggressive procedure that might involve removal of the left or right hepatic lobe and caudate lobe of the liver. A mortality rate of up to 10% has been encountered, and survival after surgical resection on this type of tumor has been disappointing<sup>[12,14,21-24]</sup>. Palliative resection of Type IV tumor is still controversial, but doctors tend to perform liver transplantation. Transartery chemoembolization (TACE) is not an ideal treatment for Type IV tumors. Since the tumor mass is hypo-vascular, only a faint tumor contrast dye will be observed by DSA (Digital Subtraction Angiography) resulting in inadequate lipiodol staining. Chemotherapy either general or intra-arterial does not improve survival<sup>[25-27]</sup>. Based on these reasons, we propose that RFA therapy should be used for the treatment of type III and IV cholangiocarcinoma.

Due to the massive and wide range of invasion of the tumor, the majority of patients with Type III and IV Klatskin's tumor present with serious obstructive jaundice on admission. Therefore, with the aid of DSA, PTCD is the first step of the sequential interventional therapy since it anticipates jaundice and decompensates liver function by using an internal and external drainage. Two weeks following the drainage, until jaundice diminishes and hepatic function is amended, RFA is carried out and that is the most critical step of this sequential interventional therapy. RFA is becoming a widely used tool for treatment of liver metastatic tumors especially since Rossi *et al* introduced new needle



**Figure 3** A case with type IV Klatskin tumor. **A:** The plain CT reveals that the tumor is in the portal hepatic region with dilatation of the left and right hepatic ducts; **B:** The patient underwent RFA under CT guidance after internal and external drainage for 2 wk; **C:** The plain CT scan reveals a liquidized and necrotic region of the tumor in the portal hepatic region without contrast enhancement 2 mo after RFA.

electrodes capable of increasing the diameter of tissue necrosis to 4-5 cm<sup>[28,29]</sup>. The tissue necrosis should include 5 mm of normal tissue around the lesion for oncological clearance.

The basic principle of RFA therapy is described below. Under CT or sonographic guidance, the electrode percutaneously punctures and is placed into the tumor tissue with single or multiple electrode probes. The cluster of electrodes at the end of the probe will emit median to high-frequency electromagnetic wave energy that may induce ionic vibrating friction of the target tissue cells resulting in the generation of heat. As the local temperature increases up to 80°C-90°C, it is sufficient to cause coagulation necrosis of the tumor tissue, and eventually, liquefaction or fibrosis. Coagulation of the peripheral vessel and tissue around the tumor will form a reactive zone. Thus, the tumor blood supplies are interrupted contributing to the prevention of metastasis.

The tumor size determines the number of sessions and duration of RFA. Furthermore, extending the range of RFA to 0.5 cm out of the normal tissue margin where possible will effectively eliminate potential minimal tumor infiltration in the normal liver tissue. This creates a free margin and may reduce the likelihood of tumor relapse.

RFA has a tendency to injure the biliary system when the lesions are near the porta hepatis. This injury may be a bile fistula or an obstruction of the biliary tract. In our study, none of these complications occurred, since PTCD was performed in all of our cases before RFA. The advantages of PTCD before RFA are that: (1) the probability of biliary tract injury decreases due to remission of the obstruction and dilation of the biliary tract after PTCD; and (2) the drain pipe which was detained in the biliary tract during PTCD can be used as a localization mark during RFA.

There are several large vessels near the porta hepatis. Thus, serious hemorrhage will occur if the puncture of the needle injures the large vessels. Hemorrhage will also occur if the dissepiment necrosis of the vessels happens during the RFA procedure due to the entry of the electrode. RFA treatment of all 11 cases in our

study did not result in hemorrhagic complications. Our experience is that the radiologist who operates the RFA procedure must possess the imaging radiology and puncture technique, as well as being able to identify the large vessels in the porta hepatis and evade them. When the electrode enters the large vessels, the temperature remains low or the curve of the temperature offers crenate type and duty curve lasts high. At this time, the power source must be turned off immediately, and the location of the needle and electrode is adjusted. The cooling effect of the blood flow in the large vessels near the lesions results in lower efficacy in tumor necrosis. Our countermeasure is to inject 1 mL of 10% saline from the side aperture of the needle every minute to enhance ion vibration and excite the instrument to enhance the duty. It is also important to prolong the procedure time to ensure that the total time of effective temperature (higher than 70°C) lasts for 10-15 min.

Evaluation of the therapeutic effects of this treatment requires analysis of the changes in the density and size of the tumor mass by CT compared with base line data. One month follow-up after RFA showed that for all 11 cases, there were varying degrees of diminishing density and there was no enhancement in the contrast scans of the tumor mass. These effects were mainly due to the tumor coagulation and necrosis. In some cases, the lesion presented with an even lower density value because of the vacuum phenomenon resulting from long-term and multi-session therapy.

Six masses had diminished in size by approximately 30%, two masses had been reduced by approximately 20% and no significant change in size was noted in three masses.

Size reduction of the tumor mass was significantly noted in six out of 11 cases, two masses had been reduced by approximately 20%, and the other three lesions remained unchanged in other five patients in one month CT follow-up. However, 6 mo after synthetic sequential IR therapy, all 11 cases presented with varying degrees of size reduction. This was probably due to the slow progressive development of the coagulated necrosis, liquefaction and fibrosis of the lesion.

To date, all 11 patients are alive. A recent follow-up shows that the survival of a group of 29 cases of Klatskin tumor treated by surgical intervention. The mortality rate in the hospital (during and after surgery) was 17%, the average hospital stay was 71 d, and a survival rate of 1 year was observed in 50% of cases. Tsukada *et al* reported the survival after RFA therapy to be 18 mo on average, ranging between 10 mo and 30 mo so far. No complications among these 11 cases were observed during or after synthetic sequential interventional therapy. Parc *et al*<sup>[12]</sup> reported a group of 39 cases of Klatskin's tumor treated by surgical intervention. Radical resection was performed in 18 cases, and the remaining 21 cases underwent palliative operation. Among the 18 cases treated with radical resection, one had type I, two had type II, eight had type III a, two had type III b and five had type IV. Four of the 18 cases developed post-operative complications and the survival rate ranged between 1 (67%) and 5 (47%) years. Of the 21 cases treated with palliative operation, the post-surgery mortality was 14% and the mean survival range was only 7 mo. Nimura<sup>[30,31]</sup> performed a liver and bile duct resection combined with Whipple's operation in five patients with hilar tumors. Two of the five patients died after surgery and the three survivors died 8, 10 and 27 mo later.

Compared with surgical intervention, the RFA treatment that we used for type III and IV Klatskin's tumor is less invasive and involves less complications. Furthermore, a higher mean survival rate (up to 18 mo) has been shown after RFA than surgical intervention. Thus, we believe that RFA is a less invasive, safe, effective and promising therapy for type III and IV Klatskin tumors. The long-term curative effect requires further study.

## COMMENTS

### Background

Hilar cholangiocarcinoma (also known as Klatskin tumor) was first reported by Gerald Klatskin in 1965. Since then, many therapeutic methods have been established to treat this type of tumor. For those patients presenting with type I and type II tumors, surgical resection is good and has a high 5-year survival rate. However, for those tumors classified as type III and IV tumors, the surgical prognosis is poor even when combined with local tumor resection and left or right hemi-lobectomy of the liver, which can itself lead to further complications. Thus, finding a surgical approach for the treatment of type III and IV Klatskin tumors is problematic.

### Research frontiers

Percutaneous image-guided radiofrequency ablation (RFA) has received increasing attention as a promising technique for the treatment of liver tumors. This technique permits the destruction of tumors without necessitating their removal, and in many cases, can be used in place of more invasive and expensive surgical treatment. Initial attempts at tissue ablation with radiofrequency have been limited to the 1.6-cm diameter coagulation necrosis obtained from a single conventional electrode. In order to achieve larger thermal necrosis, internally cooled, single or clustered electrode technique, as well as expandable needle techniques have been introduced.

### Innovations and breakthroughs

To date, all 11 patients are alive. A recent follow-up shows that the survival range after RFA therapy is 18 mo on average, ranging between 10 mo and 30 mo so far. No complications among these 11 cases were observed during or after synthetic sequential interventional therapy. Compared with surgical intervention, the RFA treatment that we used for type III and IV Klatskin's tumor

is less invasive and involves less complications.

### Applications

RFA is a less invasive, safe, effective and promising therapy for type III and IV Klatskin tumors. The long-term curative effect requires further study.

### Peer review

According to the study, we know that a new therapy for type III and IV Klatskin tumor, and RFA is less invasive, soft and have fewer complications than common surgical treatment. It is worth paying attention and we are looking forward to the better curative effect from RFA.

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S- Editor Zhong XY L- Editor Rippe RA E- Editor Lin YP



RAPID COMMUNICATION

# Delayed ethyl pyruvate therapy attenuates experimental severe acute pancreatitis *via* reduced serum high mobility group box 1 levels in rats

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0.42 vs  $9.76 \pm 0.45$ ,  $P < 0.01$ ), pulmonary histological scores ( $7.1 \pm 0.7$  vs  $8.4 \pm 1.1$ ,  $P < 0.01$ ), serum AST ( $667 \pm 103$  vs  $1368 \pm 271$ ,  $P < 0.01$ ), ALT ( $446 \pm 91$  vs  $653 \pm 98$ ,  $P < 0.01$ ) and Cr ( $1.2 \pm 0.3$  vs  $1.8 \pm 0.3$ ,  $P < 0.01$ ) levels. SAP rats had a median survival time of 44 h. Delayed EP treatment significantly prolonged median survival time to 72 h ( $P < 0.01$ ).

**CONCLUSION:** Delayed EP therapy protects against distant organ injury and prolongs survival time *via* reduced serum HMGB1 levels in rats with experimental SAP. EP may potentially serve as an effective new therapeutic option against the inflammatory response and multiple organ dysfunction syndrome (MODS) in SAP patients.

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**Key words:** Severe acute pancreatitis; Ethyl pyruvate; High mobility group box 1; Multiple organ dysfunction syndrome; Survival time

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## INTRODUCTION

Excessive activation of the inflammatory mediator cascade during severe acute pancreatitis (SAP) is a major cause of multiple organ dysfunction syndrome (MODS), which leads to a high mortality rate<sup>[1]</sup>. Therapeutic strategies targeting these inflammatory mediators are thought to be an ideal way to reduce the severity of SAP. This is difficult, however, because the cytokines, such as TNF- $\alpha$  and IL-1 $\beta$ , are released early in the development of a systemic inflammatory response<sup>[2]</sup>. This leaves a narrow therapeutic window for the administration of

therapeutics, and delayed delivery of anti-inflammatory therapeutics are not effective after the inflammatory mediator cascade has developed<sup>[2]</sup>.

High mobility group box 1 (HMGB1) protein, which has been known as a ubiquitously expressed, intracellular DNA-binding protein for about 30 years, was recently identified as a late-acting mediator of endotoxin lethality<sup>[3]</sup>. It was reported that serum HMGB1 levels increased in patients with sepsis/endotoxemia<sup>[3-5]</sup>, hemorrhagic shock<sup>[6]</sup>, acute lung injury<sup>[7,8]</sup>, rheumatoid arthritis<sup>[9]</sup> and disseminated intravascular coagulation<sup>[10]</sup>. HMGB1 is identified as a late mediator of endotoxin lethality because its systemic release during endotoxemia is delayed as compared with the rapid increase of the early proinflammatory cytokines, such as TNF- $\alpha$  and IL-1 $\beta$ . HMGB1 is released by endotoxin-stimulated macrophages only after a delay of 12-18 h. A similar delayed appearance of HMGB1 (8-32 h) was also observed in the serum of mice with endotoxemia after TNF- $\alpha$  had reached its peak and subsided<sup>[3]</sup>. Delayed anti-HMGB1 antibody dosing still confers significant protection against endotoxin lethality<sup>[3]</sup>. Strategies that target HMGB1 with specific antibodies or antagonists seem to have potential value for treating lethal systemic inflammatory diseases characterized by excessive HMGB1 release, and the delayed kinetics indicate that HMGB1 may provide a broader therapeutic window for treating those inflammatory disorders<sup>[11]</sup>.

It has been recently demonstrated that the serum levels of HMGB1 were significantly elevated in patients with SAP on admission, and were correlated with the severity of the disease<sup>[12]</sup>. Early blockade of HMGB1 attenuates the development and associated organ dysfunction in experimental SAP<sup>[13]</sup>. These indicate that HMGB1 may be an effective therapeutic target of SAP. In a previous experimental study, we demonstrated that the serum levels of HMGB1 began to rise significantly at 12 h, and were maintained in high levels up to 48 h after induction of experimental SAP in rats<sup>[14]</sup>. Thus, we conceive that delayed therapeutic delivery targeting HMGB1 might attenuate SAP in rats.

Ethyl pyruvate (EP), a stable lipophilic pyruvate derivative, is an agent that can effectively protect animals from ischemia-reperfusion-induced tissue injury<sup>[15]</sup>. EP administration significantly improves the survival of lethal hemorrhagic shock in standard models<sup>[16,17]</sup>, and significantly inhibits the systemic release of both early (TNF- $\alpha$ ) and late (HMGB1) cytokines that mediate the lethality of sepsis and systemic inflammation, even when administered 24 h after cecal puncture<sup>[18]</sup>. We hypothesize that delayed EP administration could reduce the severity of SAP in rats through inhibiting the systemic release of HMGB1.

## MATERIALS AND METHODS

### Animals

Male Wistar rats weighing 200-250 g were obtained from the Experimental Animal Center of Tongji

Medical College, Huazhong University of Science and Technology (Wuhan, China). Before the experiment, the animals were kept in rooms at 20 °C ± 2 °C in 12-h light-dark cycles for at least 1 wk to acclimate to the surroundings with free access to water and standard rat chow.

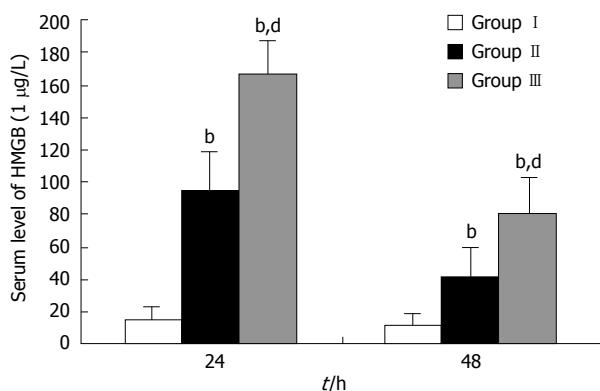
### Induction of pancreatitis

The animals were fasted with free access to water 12 h before surgery. The rats were then intra-abdominally anesthetized by 1% pentobarbital sodium (35 mg/kg body weight), and incised through a median incision of the abdomen. After the common bile duct was clamped in the hepatoduodenal ligament by a small bulldog clamp, the biliopancreatic duct was cannulated through mammary papilla from the anterior wall of the duodenum. 1 mL/kg body weight of 5% sodium taurocholate (Sigma, St. Louis, MO, USA) was injected by the cannula with an even speed of 0.1 mL/min, and the atraumatic vascular clamp was removed 10 min later. Finally, the abdominal incisions were closed in two layers. All procedures were performed using a sterile technique.

### Study protocol

After the stabilization period, 96 male rats were randomly divided into three groups (each group  $n = 32$ ), and each group was divided into two subgroups (each subgroup  $n = 16$ ). Animals in a subgroup were sacrificed at either 24 h or 48 h after surgery. Rats in group I (sham group) underwent laparotomy with manipulation of the pancreas (sham procedure) and received 40 mL/kg normal saline subcutaneously (single dose). Groups II and III underwent laparotomy with induction of SAP, and subsequently received saline every 6 h after induction of SAP. Rats in group II (delayed EP treatment group) additionally received 30 mg/kg body weight EP intravenously at 12, 18 and 30 h after induction of SAP. EP (Sigma, St. Louis, MO, USA) was prepared in solution with sodium (130 mmol/L), potassium (4 mmol/L), calcium (2.7 mmol/L), chloride (139 mmol/L), and EP (28 mmol/L) (pH 7.0). Rats in group III (SAP group) intravenously received the same volume of vehicle at the same time as in group II. Twenty four hours after induction of SAP, the surviving rats were anesthetized with ether, and blood samples were taken from the inferior vena cava to measure serum HMGB1 levels and blood biochemical parameters. The animals were sacrificed, and the right lung was obtained to evaluate lung injury. 48 h after induction of SAP, only blood samples were taken in order to measure serum HMGB1 levels.

To investigate the effect of EP on the survival time of SAP rats, 48 male rats underwent laparotomy with induction of SAP, and were randomly divided into two groups (each group  $n = 24$ ): SAP group and delayed EP treatment group. Rats in the EP treatment group received delayed EP delivery (30 mg/kg body weight) intravenously at 12, 18, 30 and 48 h after induction of SAP, and received



**Figure 1** Serum HMGB1 levels of rats. Group I: Sham group; Group II: Delayed EP treatment group; Group III: SAP group. <sup>b</sup>*P* < 0.01 vs Group I; <sup>d</sup>*P* < 0.01 vs Group II.

the above-mentioned fluid resuscitation. Rats in the SAP group received fluid resuscitation and the same volume of vehicle intravenously as in the EP treatment group. The number of surviving rats was recorded every 4 h after induction of SAP.

#### HMGB1 measurement

HMGB1 was analyzed by Western blot as described by Wang *et al*<sup>[3]</sup>. Briefly, serum was first filtered with Centricon YM-100 (Millipore) to clear the samples from cell debris and macromolecular complexes formed during clotting. Samples were then concentrated 15-fold with Centricon YM-30 and separated on 12% SDS-polyacrylamide gels. Protein was transferred to nitrocellulose membranes (Pall) and HMGB1 was analyzed by using polyclonal anti-HMGB1 antibodies (Santa Cruz) and secondary anti-goat alkaline phosphatase (Beijing Zhongshan Biotechnology). The intensity of the 30-kDa band was analyzed by densitometry. Standard curves were constructed using r-HMGB1 (Sigma, St. Louis, MO, USA).

#### Blood biochemical parameters measurements

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), and creatinine (Cr) levels were measured using a standard clinical automated analyzer.

#### Lung wet-to-dry-weight (W/D) ratios and histological scores

The right lung was wiped dry with filter paper and weighed. Then, it was dried to a constant weight at 60 °C for 72 h in an oven. W/D ratios could then be calculated. Routine paraffin sectioning was performed on the lung tissue. Pulmonary histological scores were graded using a Gloor score system (normal, 0; mild, 1; moderate, 2; severe, 3; overwhelming, 4) for intra-alveolar oedema, intra-alveolar haemorrhage, and neutrophil infiltration and these scores were then added to give a total score<sup>[19]</sup>.

#### Statistical analysis

mean ± SD values for blood biochemical parameters,

**Table 1** Blood biochemical parameters

Groups	Group I	Group II	Group III
AST (IU/L)	154 ± 20	446 ± 91 <sup>b</sup>	653 ± 98 <sup>b,d</sup>
ALT (IU/L)	51 ± 9	667 ± 103 <sup>b</sup>	1368 ± 271 <sup>b,d</sup>
BUN (mg/dL)	26 ± 3	38 ± 4 <sup>b</sup>	41 ± 4 <sup>b</sup>
Cr (mg/dL)	0.4 ± 0.1	1.2 ± 0.3 <sup>b</sup>	1.8 ± 0.3 <sup>b,d</sup>

Blood samples were obtained 24 h after induction of SAP. Group I: Sham group; Group II: Delayed EP treatment group; Group III: SAP group. <sup>b</sup>*P* < 0.01 vs Group I; <sup>d</sup>*P* < 0.01 vs Group II.

HMGB1 serum levels, lung W/D ratios and histological scores were determined. The differences between the two groups were further evaluated with the Mann-Whitney *U* test. Overall survival was calculated by the Kaplan-Meier Estimate. The log-rank test and the Breslow test were used to compare survival curves in the two groups. A *P* value < 0.05 was considered statistically significant.

## RESULTS

#### Serum HMGB1 levels

At 24 h and 48 h after induction of SAP, serum HMGB1 levels of SAP rats were higher than those of the sham group. Delayed EP administration significantly reduced the serum HMGB1 levels of SAP rats (Figure 1).

#### Lung injury

Lung W/D ratios were observed to evaluate the severity of pulmonary edema. At 24 h after induction of SAP, W/D ratios were elevated in the SAP group in comparison with the sham group ( $9.76 \pm 0.45$  vs  $5.43 \pm 0.21$ , *P* < 0.01). The lung W/D ratio of rats that received delayed EP administration was significantly lower than that of the SAP group (*P* < 0.01), although it was significantly higher than that of the sham group ( $8.22 \pm 0.42$  vs  $5.43 \pm 0.21$ , *P* < 0.01).

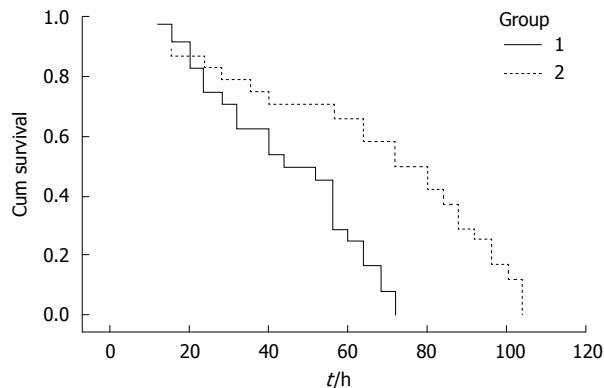
The pulmonary histological scores, an all round evaluation for lung injury, were lower in the EP treatment group than that in the SAP group ( $7.1 \pm 0.7$  vs  $8.4 \pm 1.1$ , *P* < 0.01), although they were significantly elevated in comparison with the sham group (vs  $0.5 \pm 0.1$ , *P* < 0.01).

#### Hepatic and renal dysfunction

EP treatment protected against liver and renal injury. 24 hours after induction of SAP, serum AST, ALT, BUN and Cr levels were significantly elevated in groups II and III. Delayed EP administration significantly attenuated the elevated AST, ALT and Cr levels (Table 1).

#### Survival analysis

SAP rats all died within 3 d without the delayed EP administration, and their median survival time was 44 h (95% confidence interval 29.6 h to 58.4 h). Delayed EP administration significantly prolonged the median survival time to 72 h (95% confidence interval 52.8 h to 91.2 h; Figure 2).



**Figure 2** Kaplan-Meier survival curves of SAP rats. 1: SAP group; 2: Delayed EP treatment group. Log-rank test,  $P = 0.0006$ ; Breslow test,  $P = 0.0050$ .

## DISCUSSION

Extracellular HMGB1 was recently implicated as a late mediator of delayed endotoxin lethality. High serum HMGB1 levels in patients with sepsis are associated with increased mortality, and administration of HMGB1 produces acute inflammation in animal models of lung injury and endotoxemia. During lethal endotoxemia in mice, serum HMGB1 levels accumulate 8–32 h after LPS administration<sup>[3]</sup>. Passive immunization of mice with anti-HMGB1 antibodies attenuates LPS-induced lethality, even when antibody administration is delayed until after the onset of the early proinflammatory cytokine response (2 h after endotoxin administration)<sup>[3]</sup>. The delayed kinetics of HMGB1 appearance indicates that the therapeutic window may be significantly wider than for any previously described cytokine target.

In a previous experimental study, we demonstrated that the serum levels of HMGB1 began to increase significantly at 12 h after induction of SAP, after the TNF- $\alpha$  and IL-1 $\beta$  peak had already occurred. The delayed kinetics of HMGB1 release may provide a wider therapeutic window for SAP. It was shown that EP could inhibit HMGB1 release from macrophages and prevent the accumulation of serum HMGB1 levels in mice with lethal sepsis by inhibiting NF- $\kappa$ B and p38 MAPK signaling<sup>[18]</sup>. In this study, we administered SAP rats with EP intravenously. As a result, EP significantly reduced serum HMGB1 levels in rats with SAP, even though EP delivery began 12 h after induction of SAP.

In SAP, MODS, a consequence of the systemic inflammatory response syndrome, is a contributor to high mortality in the early phase<sup>[11]</sup>. It is conceivable that the release of mediators from the excess of activated macrophages/monocytes and neutrophils may lead to remote organ injury<sup>[11]</sup>. Serum HMGB1 levels are significantly elevated in patients with SAP on admission<sup>[12]</sup>. Purified rHMG-1 is lethal to both LPS-responsive (C3H/HeN, Balb/c) and LPS-resistant (C3H/HeJ) mice, indicating that HMG-1 mediates lethal toxicity in the absence of LPS signal transduction<sup>[3]</sup>. The cytokine activity of HMGB1 has been well-documented in many cell types. In cultured human primary macrophages/monocytes, HMGB1 stimulates

the release of multiple proinflammatory cytokines, including TNF- $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-1RA, IL-6, IL-8, MIP-1 $\alpha$ , and MIP-1 $\beta$ , but not IL-10 and IL-12<sup>[20]</sup>. In cultured human microvascular endothelial cells, addition of HMGB1 induces the expression of adhesion molecules, such as ICAM-1, VCAM-1, and RAGE, as well as the release of TNF- $\alpha$  and IL-8, MCP-1, PAI-1, and tPA<sup>[21]</sup>. HMGB1 also activates human neutrophils to produce proinflammatory mediators, such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-8, suggesting an important role for HMGB1 in activation of neutrophils during inflammation<sup>[22]</sup>. HMGB1 also increases the permeability in cultured enterocytes via a nitric oxide (NO)-dependent pathway<sup>[23,24]</sup>. These indicate that HMGB1 is potent in augmenting the inflammatory response. A previous study showed the early blockade of HMGB1 attenuated organ dysfunction in experimental SAP<sup>[13]</sup>. This study indicated that HMGB1 might be a good target to prevent organ injury in SAP. In this study, to evaluate the degree of injury in distant organs, such as lung, liver and kidney, serum AST, ALT, BUN and Cr levels were measured, and lung W/D ratios were calculated. Serum AST, ALT and Cr levels, and lung W/D ratios were lower in the EP treatment group compared to those in the SAP group. This indicates that delayed EP treatment protected against distant organ injury. An additional study was performed to evaluate the effect of EP on survival times of SAP rats. As a result, delayed EP administration significantly prolonged the survival times of SAP rats.

These results give direct evidence that the beneficial effects of EP are due to downregulation of HMGB1, and reveal that EP may be a useful new therapeutic option against the inflammatory response and MODS in SAP rats, and may also be effective in SAP patients, even when anti-inflammatory therapy is delayed in the early phase. In this study, EP and reduction in serum HMGB1 levels actually reduces the severity of acute pancreatitis, and prolongs survival time of SAP rats. All rats finally die, however. Further studies should be performed to elucidate the role of HMGB1 in SAP and to determine whether a higher dose or earlier delivery of EP could improve the survival rates of SAP rats. Subsequently, clinical investigations could be carried out to study whether EP can be used for SAP patients.

## COMMENTS

### Background

Excessive activation of inflammatory mediator cascade during severe acute pancreatitis (SAP) is a major cause of the high mortality. Cytokines such as TNF- $\alpha$  and IL-1 $\beta$  are released early in the development of systemic inflammatory response. This leaves a narrow therapeutic window for administration of therapeutics and delayed delivery of that anti-inflammatory therapeutics is not effective after the inflammatory mediator cascade has developed.

### Research frontiers

Extracellular high mobility group box 1 (HMGB1) was implicated as a late mediator of endotoxin lethality. The cytokine activity of HMGB1 has been well-documented in many cell types. It was reported that serum HMGB1 levels increased in patients with sepsis/endotoxemia, hemorrhagic shock, acute lung injury, rheumatoid arthritis and disseminated intravascular coagulation. It has been recently demonstrated that the serum levels of HMGB1 correlated with

the severity of SAP.

### Innovations and breakthroughs

In a previous experimental study, the authors demonstrated that the serum levels of HMGB1 began to rise significantly at 12 h, and maintained at high levels up to 48 h after induction of experimental SAP in rats. The delayed kinetics indicates that HMGB1 may provide a broader therapeutic window for treating this lethal systemic inflammatory disease.

### Applications

Ethyl pyruvate (EP), a stable lipophilic pyruvate derivatives, is a nontoxic food additive. According to this study, it is potential to be used as an effective and low-cost therapeutic remedy for SAP patients.

### Peer review

This report is of interest and considerable potential importance, because it indicates that delayed treatment of rats with experimental SAP with EP is associated with a reduction of HMGB1 levels in blood; a decrease in lung, kidney, and liver injury; and prolonged survival. The "therapeutic window" for inhibiting this inflammatory mediator appears to be more favorable than for some other mediators which more rapidly reach a peak in the blood.

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RAPID COMMUNICATION

## Programmed death-1 expression is associated with the disease status in hepatitis B virus infection

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± 2.19%,  $P < 0.01$ ) and healthy individuals (4.63% ± 1.23%,  $P < 0.01$ ). Compared to AEHB patients (0.81% ± 0.73%), lower frequency of HBV-specific CD8+ T cells was detected in chronic hepatitis B patients (0.37% ± 0.43%,  $P < 0.05$ ). There was an inverse correlation between the strength of HBV-specific CD8+ T-cell response and the level of PD-1 expression. Besides, there was a significant positive correlation between HBV viral load and the percentage of PD-1 expression on CD8+ T cells in CHB and AEHB subjects ( $R = 0.541$ ,  $P < 0.01$ ). However, PD-1 expression was not associated with disease flare-ups as indicated by alanine aminotransferase (ALT) levels ( $R = 0.066$ ,  $P > 0.05$ ).

**CONCLUSION:** Our results confirm previous reports that HBV specific CD8+ T-cell response in the peripheral blood is more intense in patients with AEHB than in chronic hepatitis B with persistent viral infection. Moreover, there is a negative correlation between the level of PD-1 and the intensity of virus specific CD8+ T cell response.

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**Key words:** Chronic hepatitis B; Acute exacerbation of hepatitis B; Programmed death-1; Programmed death-ligand 1; Pentamer; Serum viral load; Blockade

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Ye P, Weng ZH, Zhang SL, Zhang JA, Zhao L, Dong JH, Jie SH, Pang R, Wei RH. Programmed death-1 expression is associated with the disease status in hepatitis B virus infection. *World J Gastroenterol* 2008; 14(28): 4551-4557 Available from: URL: <http://www.wjgnet.com/1007-9327/14/4551.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4551>

## Abstract

**AIM:** To define the potential role of programmed death-1/programmed death-ligand (PD-1/PD-L) pathway in different hepatitis B virus (HBV) infection disease status; we examined the expression of PD-1 on antigen specific CD8+ T cells in peripheral blood of patients with chronic hepatitis B (CHB) and acute exacerbation of hepatitis B (AEHB) infection.

**METHODS:** The PD-1 level on CD8+ T lymphocytes and the number of HBV specific CD8+ T lymphocytes in patients and healthy controls (HCs) were analyzed by staining with pentameric peptide-human leukocyte antigen2 (HLA2) complexes combined with flow cytometry. Real-time quantitative polymerase chain reaction (PCR) was used to measure the serum HBV-DNA levels.

**RESULTS:** The level of PD-1 expression on total CD8+ T cells in CHB patients (13.86% ± 3.38%) was significantly higher than that in AEHB patients (6.80%

## INTRODUCTION

Many individuals infected with hepatitis B virus (HBV) become chronic carriers and their liver disease may progress to chronic active hepatitis, cirrhosis, and hepatocellular carcinoma<sup>[1]</sup>. There is substantial evidence

to suggest that adaptive immunity has a central role in determining whether HBV infection is followed by recovery or viral persistence<sup>[2]</sup>. In particular, the cellular immune response plays a critical role in the ability of HBV-infected individuals to control viral replication<sup>[3]</sup>. Patients who develop chronic HBV infection have progressive low frequency and functional impairment of T helper cells (Th cells), both in the peripheral blood and the liver<sup>[4]</sup>. The cytotoxic T lymphocyte (CTL) response is also impaired in chronic HBV infection with high viral load<sup>[5]</sup>. However, the molecular mechanisms underlying the exhaustion of memory T-cell subsets have not yet been elucidated. Understanding the immunopathogenesis of HBV infection is crucial for the development of effective strategies to control HBV.

Our present knowledge suggests that the interaction between positive and negative costimulatory molecules expressed on T cells and antigen presenting cells is essential for the development of T cell responses<sup>[6,7]</sup>. Among the many costimulatory molecules, programmed death-1 (PD-1) and its ligands programmed death 1-ligand 1 (PD-L1) and PD-L2 constitute important pathways that regulate and fine-tune immune responses<sup>[8-10]</sup>. Recent evidence indicates that the exhausted virus-specific CD8+ and CD4+ T cells in chronic viral infection hyperexpress PD-1 molecule<sup>[11-14]</sup>. However, the infected cells that remain productive resist early apoptosis by down regulation of PD-1<sup>[15]</sup>. PD-L1 expression on monocytes and dendritic cells is also increased in HIV infected individuals<sup>[16]</sup>. These findings indicate that the PD-1/PD-L1 pathway plays a crucial role in inhibiting the function of virus-specific CD8+ T cells in chronic viral infections, in mice and humans<sup>[17]</sup> [Human immunodeficiency virus (HIV)<sup>[18-20]</sup>, hepatitis C virus (HCV)<sup>[21,22]</sup> and HBV<sup>[23]</sup>]. These studies illustrated that the level of PD-1 expression correlates with the degree of HBV-specific T-cell impairment. Thus, induction of apoptosis may be a major mechanism employed by the PD-1/PD-L costimulatory pathway to affect the outcome of virus-specific T cell response. Such negative regulation of CD8+ T cell function by PD-1/PD-L system has also been observed in HBV infection<sup>[16]</sup>.

It should be noted that the extent of HBV-specific T-cell exhaustion which influences the disease status of patients with HBV infection has thus far been analyzed only in small groups of patients, both those who were able to control an acute infection and those with established chronic infection<sup>[16]</sup>. However, little is known as to whether PD-1 expression on CD8+ T cells differs between patients with acute exacerbation of hepatitis B (AEHB) and chronic HBV infection.

To define the potential role of PD-1/PD-L pathway in acute exacerbation of HBV infection, we examined the expression of PD-1 on antigen specific CD8+ T cells in the peripheral blood in 32 patients with chronic untreated HBV infection and 11 patients with AEHB. The present study was performed by using pentamer technology combined with flow cytometry, allowing the direct visualization of HBV specific CD8+ T cells.

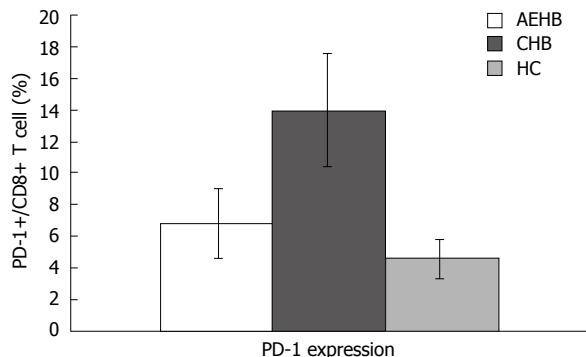
## MATERIALS AND METHODS

### Subjects

A total of 89 patients were enrolled in the study from either the outpatient clinic or the inpatient service of the Department of Infectious Diseases and Hepatology of the Union Hospital, Wuhan, China. The study group comprised of 66 chronic hepatitis B (CHB) patients [32 were human leukocyte antigen (HLA)-A2+], 23 AEHB (11 HLA-A2+) patients and 28 healthy donors. All subjects were anti-HBV treatment naive at the time of enrollment. Since APC-labeled HLA-A2 pentamer complexes specific for the HBV Core 18-27 (FLPSDFFPSU) can only identify HBV specific T cells in HLA-A2+ patients, all HBV-infected individuals subjected to fluorescent monoclonal antibodies and pentamer staining were first serologically identified as having the HLA-A2+ genotype. HLA typing was performed using flow cytometry by staining peripheral blood mononuclear cells (PBMCs) with a fluorescent anti-HLA-A2.01 antibody (Serotec). The criteria for the diagnosis of AEHB and CHB have been described previously in detail<sup>[24]</sup>. Based on the infection status, the subjects were divided into two groups: 11 patients had clinical, biochemical, and virologic evidence of AEHB infection [history of CHB, total bilirubin (TB) levels at least 10 times the upper limit of the normal, and plasma prothrombin activity (PTA) < 40%]. All patients were hepatitis B surface antigen (HBsAg) positive, and negative for antibodies against hepatitis C virus, hepatitis D virus (HDV), hepatitis E virus (HEV), HIV type-1 and HIV-2. Moreover, other causes of chronic liver disease were excluded. Patients with CHB patients had ALT levels ranging from 6 to 1485 U/liters and HBV DNA values from < 10<sup>3</sup> to 4.61 × 10<sup>8</sup> copies/mL. Patients with AEHB had TB levels ranging from 228 to 715.8 μmol/L, ALT from 36 to 174 U/liters, and HBV DNA from 4.3 × 10<sup>4</sup> to 8.9 × 10<sup>7</sup> copies/mL. A total of 28 HLA-A2+ uninfected age and sex matched healthy blood donors with normal liver functions were selected as healthy control (HC). Our study was approved by the local ethics committee, and all subjects gave a written informed consent.

### Serum viral load and ALT determination

The presence or absence of HBsAg, hepatitis B “e” antigen (HBeAg), antibody to hepatitis B surface antigen (anti-HBs), antibody to hepatitis B core antigen (anti-HBc), antibody to hepatitis B “e” antigen (anti-HBe), and antibodies to HCV, HDV, HIV-1, and HIV-2 were determined by using commercial enzyme immunoassay kits. Serum HBV viral load quantification was performed at the laboratory of Hepatology and Infectious Disease, Union Hospital by real-time polymerase chain reaction (PCR) (ABI PRISM 7300 Sequence Detector, PE Biosystems) based on the TaqMan technology using a commercial PCR diagnostic kit (Da An Gene Co. Ltd. of Zhong Shan University, Guangzhou, China). The viral DNA was extracted according to the manufacturer's protocol. Sequences of forward primer (5'-ATCCT



**Figure 1** PD-1 expression on CD8+ T cells in patients with acute exacerbation of hepatitis B (AEHB), chronic hepatitis B (CHB) and healthy controls (HC).

GCTGCTATGCCTCATCTT-3'), reverse primer (5'-ACAGTGGGGAAAGCCCTACGAA-3') and fluorogenic Taqman probes (5'-TGGCTAGTTACTAG TGCCATT-G3') were designed against a highly conserved region of the HBV genome overlapping the genes encoding the X-protein and DNA polymerase. The cycling program was: 50°C for 2 min, 93°C for 5 min, 40 cycles of 93°C for 30 s, 53°C for 30 s, 72°C for 30 s, and 72°C for 7 min. A serum sample quantified by b-DNA method was used as the standard to estimate the number of virus and as the quality control for HBV quantitative PCR. The internal control was estimated by commercialized  $\beta$ -actin kit (PE Biosystems) with reaction condition at 50°C for 2 min first and 5 min at 93°C, followed by 40 cycles of denaturation at 93°C for 30 s and annealing-extension at 65°C for 1 min. The analysis was accomplished within 2 min automatically at the end of the run. The HBV DNA cut-off value was 1000 copies/mL.

The serum ALT levels were assayed by CL-7200 Fully-auto Chemistry Analyzer provided by Shimadzu Co. Ltd, at the Department of Clinical Laboratory, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, China.

#### Isolation of PBMC

Using centrifugation on Ficoll-Hypaque density gradient (HaoYang Biological Manufacture, Tianjin, China) and RBC lysis Solution (Roche), PBMC were isolated from fresh heparinized blood (5 mL) collected from each patient, washed twice in phosphate-buffered saline and analyzed immediately.

#### Peptide-HLA class I pentamer antibodies and cell surface staining

To measure the number of virus-specific CD8+ T cells and PD-1 expression on CD8+ T-cell subsets, cells were stained with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, and allophycocyanin (APC)-labeled monoclonal antibodies or pentamer, according to the manufacturers' instructions. Briefly, a total of  $1 \times 10^6$  to  $5 \times 10^6$  freshly isolated PBMC were incubated for 30 min at room temperature with APC-labeled HLA-A2 pentamer complexes specific for the HBV Core 18-27

(FLPSDFFPSU) (Proimmune Oxford, United Kingdom) in RPMI 1640 and 10% fetal calf serum. After a wash with phosphate-buffered saline-0.1% fetal calf serum, cell surface staining was performed for 15 min in the dark by simultaneously using anti-CD8 (PE-conjugated), anti-PD-1 (FITC-conjugated) and the corresponding IgG2a isotype controls (eBioscience San Diego, CA).

#### Flow cytometry

Cells were washed twice with PBS and gated on CD8+ T cells;  $1 \times 10^6$  events in the lymphogate were collected by a BD Biosciences multi-color flow cytometer (LSR2, Becton Dickinson Biosciences). The data were analyzed by using the FACS Diva software. Pentamer-positive responses were reported as a percentage of pentamer-positive T cells among the total CD8+ T cell population. The frequency of pentamer-positive cells exceeding 0.02% of CD8+ T cells indicated a positive response.

#### Statistical analysis

We used SPSS 12.0 software to perform statistical analyses. Frequency of pentamer positive CD8+ T cells and levels of PD-1 expression in HCs and patients with different viremia levels were compared using the Mann-Whitney test. Spearman correlation analysis was used to evaluate the correlation between viral load, frequency of HBV-specific T cells and PD-1 expression. All tests were two-tailed and *P*-values less than 0.05 were considered significant.

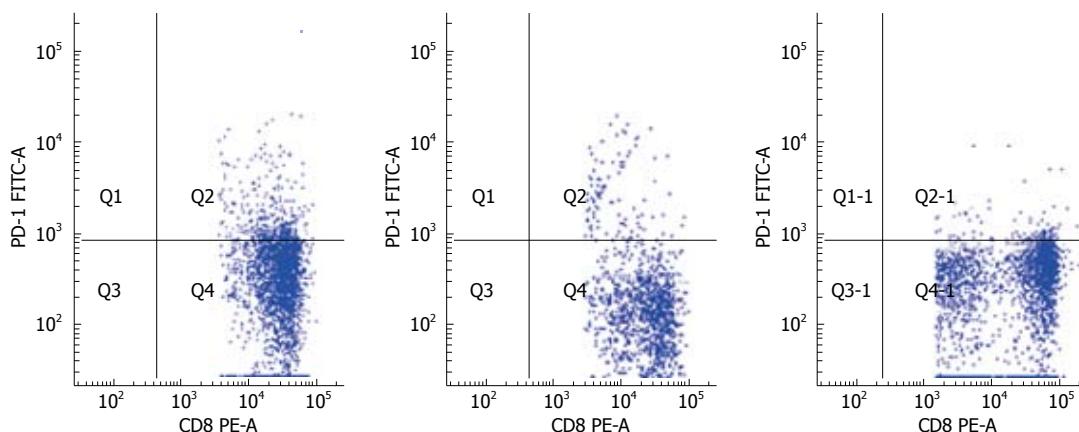
## RESULTS

#### PD-1 expression on PBMC in patients of different disease states

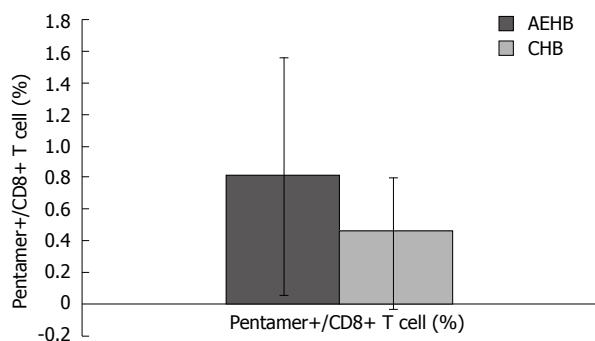
To determine PD-1 expression on CD8+ T cell population, we examined the frequency of PD-1-expression on CD8+ T-cell subsets using polychromatic flow cytometry in the three study groups: AEHB, CHB and HC. The PD-1 levels on the total CD8+ T cells in CHB patients ( $13.86\% \pm 3.38\%$ ) were significantly higher compared to AEHB patients ( $6.80\% \pm 2.19\%$ ,  $P < 0.01$ ) and healthy individuals ( $4.63\% \pm 1.23\%$ ,  $P < 0.01$ ) (Figure 1). Representative flow cytometry plots of PD-1 expression on CD8+ T cells from the peripheral blood of one health individual (5.8%) and two patients with CHB (12.71%) and AEHB (7.1%) are shown in Figure 2.

#### Comparison of HBV-specific CD8+ T cells upregulated in AEHB and CHB patients

The frequency of HBV-specific CD8+ T cells in the three study groups was assessed. The detection limit was 0.02% for CD8+ T cells specific for the core peptide spanning amino acids 18 to 27. None of the 28 healthy individuals showed a positive response with pentamer. Compared to AEHB patients ( $0.81\% \pm 0.73\%$ ), there was a lower frequency of HBV-specific CD8+ T cells ( $0.37\% \pm 0.43\%$ ) in CHB patients ( $P < 0.05$ ) (Figure 3). Representative plots from two patients with CHB (0.07%) and AEHB (0.3%) infection indicating the number of HBV-specific CD8+ T cells are shown in Figure 4.



**Figure 2** Comparison of PD-1 expression on CD8+ T cells in healthy controls (HC; 5.8%), chronic hepatitis B (CHB; 12.7%), and acute exacerbation of hepatitis B (AEHB; 7.1%).



**Figure 3** The number of HBV-specific CD8 T cells in CHB and AEHB patients.

#### **PD-1 expression in relation to different HBV-specific CD8+ T cell responses**

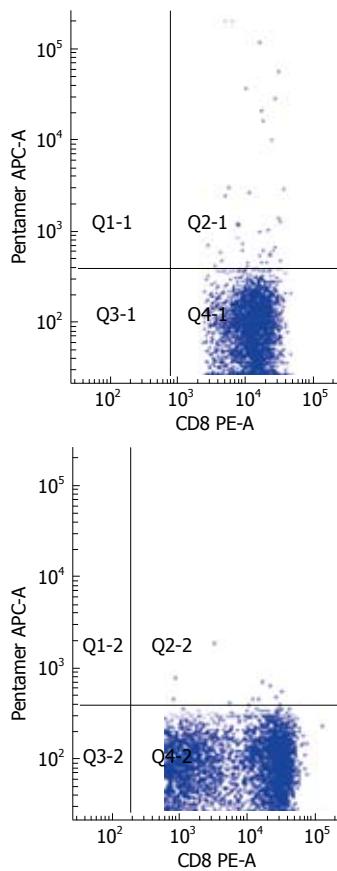
Using linear regression and Spearman's correlation analyses, we found an inverse correlation between HBV-specific CD8+ T-cell response and level of PD-1 expression in AEHB and CHB patients. In other words, the higher the PD-1 expression level, the weaker (or totally absent) HBV-specific CD8+ T-cell response was observed (Figure 5).

#### **Correlation of PD-1 expression with serum HBV DNA load**

In the present study, there was a significant positive correlation between HBV viral load and PD-1 expression on CD8+ T cells in CHB and AEHB subjects. These findings indicate that high plasma viral load correlates with PD-1 upregulation on the total CD8+ T cells in viremic individuals (Figure 6).

#### **Correlation between PD-1 expression and serum ALT levels**

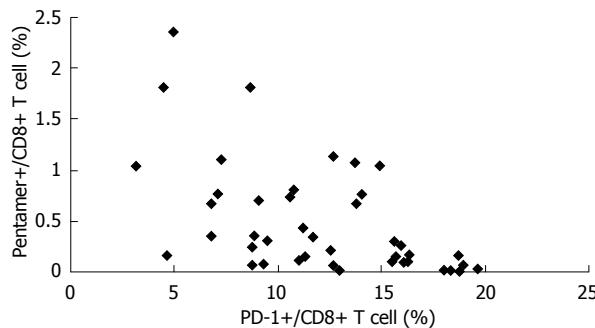
We also assessed the relationship between PD-1 expression and serum ALT levels, another predictor of HBV disease progression. There was no correlation between PD-1 expression and serum ALT levels in the study patients (Figure 7).



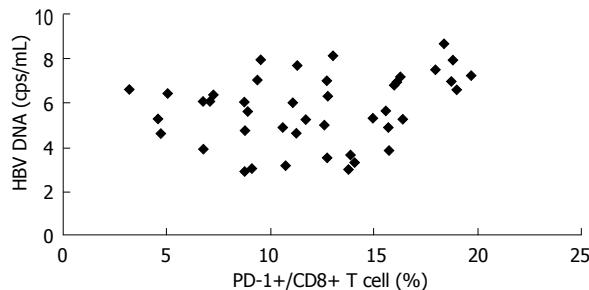
**Figure 4** The number of HBV-specific CD8 T cells in two patients with CHB (0.07%) and AEHB (0.31%) respectively.

## **DISCUSSION**

The development of chronic persistent HBV infection is usually associated with quantitative and qualitative exhaustion of functional T cells. Recent studies have shown that the negative costimulatory receptor PD-1 along with its ligand PD-L1 are upregulated on PBMC and virus specific T cells, to attenuate T cell responses which accounts for the impairment of their function<sup>[15,16]</sup>. However, under acute hepatitis B (AHB) conditions, the



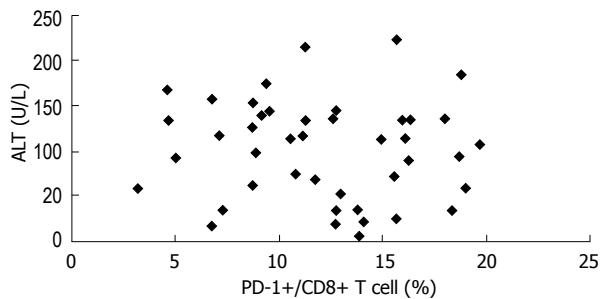
**Figure 5** Significant inverse correlation between the number of HBV-specific CD8+ T-cells and the level of PD-1 expression in patients with AEHB and CHB ( $R = 0.541, P < 0.01$ ).



**Figure 6** Positive correlation between serum viral load and level of PD-1 expression on CD8+ T cells in patients with CHB and AEHB ( $R = 0.272, P < 0.05$ ).

levels of PD-1 are significantly down regulated on PBMC or on HBV specific CD8+ T cells compared to CHB patients. Several previous studies<sup>[10,25,26]</sup> have suggested that operating co-stimulatory pathways may provide a new method to restore the impaired function of virus specific T cells and control chronic virus infection. Blocking the PD-1/PD-L1 pathway by anti-PD-L1 mAb or applying soluble PD-1 results in improvement of T cell functions such as survival, proliferation and cytokine production<sup>[27-29]</sup>. This would result in better control of the virus infection. Thus, an accurate balance between positive and negative co-stimulatory regulation such as PD-1/ PD-L pathway may contribute to the outcome of disease progression in HBV infection.

However, the mechanism by which PD-1 affects the function of CD8+ T cells in AEHB is unclear. To clarify the correlation between PD-1 expression and different presentations of HBV infection, we investigated the role of PD-1 in AEHB and chronic HBV infection, using a MHC class I pentamer specific for 18-27 epitopes, using the flow cytometry technology. Consistent with previous studies, we found that peripheral blood CD8+ T cells showed a higher PD-1 expression in CHB patients compared to AEHB patients and HCs. There was a negative correlation with impaired HBV-specific CD8+ T cell function and a positive correlation with the plasma viral load. However, we did not find an association between PD-1 expression and hepatic inflammatory indicator: serum ALT levels. Our data indicates that similar to AHB patients, CD8+ T cells in AEHB patients expressed a low level of PD-1 and



**Figure 7** Lack of significant correlation between PD-1 expression and serum ALT levels in the study patients ( $R = 0.066, P > 0.05$ ).

an enhanced level of HBV specific CD8+ T cells compared to CHB patients. These results are in line with previous reports which indicate that virus specific T cell function is upregulated during acute exacerbation of hepatitis B infection compared to during persistent CHB. Furthermore, the proportion of CD8+ T cells expressing PD-1 correlated negatively with the intensity of virus specific CD8+ T cell response.

The low levels of PD-1 expression were associated with low levels of HBV viremia. However, we did not find any correlation between PD-1 expression and disease flare-up indicator, ALT. Recent studies have shown that persistent antigenic stimulation has a suppressive effect on functional T cells. Briefly, T cells up-regulate their surface receptors such as PD-1 to restrict positive TCR signaling and, therefore, avoid the development of a strong immune response, including autoimmune diseases. However, some microorganisms may utilize this *in vivo* protection system to escape the host immune system and result in persistent viremia. However, why this phenomenon is broken in patients with AEHB remains to be investigated.

Although PD-1/PD-L pathway plays a prominent role in the regulation of T cell dysfunction, other costimulatory molecules cannot be excluded. In a recent study, it was observed that apart from PD-1, several other gene expressions were involved in promoting exhausted CD8+ T cells during chronic viral infection<sup>[13]</sup>. However, recent data indicates that PD-1 is a major factor of apoptosis sensitivity over and above other factors<sup>[9]</sup>. Future studies should be addressed to clarify the intracellular mechanisms used by the PD-1/PD-L1 pathway to influence disease status during viral infection. Importantly, the use of highly active anti-retroviral therapy (HAART), accompanied by the recovery of the host immune response, has been found to down-regulate PD-1 expression in 'typical progressor' (TP) patients infected with HIV<sup>[11,30]</sup>. Therefore, it is important to determine whether direct suppression of PD-1/PD-L pathway could provide a potentially effective treatment for persistent viral infections.

## COMMENTS

### Background

Nearly, 2 billion people are infected with hepatitis B virus (HBV) worldwide. Persistent HBV infection of the liver is associated with end-stage liver diseases

including cirrhosis and hepatocellular carcinoma. During chronic HBV infection, the effector functions of virus specific T cells often show an impaired activity indicated by low levels of cytokine production and cytotoxic activity. As a result, viruses can persist and establish long-term residency *in vivo*. However, the underlying mechanisms responsible for the induction of T-cell tolerance are not completely understood.

### Research frontiers

The aim of this study was to explore the potential role of programmed death-1/programmed death-ligand (PD-1/PD-L) pathway in antiviral immunity during HBV infection.

### Innovations and breakthroughs

This is the first report on the differences in PD-1 expression on CD8+ T cells in acute exacerbation of hepatitis B (AEHB) and chronic HBV infection.

### Applications

Blockade of PD-1/PD-L1 pathway may open a novel therapeutic strategy for restoring the function of the exhausted CD8+ T cells, and enhancing viral control during chronic viral infections. The perspective of future application: further studies to assess the intracellular mechanisms used by the PD-1/PD-L1 pathway to influence the disease status in viral infection.

### Peer review

This is a requisite investigation. The levels of PD-1 on CD8+ T cells has until now been analyzed only in small groups of patients with acute infection and established chronic infection. The manuscript provides new information about PD-1 expression on CD8+ T cells in different disease states of HBV infection including AEHB and chronic hepatitis B (CHB).

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RAPID COMMUNICATION

## Effect of admission hypertriglyceridemia on the episodes of severe acute pancreatitis

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**CONCLUSION:** The clinical features of SAP patients with HTG are largely consistent with previous studies. HTG aggravates the episodes of SAP.

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**Key words:** Clinical study; Hypertriglyceridemia; Severe acute pancreatitis; Clinical features; Outcome

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Deng LH, Xue P, Xia Q, Yang XN, Wan MH. Effect of admission hypertriglyceridemia on the episodes of severe acute pancreatitis. *World J Gastroenterol* 2008; 14(28): 4558-4561 Available from: URL: <http://www.wjnet.com/1007-9327/14/4558.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4558>

### Abstract

**AIM:** To investigate the effect of admission hypertriglyceridemia (HTG) on the episodes of severe acute pancreatitis (SAP).

**METHODS:** One hundred and seventy-six patients with SAP were divided into HTG group ( $n = 45$ ) and control group ( $n = 131$ ) according to admission triglyceride (TG)  $\geq 5.65$  mmol/L and  $< 5.65$  mmol/L, respectively. Demographics, etiology, underlying diseases, biochemical parameters, Ranson's score, acute physiology and chronic health evaluation II (APACHE II) score, Balthazar's computed tomography (CT) score, complications and mortality were compared. Correlation between admission TG and 24-h APACHE II score was analyzed.

**RESULTS:** SAP patients with HTG were younger ( $40.8 \pm 9.3$  years vs  $52.6 \pm 13.4$  years,  $P < 0.05$ ) with higher etiology rate of overeating, high-fat diet (40.0% vs 14.5%,  $P < 0.05$ ) and alcohol abuse (46.7% vs 23.7%,  $P < 0.01$ ), incidence rate of hypocalcemia (86.7% vs 63.4%,  $P < 0.01$ ) and hypoalbuminemia (84.4% vs 60.3%,  $P < 0.01$ ), 24-h APACHE II score ( $13.6 \pm 5.7$  vs  $10.7 \pm 4.6$ ,  $P < 0.01$ ) and admission serum glucose ( $17.7 \pm 7.7$  vs  $13.4 \pm 6.1$ ,  $P < 0.01$ ), complication rate of renal failure (51.1% vs 16.8%,  $P < 0.01$ ), shock (37.9% vs 14.5%,  $P < 0.01$ ) and infection (37.4% vs 18.3%,  $P < 0.01$ ) and mortality (13.1% vs 9.1%,  $P < 0.01$ ). Logistic regression analysis showed a positive correlation between admission TG and 24-h APACHE II score ( $r = 0.509$ ,  $P = 0.004$ ).

### INTRODUCTION

Hypertriglyceridemia (HTG) is a rare but well-recognized cause for severe acute pancreatitis (SAP), which has been intensively studied since Speck noted the association between hyperlipidemia and acute pancreatitis (AP) in 1865<sup>[1-4]</sup>. HTG can be a primary cause for AP which occurs in 1.3%-3.8% of AP patients, or secondary to other factors prior to the increase of lipid levels, or both<sup>[5]</sup>. Clinically, mild to moderate hyperlipidemia in AP patients, particularly in alcoholic pancreatitis patients, is often observed. However, it is difficult for clinicians to distinguish mild to moderate hyperlipidemia secondary to AP from marked HTG that primarily causes AP.

It is generally believed that a serum triglyceride (TG) level of more than 1000 mg/dL (about 11.3 mmol/L) is needed to precipitate AP, the reduction of which to well below 1000 mg/dL may effectively prevent further episodes<sup>[6]</sup>. Animal studies showed that HTG intensifies the course of AP including both edematous and necrotizing pancreatitis<sup>[7,8]</sup>. It was reported that TG would worsen pancreatic injury induced by AP when it reaches 5.65 mmol/L, thus playing a worth-noting role in predisposing mild pancreatitis to the vicious episode<sup>[9]</sup>. Extreme elevation of TG in AP patients with familial HTG can cause the so-called "hyperlipidemic abdominal crisis"<sup>[10]</sup>. However, it was also reported that

SAP patients with HTG can have pancreatic necrosis, pseudocysts, abscesses and other complications that can be seen in other types of pancreatitis with a different clinical course from other forms of AP<sup>[11]</sup>. Thus, the correlation between HTG and the severity of AP episodes is still uncertain.

In this study, the clinical features and the effect of HTG on the episodes of SAP were investigated with HTG as a coexisting medical condition of SAP.

## MATERIALS AND METHODS

### Patients

The diagnostic criteria for SAP formulated at the Bangkok World Congress of Gastroenterology 2002 in Thailand<sup>[12]</sup> were employed and organ failure was defined according to the Criteria of Clinical Diagnosis and Classification System for AP formulated by the Pancreatic Surgical Society of Chinese Medical Association in 1997<sup>[13]</sup> in this study. From March 2003 to December 2004, all patients diagnosed with SAP and admitted to West China Hospital of Sichuan University within 72 h after onset of symptoms were included. Patients were excluded if they had hepatic dysfunction or renal failure prior to the development of SAP.

### Methods

One hundred and seventy-six SAP patients admitted to our hospital were enrolled and divided into HTG group ( $n = 45$ ) and control group ( $n = 131$ ) according to admission TG  $\geq 5.65$  mmol/L and TG  $< 5.65$  mmol/L, respectively. Blood samples were collected at admission for biochemical examinations in Department of Laboratory Medicine of our hospital.

All patients received standardized comprehensive treatments<sup>[12]</sup>. The main protocols of treatment throughout the study were intensive care, oxygen inhalation, fasting, intermittent gastrointestinal decompression and fluid infusion. The balance of internal environment was maintained, prophylactic antibiotics were used for 7–14 d, H<sub>2</sub> receptor antagonists or proton pump inhibitors were given for 7 d. When the patients developed respiratory failure, a respirator was employed to assist respiration. When the patients developed hypoalbuminaemia, 20% of human serum albumin in 50 mL was used daily until the serum albumin value became normal. When serum lipid value was decreased to normal, fat emulsion was added in parenteral nutrition. During hospitalization, microbiological tests of sputum, urine, feces, or blood were performed, when the following susceptible clinical symptoms or signs appeared: body temperature  $\geq 38.5^{\circ}\text{C}$  and white blood cell (WBC) count  $\geq 20 \times 10^9 / \text{L}$ , signs of peritoneal irritation (area) in more than 2 quadrants, and intractable malnutrition. Contrast-enhanced computed tomography (CECT) was performed to determine necrotic infection of (peri) pancreas. For those who were unsuitable for CECT evaluated by the investigator, magnetic resonance imaging was alternatively eligible. Ultrasound-guided

**Table 1 Clinical features of patients in HTG and control groups**

	HTG group (n = 45)	Control group (n = 131)
Sex (Male/Female)	27/18	72/59
Age (mean $\pm$ SD, yr)	40.8 $\pm$ 9.3 (24–61) <sup>a</sup>	52.6 $\pm$ 13.4 (22–82)
Etiology, n (%)		
Overeating and high fat diet	18 (40.0) <sup>a</sup>	19 (14.5)
Alcohol abuse	21 (46.7) <sup>b</sup>	31 (23.7)
Gallstones	5 (11.1) <sup>b</sup>	37 (28.2)
L-Asparaginase chemotherapy	2 (4.4)	0
Pregnancy	6 (13.3)	0
Underlying diseases, n (%)		
Hypertension	6 (13.3)	15 (11.5)
Coronary heart disease	3 (6.7)	7 (5.3)
Atherosclerosis	3 (6.7)	9 (6.9)
Familial hyperlipidemia	6 (13.3)	11 (8.4)
Admission biochemical		
Serum glucose (mmol/L)	17.7 $\pm$ 7.7 <sup>b</sup>	13.4 $\pm$ 6.1
Hypoalbuminaemia (%)	38 (84.4) <sup>b</sup>	79 (60.3)
Hypocalcaemia (%)	39 (86.7) <sup>b</sup>	83 (63.4)
Hypopotassemia (%)	16 (35.6)	50 (38.2)
Hyponatremia (%)	26 (57.8)	59 (45.0)
Ranson's score (mean $\pm$ SD)	4.7 $\pm$ 1.9	4.9 $\pm$ 2.0
24-h APACHE II score (mean $\pm$ SD)	13.6 $\pm$ 5.7 <sup>b</sup>	10.7 $\pm$ 4.6
Balthazar's CT score (mean $\pm$ SD)	5.4 $\pm$ 2.3	6.3 $\pm$ 5.4

<sup>a</sup>P < 0.05, <sup>b</sup>P < 0.01 vs control group.

fine needle aspiration (FNA) was performed for microbiological testing when air bubbles appeared in necrotic tissue of the (peri) pancreas. Bacterial infection was confirmed by positive culture or smear examination. Fungal infection was confirmed by positive fungi in no less than 2 different specimens by culture or smear examination.

The sex, age, etiology, underlying diseases, biochemical parameters and incidence of complications including acute respiratory distress syndrome (ARDS), renal failure, acute hepatitis, shock, encephalopathy, infection rate, and mortality, were confirmed by one of the investigators using a standard data collection instrument. The Ranson's score, 24-h APACHE II score and admission Balthazar's CT score were calculated by a single investigator.

### Statistical analysis

Data were expressed as mean  $\pm$  SD or percentage. Data in normal distribution were analyzed using *t*-test. Data in abnormal distribution were analyzed using Wilcoxon rank sum test. Categorical data were analyzed using chi-square test. The correlation between serum TG and 24-h APACHE II score was analyzed using Logistic regression. P < 0.05 was considered statistically significant.

## RESULTS

### Clinical features

The clinical features of the patients in the HTG and control groups are summarized in Table 1. There were no statistical differences in sex distribution and incidence

**Table 2** Incidence of complications and mortality in HTG and control groups *n* (%)

	HTG group ( <i>n</i> = 45)	Control group ( <i>n</i> = 131)
Complications		
ARDS	29 (64.4)	61 (46.6)
Renal failure	23 (51.1) <sup>b</sup>	22 (16.8)
Acute hepatitis	20 (44.4)	38 (29.0)
Shock	17 (37.9) <sup>b</sup>	19 (14.5)
Encephalopathy	10 (22.2)	24 (18.3)
Infection	17 (37.4) <sup>b</sup>	24 (18.3)
Death	14 (31.1) <sup>b</sup>	12 (9.1)

<sup>b</sup>*P* < 0.01 vs control group.

rate of underlying diseases between the two groups. Patients in the HTG group were younger (*P* < 0.05) with a higher rate of overeating and a high fat diet (*P* < 0.05) and alcohol abuse (*P* < 0.01) and less gallstones (*P* < 0.01). The Ranson's score and initial Balthazar's CT score were not statistically different between the two groups, but the 24-h APACHE II score was higher in the HTG group than in the control group (*P* < 0.01). The incidence rate of hypokalemia and hyponatremia had no difference and was higher in the HTG group than in the control group (*P* < 0.01), and admission serum glucose was higher in the HTG group than in the control group (*P* < 0.01).

### Complications and mortality

There were no statistical differences in complications such as ARDS, acute hepatitis and encephalopathy between the two groups. However, more complications, such as renal failure, shock and infection occurred in the HTG group than in the control group (*P* < 0.01). The mortality was higher in the HTG group than in the control group (*P* < 0.01) (Table 2).

## DISCUSSION

HTG-associated SAP is an uncommon, but potentially life-threatening disease<sup>[14]</sup>. HTG may be primary in origin (hereditary or sporadic genetic disorder of metabolism) or secondary (associated with an identifiable disease or condition and is reversible with control or eradication of that disease or condition) to other factors or both<sup>[15-17]</sup>. Although the exact pathogenesis of HTG-associated AP is not clear, it is thought to result from toxic injury to acinar cells and capillary endothelia by excessive free fatty acids from hydrolysis of TG<sup>[18,19]</sup>. Competitive oxidation of ethanol is also responsible for SAP by aggravating the level of serum lipids<sup>[20]</sup>. L-asparaginase-induced HTG is suggested as a possible mechanism of SAP<sup>[21,22]</sup>. HTG is the cause for gestational pancreatitis in 56% cases<sup>[23]</sup>. Elevated estrogen increases the synthesis of TG and depresses plasma postheparin lipolytic activities by inhibiting the removal efficiency of TG, and high-fat intake may be a cause for AP during pregnancy<sup>[24-27]</sup>, which are largely consistent with the results of our study, showing that the patients with

HTG were younger with more diet-associated etiologies including overeating, high-fat diet and alcohol abuse. SAP patients with HTG had a higher incidence of hypoalbuminaemia and hypocalcemia and a higher level of admission serum glucose, which may be associated with the aggravation of HTG, resulting from severe stress response and metabolic disorders<sup>[28]</sup>.

The role of HTG in modulating disease course of AP is still controversial. Although studies demonstrated that HTG has no significant correlation to complications of disease or overall end-of-episode severity in AP patients<sup>[3,12]</sup>. It was reported that HTG is independently associated with the severity of AP and plays a role in the aggravation of acute necrotizing pancreatitis<sup>[29-31]</sup>. The results of our study show that the incidence of admission hypocalcaemia, a predictable index of SAP<sup>[32]</sup>, and the 24-h APACHE II score were higher in the HTG group than in the control group. The complications, such as renal failure, shock and infection, and the mortality were higher in the HTG group than in the control group, indicating that HTG aggravates SAP, leading to systemic complications and a high mortality rate of SAP.

In conclusion, the clinical features of HTG-associated SAP are largely consistent with previous studies. HTG aggravates SAP.

## COMMENTS

### Background

Hypertriglyceridemia (HTG) is associated with severe acute pancreatitis (SAP), which has long been recognized. HTG may be a primary etiology of SAP, and it is difficult for clinicians to identify it. Some previous studies suggest that HTG is independently associated with the severity of acute pancreatitis (AP) and plays a role in the aggravation of acute necrotizing pancreatitis. However, its mechanism underlying such a role is still controversial.

### Research frontiers

HTG is a rare cause for pancreatitis. An elevated TG level is needed to precipitate the episode of AP, which is called "hyperlipidemic abdominal crisis". Moreover, recurrent pancreatitis may occur in patients with familial hyperlipoproteinemia. Which patients progress to the extremely dangerous state during SAP and specific strategy against its deterioration and recurrence are the major research field.

### Innovations and breakthroughs

It is generally believed that the serum triglyceride (TG) level of more than 1000 mg/dL (about 11.3 mmol/L) is needed to precipitate AP, but the clinicians cannot identify mild to moderate HTG secondary to SAP with marked HTG that causes AP in the acute phase. This research used admission HTG as a coexisting factor for SAP patients at admission.

### Applications

At present, the exact role of HTG in the development of SAP remains unclear. Our study, conducted in one of the largest institutions in China, showed that HTG could aggravate SAP and leads to a worse outcome of SAP patients, thus providing applicable and valuable evidence for prognostic evaluation of pancreatitis.

### Terminology

AP is an acute inflammatory process in the pancreas involving local or other organ systems. Severe AP is defined as the occurrence of organ failure. HTG is defined as an abnormal concentration of TG in the blood.

### Peer review

This research investigated the effect of admission HTG on the severity of SAP, by regarding admission HTG as a coexisting factor for SAP. The study was well designed and its results are valuable.

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RAPID COMMUNICATION

## Correlation between expression and differentiation of endocan in colorectal cancer

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TNM stage. However, the expression of endocan was positively correlated with the tissue differentiation in colorectal cancer.

**CONCLUSION:** The expression of endocan is down-regulated in colorectal cancer and is positively correlated with the tissue differentiation in colorectal cancer, suggesting that the expression of endocan is associated with development and differentiation of colorectal cancer.

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**Key words:** Endocan; Colorectal cancer; Differentiation; Expression; In situ hybridization

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## Abstract

**AIM:** To investigate the expression frequency of endocan in colorectal cancer and analyze the relationship between endocan expression and clinical parameters and to study the role of endocan in colorectal carcinogenesis.

**METHODS:** Expression of endocan in 72 tumor tissue samples of colorectal cancer as well as in 27 normal mucous membrane tissue samples was analyzed using *in situ* hybridization, immunohistochemistry on tissue microarray, Western blot and reverse-transcript polymerase chain reaction (RT-PCR).

**RESULTS:** The expression of endocan was higher in normal colon and rectum tissue samples than in cancerous tissue samples (mRNA = 92.6%, protein = 36%), and was lower in colorectal cancer tissue samples (mRNA = 70.4%, protein = 36.1%). No correlation was found between staining intensity and clinical parameters such as sex, age, tumor size and

## INTRODUCTION

Colon and rectum cancers accounted for about 1 million new cases in 2002 (9.4% of the world total)<sup>[1]</sup>, There is at least a 25-fold variation in the occurrence of colorectal cancer around the world. The incidence of colorectal cancer increases rather rapidly in countries where the overall risk was formerly low (especially in Japan, but also elsewhere in Asia)<sup>[2]</sup>. Although it has been found that many factors are correlated with genesis and development of colon and rectum cancers, it cannot explain all the clinical and pathological manifestations. It is critical to investigate new factors which are intimately correlated with initiation and development of colorectal cancer.

Endocan, previously called endothelial cell-specific molecule-1 (ESM-1)<sup>[3]</sup>, is over expressed in human tumors, and its serum levels are elevated in late-stage lung cancer and experimental tumor, as measured by enzyme-linked immunoassay or by

immunohistochemistry. mRNA level of endocan is also recognized as one of the most significant molecular signatures with a poor prognosis of several types of cancer including lung cancer. Over expression of this dermatan sulphate proteoglycan is also directly involved in tumor progression as observed in mouse models of human tumor xenografts. These results suggest that endocan is a biomarker of inflammatory disorders and tumor progression as well as a validated therapeutic target in cancer.

We studied the expression of endocan in colon and rectum tissue samples. The results of this study indicate that endocan expression is down-regulated in colorectal cancer and positively correlated with the differentiation of colorectal cancer. Changes in endocan expression represent an important step in development and differentiation of colorectal cancer.

## MATERIALS AND METHODS

### **Patients and samples**

Seventy-two colorectal cancer patients, who consecutively underwent radical surgical resection at Anhui Medical University Hospital from the year 2001 to 2003, were recruited into this study. Tumor and mucosa samples were embedded in paraffin after 16 h formalin fixation. None of the patients (23 males, 49 females, mean age 54 years, range 17-87 years) received any anticancer therapy. According to the TNM classification<sup>[4]</sup>, 43 cases were at stages I and II, 29 cases at stages III and IV. Well- and moderately-differentiated adenocarcinoma was found in 57 patients and poorly-differentiated adenocarcinoma was observed 15 patients<sup>[5]</sup>, and strong lymphoid infiltrate including lymphoid follicles with germinal centers was demonstrated in 39 patients.

### **In situ hybridization**

**cRNA probe labeling:** The sequences of specific primers for endocan are as follows: sense, 5'-AGCTGGAATTCCATGAAGAG (20 bp) and antisense, 5'-TCTCTCAGAAAGCTTAGCCG (20 bp)<sup>[3]</sup>. PCR was performed to amplify endocan DNA, and the PCR product was ligated into the pGEM-T-Vector to get the recombinant plasmid pGEM-T-endocan. The recombinant plasmid was transformed into *E.coli*, amplified and digested with the restriction endonuclease (*Eco*RI and *Hind*III). The objective gene (V-gene) was purified using a DNA gel extraction kit to obtain the probes for the following digoxigenin-labeling and detected according to the manufacturer's instructions.

**Hybridization:** All specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. A series of 5-μm thick sections were cut for analysis. In situ hybridization was performed as previously described<sup>[6]</sup> with certain modifications, using digoxigenin-labeled antisense cDNA probes. Briefly, the sections were dried at 60°C for 4 h, dewaxed, rehydrated and pretreated with DEPC-treated PBS containing 100 mmol/L glycine and 0.3% Triton X-100, respectively. The sections were then

permeabilized with 20 μg/mL RNase-free proteinase K (boster, Wuhuan, China) for 20 min, incubated at 37°C for at least 20 min with prehybridization buffer. Each section was overlaid with 30 μL hybridization buffer containing a 10 ng digoxigenin-labeled cDNA probe and incubated at 42°C overnight. After hybridization, the section was incubated with digoxigenin antibody (75 mU/mL) for 2 h. The positive signal for endocan mRNA was detected using DAB as a substrate. The presence of brown staining in the cytoplasm was considered positive.

### **Protein extraction from paraffin-fixed tissue**

Paraffin-fixed tissue was cut into 50 5-μm thick sections for protein extraction and mounted onto plain glass slides. Three 5-μm thick sections for protein extraction were deparaffinized in xylene, rehydrated in graded ethanol, immersed in distilled water, and air-dried. To exclusively collect 5 mm × 5 mm cancer tissues, the targeted areas were cut microscopically with a fine needle for observation of the morphology of HE-stained sections under a microscope. After the tissue sections on the glass slide were immersed in distilled water, only the targeted areas of cancer tissue were separated from the glass slide and recovered. Adenoma tissue was also cut into sections and collected in the same manner. Normal mucosa was recovered from 5 cm-long sections of full-depth colorectal wall with a fine needle as previously described<sup>[7,8]</sup>.

### **Immunohistochemistry**

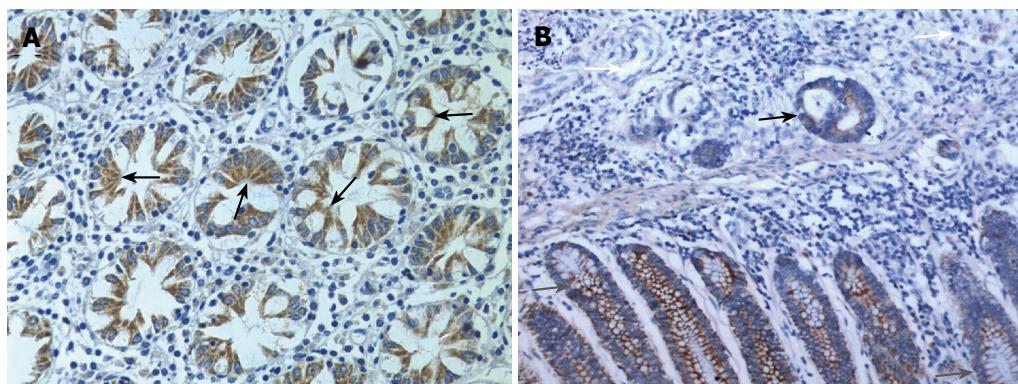
The pathology of colorectal carcinoma was performed on 5-μm thick sections of 10% formalin-embedded samples with a S-P kit. Slides were boiled in 10 mmol/L citrate buffer (pH 6.0) for 10 min to allow antigen retrieval before a 12-h incubation at 4°C with primary antibody against endocan (Santa Cruz). The mean percentage of positive tumor cells was determined in ten areas at a high magnification (× 400) and graded from 0 to 4 (0 ≤ 5% positive cells, 1 = 6%-25%, 2 = 26%-50%, 3 = 51%-75%, and 4 = 76%-100%, 0 = negative, 1-4 = positive). Negative controls were obtained by omitting the primary antibody. Each normal mucosa sample, as an internal positive control, was simultaneously analyzed. Slides were read by two observers blinded to the clinical data.

### **Reverse-transcript polymerase chain reaction (RT-PCR)**

Two micrograms of total RNA was prepared from colon and rectal tissues, randomly primed, and reverse transcribed with Superscript II (Gibco). The sequences of specific primers used for endocan are as follows: sense, 5'-CTCAGGCATGGATGGCATGAAGTG-3'; antisense, 5'-GAGACCCGGCAGCATTCTCTT TCA-3'; and β-actin: sense: 5'-ACTCTCCAGCCTTC CTTC-3' and antisense: 5'-ATCTCCTTCTGCATC CTGTC-3'. After a hot start at 94°C, 35 PCR cycles were performed, each cycle consisting of annealing at 57°C for 45 s and extension at 72°C for 45 s.

### **Western blot analysis**

Twenty micrograms of protein was incubated in a



**Figure 1** Expression of endocan in normal mucous membrane and cancer tissues of colon and rectum tissues. **A:** Endocan was expressed in the cytoplasm of the epithelial cell and it had a high polarity under the nucleus; **B:** The expression of endocan in colon and rectum tissues. This section show that it had a high expression in mucous membrane by the side of carcinoma tissue (gray arrow), also in well and moderately-differentiated colon carcinoma tissues (black arrow), but a weak expression in poorly differentiated carcinoma tissues (white arrow).

**Table 1 Differential expression of *endocan* mRNA and protein in normal and colon cancer tissues**

Type	<i>n</i>	Expression of <i>endocan</i> mRNA		Positive (%)	<i>P</i>
		+	-		
Normal mucous membrane	27	25	2	92.6	0.001
Colon carcinoma tissue	72	24	48	33.3 ( $\chi^2 = 25.266$ )	

loading buffer (125 mmol/L Tris-HCl, pH 6.8, 10%  $\beta$ -mercapto-ethanol, 4.6% SDS, 20% glycerol and 0.003% bromophenol blue) for 5 min at 100°C, separated by sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and electroblotted to PVDF membrane (BioRad). After non-specific binding sites were blocked for 1 h with 5% nonfat milk in TPBS (PBS contained 0.05% Tween 20), the membrane was incubated overnight at 4°C with primary antibody. After washing 3 times in TPBS, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG for 2 h at room temperature, and washed twice with TPBS. Immunoblot was detected by autoradiography using an enhanced chemoluminescence detection kit.

#### Statistical analysis

Chi-square test and *F*-test were used to compare the categorical data. SPSS 11.0 was used to analyze the data.

## RESULTS

### Expression of endocan in colon mucous membrane and colorectal cancer specimens

*In situ* hybridization analysis showed that endocan mRNA was expressed in the cytoplasm of epithelial cells in mucous membrane of colon and rectum and in well- and moderately-differentiated colorectal cancer. However, endocan mRNA expression was down-regulated in poorly-differentiated colorectal cancer (Figures 1 and 2).

Meanwhile, we performed immunohistochemical staining for endocan protein with a monoclonal antibody against human endocan. The endocan protein expression

**Table 2 Differential expression of endocan protein in normal and colorectal cancer tissues**

Type	<i>n</i>	Expression of endocan protein		Positive (%)	<i>P</i>
		+	-		
Normal mucous membrane	27	19	8	70.4	0.005
Colon carcinoma tissue	72	26	46	36.1 ( $\chi^2 = 7.965$ )	

was concordantly regulated by mRNA.

The statistical results demonstrated that endocan was differentially expressed in normal colon mucosa and carcinoma tissue samples. The expression rate of endocan was 92.6% (25/27) in normal colon mucosa tissue samples and 36.1% (24/72) in colorectal cancer tissue samples, and was significantly lower in cancerous tissue samples than in normal tissue samples (*P* = 0.001, Table 1). Endocan protein was identically expressed as mRNA; The expression rate was 70.4% (19/27) in normal colon and rectum mucosa tissue samples and 36.1% (26/72) in colorectal cancer tissue samples. Endocan was also differently expressed in normal and colorectal cancer tissue samples (*P* = 0.005, Table 2).

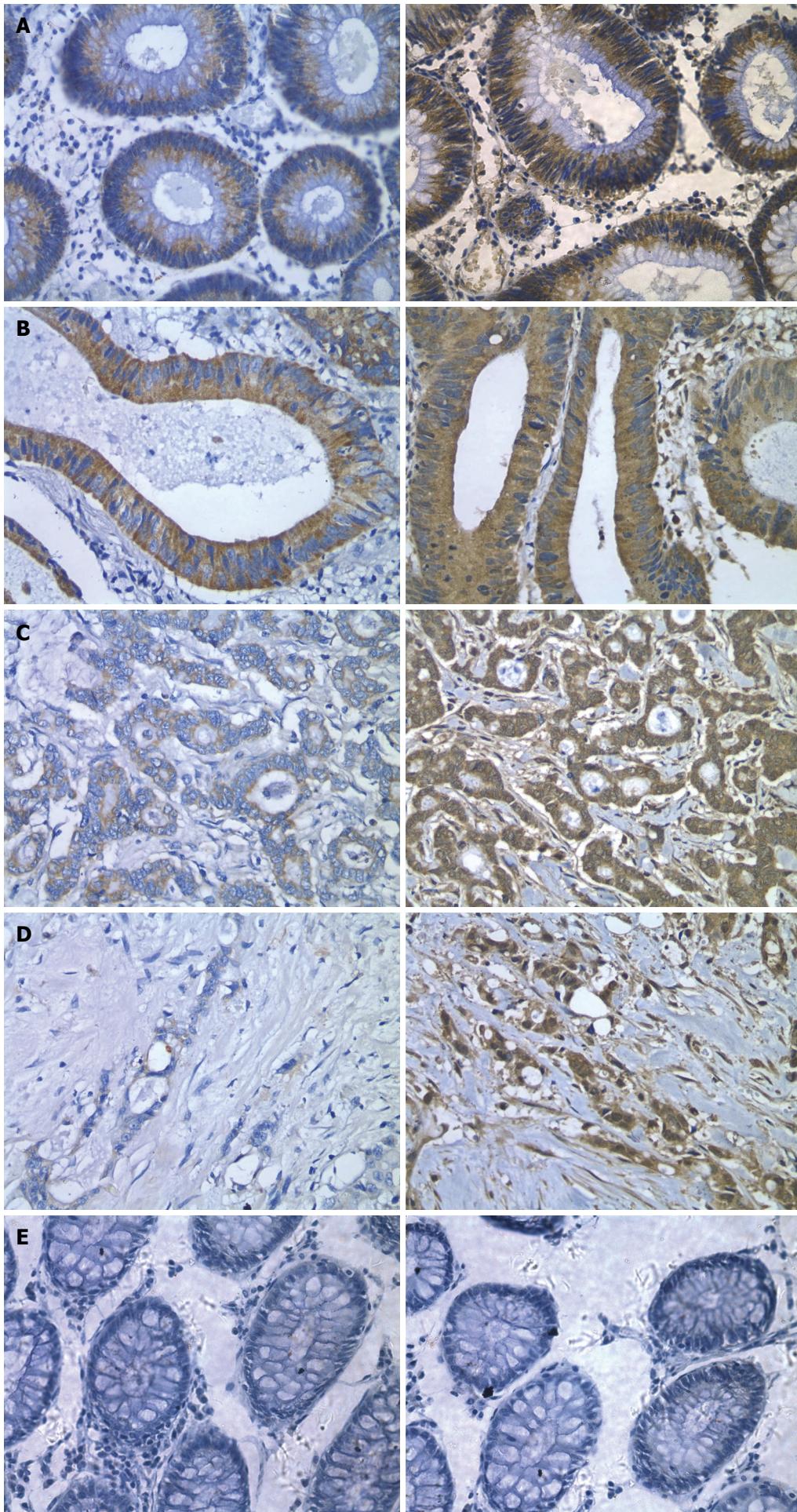
### Correlation between expression of endocan and differentiation of colorectal cancer

The expression of mRNA and protein in colorectal cancer tissue samples was not correlated with age, gender, clinical stage, tumor size or lymph node metastasis, but positively correlated with the differentiation of tumors (Table 3).

RT-PCR and Western blot were performed to further observe the relationship between the expression levels of endocan and differentiation of colorectal cancer (Figure 3). Both endocan transcript and translation were detected in colon mucous membrane and in well- and moderately-differentiated colon carcinoma, but scarcely detected in poorly-differentiated carcinoma.

## DISCUSSION

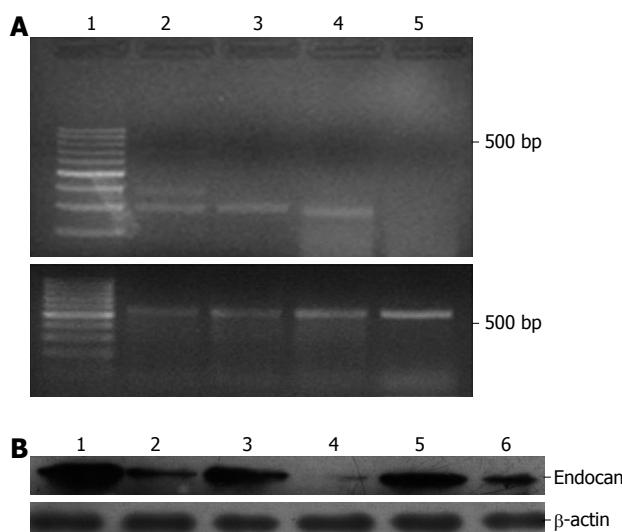
Endocan was originally cloned from a human endothelial



**Figure 2** The expression of endocan in normal mucous membrane and different differentiation colon carcinoma tissues. It had a high expression in well and moderately-differentiated colon carcinoma tissues, but a weak expression in poorly differentiated carcinoma tissues. **A:** The expression of endocan in normal mucous membrane; **B:** The expression of endocan in well-differentiation colon carcinoma tissue; **C:** The expression of endocan in well-differentiation colon carcinoma tissue; **D:** The expression of endocan in poorly differentiated colon carcinoma tissue; **E:** Negative control. Left: *in situ* hybridization; Right: immunohistochemistry.

**Table 3** Correlation of *endocan* mRNA and protein expression with clinical pathological parameters

Group	n	Endocan mRNA				Endocan protein				
		+	-	Positive (%)	P	+	-	Positive (%)	P	
Age	≤ 54	34	10	24	29.4	0.676 ( $\chi^2 = 0.174$ )	11	23	32.4	0.702 ( $\chi^2 = 0.146$ )
	> 54	38	14	24	36.8		15	23	39.5	
Sex	Male	23	9	14	39.1	0.919 ( $\chi^2 = 0.01$ )	10	13	43.5	0.530 ( $\chi^2 = 0.395$ )
	Female	49	17	32	34.7		16	33	32.7	
Size	≤ 3	11	3	8	27.3	0.643 <sup>a</sup>	5	6	45.5	0.483 <sup>a</sup>
	> 3	61	21	40	34.4		21	40	34.4	
Infiltration	Full-thickness	64	21	43	32.8	0.791 <sup>a</sup>	21	43	32.8	0.099 <sup>a</sup>
	Non-full-thickness	8	3	5	37.5		5	3	62.5	
Metastasis	Nonmetastatic tumor	27	8	19	29.6	0.796 ( $\chi^2 = 0.067$ )	9	18	33.3	0.899 <sup>a</sup>
	Metastatic tumor	45	16	29	35.6		17	28	37.8	
Grade	Differentiated (well + moderately)	57	23	34	40.4	0.031 ( $\chi^2 = 4.642$ )	26	31	45.6	0.003 ( $\chi^2 = 8.824$ )
	Poorly differentiated	15	1	14	6.7		1	14	6.7	
TNM stage	I and II	43	18	25	41.9	0.324 ( $\chi^2 = 0.973$ )	18	25	41.9	0.324 ( $\chi^2 = 0.973$ )
	III and IV	29	8	21	27.6		8	21	27.6	

<sup>a</sup>P < 0.05 vs controls.

**Figure 3** The expression of endocan in colon and rectum tissues. **A:** RT-PCR analysis of *endocan* mRNA in colon and rectum tissues. *Endocan* mRNA was highly expressed in normal colon and rectum tissue and well and mid-differentiated colorectal cancer tissues, but was down regulated in poorly differentiated colorectal cancer tissues. Endocan expression (up) and  $\beta$ -actin expression (down). 1: 100 bp Marker; 2: Normal colon and rectum mucous membrane; 3: Well-differentiated differentiated colorectal cancer tissue; 4: Moderately colorectal cancer tissue; 5: Poorly differentiated colorectal cancer tissue. **B:** Expression of endocan by Western blot. Endocan was detected at high expression levels in normal colorectal and well-differentiated tissues; Moreover, there was a down regulation in the poorly differentiated colorectal cancer tissues. Lane 1, 3, 5: Normal mcosa, well-differentiated tissues; lane 2, 4, 6: Poorly-differentiated tissues.

cell cDNA library by Lassalle and collaborators in 1996<sup>[3]</sup>. This molecule is the product of a single gene, localized on human chromosome 5 at the position 5q11.2, that is organized into 3 exons separated by 2 introns. It encodes for a soluble proteoglycan of 50 kDa containing a mature polypeptide of 165 amino acids and a single dermatan sulphate chain, covalently linked to the serine residue at position 137<sup>[9]</sup>.

Endocan, as a proteoglycan, plays an important role in several pathophysiological processes including

inflammatory disorders<sup>[10-15]</sup> and tumor progression, and in the control of fundamental cellular processes, such as adhesion<sup>[16]</sup>, migration and angiogenesis. Inflammatory cytokines, such as TNF- $\alpha$  and LPS<sup>[17]</sup>, and pro-angiogenic growth factors, such as VEGF<sup>[18]</sup>, HGF/SF<sup>[19-22]</sup> and FGF-2<sup>[23,24]</sup>, strongly stimulate the expression and secretion of endocan in human endothelial cells.

Endocan has been identified as a potential novel endothelial cell marker and a new target for cancer therapy. It was reported that high endocan mRNA levels correlate with a poor prognosis and metastasis of several types of cancer, including breast, renal and lung cancer<sup>[1,25,26]</sup>. A study of 78 breast cancer patients, with the aim to define the optimal prognosis classifier, was performed on 70 genes according to standard prognostic criteria, showing that endocan over expression in breast cancer is associated with a higher risk of metastasis and death within 5 years<sup>[27]</sup>. Furthermore, 1234 genes that have been identified are differentially expressed in renal cell carcinoma, and endocan mRNA level is 3-fold higher in renal cell carcinoma samples than in normal tissue samples<sup>[28]</sup>. This up-regulation of endocan expression also correlates with increased tumor vasculature and inflammation in renal cancer, which is actually the ninth most common malignancy in Western countries, and no effective treatment is available for it. Similarly, a recent extensive hybridization study showed that the endocan gene is one of the most highlighted genes, with at least a 2-fold increase in all the 8 renal cell carcinoma samples analyzed, compared to normal tissue samples<sup>[29]</sup>. Interestingly, a parallel up-regulation was also revealed for VEGF and c-Met proto-oncogene receptor for HGF/SF, both of which are heavily implicated in angiogenesis. A comparable study, by dot blotting and hybridization showed that endocan is dramatically up-regulated in several (5/14) renal cell carcinoma biopsies, and is correlated with both VEGF and VEGF receptor gene expressions<sup>[30]</sup>. A gene profiling study of tissues from 23 lung cancer patients demonstrated that endocan is one of the significant poor prognosis classifiers among the 42

genes associated with a high risk of cancer-related death.

Endocan was less reported in colon and rectum tissues. Moreover, little is known about its molecular mechanism. We mapped the regulation of endocan expression in normal membrane mucosa and colorectal cancer tissue samples. Our results reveal that endocan was significantly expressed at transcriptional and translational level in normal colorectal mucous membrane and in well- and moderately-differentiated colorectal cancer, but weakly expressed in poorly-differentiated colorectal cancer. Meanwhile, RT-PCR and Western blot also showed that the expression of endocan was upregulated in normal colon and rectum tissue samples, and down-regulated in poorly-differentiated colorectal cancer tissue samples.

All these data show that endocan is differently expressed in colon and rectum tissue and other tissues. According to the previous results, endocan is almost not expressed in normal human tissue except in lung tissue. Our study showed that endocan was also expressed in normal colon and rectum tissue, but its expression was down-regulated in colorectal cancer, suggesting that regulation may be complex in colon and rectum. As we know, there are a lot of germs in human colon. Most of the outer germs are killed by gastric acid when they get into the stomach through the mouth. In the upper part of the small intestine, the number of germs is also small. However, this number increases gradually at the end of the ileum and reaches its maximum in the colon, where the contents is neutral or alkaline and movement is slower, thus making the germs propagate at a fast pace. There are  $10^9$ - $10^{11}$  germs per gram of colon contents. However, these germs can decompose protein, which is called degradation. In this process, the germs also produce some virulent substances, amino acids, peptide, amine, and hydrogen sulphide and proper indole, all of which can activate macrophages and monocytes to secrete a large number of cell factors, such as IL-1 and TNF- $\alpha$ , which can stimulate expression of endocan. That is why we can detect a high expression of endocan in normal colon and rectum tissue. However, the expression of endocan was down-regulated in poorly-differentiated colorectal cancer, suggesting that endocan may be closely related with differentiation and development of colorectal cancer.

## COMMENTS

### Background

Endocan plays a key role in the regulation of certain processes, such as cell adhesion, inflammatory disorders and tumor progression and correlates with poor prognosis and metastasis in several types of cancer, including breast, renal and lung cancer, indicating that endocan may also play a role in the pathology of cancer cells and/or may be a tumor marker. However, few studies are available on endocan expression in colorectal tissue. This study was to map endocan expression in colorectal tissue and analyze the relationship between endocan expression and tumor differentiation.

### Research frontiers

Colorectal cancer accounted for about 1 million new cases in 2002 and its incidence increases. The results of this study indicate that endocan may be used as a special molecule in the early diagnosis and treatment of colorectal

cancer.

### Innovations and breakthroughs

The results of this study show that endocan expression plays a role in the pathogenesis of colorectal cancer.

### Applications

The expression level of endocan plays an important role in the pathogenesis of colorectal cancer. Endocan may be used in the treatment of colorectal cancer in clinical practice.

### Peer review

The authors showed that the expression level of endocan was lower in colon cancer tissue than in normal colon tissue. The study was well-designed and the data are original and informative.

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RAPID COMMUNICATION

# Diagnosis and treatment of spontaneous colonic perforation: Analysis of 10 cases

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**Author contributions:** Ni HK and Yang B contributed equally to perform all surgical cases; Ni HK designed research; Ni HK and Yang B performed research; Ni HK analyzed data and wrote the paper.

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treatment. The prognosis is bad and the mortality rate after surgery is high.

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**Key words:** Spontaneous; Perforation; Colon; Treatment; Surgery

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## Abstract

**AIM:** To investigate the etiology, diagnosis and treatment of spontaneous perforation of the colon.

**METHODS:** The clinical data of 10 cases of spontaneous perforation of the colon, observed at Fuding hospital from January 2004 to December 2007, were analyzed retrospectively.

**RESULTS:** The mean age at onset was 65 years (range from 45 to 73). Seven patients had a history of chronic constipation. All patients complained of sudden lower abdominal pain. The perforation occurred after colostomy and administration of senna leaves in two patients. Nine patients had signs of peritoneal irritation. Seven cases underwent abdominal paracentesis, which was diagnostic in six. Only one case was definitely diagnosed prior to surgery. One patient underwent neoplasty of the colon, another a partial resection of colon, six a neoplasty of the colon plus sigmoid colostomy, and two underwent Hartmann surgery. All perforation sites were opposite to the mesenteric edge. The perforation sites were located on descending colon in one case, sigmoid colon in three cases, and rectosigmoid colon in six cases. In five patients, surgical pathological examination was consistent with the microscopical changes of colonic perforation caused by feces. Three patients died after surgery.

**CONCLUSION:** Spontaneous perforation of the colon most commonly occurs among the elderly with chronic constipation. Abdominal paracentesis is helpful for the diagnosis. The perforation site is located opposite to the mesenteric edge. Sigmoid colon and rectosigmoid colon are the most frequent locations. Neoplasty of the colon and sigmoid colostomy are the most frequent

## INTRODUCTION

Spontaneous perforation of the colon is defined as a sudden perforation of the normal colon in the absence of diseases such as tumors, diverticulosis or external injury<sup>[1]</sup>. It is rare, often misdiagnosed and has a high mortality rate. It is seldom reported in the literature. In this study, we collected 10 cases of such patients during 2004 to 2007, and analyzed their clinical features in order to improve the understanding of this disease. The present study may be helpful for the diagnosis and treatment of spontaneous colonic perforation.

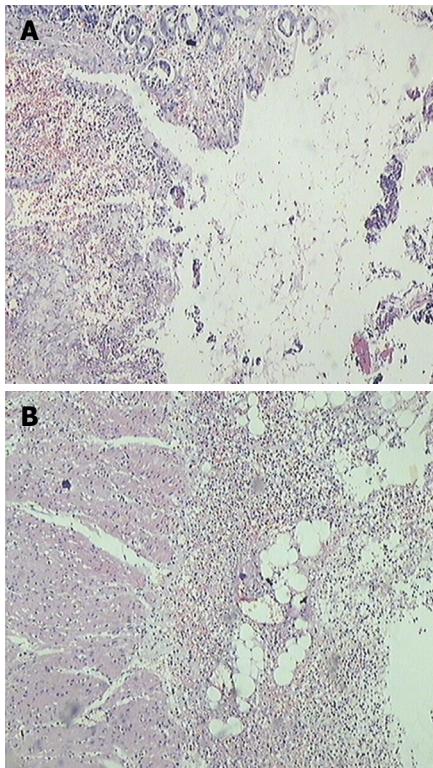
## METHODS AND MATERIALS

Ten cases of spontaneous perforation of the colon were collected at Fuding hospital from January 2004 to December 2007. The clinicopathological data, including gender, age, past history, symptoms, physical examination, diagnostic assays, pathological examination and surgical information as well as outcome were analyzed retrospectively to assess the diagnosis and treatment.

## RESULTS

### Clinical data

There were 8 males and 2 females. The mean age at onset of the disease was 65 years (range, 45 to 73).



**Figure 1** A: Inflammatory sphacelus and abundant neutrophil infiltration at the edge of the ulcer (HE, x 40); B: Numerous neutrophils infiltrate the muscular layer of the colon and the disrupted smooth muscle (HE, x 40).

Seven patients had a history of chronic constipation. All patients reported a sudden lower abdominal pain. Three patients had a history of oral administration of nonsteroid anti-inflammatory drugs (NSAIDs) to relieve abdominal pain. Five patients had possible correlated factors: in three, onset occurred after defecation and in the other two it did after coloclysis and administration of senna leaves. Three patients had symptoms compatible with shock prior to surgery. Physical examination showed signs of peritoneal irritation in 9 cases, with tension of all abdominal muscles, tenderness and rebound tenderness. The abdominal X-ray did not reveal any abnormal findings in 4 patients. Seven patients underwent diagnostic abdominal paracentesis. Feculent material was aspirated in six patients. Only one patient was definitely diagnosed before surgery. Five patients were misdiagnosed as having gastric or duodenal perforation, colorectal tumor in two other cases, acute gangrenous perforating appendicitis in one case and acute pancreatitis in one case.

### Surgery

All patients underwent emergency surgical intervention. The time interval between onset and surgery ranged from 15 to 96 h. Seven patients underwent surgery 6 h after hospital admission. One patient underwent neoplasty of the colon, one a partial resection of the colon, six a neoplasty of the colon plus a sigmoid colostomy, and two had Hartmann surgery. All perforation sites were opposite to the mesenteric edge and were located on the descending colon in one case,

the sigmoid colon in three, and the rectosigmoid colon in the remaining six.

### Pathological examination

Five patients had surgical pathological examination. Macroscopic examination showed that the perforation sites were all located opposite to the mesenteric edge, had circular shape, and ranged from 2 to 3 cm in diameter. Microscopic examination showed necrosis of the whole wall of the colon. The perforation site was characterized at its surface by inflammatory sphacelus. Granulation tissue grew from the bottom. Submucosal edema, diffuse hemorrhage and abundant neutrophil infiltration were found in the surrounding colonic mucosa, consistent with the microscopical changes typical of colonic perforation caused by feces (Figure 1).

### Prognosis

Three patients died after surgery because of multiple organ failure caused by septic shock. The other seven patients survived and six patients who received neoplasty and sigmoid colostomy underwent another surgical intervention to close the stoma 3 months thereafter.

## DISCUSSION

### Cause of spontaneous colonic perforation

The cause of spontaneous colonic perforation is usually unclear. In general, colonic perforation caused by feces is the most frequent occurrence. The disease has often been seen in patients with chronic constipation. In these cases, the solid feculent mass compresses the colonic wall, diminishes the blood supply and leads to ischemia and necrosis of colonic mucosa, which forms marked feculent ulcer changes. The ulcer might lead to colonic rupture in some cases<sup>[2-5]</sup>. Maurer *et al*<sup>[3]</sup> have proposed the diagnostic criteria of feculent colonic perforation: (1) Rounded shape, more than 1 cm in diameter; (2) The colon is full of stool, which diffuses to the abdominal cavity through the perforation; (3) Ischemia and necrosis of colonic mucosa leading to feculent ulcer and acute inflammatory reaction surrounding the perforation site can be seen at microscopical examination; (4) External injury or other diseases such as obstruction, tumors and diverticulosis must be excluded. All five cases with pathological examination were consistent with the above criteria. Maurer *et al*<sup>[3]</sup> also proposed that the feculent ulcer may present at multiple sites. The proportion of cases with multiple ulcers in the same colonic segment is about 28%.

Another cause is idiopathic colonic perforation. The pressure within the colonic lumen increases and distributes asymmetrically, leading to an excess pressure increase at the level of the angle<sup>[6]</sup>. The colonic wall is hyperdilated, becomes excessively thin and the perforation occurs. Compared to feculent perforation, idiopathic colonic perforation has the following features: (1) The perforation is linear; (2) Feculent ulcer cannot be seen at microscopic examination. The mucosal edge is clear and does not extend to the serosa. The broken

ends of the muscular layer are regular<sup>[7]</sup>. Although these two conditions are different both macroscopically and microscopically, they are occasionally difficult to distinguish at surgery. Surgical pathological examination is necessary to make a definite diagnosis<sup>[7]</sup>.

### **The most frequent sites of spontaneous colonic perforation**

The most frequent location is opposite to the mesenteric edge of the sigmoid colon and recto-sigmoid colon. Maurer *et al*<sup>[3]</sup> reported that 52 out of 81 cases (64%) of feculent perforation occurred at the above sites. In the study of Kasahara *et al*, 68% (44/65) of idiopathic colonic perforation were located at those sites<sup>[7]</sup>. In the present study, 9 patients had the same characteristics. This phenomenon may be due to the special physiological and anatomical features of sigmoid colon. There is no ramus anastomoticus between the lowest branch of sigmoid arteries and the superior rectal artery, which causes a physiological ischemia. When some stiff stool goes through sigmoid colon, the colonic wall is compressed and leads to the hindrance of blood supply. The blood supply to the opposite side of the mesentery is poor. The stool is more likely to stay in the rectosigmoid colon because of the confined colonic cavity. The smooth muscle contracts, which leads to the increase of the pressure of colonic cavity<sup>[8-12]</sup>.

This disease is more frequent in the elderly and the mean age at onset is more than 60. About 61% to 81% of patients had constipation history<sup>[2,4]</sup>. It is often misdiagnosed because doctors are unaware about this disease. Only 10% of patients are definitely diagnosed prior to surgery<sup>[6]</sup>. In the present study, only one patient (1/10) was definitely diagnosed before surgery. It is very important to increase the awareness about this disease in order to improve the accuracy of diagnosis. We think that the possibility of this disease should be taken into consideration in elderly patients who have chronic constipation, when they have a history of induction of increased intra-abdominal pressure, present with sudden abdominal pain spreading to the whole abdomen and have peritoneal irritation signs<sup>[13,14]</sup>. In this study, the abdominal paracentesis was diagnostic in 6 out of 7 patients. Therefore, abdominal paracentesis is a valuable tool for the diagnosis of patients with this complication<sup>[15]</sup>.

### **Surgical treatment of spontaneous colonic perforation**

The mortality rate of this disease is as high as 35% to 47%<sup>[2,7]</sup>. In case of perforation, innumerable bacteria spread from the colon into the abdominal cavity and cause acute diffuse peritonitis. Bacterial toxins are absorbed and lead to infectious shock and then multiple organ failure. So, patients should undergo surgery as soon as the disease is definitely diagnosed<sup>[16-20]</sup>. The types of surgery are different depending on the time of onset, degree of peritonitis, general physical condition and lesion of the colon. The following types of surgery are common: neoplasty, colostomy, neoplasty plus proximal colostomy, Hartmann surgery<sup>[21-23]</sup>. Neoplasty

plus proximal colostomy is the most popular since it is safe and time-saving. Six patients underwent neoplasty plus proximal colostomy in the present study. Serpell *et al*<sup>[2]</sup> found that the mortality and complication rates after Hartmann surgery were lower than in case of other operations because Hartmann surgery dissects the affected colon. Maurer *et al*<sup>[3]</sup> proposed that feculent ulcer had multiple origins and, therefore, other segments of the colon should be explored during the operation. If the colonic wall is dilated or thinner, it should be resected. Subtotal colectomy may be essential for some cases, which can spare time-consuming cololysis during the operation and avoid possible later re-perforation of the affected colon<sup>[24,25]</sup>.

Spontaneous colonic perforation is noteworthy due to its high mortality rate. The possibility of this disease should be taken into consideration in elderly patients having chronic constipation and bed-ridden for long periods of time. Doctors should be careful when administering enema and cathartics.

## **COMMENTS**

### **Background**

Spontaneous perforation of the colon most commonly occurs in the elderly, is usually misdiagnosed before surgery and leads to a high mortality rate after surgery. Early correct diagnosis, early surgery and appropriate surgical treatment options are the key to improve the prognosis. Effective measures should be carried out to prevent this disease in high-risk patients. This study may help the surgeon to recognize this rare entity.

### **Research frontiers**

This article focuses on a rare disease, i.e. acute abdomen due to spontaneous perforation of the colon. It is helpful to increase awareness about spontaneous perforation of the colon.

### **Innovations and breakthroughs**

It is very important to increase the knowledge about this disease in order to improve the diagnostic accuracy. The authors think that the diagnosis of this disease should be considered in elderly patients with chronic constipation, who induce an increase of the intra-abdominal pressure, present with sudden abdominal pain spreading to the whole abdomen and have signs of peritoneal irritation. In this study, the results of abdominal paracentesis were positive for 6 out of 7 patients. Therefore, abdominal paracentesis is valuable for the diagnosis of patients with this complication.

### **Applications**

The spontaneous colonic perforation should be correctly diagnosed, due to its high mortality rate. Doctors should be careful when administering enema and cathartics.

### **Peer review**

This is an interesting series of patients with a rare condition. The authors performed a single study center report of 10 patients. They analyzed the clinicopathological data retrospectively and assessed the diagnostic procedures and the treatment. In addition, they point out to surgeons the importance of recognizing this rare cause of acute abdomen with a high mortality rate.

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CASE REPORT

# Anabolic steroid abuse causing recurrent hepatic adenomas and hemorrhage

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## INTRODUCTION

Anabolic steroid abuse is common among athletes and is associated with a number of medical complications<sup>[1-3]</sup>. Reported hepatic complications include cholestasis, elevation of aminotransferases, jaundice, benign hepatic adenomas, and rare cases of hepatocellular carcinoma<sup>[4-6]</sup>. Histologic findings include peliosis hepatitis, a lesion characterized by hepatic sinusoidal dilatation that is often cystic<sup>[7,8]</sup>. Rupture of these cysts can cause fatal internal hemorrhage<sup>[9]</sup>. We report the first case of adenoma regrowth and hemorrhage after relapse of androgen abuse.

## CASE REPORT

A 27-year-old man with a 5-year history of anabolic steroid abuse presented to the emergency room with 2 d of midepigastric pain and nausea. His only medications were oral androstenedione and intramuscular nandrolone. He was a police officer and competitive bodybuilder. He denied use of alcohol, tobacco, and intravenous drugs. Physical examination disclosed midepigastric tenderness and tender hepatomegaly. Laboratories were notable for 2.2 mg/dL total bilirubin, 1.3 mg/dL direct bilirubin, 2457 U/L ALT, 431 U/L AST, and 275 U/L alkaline phosphatase. Hematocrit was 50.5%. Abdominal computed tomography (CT) on admission showed a round, heterogeneous-appearing 9.9 cm × 9.6 cm mass in the left lobe of the liver. Magnetic resonance imaging (MRI) with gadolinium contrast demonstrated multiple hepatic masses, the largest of which measured 10.6 cm × 10.6 cm. The largest mass had an enhancing capsule and demonstrated signal heterogeneity, characteristic of an adenoma with intralesional hemorrhage (Figure 1A). The patient underwent left lateral hepatic segmentectomy with open cholecystectomy. Pathologic examination revealed an adenoma with peliosis hepatitis, 25 cm in diameter. The patient was instructed to discontinue steroid use. On MRI 3 mo later, the adenomas appeared 40% smaller (Figure 1B). The patient subsequently

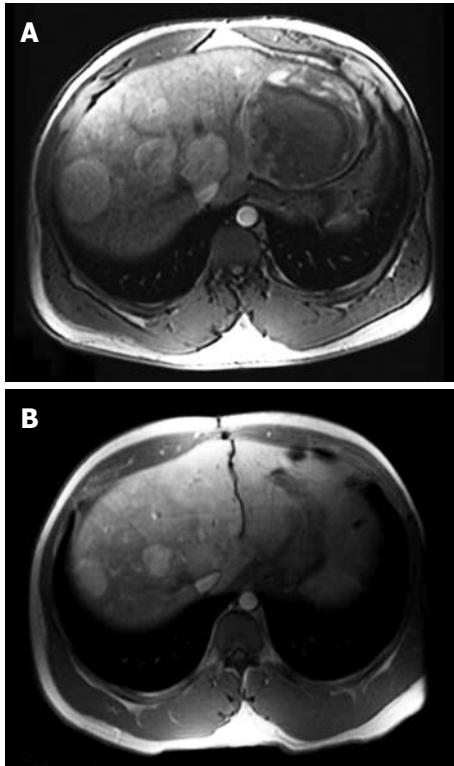
## Abstract

Anabolic steroid abuse is common among athletes and is associated with a number of medical complications. We describe a case of a 27-year-old male bodybuilder with multiple hepatic adenomas induced by anabolic steroids. He initially presented with tumor hemorrhage and was treated with left lateral hepatic segmentectomy. Regression of the remaining tumors was observed with cessation of steroid use. However, 3 years and a half after his initial hepatic segmentectomy, he presented with recurrent tumor enlargement and intraperitoneal hemorrhage in the setting of steroid abuse relapse. Given his limited hepatic reserve, he was conservatively managed with embolization of the right accessory hepatic artery. This is the first reported case of hepatic adenoma regrowth with recidivistic steroid abuse, complicated by life-threatening hemorrhage. While athletes and bodybuilders are often aware of the legal and social ramifications of steroid abuse, they should continue to be counseled about its serious medical risks.

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**Key words:** Anabolic steroids; Adenoma; Liver; Hemorrhage

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**Figure 1** T1-weighted magnetic resonance imaging (MRI) of multiple hepatic adenomas. **A:** MRI at initial presentation demonstrates a heterogeneous-appearing, well-circumscribed mass measuring 10.6 cm x 10.6 cm in segments 2 and 3 of the liver and several smaller masses in the right liver. The largest mass has an enhancing capsule and demonstrates areas of internal T1 hyperintensity and hypointensity, as well as T2 hyperintensity, characteristic of an adenoma with intralesional hemorrhage; **B:** Three months after left lateral segmentectomy and steroid cessation, the lesions in the right liver appear approximately 40% smaller and enhance more homogeneously on T1-weighted MRI, indicating regression. Images have been electronically adjusted to illustrate lesions.

resumed oral androstenedione only.

Approximately 3 years and a half after his first presentation, the patient returned to the emergency room with sudden-onset, right-upper quadrant pain in the setting of recurrent injection nandrolone use 6 wk earlier. Vital signs were within normal limits, and there was tender hepatomegaly. Laboratories were notable for ALT 625 U/L, AST 398 U/L, and normal alkaline phosphatase and total bilirubin. Prothrombin time (PT) was 13.1 s; international normalized ratio (INR) was 1.1. The hematocrit was 38.7%. Abdominal CT revealed several lesions in the right lobe of the liver, the largest of which had increased in size to 7.7 cm x 7.2 cm and demonstrated intralesional hemorrhage, accompanied by a subcapsular hematoma and intraperitoneal hemorrhage. CT angiogram on hospital day 2 showed no contrast extravasation, but the hematocrit dropped to 24.9%. On hospital day 3, the right upper quadrant pain worsened, and he became tachycardic. Repeat abdominal CT showed expansion of the hematoma, with new anterior subcapsular and subphrenic components (Figure 2). The hematocrit was 24.4%. Because of his limited hepatic reserve and ongoing steroid abuse, he was felt to be a poor candidate for either hepatic



**Figure 2** Abdominal CT at second presentation with abdominal pain after resumption of steroid abuse. A heterogeneous-appearing, right hepatic mass measuring 7.2 cm x 7.7 cm and a large subcapsular hematoma are seen, indicating that one of the hepatic adenomas has enlarged since the previous presentation and has hemorrhaged spontaneously. Image has been electronically altered.

resection or liver transplantation. He, therefore, underwent angiographic embolization of the accessory right hepatic artery. Four units of packed red blood cells were transfused. The serum ALT exceeded 10000 U/L after the procedure but declined over several days. After transient oliguric renal failure, he was discharged to home on post-procedural day 5.

## DISCUSSION

We report a rare case of hepatic adenoma regrowth with recidivistic steroid abuse, complicated by life-threatening hemorrhage. This case underscores the potentially life-threatening complications of anabolic steroid abuse, and calls for a high index of suspicion among health care providers for hepatic complications if a history of steroid use is elicited.

The risk of androgen-associated liver tumors appears to correlate with the cumulative androgen dose and the potency of the steroid used<sup>[10]</sup>. Our patient self-administered both oral androstenedione, which has relatively weak androgenic potential, and parenteral nandrolone, which is particularly potent due to C10 hydroxylation. Since androstenedione has not been associated with liver tumors, it is likely that the nandrolone promoted development of his hepatic adenomas. This is consistent with the recurrence of his symptoms soon after resumption of nandrolone.

Both nandrolone and androstenedione have been classified as Schedule III controlled substances in recognition of their abuse potential<sup>[11,12]</sup>. Despite these legal restrictions, anyone can still obtain these drugs with little difficulty over the Internet.

By resuming anabolic steroid consumption after his first hospitalization, our patient clearly demonstrated a pattern of substance abuse. Risk factors for anabolic steroid abuse in male bodybuilders include body-image disturbances, history of childhood conduct disorder, and poor father-son relationships<sup>[13]</sup>. Had our patient's condition deteriorated and necessitated consideration

of liver transplantation, he would have been required to demonstrate a commitment to abstinence from steroids, in a manner analogous to the alcoholic patient<sup>[14]</sup>.

Patients and physicians must be reminded that the sequelae of anabolic steroid abuse are life threatening. While athletes and bodybuilders are often aware of the legal and social ramifications of steroid abuse, they should also be counseled about its serious medical risks. In the context of an addictive behavior pattern, assiduous surveillance for neoplasms should also be undertaken.

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CASE REPORT

## Cerebral venous thrombosis and heparin-induced thrombocytopenia in an 18-year old male with severe ulcerative colitis

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## INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) with unknown aetiology, which is localized in the colon. It affects both adults and children, and approximately 10% of the patients are diagnosed during childhood<sup>[1]</sup>. Drugs used to induce its remission consist mainly of 5-aminosalicylic acid (5-ASA) and steroids, and 5-ASA as its maintenance therapy<sup>[2]</sup> is often used. In more severe or steroid refractory cases, immune modulating therapy with azathioprine or 6-mercaptopurine, may be used, although the evidence for this is less convincing as compared to the treatment of Crohn's disease (CD)<sup>[3]</sup>. Despite intensive pharmacological treatment, relapse is not uncommon. Extra intestinal manifestations are reported to occur in about 40% of adult patients with UC<sup>[4]</sup>. Figures for children are lower.

There are several risk factors for cerebral venous thrombosis (CVT), such as hormones (e.g. contraceptives and pregnancy), different kinds of hereditary thrombophilia and local factors including tumors<sup>[5]</sup>. In childhood, other risk factors such as local head/neck infections, sepsis and dehydration due to systemic illness have been described<sup>[6]</sup>. CVT is associated with a significant morbidity and mortality in children, and antithrombotic therapy with heparin in the acute phase followed by warfarin is recommended both in

## Abstract

The risk of thromboembolism is increased in inflammatory bowel disease and its symptoms may be overlooked. Furthermore, its treatment can be complex and is not without complications. We describe a case of an adolescent boy who developed a cerebral sinus venous thrombosis during a relapse of his ulcerative colitis and who, while on treatment with heparin, developed heparin-induced thrombocytopenia (HIT). The treatment was then switched to fondaparinux, a synthetic and selective inhibitor of activated factor X.

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**Key words:** Inflammatory bowel disease; Ulcerative

children and in adults<sup>[5,7]</sup>. However, CVT in children and adolescents with IBD is, only sparsely described. Hence, we describe a case of an adolescent boy who developed a cerebral sinus venous thrombosis during a relapse of his UC, and who, while on treatment with heparin, developed heparin-induced thrombocytopenia (HIT).

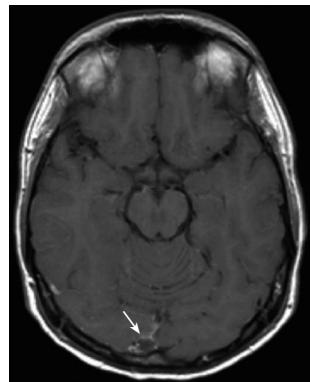
Written consent was received from the patient.

## CASE REPORT

The patient was an 18-year old male who presented with extensive ulcerative colitis at the age of 12.5 years. He was initially treated with steroids and olsalazine. He went into remission within 3 mo. During the following years, it was complicated with several relapses, which were treated with repeated courses of prednisolone. Thus, two years after diagnosis, azathioprine (AZA) was started at a dosage of 1 mg/kg. His clinical condition was quite stable until the age of 17 years when an upper respiratory infection induced another relapse with worsening of the patient's general condition and a pronounced weight loss. A high dose of prednisolone (i.e., 40 mg daily) was restarted and the AZA dose was further increased (approximate 2 mg/kg). One month after the introduction of steroids, the patient developed unilateral peritonsillitis and was admitted to hospital for incision and intravenous penicillin G treatment. The dose of prednisolone was then tapered to 10 mg daily. The pharyngeal symptoms resolved and the patient was discharged on the third day. However, 5 d after discharge, he was readmitted due to a three-day history of severe headache, accompanied with nausea and vomiting. His colitis was clinically improved, apart from continuing daily episodes of loose stools.

The vital signs were normal. Neurological examination did not show any abnormalities or clinical signs of meningitis. During the first days after admission, the patient's headache deteriorated. A CT-scan of the head was performed and interpreted as normal. Hence, a lumbar puncture performed surprisingly revealed an intralumbar pressure of 49 cmH<sub>2</sub>O (< 20). The spinal fluid analyses were otherwise normal. An eye fundoscopy revealed bilateral papilloedema and a diagnosis of pseudotumor cerebri induced by the steroid treatment was suspected. In order to reduce the cerebrospinal pressure, 10 mL of cerebrospinal liquid was withdrawn. After an asymptomatic period of about 14 h, the patient's general appearance deteriorated with recurring severe headache.

Repeated neurological examinations did not reveal any focal symptoms. A magnetic resonance imaging of the brain (MRI) showed a CVT in the right sinus transversum and confluence area (Figure 1). D-dimer was clearly elevated. Treatment with intravenous heparin infusion was initiated with a loading dose of 5000 IU, followed by a continuous heparin infusion adjusted according to aPTT (approximately 27 IU/kg per hour was required to maintain aPTT 2-3 times the baseline value). Despite an ongoing active colitis, anticoagulant therapy was considered safe.



**Figure 1** T1 contrast enhanced transverse image showing a focal dark area (arrow) in the right transverse sinus and confluence, which are consistent with a cerebral sinus thrombosis.

In the following 7 d, the patient's general condition improved, although his appetite was poor and the blood-stained diarrhoea continued. Mild iron deficiency anaemia was noted, but vitamin B12 and homocysteine levels were normal. Parenteral nutrition was given as a nutritional support.

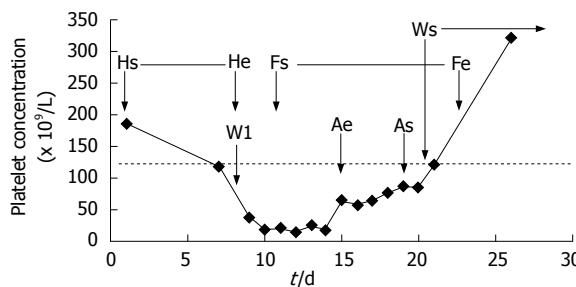
On day 7, warfarin was introduced under concomitant continuous heparin infusion. A rapid platelet count fall of 80% in 48 h was noted. Hence, azathioprine was temporarily stopped due to its known risk of thrombocytopenia. Heparin-induced thrombocytopenia (HIT) was suspected, warfarin was stopped and heparin infusion was switched to fondaparinux (Arixtra®, GlaxoSmithKline). The starting dose of fondaparinux was 7.5 mg, which was tapered to 5 mg after 3 d. The diagnosis was later confirmed by the detection of IgG-antibodies against heparin with consistent criteria for HIT<sup>[8]</sup>. The patient received a total of 6 units of platelet transfusion over a period of 4 d when the platelet count was < 18 × 10<sup>9</sup>/L, due to the high expected risk of bleeding in this patient with colitis. Despite this, he had blood in his stools on several occasions during this period. The platelet count started to rise 7 d after heparin was stopped. When the platelet count reached > 80 × 10<sup>9</sup>/L, azathioprine and warfarin were reintroduced and after 2 d of therapeutic INR between 2 and 3, fondaparinux was stopped after 9 d of treatment. The course of thrombocytopenia and medical intervention are presented in Figure 2.

During the course of CVT and HIT, a low dose of prednisolone and olsalazine was continued.

A follow-up CT scan 6 mo after the CVT diagnosis showed normal blood flow in all cerebral venous sini with no clinical neurological sequels.

A thorough work-up 7 mo after discharge showed that antithrombin, fibrinogen, lipoprotein (a), factor V gene mutation, prothrombin gene mutation, PAI-I, antiphospholipid antibodies, homocysteine, were all normal. Warfarin treatment was stopped after 8 mo of treatment. Thereafter, protein S, protein C, lupus anticoagulants, and FVIII were tested normal.

The colitis eventually went into clinical and biochemical remission. The patient was hesitant to undergo another colonoscopy until 14 mo later, when the biopsies showed a mild diffuse inflammatory activity and some architectural mucosal changes.



**Figure 2** Platelet count expressed in  $10^9/L$  blood (normal range, 125-340) from readmission until discharge showing the progress of thrombocytopenia. The letters denote start or end of heparin (Hs and He), fondaparinux (Fs and Fe), warfarin (Ws), and azatioprine (Ae and As). W1 denotes only one dose. Dotted line denotes a level of thrombocytes lower than their normal range.

## DISCUSSION

The increased risk of thromboembolism (TE) in patients with active IBD is well established<sup>[9-13]</sup> with a 6.5% incidence of thrombosis or a 3-4 fold higher risk than in the general population<sup>[14]</sup>. TE can also occur in the pediatric age group<sup>[15]</sup>. The potential mechanisms underlying the prothrombotic state in IBD are hypercoagulation (elevated FVIII, fibrinogen, decrease in antithrombin, protein S and protein C), hypofibrinolysis [elevated PAI-1 and lipoprotein (a)], platelet abnormalities, endothelial dysfunction (increased von Willebrand factor) and immunological abnormalities (antiphospholipid antibodies)<sup>[14,16]</sup>. The common genetic risk factors for TE factor V mutation (Factor V Leiden) and prothrombin mutation (G20210A) are not overrepresented in patients with TE and IBD compared to other patients with TE<sup>[16]</sup>.

It has been suggested that hyperhomocysteinemia is a risk factor for vascular disease and a mediator of TE in adults and occurs more commonly in both pediatric and adult IBD patients than in healthy controls<sup>[17,18]</sup>, which probably reflects the nutritional status with depleted levels of folate, cobalamin (B12) and pyridoxine (B6)<sup>[19]</sup>. The increased risk of TE in IBD associated with hyperhomocysteinemia is, however, questioned<sup>[19]</sup>. Other acquired risk factors for TE that can affect patients with IBD are steroid treatment, surgery, immobilisation, dehydration, central venous catheters<sup>[16]</sup>, iron deficiency and infections<sup>[6]</sup>.

In our patient, the risk factors for the development of CVT were relapse of the disease, treatment with high doses of steroids and development of peritonitis, surgical incision, dehydration, and iron deficiency anemia. Despite the risk factors, the diagnosis of CVT was delayed mainly due to this complication which is rather rare. It is important to raise the question about sinus thrombosis in the request form to the radiology department since not only unenhanced CT, but also contrast CT needs to be performed for most cases.

Cerebral venous thrombosis is a rare, but serious complication of IBD and seems to be more common in UC than in CD patients<sup>[13]</sup>. Its prognosis is variable with a high risk of residual symptoms in affected children and adolescents. Even death has been reported<sup>[13]</sup>. The most frequent symptom is headache, which occurs in

75%-96% of patients. The headache is often severe and diffuse, usually preceding the appearance of neurological deficits. A combination of focal deficits, headache, seizures and altered consciousness is very suggestive of CVT<sup>[5]</sup>. Apart from supportive care, anticoagulation therapy with heparin is the first line therapy for mild to moderate cases. Thrombolysis using recombinant tissue-type plasminogen activator (rt-PA) or urokinase has also been tried with various successes<sup>[20]</sup>. Due to the potential risks, expert guidelines recommend that local thrombolysis using urokinase<sup>[20]</sup> or (rt-PA)<sup>[5]</sup> should be restricted to comatose patients or patients who deteriorate despite anticoagulant therapy.

HIT is defined as an immune-mediated side effect of heparin therapy, which causes a drop in platelet count of  $\geq 50\%$ <sup>[21]</sup>. HIT usually occurs after 5-10 d of heparin treatment, but a faster drop may be seen if heparin has been given earlier. Apart from thrombocytopenia, venous or arterial thrombosis can occur, even before thrombocytopenia. Other symptoms observed in children with HIT are acute thoracic pain, respiratory distress, anaphylactic shock and prolonged fever<sup>[8]</sup>. An auto-immune response to platelet surface factor 4 (PF4) in complex with heparin is the major pathophysiological factor<sup>[22]</sup>. These complexes lead to cellular activation and development of thrombocytopenia or thrombosis<sup>[22]</sup>. In children, this is probably as common as in adults. In a recent review by Risch and co-workers, the incidence of HIT in children is estimated to be 0%-2.3%<sup>[21]</sup>. Approximately 90 pediatric HIT cases have been reported in the literature until now<sup>[21]</sup>. Children or neonates treated in intensive care units after cardiac surgery and adolescents treated with unfractionated heparin after thromboembolism constitute the highest risk of developing HIT in the pediatric population<sup>[21]</sup>. Although a rapid increase in platelet levels after cessation of heparin is observed, stopping heparin, as a sole treatment, is not recommended since unfavourable outcome has been reported in over 40% of patients due to the risk of thrombosis. Hence, alternative anticoagulant therapy is recommended. Danaparoid (heparinoid) inhibits mainly factor Xa, and to a lesser extent prothrombin, lepirudin (thrombin inhibitor), and argatroban (thrombin inhibitor) are the recommended drugs for the treatment of HIT. Treatment of HIT with danaparoid has a risk of cross reactivity. Lepirudin and argatroban are expensive, administered as continuous intravenous infusion, and require frequent aPTT testing. However, for our patient, fondaparinux was used as anti-coagulant therapy. Fondaparinux is a synthetic and selective inhibitor of activated factor X (Xa), administered as subcutaneous injection once daily. The antithrombotic effect is achieved by a selective binding of fondaparinux to antithrombin, which increases 300-fold the endogenous neutralisation by antithrombin on factor Xa. Hence, it inhibits the production of thrombin and the development of thrombosis<sup>[23]</sup>. Fondaparinux does not appear to interact with HIT-related antibodies to induce platelet activity and aggregation according to *in vitro* tests and small clinical trials<sup>[24]</sup>.

For our patient, all potential future surgical

procedures and longer periods of immobilization should be accompanied with anti-embolic prophylactic treatment. However, due to his episode of HIT, heparin and low molecular weight heparin (LMWH) is contraindicated and alternative anticoagulant treatment must be used.

Patients with severe IBD are frequently treated with AZA, which may cause a problem during anticoagulant therapy for TE. Scarce reports are available on the AZA treatment which may interact with warfarin by diminishing its effect. Hence, the dose of warfarin may have to be increased 3-4 fold in order to achieve an optimal PK INR level<sup>[25]</sup>.

Thromboembolic complications of IBD are not uncommon. If headache occurs during severe relapse of thromboembolism, CVT must be excluded. A CT scan with and without contrast is recommended as the first initial investigation. Platelet levels should be closely monitored during heparin treatment. When heparin is used, LMWH should be considered due to its lower risk of HIT than unfractionated heparin<sup>[26]</sup>. However, heparin has advantages in patients with a high risk of bleeding due to shorter T<sub>1/2</sub>. More studies concerning fondaparinux in treatment of patients with HIT are needed. It is necessary to evaluate the screening methods for identification of IBD patients with a high risk of thromboembolic complications. Furthermore, the efficacy and safety of prophylactic antithrombotic treatment of children with severe IBD must be evaluated in controlled clinical trials.

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CASE REPORT

## Septic thrombophlebitis of the porto-mesenteric veins as a complication of acute appendicitis

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### Abstract

Pylephlebitis, a rare complication of acute appendicitis, is defined as thrombophlebitis of the portal venous system. Pylephlebitis usually occurs due to secondary infection in the region drained into the portal system. We report a case of pylephlebitis caused by acute appendicitis. The patient was transferred from a private clinic 1 wk after appendectomy with the chief complaints of high fever and abdominal pain. He was diagnosed with pylephlebitis of the portal vein and superior mesenteric vein by CT-scan. The patient was treated with antibiotics and anticoagulation therapy, and discharged on the 25th day and follow-up CT scan showed a cavernous transformation of portal thrombosis.

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**Key words:** Acute appendicitis; Pylephlebitis; Antibiotics; Anti-coagulation therapy

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Chang YS, Min SY, Joo SH, Lee SH. Septic thrombophlebitis of the porto-mesenteric veins as a complication of acute appendicitis. *World J Gastroenterol* 2008; 14(28): 4580-4582 Available from: URL: <http://www.wjgnet.com/1007-9327/14/4580.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4580>

### INTRODUCTION

Pylephlebitis is defined as thrombophlebitis of the portal venous system associated with intraperitoneal septic conditions, such as colonic diverticulitis, acute appendicitis, and cholangitis. Pylephlebitis is considered a seriously lethal condition.

Recent advances in antibiotic therapy have made the occurrence of pylephlebitis very rare. However, the mortality rate remains high because non-specific symptoms and low index of suspicion usually delay the diagnosis of pylephlebitis. The early use of optimal diagnostic modalities and surgical interventions are essential to ensure the survival of patients.

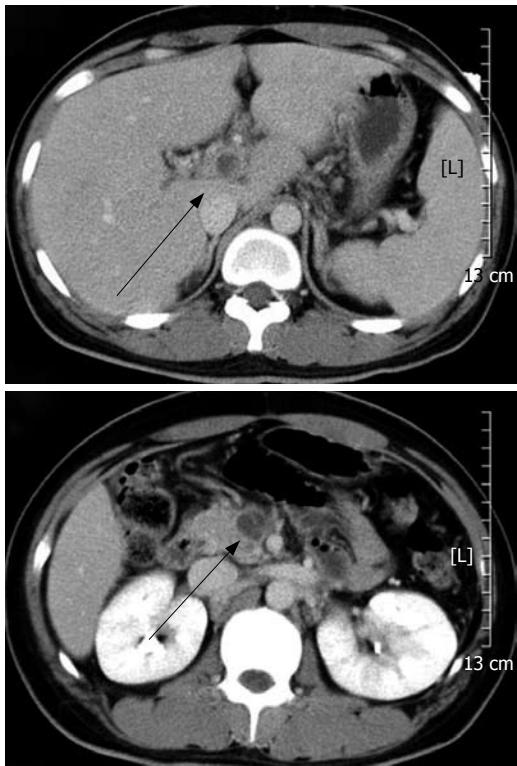
We describe a case of thrombophlebitis of the portal vein and superior mesenteric vein as a complication of acute appendicitis, which was successfully treated with antibiotics and anticoagulation therapy.

### CASE REPORT

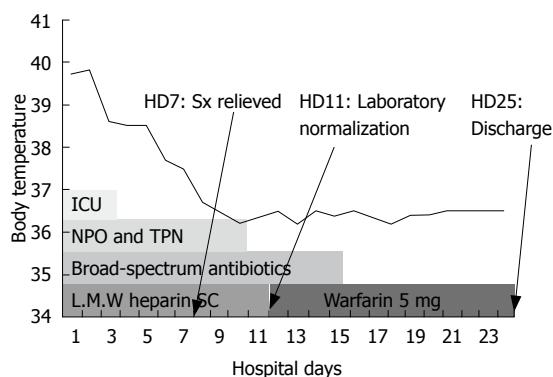
A 26-year-old man was transferred to the emergency department with the complaints of high fever and severe abdominal pain for 4 d. He underwent appendectomy for acute appendicitis 1 wk earlier. During the hospital stay, he had persistent fever up to 40°C and diarrhea, and was treated with antibiotics, fluids and anti-pyretics.

On arrival, he had an elevated temperature of 39.7°C and complained of severe epigastric pain. His blood pressure was 140/80 mmHg, his pulse rate was 114/min, and the respiration rate was 24/min. Physical examination noted epigastric pain and tenderness, but could not find rebound tenderness and muscle guarding. Laboratory results showed mild leukocytosis (11500/mm<sup>3</sup>), anemia (9.9 g/dL hemoglobin), and elevated bilirubin (1.6/0.9 mg/dL total/direct bilirubin), alkaline phosphatase (213 U/L), and γ-GT (73 U/L).

Abdominal CT scan demonstrated thrombus formation in the portal vein extending from the superior mesenteric vein (SMV; Figure 1). He was admitted to the intensive care unit and treated with systemic IV antibiotics (the 3rd generation cephalosporin and metronidazole, chosen by empirical therapy) and anticoagulation therapy (a subcutaneous injection of low molecular heparin, 1 mg/kg every 12 h). He was given nothing by mouth and parenteral nutrition was initiated to decrease the portal blood flow from the mesenteric vein.



**Figure 1** CT scan showing total occlusion of the portal vein with a thrombus (arrow) extending to the superior mesenteric vein.

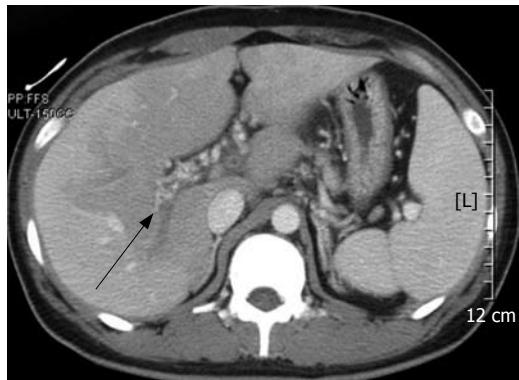


**Figure 2** Clinical progress and treatment course of the patient. ICU: Intensive care unit; NPO: Non per os; TPN: Total parenteral nutrition; HD: Hospital day; L.M.W. heparin SC: Low molecular weight heparin subcutaneous injection.

By the 3rd hospital day, his symptoms were gradually improved and the liver dysfunction and leukocytosis were normalized on the 11th day. Thereafter, oral feeding was started. No microorganisms were identified in his blood culture specimen. On the 12th hospital day, low molecular weight heparin was replaced with warfarin (5 mg/d) and continued until one month after discharge (Figure 2).

The follow-up CT scan taken 2 wk after admission showed a cavernous transformation of portal vein thrombus and improved SMV thrombosis (Figure 3).

The patient was discharged on the 25th day without complications. A follow-up CT scan after 6 mo showed a slightly increased cavernous transformation of the portal vein and marked improvement of the SMV thrombosis.



**Figure 3** Arrow indicates the cavernous transformation of the portal vein on follow-up CT scan.

He appeared healthy and had no clinical and laboratory abnormalities on follow-up.

## DISCUSSION

Although pylephlebitis is a rare complication derived from septic conditions of the portal drainage area most commonly caused by colonic diverticulitis, it occurs in association with acute appendicitis, inflammatory bowel disease, suppurative pancreatitis, acute cholangitis, bowel perforation, and pelvic infection<sup>[1-3]</sup>.

This disease entity occurred in 0.4% of patients with acute appendicitis before 1950, but it has become very rare due to major advances in antibiotic therapy and surgical treatment<sup>[4]</sup>. However, the reported mortality rate of pylephlebitis is 30%-50%, partly due to a delay in diagnosis from its atypical clinical findings and a low index of suspicion<sup>[1,5]</sup>.

Reported cases of pylephlebitis are mainly young children, with a mortality rate of up to 50%<sup>[4,6-8]</sup>. Children are particularly at a great risk of appendiceal perforation because the diagnosis is delayed. As a result, young children are prone to develop pylephlebitis.

The clinical features of pylephlebitis are non-specific. High fever, chills, malaise, right upper quadrant pain, and tenderness are the initial clinical manifestations. Balthazar and Gollapudi<sup>[9]</sup> reported that only 30% of patients with pylephlebitis present with localizing clinical signs of a primary source of sepsis. Laboratory findings, such as leucocytosis and mild abnormalities of liver function tests, are usually non-specific, but jaundice is rare except in case of multiple liver abscesses<sup>[4,5,7,10]</sup>. Our patient was treated conservatively for epigastric pain, high fever, and diarrhea after appendectomy in a private clinic, as there was no suspicion of pylephlebitis.

Blood cultures revealed no microorganisms in our case. Baril *et al*<sup>[5]</sup> reported that bacteremia is present in less than one-half of patients, whereas Balthazar and Gollapudi<sup>[9]</sup> reported that up to 80% of patients have positive blood cultures, and *Escherichia coli*, *Bacteroides fragilis*, *Proteus mirabilis*, *Klebsiella pneumoniae*, and *Enterobacter* spp. are the most common microorganisms isolated<sup>[4,7,9,10]</sup>.

Modern diagnostic imaging techniques help the early

diagnosis of acute phase pylephlebitis. The sensitivity and specificity of CT scans for pylephlebitis are not known. However, CT scans could simultaneously detect the primary source of infection, extent of pylephlebitis, and intrahepatic abnormalities, such as liver abscesses. Thus, CT scan is the most reliable initial diagnostic choice<sup>[9-11]</sup>. Air bubbles or thrombi of the portal venous system are the critical CT findings of pylephlebitis<sup>[9]</sup>. Ultrasound scan with color flow Doppler is also a sensitive test for confirming partial patency of the portal vein and portal vein thrombosis<sup>[7]</sup>.

Once a diagnosis of pylephlebitis is established, appropriate treatment should be initiated as soon as possible.

The principal of treatment for pylephlebitis is to remove the source of infection and eradicate the toxic microorganisms using appropriate antibiotics. Immediate surgical intervention is necessary in most cases, but Stitzenberg *et al*<sup>[8]</sup> reported that interval laparoscopic appendectomy can be performed 3 mo after treatment with antibiotics and anticoagulants. Regarding the treatment of portal thrombosis, Nishimori *et al*<sup>[11]</sup> reported that surgical thrombectomy can be performed through the ileocolic vein using a Fogarty catheter, but most reported cases are treated with systemic antibiotics and anticoagulants. A minimum of 4 wk of antibiotic therapy is usually recommended and patients presenting with a hepatic abscess should receive at least 6 wk of antibiotic therapy<sup>[7,10]</sup>.

The effectiveness of anticoagulants in the treatment of pylephlebitis is still controversial. We administered low molecular heparin for 11 d initially and warfarin for 1.5 mo. Condat *et al*<sup>[12]</sup> recommended early anticoagulants therapy because the recanalization of the portal system was significantly higher in the anticoagulation group compared to the control group. Baril *et al*<sup>[5]</sup> insisted that anticoagulants should be considered carefully because complications could present in 20% of patients, and it is not necessary in patients with thrombus isolated to the portal vein, but could be used for prevention of intestinal ischemia in patients with involvement of the superior or inferior mesenteric vein. Lim *et al*<sup>[10]</sup> recommended anticoagulation for the prevention

of septic pulmonary embolism from infected portal thrombi.

In summary, pylephlebitis is a rare, but fatal complication of acute appendicitis. Therefore, when pylephlebitis is suspected, immediate CT scan and antibiotic therapy, with or without surgical intervention, should be started to ensure the survival of patients.

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CASE REPORT

## Direct invasion to the colon by hepatocellular carcinoma: Report of two cases

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**Author contributions:** Hirashita T, Kai S, Shibata K, Sasaki A, and Nakashima K were involved in the treatment of these patients; Iwaki K contributed to the pathological examination; Hirashita T, Ohta M, and Kitano S wrote the paper.

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Hirashita T, Ohta M, Iwaki K, Kai S, Shibata K, Sasaki A, Nakashima K, Seigo K. Direct invasion to the colon by hepatocellular carcinoma: Report of two cases. *World J Gastroenterol* 2008; 14(28): 4583-4585 Available from: URL: <http://www.wjgnet.com/1007-9327/14/4583.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4583>

### INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common tumors worldwide<sup>[1]</sup>. Direct invasion to the gastrointestinal (GI) tract by HCC is uncommon, with a reported incidence of 0.5%-2% among clinical HCC cases<sup>[2,3]</sup>. GI bleeding or stenosis due to HCC invasion is very uncommon. In such cases, the best treatment remains controversial<sup>[4]</sup>.

Due to improved instruments, techniques, and perioperative management, surgical resection is now safely performed in patients with advanced HCC. Therefore, it is also possible to resect HCC with direct invasion to the GI tract. Here, we present two cases of HCC with direct invasion to the colon that were treated by surgical resection.

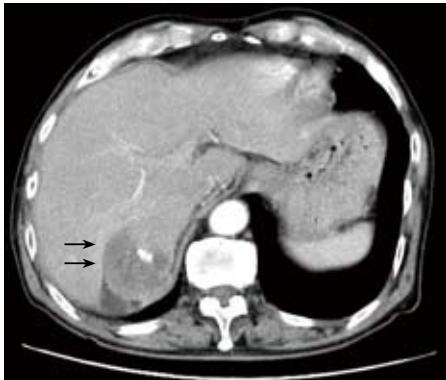
### CASE REPORT

#### Case 1

A 79-year-old man with chronic hepatitis C has been followed up since 1983. In August 1998, computed tomography (CT) revealed a 4-cm tumor in the caudate lobe of the liver, which was diagnosed as HCC. The lesion was treated by transarterial chemo-embolization (TACE). In February 2000, the patient suffered from epigastralgia, and was admitted to our hospital. CT revealed that the liver tumor increased to 7.5 cm in diameter and directly invaded the transverse colon (Figure 1). Liver function tests revealed no abnormalities. The serum  $\alpha$ -fetoprotein (AFP) level was 331 ng/mL, and the protein induced by vitamin K absence or antagonist 2 (PIVKA-2) level was within normal range.

### Abstract

Although hepatocellular carcinoma (HCC) is a common tumor, direct invasion of the gastrointestinal tract by HCC is uncommon. Recently, we encountered two cases of HCC with direct invasion to the colon. The first patient was a 79-year-old man who underwent transarterial chemo-embolization (TACE) for HCC 1.5 years prior to admission to our hospital. Computed tomography (CT) showed a 7.5-cm liver tumor directly invading the transverse colon. Partial resection of the liver and transverse colon was performed. The patient survived 6 mo after surgery, but died of recurrent HCC. The second patient was a 69-year-old man who underwent TACE and ablation for HCC 2 years and 7 months prior to being admitted to our hospital for melena and abdominal distension. CT revealed a 6-cm liver tumor with direct invasion to the colon. The patient underwent partial resection of the liver and right hemicolectomy. The patient recovered from the surgery. But, unfortunately, he died of liver failure due to liver cirrhosis one month later. Although the prognosis of HCC that has invaded the colon is generally poor due to the advanced stage of the disease, surgical resection may be a favorable treatment option in patients with a good general condition.



**Figure 1** Computed tomography images showing a 7.5-cm liver tumor (arrows) arising from the caudate lobe in case 1(A), which appears to invade the transverse colon directly (arrows) (B).



**Figure 2** Macroscopic appearance of the surgical specimen in case 1. The liver tumor invades the colon (arrows).

HCC invading the transverse colon was diagnosed and partial resection of the liver and transverse colon was performed. In the resected specimen, a 96 mm × 58 mm liver tumor invading the transverse colon was found (Figure 2). Histopathologic examination of the specimen also showed poorly-differentiated HCC with direct invasion to the colon. The postoperative course was uneventful, and the patient was discharged on postoperative day 21. He survived symptom free for 6 mo and died of recurrent HCC.

## Case 2

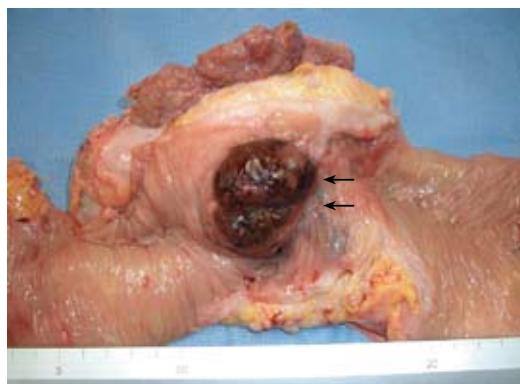
A 69-year-old man has been treated since 2000 for liver cirrhosis due to hepatitis C. In July 2004, CT revealed a 4-cm tumor in segment 6 of the liver, which was diagnosed as HCC. The lesion was treated by radiofrequency ablation (RFA) and TACE. In February 2007, the patient suffered from melena and abdominal distension and was admitted to our hospital. CT revealed that the tumor increased to 6 cm in diameter and directly invaded the diaphragm and the hepatic flexure of the colon (Figure 3). The ascending colon was dilated due to stenosis of the colon. Colonoscopic examination revealed a hemorrhagic and lobulated tumor in the hepatic flexure of the colon (Figure 4). Laboratory tests revealed 2.9 g/dL serum albumin, 1.9 mg/dL serum total bilirubin, 52.1% prothrombin activity, and 17.3% indocyanine green retention rate at 15 minutes,



**Figure 3** Computed tomography images showing a 6-cm liver tumor invades the colon and diaphragm (arrows) in case 2.



**Figure 4** Colonoscopic view showing a hemorrhagic and lobulated tumor with a smooth surface is seen in the ascending colon in case 2.



**Figure 5** Macroscopic appearance of the surgical specimen in case 2. The liver tumor invades the colon (arrows).

15 ng/mL serum AFP, and 370 AU/mL PIVKA-2. HCC invading the hepatic flexure of the colon was diagnosed, and partial resection of the liver, right hemicolectomy and partial excision of the diaphragm were performed. In the resected specimen, a 65 mm × 47 mm liver tumor, which invaded the hepatic flexure of the colon, was found (Figure 5). Histopathologic examination of the specimen also showed moderately differentiated HCC with direct invasion to the colon and diaphragm. The patient recovered from the operation, and had no evidence of HCC recurrence, but unfortunately, he died of liver failure due to liver cirrhosis 1 mo later.

**Table 1** Reported cases with invasion to the colon by HCC

Authors	Age (yr)/Sex	Viral infection	Symptom	Endoscopic shape	Tumor size (mm)	Previous treatment for HCC	Treatment	Prognosis
Hashimoto M <sup>[8]</sup> (1996)	72/F	HCV	Melena	Ulcerated	45	TAE (7 times)	Operation	4 mo alive
Chen CY <sup>[9]</sup> (1997)	71/M	Negative	Bloody stool	Lobulated	200	-	-	6 mo dead
Lin CP <sup>[2]</sup> (2000)	59/M	HCV	Bloody stool	Polypoid	80	TAE (3 times)	-	1.2 mo dead
Lin CP <sup>[2]</sup> (2000)	67/M	HBV	Stool OB (+)	Not observed	150	Operation TAE	-	1.5 mo dead
Lin CP <sup>[2]</sup> (2000)	69/M	HBV	Stool OB (+)	Not observed	200	-	-	1.2 mo dead
Lin CP <sup>[2]</sup> (2000)	63/M	Negative	Bloody stool	Not observed	200	-	-	4.0 mo dead
Strivastava DN <sup>[10]</sup> (2000)	32/M	HBV	Bloody stool	Not observed	n.d.	TAE	TAE	0.7 mo dead
Zech CJ <sup>[11]</sup> (2006)	57/M	HBV HCV	Abdominal pain	Not observed	n.d.	TACE (6 times)	Operation	ND
Our case	79/M	HCV	Epigastralgia	Not observed	75	TACE	Operation	6.0 mo dead
Our case	69/M	HCV	Melena	Lobulated	55	RFA TACE	Operation	1.0 mo dead

HBV: Hepatitis B virus; HCV: Hepatitis C virus; Stool OB (+): Stool positive for occult blood; TAE: Transarterial embolization; TACE: Transarterial chemoembolization; ND: Not described.

## DISCUSSION

HCC, one of the most common malignant tumors worldwide, is responsible for more than 250 000 deaths annually<sup>[1]</sup>. In some autopsy series, extrahepatic metastasis to the lung, lymphnodes, bone, heart, or adrenal glands has been found in 30%-75% of advanced HCC cases<sup>[5]</sup>. HCC with direct invasion to other organs can occur, with the most frequent sites being the diaphragm and gallbladder<sup>[6]</sup>. HCC only rarely invades the GI tract, the reported incidence is 0.5%-2% of clinical HCC cases and 4% of autopsy cases<sup>[2,3,7]</sup>. GI bleeding or stenosis due to HCC invasion is very uncommon<sup>[8]</sup>. The most frequently invaded GI tract sites are the duodenum and stomach<sup>[2]</sup> and invasion into the colon is very rare. To date, only eight cases of invasion to the colon by HCC have been reported in the English literature (Table 1)<sup>[2,3,9-11]</sup>. Among the 10 patients (including our two cases), the most frequent symptom was bloody stool (8 of 10 patients, 80%). Seven patients (70%) underwent transarterial embolization (TAE) or TACE for HCC prior to development of invasion. Surgical resection or supportive care was almost selected in almost all cases. However, the outcomes were very poor, and the median survival was only 2.5 mo.

GI tract invasion by HCC sometimes occurs after TAE or TACE<sup>[8]</sup>. TAE and TACE can induce exophytic growth of the HCC due to an inflammatory reaction and change in the extrahepatic blood supply. As a result, HCC may invade adjacent organs such as the diaphragm, stomach, duodenum, and colon.

TAE or TACE is not an effective treatment for GI invasion by HCC. RFA is difficult to perform in patients with GI tract invasion because of the risk of GI tract perforation. Fujii *et al*<sup>[12]</sup> reported that the median survival time of patients with GI tract invasion treated by surgical resection, nonsurgically, or by supportive therapies is 9.7 mo, 3.0 mo and 1.2 mo, respectively. Therefore, surgical resection may be the most effective treatment for GI tract invasion by HCC. In addition, surgical techniques including liver resection and perioperative management have recently improved. Surgical resection is probably the best treatment option for HCC invading the GI tract if the patient's general condition including liver function is good.

In conclusion, although the prognosis of colonic invasion of HCC is generally poor due to the advanced stage of the disease, surgical resection may be a favorable treatment option in patients with a good general condition.

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LETTERS TO THE EDITOR

## Ten mg dexrabeprazole daily is as effective as 20 mg dexrabeprazole daily

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### Abstract

Ten mg dexrabeprazole daily has been shown to be more effective than 20 mg rabeprazole daily against gastroesophageal reflux disease (GERD). This report shows that the efficacy of 10 mg dexrabeprazole daily is equivalent to that of 20 mg dexrabeprazole daily against GERD. This implies that a dose of 10 mg dexrabeprazole is sufficient to block the maximum amount of proton pumps without any need to double the dose as suggested with rabeprazole.

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**Key words:** Dexrabeprazole; Rabeprazole; Gastroesophageal reflux disease

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Kanakia R, Jain S. Ten mg dexrabeprazole daily is as effective as 20 mg dexrabeprazole daily. *World J Gastroenterol* 2008; 14(28): 4586-4587 Available from: URL: <http://www.wjgnet.com/1007-9327/14/4586.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4586>

### TO THE EDITOR

We read with interest the article by Pai *et al* on the efficacy of 10 mg dexrabeprazole<sup>[1]</sup>. However, the effects of rabeprazole on acid secretion are dose-dependent,

and an increase in gastric pH, coupled with a reduction in oesophageal acid exposure, has been seen in gastroesophageal reflux disease (GERD) patients receiving 20 mg or 40 mg rabeprazole once daily<sup>[2]</sup>. Shimatani *et al*<sup>[3]</sup> showed that 20 mg rabeprazole, twice daily, may result in better acid suppression than 10 mg rabeprazole, twice daily, in GERD patients<sup>[3]</sup>. Hence, we wanted to find out whether 20 mg dexrabeprazole daily would provide a greater efficacy than 10 mg dexrabeprazole daily against GERD.

This was a randomized, double-blinded, comparative study in clinical setting, approved by the institutional review board and conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. Males and non-pregnant and non-lactating females between the age of 18-65 years, clinically diagnosed with GERD, were included after a written informed consent was obtained from each of them. Excluded from the study were those with abnormal laboratory tests at baseline (including liver enzymes greater than twice the upper limit of normal), those who were refractory to a 2-mo course of H<sub>2</sub>-blocker or PPI therapy for GERD treatment, those who took PPI within 14 d of screening or a H<sub>2</sub>-blocker or a prokinetic agent within 7 d of screening, those who required daily use of NSAIDs, oral steroids, aspirin or were unable to discontinue the use of anticholinergics, cholinergics, spasmolytics, opiates or sucralfate, and those with poorly controlled associated disease, such as heart disease, coagulation disorders, thyroid disorders. Patients having a history of infectious or inflammatory conditions of the intestine (including inflammatory bowel disease), malabsorption syndrome, obstruction, gastrointestinal malignancy, gastric or intestinal surgery including vagotomy, Barrett's esophagus, esophageal stricture, pyloric stenosis, scleroderma or a history of hypersensitivity to any of the PPIs, were also excluded from the study. Enrolled patients were randomized to receive 10 mg dexrabeprazole once daily (D10-OD), 10 mg dexrabeprazole twice daily (D10-BD) or 20 mg dexrabeprazole daily (D20-OD) for 28 d. Visual analog scale (VAS, 0-100) was used to assess the severity of GERD symptoms. A total of 136 patients were enrolled and all completed the study. No difference was found in the baseline demographics of the patients.

A significant reduction ( $P < 0.001$ , Tukey-Kramer multiple comparison test) from baseline (day 0, before therapy) VAS scores of heartburn and regurgitation was

**Table 1** Improvement in Visual Analog Scale (VAS) scores of symptoms (values expressed as mean  $\pm$  SD)

	Day 0			Day 14			Day 28		
	D10-OD (A, n = 74)	D10-BD (B, n = 34)	D20-OD (C, n = 28)	D10-OD (A, n = 74)	D10-BD (B, n = 34)	D20-OD (C, n = 28)	D10-OD (A, n = 74)	D10-BD (B, n = 34)	D20-OD (C, n = 28)
Heartburn	48.5 $\pm$ 22.2	59.7 $\pm$ 12.4	58.2 $\pm$ 13.6	25.1 $\pm$ 16.2 <sup>b</sup>	38.2 $\pm$ 13.1 <sup>b</sup>	32.1 $\pm$ 15.5 <sup>b</sup>	7.6 $\pm$ 15.2 <sup>b</sup>	13.8 $\pm$ 10.7 <sup>b</sup>	12.9 $\pm$ 14.4 <sup>b</sup>
Between group	0.007 <sup>1</sup>	0.007	0.033	0.0001 <sup>1</sup>	0.0001	0.052	0.034 <sup>1</sup>	0.034	0.114
P values	0.033 <sup>2</sup>	0.652 <sup>3</sup>	0.652	0.052 <sup>2</sup>	0.078 <sup>3</sup>	0.078	0.114 <sup>2</sup>	0.779 <sup>3</sup>	0.779
Regurgitation	45.9 $\pm$ 20.4	57.6 $\pm$ 12.6	56.4 $\pm$ 14.5	21.9 $\pm$ 16.2 <sup>b</sup>	35.9 $\pm$ 12.1 <sup>b</sup>	30.7 $\pm$ 16.8 <sup>b</sup>	6.2 $\pm$ 14.2 <sup>b</sup>	11.8 $\pm$ 10.3 <sup>b</sup>	10 $\pm$ 13.6 <sup>b</sup>
Between group	0.003 <sup>1</sup>	0.003	0.014	< 0.0001 <sup>1</sup>	< 0.0001	0.017	< 0.0001 <sup>1</sup>	< 0.0001	0.225
P values	0.014 <sup>2</sup>	0.729 <sup>3</sup>	0.729	0.017 <sup>2</sup>	0.162 <sup>3</sup>	0.162	0.225 <sup>2</sup>	0.556 <sup>3</sup>	0.556

<sup>b</sup>P < 0.001 vs baseline values (Tukey-Krammer Multiple comparison test). Between-group difference (T-test): <sup>1</sup>A vs B; <sup>2</sup>A vs C; <sup>3</sup>B vs C.

seen in all the treatment groups on day 14 with a further reduction on continuing the therapy until 28 d. Improvement in the VAS scores in the D10-OD group was significantly better than in the D10-BD and D20-OD groups on day 14 with no significant difference between the D10-BD and D20-OD groups. On day 28, the D10-OD group showed significantly higher improvement than the D10-BD group with no significant differences between the D10-OD & D20-OD and the D10-BD & D20-OD groups (Table 1). Percentage of patients with  $\geq 50\%$  relief in symptoms of heartburn and regurgitation on day 28 was 86.5% and 91.9% in the D10-OD group, 91.2% and 97.1% in the D10-BD group, 89.3% and 92.9% in the D20-OD group, respectively. No between-group difference in proportion of patients with  $\geq 50\%$  relief was observed ( $P > 0.05$ , chi-square test). None of the patients reported any adverse drug reaction and no differences were seen in baseline laboratory parameters after therapy, indicating that dexrabeprazole at different doses was well-tolerated.

The results of this study demonstrates that the efficacy of 10 mg dexrabeprazole daily is equivalent to that of 20 mg dexrabeprazole daily in relieving symptoms of GERD. This implies that 10 mg dexrabeprazole daily is potent and sufficient enough to block the maximum amount of proton pumps, thus precluding the need to use higher doses as has been suggested with rabeprazole.

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- 1 Pai V, Pai N. Randomized, double-blind, comparative study of dexrabeprazole 10 mg versus rabeprazole 20 mg in the treatment of gastroesophageal reflux disease. *World J Gastroenterol* 2007; **13**: 4100-4102
- 2 Langtry HD, Markham A. Rabeprazole: a review of its use in acid-related gastrointestinal disorders. *Drugs* 1999; **58**: 725-742
- 3 Shimatani T, Moriwaki M, Xu J, Tazuma S, Inoue M. Acid-suppressive effects of rabeprazole: comparing 10mg and 20mg twice daily in Japanese Helicobacter pylori-negative and -positive CYP2C19 extensive metabolisers. *Dig Liver Dis* 2006; **38**: 802-808

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## Meetings

### Events Calendar 2008-2009

FALK SYMPOSIA 2008  
January 24-25, Frankfurt, Germany  
Falk Workshop: Perspectives in Liver Transplantation

International Gastroenterological Congresses 2008  
February 14-16, Paris, France  
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies  
[www.easl.ch/hepatitis-conference](http://www.easl.ch/hepatitis-conference)

February 14-17, Berlin, Germany  
8<sup>th</sup> International Conference on New Trends in Immunosuppression and Immunotherapy  
[www.kenes.com/immuno](http://www.kenes.com/immuno)

February 28, Lyon, France  
3<sup>rd</sup> Congress of ECCO - the European Crohn's and Colitis Organisation Inflammatory Bowel Diseases 2008  
[www.ecco-ibd.eu](http://www.ecco-ibd.eu)

February 29, Québec, Canada  
Canadian Association of Gastroenterology  
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK  
British Society of Gastroenterology Annual Meeting  
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China  
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea  
Asian Pacific Association for the Study of the Liver  
18<sup>th</sup> Conference of APASL: New Horizons in Hepatology  
[www.apaslseoul2008.org](http://www.apaslseoul2008.org)

March 29-April 1, Shanghai, China  
Shanghai-Hong Kong International Liver Congress  
[www.livercongress.org](http://www.livercongress.org)

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco  
OESO 9<sup>th</sup> World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas  
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA  
SAGES 2008 Annual Meeting - part of Surgical Spring Week  
[www.sages.org/08program/html/](http://www.sages.org/08program/html/)

April 18-22, Buenos Aires, Argentina  
9<sup>th</sup> World Congress of the International Hepato-Pancreato Biliary Association  
Association for the Study of the Liver  
[www.ca-ihpba.com.ar](http://www.ca-ihpba.com.ar)

April 23-27, Milan, Italy  
43<sup>rd</sup> Annual Meeting of the European Association for the Study of the Liver  
[www.easl.ch](http://www.easl.ch)

May 2-3, Budapest, Hungary  
Falk Symposium 164: Intestinal

### Disorders

May 18-21, San Diego, California, USA  
Digestive Disease Week 2008

May 21-22, California, USA  
ASGE Annual Postgraduate Course Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion  
E-mail: education@sgo.org

June 4-7, Helsinki, Finland  
The 39<sup>th</sup> Nordic Meeting of Gastroenterology  
[www.congrex.com/ngc2008](http://www.congrex.com/ngc2008)

June 5-8, Sitges (Barcelona), Spain  
Semana de las Enfermedades Digestivas  
E-mail: sepd@sepdi.es

June 6-8, Prague, Czech Republic  
3<sup>rd</sup> Annual European Meeting: Perspectives in Inflammatory Bowel Diseases  
E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey  
ESGAR 2008 19<sup>th</sup> Annual Meeting and Postgraduate Course  
E-mail: fca@netvisao.pt

June 11-13, Stockholm, Sweden  
16<sup>th</sup> International Congress of the European Association for Endoscopic Surgery  
E-mail: info@aes-eur.org

June 13-14, Amsterdam, Netherlands  
Falk Symposium 165: XX International Bile Acid Meeting, Bile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic  
Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management  
E-mail: idca2008@guantar.cz

June 25-28, Barcelona, Spain  
10<sup>th</sup> World Congress on Gastrointestinal Cancer  
Imedex and ESMO  
E-mail: meetings@imedex.com

June 25-28, Lodz, Poland  
Joint Meeting of the European Pancreatic Club (EPC) and the International Association of Pancreatology (IAP)  
E-mail: office@epc-iap2008.org  
[www.e-p-c.org](http://www.e-p-c.org)  
[www.pancreatology.org](http://www.pancreatology.org)

June 26-28, Bratislava, Slovakia  
5<sup>th</sup> Central European Gastroenterology Meeting  
[www.ceurgem2008.cz](http://www.ceurgem2008.cz)

July 9-12, Paris, France  
ILTS 14<sup>th</sup> Annual International Congress  
[www.ilts.org](http://www.ilts.org)

September 10-13, Budapest, Hungary  
11<sup>th</sup> World Congress of the International Society for Diseases of the Esophagus  
E-mail: isde@isde.net

September 13-16, New Delhi, India  
Asia Pacific Digestive Week  
E-mail: apdw@apdw2008.net

APDW 2008  
September 13-16, New Delhi, Indian Organized: Indian Society of Gastroenterology

III FALK GASTRO-CONFERENCE

September 17, Mainz, Germany  
Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany  
Falk Symposium 166: GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic  
Prague Hepatology Meeting 2008  
[www.czech-hepatology.cz/phm2008](http://www.czech-hepatology.cz/phm2008)

September 20-21, Mainz, Germany  
Falk Symposium 167: Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France  
Third Annual Meeting  
European Society of Coloproctology  
[www.escp.eu.com](http://www.escp.eu.com)



October 8-11, Istanbul, Turkey  
18<sup>th</sup> World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists  
E-mail: orkun.sahin@serenas.com.tr

October 18-22, Vienna, Austria  
16<sup>th</sup> United European Gastroenterology Week  
[www.negf.org](http://www.negf.org)  
[www.acv.at](http://www.acv.at)

October 22-25, Minnesota, USA  
Anstralian Gastroenterology Week 2008  
E-mail: gesa@gesa.org.au

October 22-25, Brisbane, Australia  
71<sup>st</sup> Annual Colon and Rectal Surgery Conference  
E-mail: info@colonrectalcourse.org

October 31-November 4, Moscone West Convention Center, San Francisco, CA  
59<sup>th</sup> AASLD Annual Meeting and Postgraduate Course  
The Liver Meeting Information: [www.aasld.org](http://www.aasld.org)

November 6-9, Lucerne, Switzerland  
Neurogastroenterology & Motility Joint International Meeting 2008  
E-mail: ngm2008@mci-group.com  
[www.ngm2008.com](http://www.ngm2008.com)

November 12, Santiago de Chile, Chile  
Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

November 28-29, Cairo, Egypt  
1<sup>st</sup> Hepatology and Gastroenterology Post Graduate Course  
[www.egyptgastrohep.com](http://www.egyptgastrohep.com)

December 7-9, Seoul, Korea  
6<sup>th</sup> International Meeting Hepatocellular Carcinoma: Eastern and Western Experiences  
E-mail: sglee@amc.seoul.kr

INFORMATION FOR ALL  
FALK FOUNDATION e.V.  
E-mail: [symposia@falkfoundation.de](mailto:symposia@falkfoundation.de)  
[www.falkfoundation.de](http://www.falkfoundation.de)

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January 18-19, March 28-29, June 6-7, October 3-4

N.O.T.E.S  
April 3-5, November 27-29  
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June 27-28, November 7-8  
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E-mail: [info@eits.fr](mailto:info@eits.fr)

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Meeting of the British Society of Gastroenterology (BSG)  
E-mail: [bsg@mailbox.ulcc.ac.uk](mailto:bsg@mailbox.ulcc.ac.uk)

May 17-20, Denver, Colorado, USA  
Digestive Disease Week 2009

November 21-25, London, UK  
Gastro 2009 UEGW/World Congress of Gastroenterology  
[www.gastro2009.org](http://www.gastro2009.org)



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For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London, The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.



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- 3 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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- 4 Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

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- 5 Vallancien G, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

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- 7 Geraud G, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRS A Careaction* 2002; 1-6 [PMID: 12154804]

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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**Electronic journal** (list all authors)

- 15 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/eid.htm>

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[1]Passed away on October 20, 2007

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EDITORIAL

# Stem cells for end stage liver disease: How far have we got?

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## Abstract

End stage liver disease (ESLD) is a health problem worldwide. Liver transplantation is currently the only effective therapy, but its many drawbacks include a shortage of donors, operative damage, risk of rejection and in some cases recidivism of the pre-transplant disease. These factors account for the recent growing interest in regenerative medicine. Experiments have sought to identify an optimal source of stem cells, sufficient to generate large amounts of hepatocytes to be used in bioartificial livers or injected *in vivo* to repair the diseased organ. This update aims to give non-stem cell specialists an overview of the results obtained to date in this fascinating field of biomedical research.

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**Key words:** End stage liver disease; Liver failure treatment; Stem cells; Regenerative medicine

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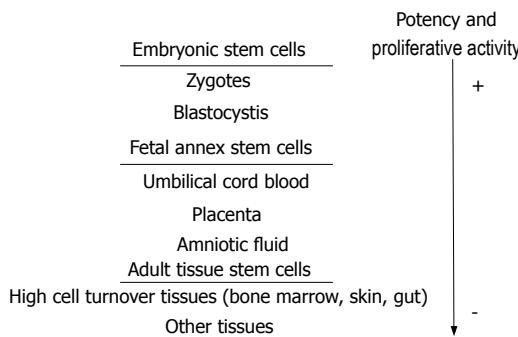
## INTRODUCTION

A stem cell is an undifferentiated cell capable of renewing itself throughout its life and of generating one or more types of differentiated cells. While embryonic stem cells (ESCs) are the only ones to be totipotential, adult tissues with high cellular turnover (e.g. skin, gut mucosa and bone marrow) retain a population of stem cells with restricted differentiation potential that constantly supply the tissue with new cells (Figure 1).

End stage liver disease (ESLD) is the final stage of acute or chronic liver damage and is irreversibly associated with liver failure. ESLD can develop rapidly, over days or weeks (acute and sub-acute liver failure, respectively), or gradually, over months or years (chronic liver failure)<sup>[1]</sup>. Currently, liver transplantation is the most effective therapy for patients with ESLD<sup>[2]</sup>. However, its potential benefits are hampered by many drawbacks, such as the relative shortage of donors, operative risk, post-transplant rejection, recidivism of the pre-existing liver disease, and high costs.

In this scenario, stem cell therapy sounds particularly attractive for its potential to support tissue regeneration requiring minimally invasive procedures with few complications. This field of research, which represents the ground from which the new discipline of “regenerative medicine” has germinated, has rapidly developed in recent years, arising great interest among scientists and physicians, and frequently appearing in newspapers headlines touting miracle cures, but arising ethical crises as well<sup>[3]</sup>. The most debated issue pertains to the use of human ESCs, as it implies, with current technologies, the destruction of human embryos. Opponents of ESC research argue that ESC research represents a slippery slope to reproductive cloning, and can fundamentally devalue human life. Contrarily, supporters argue that such research should be pursued because the resultant treatments could have significant medical potential. It is also noted that excess embryos created for *in vitro* fertilization could be donated with consent and used for the research<sup>[4]</sup>.

The ensuing debate has prompted authorities around the world to seek regulatory frameworks and highlighted the fact that stem cell research represents a social and ethical challenge. Thus, current legislation on ESC use widely varies, with some countries being more permissive (such as UK, Netherlands, Spain and France)



**Figure 1** Stem cell hierarchy in humans. While embryonic stem cells are the only ones to be totipotent, adult tissues with high cellular turnover (e.g. skin, gut mucosa and bone marrow) retain a population of stem cells with restricted differentiation potential that constantly supply the tissue of new cells.

than others (such as North America and most of the North European countries)<sup>[4]</sup>.

## GENERAL ISSUES

### How might stem cells help?

An ongoing debate involves the mechanisms by which stem cells might restore the function of a diseased organ. While some research groups support the hypothesis of stem cell integration into the tissue through "transdifferentiation" or "fusion" with resident parenchymal cells, others favour stem cells helping local cells through soluble factors production.

### How might stem cells be implanted?

The way of stem cell administration to a diseased organ widely varies in different studies, from local (direct vascular delivery) to peripheral (injection in a peripheral vein) route. Moreover, attempts to increase the number of circulating stem cells by administering growth factors have been made. Which way is best it is still unclear, and further studies are needed to clear doubts.

### What is the transferability of the data obtained from animal models to human disease?

Most data come from experiments performed in rodents, in which an organ is injured, either chemically or surgically, to study the effect of subsequent stem cell administration. Whilst animal studies are quite numerous, human usage of stem cells is still far from being everyday practice, particularly in the setting of ESLD. The translation of animal data to human disease has to be taken with great caution, and the validation of basic investigations still requires further extensive research.

### How far are we with stem cell purity and function and stability of their products?

The techniques for both ESC line isolation and adult stem cell separation from tissues need to be refined, since separation from stromal contaminating components is still not optimal. Moreover, although mature cells have been obtained by stem cell transdifferentiation *in vitro*,

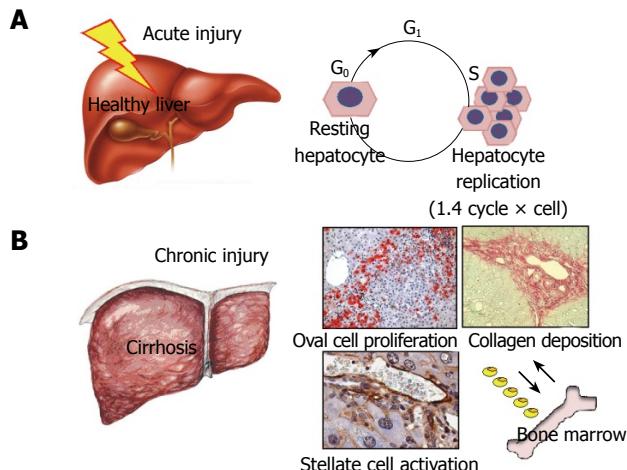
their ability to express the entire repertoire of specific biological functions and maintain them over time has not been clearly demonstrated as yet.

## LIVER REGENERATION

Under physiological conditions, the liver does not need any external source of cells to repair injury, as resting hepatocytes have the ability to re-enter the cell cycle rapidly and efficiently after an injury has occurred. Nevertheless, in persistent liver injury, as is the case with chronic liver diseases in humans, the sustained proliferative stress prematurely ages the hepatocytes and exhausts their ability to replicate. In this context, hepatic progenitor cells (or "oval cells", as they are called in rodents where they were first described) appear as a rich population of small round cells spreading from the periportal area into the parenchyma<sup>[5]</sup>. Oval cells have been demonstrated to be bipotential progenitors able to generate both hepatocytes and biliary cells<sup>[6-8]</sup>. They are thought to reside in the terminal branches of the intrahepatic biliary tree (e.g. the canals of Hering)<sup>[8]</sup> and support liver regeneration when hepatocyte proliferation is ineffective in absolute or relative terms. Rodent oval cells have proved effective in repopulating the diseased liver, but a clearly positive effect on liver function has yet to be fully demonstrated. By contrast, there is evidence that, as bipotential progenitors, oval cells can give rise to both hepatocellular- and cholangiocarcinoma<sup>[9]</sup>. The lack of an exclusive oval cell marker makes this cell population elusive and this has aroused much speculation. Some years ago, the finding of CD34 and Sca-1 hematopoietic markers on oval cells gave rise to the theory of an active trafficking of stem cells between the bone marrow (BM) and the liver and a potential involvement of the BM in liver regeneration during injury<sup>[10]</sup>. Although extremely attractive, this hypothesis is the topic of ongoing debate (Figure 2).

## BONE MARROW-DERIVED STEM CELLS

All the experimental strategies and conceptual paradigms applicable to stem cells in general were initially defined in haematopoietic stem cells (HSCs) residing in the BM. Not being at the top of the stem cell hierarchy, HSCs were initially thought to possess a restricted differentiation potential and therefore to be able to generate only cells of the haematopoietic system. This theory was questioned after studies in BM transplanted patients demonstrated the presence of donor-derived epithelial cells in some extra-haematological tissues, including the liver<sup>[11]</sup>. The hypothesis of a "germ layer-unrestricted plasticity" of HSCs rapidly captured the attention of investigators interested in regenerative medicine. There are several potential advantages of using adult rather than embryonic stem cells to regenerate tissues including fewer ethical concerns, better known biological behaviour, easier accessibility and, therefore, lower costs.



**Figure 2** Under physiological conditions, the liver does not need any external source of cells to repair injury, as resting hepatocytes have the ability to re-enter the cell cycle rapidly and efficiently after an injury has occurred (A). In persistent liver injury, as is the case with liver cirrhosis, the sustained proliferative stress prematurely ages the hepatocytes and exhausts their ability to replicate. In this context hepatic progenitor cells, or oval cells as they are called in rodents where they were first described, appear as a rich population of small round cells spreading from the periportal area into the parenchyma. The contribution of bone marrow derived stem cells to tissue regeneration in chronic liver diseases is still debated (B).

Both rodent and human HSCs have been induced to differentiate into hepatocytes *in vitro*. Most of the protocols to induce CD34+ HSCs differentiation into hepatocytes employed growing media conditioned with growth factors and mitogens [e.g. hepatocyte growth factors (HGF), fibroblast growth factor (FGF) and oncostatin M] and culture layers specific for hepatocyte growth, like matrigel. To reproduce the pathophysiological conditions of liver injury, some studies also employed cholestatic serum or co-culture with chemically damaged liver tissue<sup>[12-14]</sup>. Although these studies showed some HSC “transdifferentiation” into hepatocytes, the reported percentage of hepatocytes derived from HSCs did not exceed 5%. Thus, HSCs exhibit a limited differentiation potential that make them non-optimal candidates for tissue regeneration purposes. The cost of repeated cultures needed to obtain sufficient amounts of hepatocytes from HSC would presumably be too high for cell therapy-based applications.

Another population of stem cells in adults resides in the bone marrow stroma. Bone marrow mesenchymal stem cells (BMMSCs), as they are termed, represent the non-haematopoietic fraction of the bone marrow. *In vitro*, they are adherent, clonogenic, non-phagocytic and fibroblastic in habit. Under proper experimental conditions, they are able to differentiate into bone, cartilage, adipose and fibrous tissue, and hematopoietic supporting tissue<sup>[14]</sup>. There is also evidence that BMMSCs can undergo unorthodox differentiation, giving rise to cells with visceral mesoderm, neuroectoderm and endoderm characteristics. When transplanted, these cells can engraft in bone, muscle, brain, lung, heart, liver, gastrointestinal tract and haematopoietic tissue, and could even contribute to most somatic

cell types when injected into an early blastocyst<sup>[15]</sup>. *In vitro* experiments have shown that human and rodent BMMSCs grown on matrigel and supplemented with HGF and FGF-4 differentiate into mature hepatocytes, with a differentiation rate ranging from 30% to 80%<sup>[16,17]</sup>. BMMSCs that acquire the hepatocyte phenotype *in vitro*<sup>[18]</sup> also exhibit typical hepatocyte functions, including albumin production, glycogen storage, urea secretion, low density-lipoprotein uptake and phenobarbital-inducible cytochrome-P450 activity. BMMSCs likely represent pluripotent stem cells that remain in adult life and experimental evidence suggests that they might be a reliable cellular source to generate hepatocytes for use in cell therapy.

Flanking *in vitro* experiments, *in vivo* tests with BM-derived stem cells have also been performed to treat the diseased liver. Most data have been obtained in rodent models where liver damage was induced by either a hepatospecific necrotic insult (e.g. carbon tetrachloride (CCl<sub>4</sub>), allyl-alcohol or fumarylacetacetate hydrolase (FAH) genetically induced deficiency) or a proliferative stimulus like partial hepatectomy and bile duct ligation. Retrorsine or 2-acetyl-aminofluorene, two liver toxins enhancing oval/progenitor cell proliferation, have frequently been used to simulate chronic liver damage<sup>[19-22]</sup>. Another model of chronic hepatocellular injury used to study the role of BM in liver regeneration is the hepatitis B surface antigen (HBsAg) transgenic mouse model<sup>[23]</sup>.

The results obtained in rodents have frequently been puzzling. What generally emerges is that BMSC engraftment into the damaged liver widely varies, ranging from 0.16% to about 50% in different experiments<sup>[24-27]</sup>. Even though the cellular mechanisms responsible for these variable results are not known, transdifferentiation into hepatocytes occurs at a very low level when CD34+ HSCs are administered to rodents treated with liver toxins<sup>[28]</sup>. On the other hand, cell fusion between hepatocytes and stem cells from the myelomonocytic lineage of the BM (e.g. the precursors of circulating macrophages) has been shown to underlie liver regeneration after BM administration in the FAH-deficient mouse<sup>[20,21]</sup>. A genetic advantage of the transplanted BM stem cells with respect to resident enzyme-deficient hepatocytes likely accounts for the higher level of engraftment and tissue repopulation observed in this model.

Whereas hepatocyte formation from BM cells *in vivo* has proved to be poorly effective, some studies have postulated a much more important role for BM derived stem cells in liver tissue remodelling and fibrosis resolution. In mice injured with CCl<sub>4</sub> and thioacetamide, Russo *et al*<sup>[29]</sup> demonstrated that the BM-derived stem cell contribution to parenchymal regeneration was marginal (0.6%), but they substantially contributed to hepatic stellate cell (68%) and myofibroblast (70%) populations, which were able to influence the liver fibrotic response to toxin injury. In a sex-mismatched bone marrow transplantation model, both stellate cells and myofibroblasts of donor origin found in the

recipient liver did not originate through cell fusion with the indigenous hepatocytes, but largely derived from the circulating BMMSCs<sup>[29]</sup>. Lastly, Duffield *et al*<sup>[30]</sup> showed in rodents that BM-derived macrophages are likely to be crucial in regulating the liver fibrotic response to injury in a time-dependent manner, since depletion of these cells before injury reduces the fibrotic response, whereas their depletion during the recovery phase is associated with a greater fibrosis.

In contrast to the many studies performed in animals, those on BM-derived stem cell administration to patients with liver diseases can be counted on one hand. They can be divided into studies performed in patients with and without an underlying chronic liver disease. In patients with liver malignancies arisen on a "healthy" liver, the intraportal injection of CD133+ BM stem cells (a subpopulation of stem cells with both haematopoietic and endothelial progenitor characteristics) improved liver regeneration after extensive resection and segmental portal vein embolization<sup>[31]</sup>. This procedure was safe and highly effective in terms of liver mass recovery. Looking at future applications, this technique may offer the chance to treat the so-called "small-for-size" liver failure, a dramatic event occurring in transplanted patients who received either a small or a split liver. Few studies dealing with stem cell therapy in patients with liver cirrhosis<sup>[32-34]</sup> have been published to date. Gordon *et al*<sup>[32]</sup> injected CD34+ HSCs directly into the liver vascular system of patients with cirrhosis, whereas Terai *et al*<sup>[32]</sup> injected autologous BM through a peripheral vein. Albeit the small number of patients and lack of a control group<sup>[34]</sup>, both studies demonstrated a slight improvement in liver function and clinical conditions. These results seem to confirm, at least in part, the results obtained in the many experiments performed in rodents showing some role of BM-derived stem cells in liver repair. In our experience<sup>[35]</sup>, the administration of granulocyte colony stimulating factor (G-CSF) to mobilize BM-stem cells to the peripheral blood did not modify the residual liver function in patients with compensated liver cirrhosis. However, the procedure was safe, and may represent a good way to obtain autologous stem cells for cell therapy applications.

## EMBRYONIC STEM CELLS

Due to the difficulty in controlling their huge proliferative and differentiative potential, and major ethical concerns, the use of human ESCs is currently limited to *in vitro* and animal studies. Biotechnology industries and research laboratories are committed to devise effective protocols to optimize the ability of ESCs to differentiate into functional hepatocytes. The final goal is relevant on both scientific and clinical grounds. A suitable source of hepatocytes is what is lacking for the implementation of bio-artificial liver (BAL) technology. Effective protocols are needed not only to promote ESC differentiation into hepatocytes, but also to determine the expression of hepatic functions such as albumin secretion, indocyanine green uptake and release,

glycogen storage and p450 metabolism<sup>[36]</sup>.

Cytokines and growth factors such as HGF and FGF have been shown to promote ESC differentiation and growth<sup>[37]</sup>. In addition, it has been demonstrated that sodium butyrate, a non-proteinaceous compound, supports the action of these factors<sup>[38]</sup>. Hay *et al*<sup>[39]</sup> developed a multistage system in which HGF was used without the requirement of sodium butyrate, and human ESCs differentiated into hepatocyte-like cells without embryoid body formation. Use of an extracellular synthetic or natural matrix can be relevant, as shown by Ishizaka *et al*<sup>[40]</sup> in a three-dimensional system in which hepatocytes developed from mouse ESCs transfected with the hepatocyte nuclear factor-3 beta on a 3-D matrix scaffold. 3-D matrix scaffolds have been reported to be superior to the more commonly used 2-D monolayer culture in inducing differentiation into hepatocytes<sup>[41]</sup>. This is not surprising, as 3-D matrix scaffolds better reproduce the architecture of the liver parenchyma, which is essential for normal tissue function.

Another effective way to obtain hepatic differentiation is genetic modulation. This can be achieved by transfecting stem cells with recombinant DNA encoding for hepatospecific proteins. Adding collagen, appropriate cytokines and growth factors has an important effect on hepatocyte differentiation<sup>[42]</sup>. Recently Agarwal *et al*<sup>[43]</sup> proposed a new differentiation protocol for the generation of high-purity (70%) hepatocyte cultures: the differentiation process was largely uniform, with cell cultures progressively expressing increasing numbers of hepatic lineage markers and functional hepatic characteristics. When transplanted in mice with acute liver injury, the human ESC derived endoderm differentiated into hepatocytes and repopulated the damaged liver.

## FETAL ANNEX STEM CELLS

Cord blood contains multiple populations of embryonic-like and other pluripotential stem cells capable of originating hematopoietic, epithelial, endothelial, and neural tissues both *in vitro* and *in vivo*. The isolation of HSCs and MSCs from cord blood is a relatively new procedure and only few studies have been published<sup>[44,45]</sup>.

Saez-Lara *et al*<sup>[46]</sup> transplanted CD34+ HSCs derived from human cord blood into rats with liver cirrhosis without achieving a significant rate of engraftment, as GFP-positive cells were clearly eliminated. By contrast<sup>[47]</sup>, low-density mononuclear cells obtained from human cord blood transplanted *in utero* in fetal rats generated functional hepatocytes that persisted in the fetal recipient liver at least 6 months after birth. This humanized animal model provides a very interesting approach to *in vivo* investigation of human cord blood stem cell differentiation into hepatocytes. Hong *et al*<sup>[48]</sup> described the ability of human umbilical cord blood MSCs (CD34-) to differentiate into hepatocytes when cultured in pro-hepatogenic conditions. The differentiation rate in their protocol was about 50%, and the hepatocytes obtained

were capable of incorporating low-density lipoprotein, considered one of the most typical hepatocyte functions. More recently, Campard *et al*<sup>[49]</sup> demonstrated that human cord matrix stem cells cultured with growth factors show hepatocyte characteristics like cytochrome P450-3A expression, glycogen storage and urea production. In addition, when transplanted into hepatectomized immune-deficient mice, small clusters of human cells expressing albumin and alpha-fetoprotein appear, thereby demonstrating the good engraftment and differentiation capacity of the transplanted cells<sup>[50,51]</sup>.

Placenta is another potential source of stem cells. Placenta-derived stem cells (PDSCs) are fibroblast-like cells that attach to a plastic surface. Like BMMSCs, they can be expanded for more than 20 population doublings and induced to differentiate into cells of various mesenchymal tissues. Chien *et al*<sup>[52]</sup> recently cultivated PDSCs derived from human placentae in hepatic differentiation media, and obtained cells with hepatocyte morphology expressing specific hepatocyte functions. In comparison with stem cells isolated from other tissues there are no ethical problems associated with the study of PDSCs as the collection of placenta samples does not harm mother or infant. The ability of PDSCs to differentiate and their straightforward handling could make them an appropriate source for cell-based applications.

## CONCLUSION

Under proper experimental conditions, adult, embryonic and fetal annex stem cells have been shown to be able to differentiate into hepatocytes. At present, most biotechnology industries and research laboratories are working to optimize the differentiation protocols. In the future, stem cell-derived hepatocytes will likely be used in BAL employed as “bridge therapy” for patients with liver failure awaiting transplantation or to recover liver function. Intrahepatic injection of stem cell-derived hepatocytes might also be useful in patients with acute liver failure.

In chronic liver diseases, which account for the majority of cases of liver failure worldwide, the future of stem cell therapy is still uncertain. Liver failure occurring in patients with chronic liver disease, namely cirrhosis, is not only due to the lack of healthy cells, but also to the disruption of tissue architecture and progressive accumulation of inflammatory cells and fibrosis. While “brand new” hepatocytes derived from stem cells may temporarily support the impaired liver function, they would hardly be able to restore the original liver structure and eliminate collagen deposition. Thus, further strategies are needed. A better understanding of the mechanisms leading to collagen deposition and re-adsorption, and the development of new antifibrotic agents, combined with effective antiviral agents for patients with viral hepatitis, will be critical for the success of cell-based therapy in chronic liver failure.

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CLINICAL PRACTICE GUIDELINES

## Endoscopic resection of superficial gastrointestinal tumors

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### Abstract

Therapeutic endoscopy plays a major role in the management of gastrointestinal (GI) neoplasia. Its indications can be generalized into four broad categories; to remove or obliterate neoplastic lesion, to palliate malignant obstruction, or to treat bleeding. Only endoscopic resection allows complete histological staging of the cancer, which is critical as it allows stratification and refinement for further treatment. Although other endoscopic techniques, such as ablation therapy, may also cure early GI cancer, they can not provide a definitive pathological specimen. Early stage lesions reveal low frequency of lymph node metastasis which allows for less invasive treatments and thereby improving the quality of life when compared to surgery. Endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD) are now accepted worldwide as treatment modalities for early cancers of the GI tract.

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**Key words:** Superficial gastrointestinal cancers; Endoscopic mucosal resection; Endoscopic submucosal dissection; Lymph node spreading; Esophagus; Stomach; Colorectal

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### INTRODUCTION

Early gastrointestinal (GI) cancers are defined as lesions limited to the mucosa or submucosa without invading the muscularis propria, regardless of the presence of lymph node metastases. Since 10 years, endoscopic resection is now an alternative to surgery. Surgical resection of early GI cancers offers an excellent (90%-100%) chance of cure based on several series<sup>[1,2]</sup>. Any major surgical intervention, however, carries risks of complications including wound infection, prolonged hospital stay, anesthetic complications and death. This is especially problematic in elderly patients or those patients with concomitant severe organ dysfunction including heart failure, kidney failure, and lung disease. For this reason, endoscopic therapy may provide an attractive and less invasive treatment option that may ultimately prove to be safer in this select subgroup of patients, and may be able to translate to the general population. These techniques include endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD) which are now accepted as treatments for early GI cancers in selected cases.

Therapeutic endoscopy plays a major role in the management of GI neoplasia. Its indications can be generalized into four broad categories; (1) to remove neoplastic lesion; (2) to obliterate neoplastic lesion; (3) to palliate malignant obstruction; (4) to treat bleeding. Only endoscopic resection allows complete histological staging of the cancer, which is critical as it allows stratification and refinement for further treatment. Although other endoscopic techniques, such as ablation therapy, may also cure early GI cancer, they can not provide a definitive pathological specimen.

Early stage lesions reveal low frequency of lymph node metastasis which allows for less invasive treatments and thereby improving the quality of life when compared to surgery<sup>[3]</sup>. EMR and ESD are now accepted worldwide as treatment modalities for early cancers of the GI tract<sup>[3-5]</sup>.

Early GI cancers (except the esophagus) are defined as being limited to the mucosa or submucosa, but not invading the muscularis propria, regardless of the presence of lymph node metastases. A macroscopic classification of these lesions was first established by Japanese endoscopists in 2002 (Table 1) and has now been accepted worldwide<sup>[5]</sup>.

Detection and diagnosis of early GI cancer can be

**Table 1** Japanese classification of GI cancers

Classification of GI cancers	
Type 0	Superficial, flat with minimal elevation/depression
Type 0-I	Protruding type
Type 0-IIa	Superficial elevated type
Type 0-IIb	Flat type
Type 0-IIc	Superficial depressed type
Type 0-III	Excavated type
Type 1	Polypoid tumors on wide base, distinct demarcation from surrounding mucosa
Type 2	Ulcerated with sharply demarcated and raised borders
Type 3	Ulcerated without definite margins, infiltrating into adjacent wall
Type 4	Diffusely infiltrating lesion without marked ulceration
Type 5	Non classifiable into any of above types

difficult because of the less well defined subtle findings. Chromoendoscopy is an important adjunct technique to enhance visualization of superficial early cancers and to define their borders. Indigo carmine solution (0.2%), a contrast dye, is commonly used in the stomach to highlight the contours and topography of the lesion by entering mucosal depressions and crevices, thus enabling the biopsy of the minute lesions. Recently, narrow band image (NBI) and autofluorescence images (AFIs) are introduced as a virtual chromoendoscopy.

The depth of invasion is measured microscopically and the risk of lymph node metastasis is known to be related with a defined micrometric cutoff. In squamous cell carcinoma (SCC) of the esophagus, when infiltration is less than 200 µm, the risk of nodal metastases is low<sup>[6]</sup>. For early adenocarcinoma in Barrett's esophagus (BE) and early gastric cancer (EGC), a submucosal infiltration micrometric cutoff of 500 µm has been proposed, as the risk of nodal metastases appears low<sup>[7]</sup>. In contrast, a 20%-25% risk of node involvement with submucosal infiltration in Barrett's cancer has been reported in the West. In the colorectum, the risk of lymph node metastasis is negligible when the tumor invasion is less than 1000 µm<sup>[8]</sup>.

## EMR TECHNIQUES

Several different EMR techniques have been described, the most common of which are detailed below.

### Strip biopsy

Strip biopsy requires the use of a two-channel endoscope. The lesion is lifted with submucosal injection in the standard fashion. A snare and forceps are introduced through each channel of the endoscope. The forceps are used to guide the lesion in to the snare, which is then closed around the base of the lesion and resected using standard electrocautery<sup>[1]</sup>.

### Endoscopic double snare polypectomy

A double-channeled endoscope is required for this procedure. Snares are introduced through both channels of the endoscope, one passing through the open loop of the other. The lesion is lifted and

strangulated with the first snare, and resected below with the second snare<sup>[9]</sup>.

### EMR using cap fitted endoscope

A transparent cap with a prelooped snare on its distal tip is placed on the end of an endoscope. The lesion is lifted with a submucosal injection in standard fashion. Suction is then used to draw the lesion into the cap, and the snare is closed on the base. It is then released from the cap by breaking suction, and then removed similarly to any polypoid lesion<sup>[10]</sup>.

### EMR: Ligation device

A standard variceal ligation device is placed on the tip of the endoscope. The lesion may be injected submucosally for lifting. The lesion is then suctioned into the ligation device and a rubber band is deployed. A snare is then used to resect the lesion, usually below the level of the rubber band<sup>[11]</sup>.

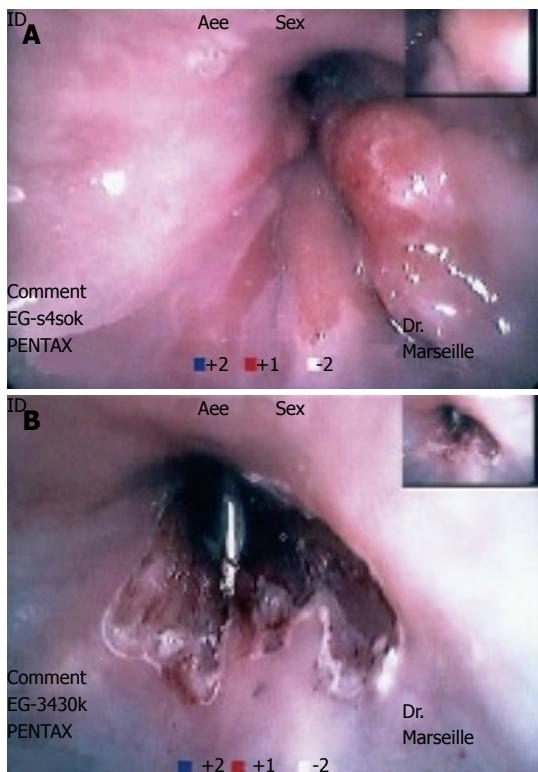
## ESD

ESD is a relatively new technique that has been used to resect larger (greater than 20 mm) size mucosal lesions in the stomach. The target lesion is first marked after careful examination with coagulation current from the tip of a knife approximately 5 mm around the margins of the tumor. The entire marked area is then elevated with a sub-mucosal injection. A needle knife is then used to cut along the outside of the margins, while the lesion is still elevated. Dissection along the submucosal plane is then performed using tools such as a hook knife or flex knife<sup>[12-14]</sup>. This technique takes significantly longer to perform than traditional EMR techniques, and requires the endoscopist to be experienced with these various tools. Larger studies are needed to assess the viability and complete resection rates with submucosal dissection.

## RESULTS OF ENDOSCOPIC RESECTION

### SCC of the esophagus

Multiple synchronous lesions as well as metachronous lesions have been reported up to 31% of patients with esophageal SCC<sup>[15]</sup>. Five-year survival rate is up to 95% after EMR in patients with superficial SCC without lymph node metastasis in m1 and m2 SCC of the esophagus<sup>[16]</sup>. Recently, an expanded indication for EMR in patients with superficial esophageal carcinoma m3 or sm1 has been proposed<sup>[17]</sup>. A study from Germany consisting of 12 HGD and 53 mucosal SCC of the esophagus, reported on the treatment using a ligation EMR technique<sup>[17]</sup>. This was the first Western study which showed similar results to those of the Japan in early esophageal SCC. They reported that complete resection was achieved in 11/12 patients of HGD and 51/53 patients of mucosal SCC. Although recurrence was observed in 16 patients after EMR, the lesions were completely resected after further endoscopic treatment. Complications occurred in 15/65 patients all being



**Figure 1** Circumferential endoscopic resection of BE with high grade dysplasia. **A:** Polypoid tumor on BE; **B:** Post-resection endoscopic aspects.

esophageal strictures which were successfully managed by endoscopy. Seven year survival rate was 77%.

#### **High grade dysplasia and early carcinoma developed in BE**

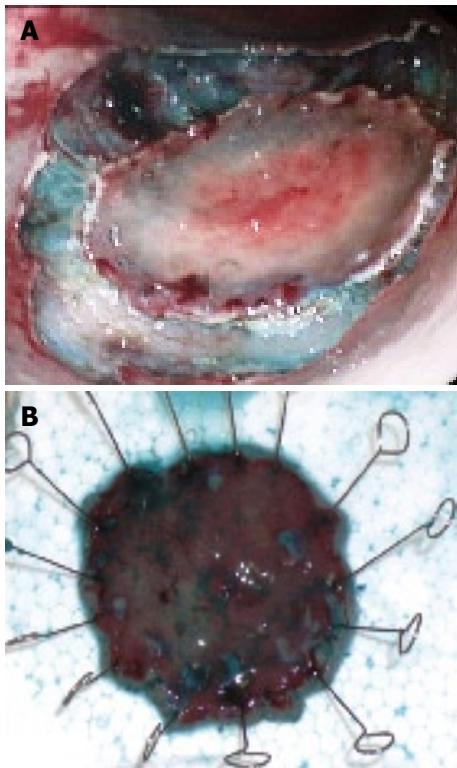
Endoscopic therapy aims to remove the dysplastic Barrett's epithelium allowing restoration of squamous epithelium. EMR could be a therapeutic alternative to surgical esophagectomy which carries substantial morbidity and mortality<sup>[18]</sup>. When HGD is diagnosed in short segment BE (BE shorter than 30 mm in length), EMR would be considered to remove all the metaplastic epithelium. A study reported that visible areas of HGD in long segment BE (BE longer than 30 mm in length) may be removed by EMR, followed by photodynamic therapy to destroy invisible foci<sup>[19]</sup>. Although strictures occurred in 30% of patients, 17 superficial esophageal cancers were removed by combining EMR and photodynamic therapy. A study from Germany reported that circumferential EMR was carried out by using a simple snare technique without a cap in 12 patients with BE containing multifocal high-grade intraepithelial neoplasia or intramucosal cancer<sup>[20]</sup>. Five had multifocal lesions while two developed strictures that required bougienage. There was no recurrence during the median follow-up of 9 mo. Our study reported on circumferential EMR (Figure 1) performed in 21 patients with HGD or mucosal cancer<sup>[21]</sup>. Three patients needed additional therapy such as surgery or chemotherapy due to residual disease after the endoscopic resection. In addition, two local recurrences were retreated by

EMR. A study from Netherlands, consisting of 77 esophagectomy specimens containing HGD or T1 adenocarcinoma, reported that lymph node metastasis occurred in 23% sm2 and 69% sm3 tumors, but not in m1, m2, m3, and sm1 lesions<sup>[22]</sup>. They concluded that m1, m2, m3, and sm1 lesions could be treated endoscopically if the lesions are less than 30 mm, well differentiate type adenocarcinoma, and without lymphangitic invasion. However, care must be taken with the initial diagnosis of endoscopic biopsy since significant changes in diagnosis occur after EMR such as downgrading from HGD to BE without dysplasia or being reclassified from benign to malignant diagnosis<sup>[23]</sup>. Ell *et al* reported that suck-and-cut EMR technique was performed in 100 consecutive patients with low-risk adenocarcinoma of the esophagus arising from BE, and complete local remission was achieved in 99/100 patients<sup>[24]</sup>. During the mean follow-up period of 36.7 mo, recurrent or metachronous carcinomas were found in 11% of the patients, but were successfully treated by repeated EMR. Five-year survival rate was 98%.

Recently, a study tried to define prognostic factors of recurrence after Endoscopic Resection of early carcinoma in BE. The aim of this study was to evaluate the value of p53 and Ki-67 immunohistochemistry in predicting the cancer recurrence in patients with Barrett's esophagus-related cancer referred to EMR. Mucosectomy specimens from 41 patients were analyzed. All endoscopic biopsies prior to EMR presented high-grade dysplasia and cancer was detected in 23 of them. Ki-67 and p53 immunoreactivity were classified as superficial, deep or mixed. EMR samples confirmed cancer in 21/23 (91.3%) cases. In these cases, p53 immunohistochemistry revealed a mixed positivity for the great majority of these cancers (90.5% vs 20%;  $P < 0.001$ ), and Ki-67 showed a mixed pattern for all cases (100% vs 30%;  $P < 0.001$ ); on the contrary, patients without cancer revealed a superficial or negative pattern for p53 (80% vs 9.5%;  $P < 0.001$ ) and Ki-67 (70% vs 0%;  $P < 0.001$ ). During a mean follow-up of 31.6 mo, 5 (12.2%) patients developed six episodes of recurrent or metachronous cancer. Previous EMR samples did not show any significant difference in the p53 and Ki-67 expression for patients developing cancer after endoscopic treatment<sup>[25]</sup>.

#### **Endoscopic resection of early gastric cancer**

Complete resection rates (defined as tumor free horizontal and vertical margins, submucosal invasion  $< 500 \mu\text{m}$ , and no lymphatic invasion) have been reported to be between 74% and 97% with the use of EMR, with lower rates of complete resection reported when expanded indications for EMR were used and when lesions were resected piecemeal instead of *en bloc* (Figure 2)<sup>[25-27]</sup>. Although no head to head prospective trials have been performed looking at long-term survival for EMR *versus* surgery, 2 and 5-year survival rates for EMR are 95% and 100%, respectively, whereas they are 100% and 100% for patients who underwent surgery<sup>[27]</sup>.



**Figure 2** ESD of early gastric cancer. **A:** Peripheral dissection of superficial gastric cancer; **B:** Resected specimen.

Similarly, other investigators have shown no differences in survival rates after 5 and 10-year periods for EMR compared with surgery<sup>[28]</sup>.

No cases of mortality due to EMR have been reported in various case series. The most serious complications of EMR include bleeding and perforation. Reported rates of bleeding vary from 1% to 20% and appear to be more frequent when resecting large lesions and when resecting lesions piecemeal<sup>[29,30]</sup>. One study reported surgical postoperative complications of 14.7% and a 0.7% mortality rate<sup>[31]</sup>. Recurrence rates in a recent study published by Ono *et al*<sup>[32]</sup> were noted to be 2% in those who had complete resection (5 of 278 cases) while there was a 13% recurrence rate in the group of patients whose resection could not be deemed complete histologically (9 of 67 cases). Local recurrences for incomplete resections in the same study are reported to be approximately 37%<sup>[32]</sup>. It should be noted that a global advantage of surgical resection is a near complete cure for early gastric cancers.

Only one study has addressed the issue of quality of life, and EMR appears to have a better post procedure quality of life compared with surgical gastrectomy<sup>[33]</sup>. No cost benefit analyses have been performed. Surgical intervention may have a high initial cost burden, however repeat surveillance endoscopies may add up to a significant cost as well.

#### **Endoscopic resection of duodenal tumors**

EMR has been used for ampullary and peri-ampullary neoplasias and sub-epithelial lesions including stromal cell tumors, cysts, and neuroendocrine tumors.

Although endoscopic resection can provide a wide tumor resection with a negative resection margin, it is not yet recommended as a curative therapy for early stage ampulla of Vater cancer because of the high lymphovascular invasion rate<sup>[34]</sup>. A study reported a higher risk of bleeding (33%) among 27 duodenal EMR after complete resection<sup>[35]</sup>.

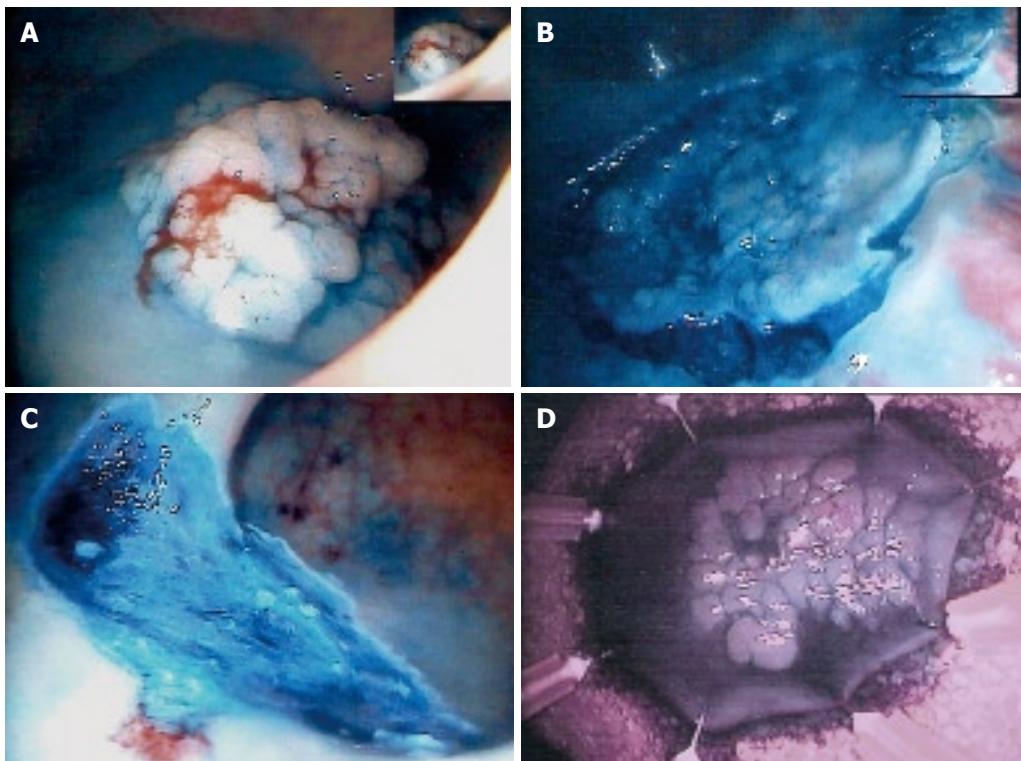
#### **Endoscopic resection of submucosal tumors**

EMR technique may also be applied to submucosal tumors in order to achieve histologic diagnosis and to achieve complete removal. In a German study, complete resection was achieved in 19/20 patients with submucosal esophageal tumors using a rubber band or a simple snare<sup>[36,37]</sup>. Bleeding occurred in 40% of the cases and was successfully managed by endoscopic hemostasis. Rosch *et al* attempted endoscopic *en bloc* resection of mucosal and submucosal tumors of the UGI tract, using the IT knife<sup>[38]</sup>. In this pilot series, complete removal was achieved in 25% of the mucosal and 36% of the submucosal lesions of 37 lesions; 13 in esophagus, 24 in stomach and 1 in duodenum. Perforation occurred in one case, and was managed conservatively with endoscopic clipping.

#### **Endoscopic resection of colorectal tumors**

EMR and ESD are being successfully used for early stage colon cancers, flat adenomas, large superficial colorectal tumors, and rectal carcinoids<sup>[39]</sup>. Lymph node metastasis in T1 colorectal carcinoma occurs only after infiltrating submucosa and is correlated to the depth of submucosal penetration by the tumor<sup>[40]</sup>. This supports the therapeutic effectiveness of endoscopic removal of polyps and flat lesions that are confined to the mucosa, regardless of their size. On the other hand, colorectal laterally spreading tumor (LST) classified as granular (LST-G) and non granular type (LST-NG), are defined as lesions larger than 10 mm in diameter, with a low vertical axis, extending along the luminal wall<sup>[41]</sup>. For *en bloc* resection of flat lesions larger than 20 mm, conventional EMR is inadequate because of incomplete removal and frequent local recurrence. When analyzing the endoscopic features of 257 LSTs in order to assess which features correlated with the depth of invasion, unevenness of nodules, presence of large nodules, size, histological type, and presence of depression in the tumor were significantly associated with the depth of invasion<sup>[41]</sup>. In addition, LST-NG showed a higher frequency of sm invasion than LST-G (14% vs 7%)<sup>[42]</sup>. Presence of a large nodule in LST-G type was associated with higher sm invasion while pit pattern, sclerous wall change, and larger size were significantly associated with higher sm invasion in LST-NG type. Therefore, it is advisable to perform endoscopic piecemeal resection for LST-G type with the area including the large nodule resected first. Besides, LST-NG type should be removed by *en bloc* resection (Figure 3) because of the higher potential of sm invasion when compared to that of the LST-G type<sup>[42]</sup>.

A study from Germany reported that complications



**Figure 3** ESD of a flat villous rectal polyp with sm1 carcinoma. **A:** Flat villous adenoma of the sigmoid colon; **B:** Peripheral dissection; **C:** Resected area; **D:** Resected specimen.

occurred in two patients of 57 patients after EMR in large colorectal neoplasia between 10 mm and 50 mm<sup>[43]</sup>. Recurrence rate following EMR ranges from 0% to 40% which could be reduced when combined by argon plasma coagulation. However, a recent study from Poland revealed that argon plasma coagulation did not reduce the recurrence rate compared to polypectomy alone<sup>[44]</sup>. Besides, another study reported that EMR was performed for 139 SP in 136 patients by snare polypectomy, and invasive carcinoma was found in 17 cases<sup>[45]</sup>. After 12 mo of follow up after EMR, no local recurrence was detected in 7 patients with invasive carcinoma without surgery. Another study from UK reported on 30 large colorectal polyps which were treated by *en bloc* resection in 22 cases and by piecemeal resection in 8 cases<sup>[45]</sup>. Histologically, the lesions were predominantly adenomatous polyps, but 7 cases revealed incidental focus of adenocarcinoma. Although bleeding occurred in 2 cases, there was no bowel perforation. There was no evidence of recurrence during the median follow-up of 21 mo.

In a prospective cohort study in Italy, IT knife was used for EMR of large colorectal polyps larger than 3 cm which are unsuitable for standard polypectomy<sup>[46]</sup>. The results showed the likelihood of complete *en bloc* resection of mucosal lesions improved by new approach with IT knife when compared with previous studies on colonic EMR, even for lesions located in difficult positions or larger than 30 mm. *En bloc* resection was achieved only in 55.1% of the lesions and piecemeal resection was performed in the rest of the cases. Although complete tumor removal was achieved in 19 patients, 13 had LGD, 15 had HGD, and one had a tumor invading the submucosa. Complications occurred

in four patients which were all managed conservatively. Local recurrences were detected in five patients and were treated by argon plasma coagulation and snare polypectomy. There was no recurrence during the median follow-up period of 15.7 mo<sup>[47]</sup>.

## CONCLUSION

EMR and ESD techniques should be considered as elective treatment modality for early GI cancers as long as it is performed under the right indications by an expertise.

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## Chronic liver disease in Aboriginal North Americans

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### INTRODUCTION

Aboriginal North Americans, which include American Indians and Alaska Natives (AI/ANs), Canadian First Nations, and native Greenlanders, are disproportionately affected by chronic liver disease (CLD). CLD for the purpose of this review includes the diagnoses of cirrhosis, end-stage liver disease (ESLD) and its complications, and chronic elevation of transaminase enzymes. In the United States, CLD was the twelfth leading cause of death in the general population in 2003, but was the fifth leading cause of death in AI/AN populations<sup>[1]</sup>. Likewise, cirrhosis is a disproportionate cause of death in Canadian First Nations<sup>[2]</sup>. Very little is known about the prevalence and incidence of CLD in native Greenlanders, although chronic hepatitis B incidence is known to be high in this population<sup>[3]</sup>. Of great concern, between 1990 and 1998, the overall annual CLD mortality rate in the United States declined by 4.5% but increased 11% among AI/AN populations<sup>[4]</sup>. Despite these striking disparities, studies examining the incidence, prevalence and etiology of CLD in Aboriginal North Americans are scarce. The goal of this paper is to summarize the etiology, natural history, and mortality rates of CLD in Aboriginal North Americans.

### Abstract

A structured literature review was performed to detail the frequency and etiology of chronic liver disease (CLD) in Aboriginal North Americans. CLD affects Aboriginal North Americans disproportionately and is now one of the most common causes of death. Alcoholic liver disease is the leading etiology of CLD, but viral hepatitis, particularly hepatitis C, is an important and growing cause of CLD. High rates of autoimmune hepatitis and primary biliary cirrhosis (PBC) are reported in regions of coastal British Columbia and southeastern Alaska. Non-alcoholic liver disease is a common, but understudied, cause of CLD. Future research should monitor the incidence and etiology of CLD and should be geographically inclusive. In addition, more research is needed on the treatment of hepatitis C virus (HCV) infection and non-alcoholic fatty liver disease (NAFLD) in this population.

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**Key words:** Hepatitis C virus; Hepatitis B virus; American Indian; Alaska Native; Chronic liver disease

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### LITERATURE RETRIEVAL

English language articles were identified by a search of the PubMed database from 1966 to August 2007. Because articles on Aboriginals from Mexico and Central America were frequently in non-English, we restricted our study to Aboriginals in the United States, Canada, and Greenland. Search terms included the following 7 individual medical conditions: chronic hepatitis, alcoholic liver disease, liver cirrhosis, fatty liver, hemochromatosis, hepatolenticular degeneration, and hepatitis A. Variants of these terms were also used; for example, in addition to fatty liver, other key words included non-alcoholic steatohepatitis (NASH). To identify publications focused on Aboriginal peoples, our search terms were American Indian(s), Alaska Native, native Alaskan, Native American, North American Indian(s), Greenland, and Inuit(s). Next, the bibliographies of the retrieved

**Table 1** Chronic liver disease mortality rates by etiology, Aboriginal group, and nation (number of deaths per 100 000)

Etiology	AI/AN	General US population	General Canadian population
Alcohol	19	4.6	3
Hepatitis C	2.3	1.7	1.3
Hepatitis B	0.6	0.4	1.3 <sup>1</sup>
Primary biliary cirrhosis	0.1	0.2	N/A
No identified cause	7.7	5.1	N/A
Overall	25.5-28.7	10.4-11.6	6.2-15.4

<sup>1</sup>Canadian data did not include separate categories for hepatitis B and C. Data not available on Canadian First Nations peoples and native Greenlanders specifically. Data adapted from Statistics Canada<sup>[7]</sup> and Vong<sup>[4]</sup>.

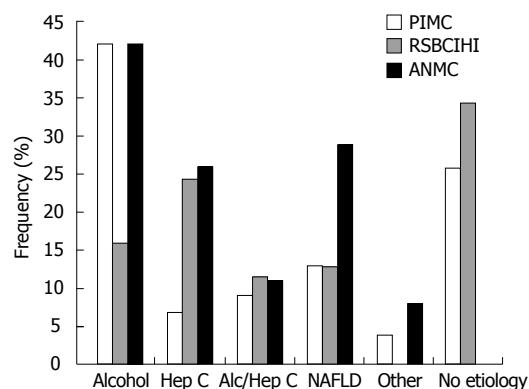
publications and those already in the authors' possession were examined for relevant articles. Finally, we used the above terms to search other databases such as usa.gov and the Native Health Database (U of New Mexico, <http://hsc.unm.edu/library/nhd/>).

In this review, we refer to the 7 disorders above as CLD. A meta-analysis, or other forms of systematic analyses, was not possible to conduct owing to the number of CLDs, variations in study designs, methods (particularly participant selection and case definitions), heterogeneous nature of the population, pockets of certain liver diseases, and the quality of the investigations.

Studies assessing patients with at least 1 CLD that included information on at least one North American Aboriginal population were included. In addition, we excluded studies which had no confirmed diagnosis, such as self-reported symptoms. We focused on publications that reported three kinds of data: prevalence, etiology, and patient characteristics. Prevalence data are summarized in Table 1. Next, we outlined information on discrete diagnoses. These studies, which are more clinically useful than population studies, are shown in Table 2. For each study, information was abstracted on the patient groups and the diagnostic criteria applied, the methods, and the major findings. The sources of the authors' funding had no role in the collection or interpretation of the data. Based on these tables, we summarized information for the sections below.

## EPIDEMIOLOGY OF CLD

Two studies from the past decade reported the overall, age-adjusted death rates due to CLD in AI/AN populations was 25.5-28.7 per 100 000 compared to 10.4-11.6 per 100 000 in the general population<sup>[4,5]</sup>. Moreover, CLD disproportionately strikes young adults; it was the second-leading cause of death among AI/ANs 25-44 years old<sup>[1]</sup>. In another study, researchers classified underlying conditions contributing to death by ICD-9 (International Statistical Classification of Diseases, version 9) codes as listed on death certificates<sup>[4]</sup>. In 1998, the top contributors to CLD mortality in AI/AN were: alcohol (65%), hepatitis C virus (HCV) (8%), hepatitis B virus (HBV) (2%), and primary biliary cirrhosis (PBC)



**Figure 1** Frequency of specific etiologies for chronic liver disease among AI/AN. Phoenix Indian Medical Center (PIMC), Phoenix, Arizona, 2000-2002, n = 1496. Riverside San Bernardino County Indian Health, Inc (RSBCIHI), Riverside, California, 2002-2003, n = 344. Alaska Native Medical Center (ANMC), Anchorage, AK, 2003-2004, n = 1903. Data adapted from<sup>[6,7]</sup> with permission. Not all categories are mutually exclusive in ANMC.

(1%). Notably, 24% of the deaths had no attributable cause.

Among AI/AN patients living with CLD, researchers observed a similar theme of high disease burden from viral hepatitis and alcohol, in conjunction with a large proportion of unexplained liver disease. In a study of AI/ANs obtaining outpatient and inpatient services in Phoenix, AZ, San Bernardino, CA, and Anchorage, AK, 5%-7% of AI/ANs had evidence of CLD<sup>[6,7]</sup>. These studies defined CLD as two elevated AST, ALT, or total bilirubin levels separated by 6 mo. Alcohol and HCV were the most common causes of CLD; however, non-alcoholic fatty liver disease (NAFLD) was also a major etiology and a significant proportion had no identified etiology (Figure 1).

In the general population of Canada, CLD is the 13th most common cause of death<sup>[8]</sup>, accounting for 6.0-15.4 deaths per 100 000. Specific mortality data from CLD in Canadian First Nations was not available in published form or directly from Statistics Canada.

No mortality statistics are available for Greenland. However, in a study of hospitalized patients, the prevalence of cirrhosis, as determined by ICD-10 (International Statistical Classification of Diseases, version 10) discharge diagnoses, was lower in Greenlanders (0.19%) than in a similar cohort in Denmark (0.54%)<sup>[9]</sup>. This finding was unexpected because Inuit Greenlanders are known to have high rates of viral hepatitis and thought to consume more alcohol on average than their Danish counterparts living in Greenland.

CLD in Aboriginal North American populations is heterogeneous, with certain causes of CLD found in unique geographic regions, such as HBV in the circumpolar regions and PBC in coastal British Columbia (Figure 2).

## HEPATITIS A

Before the implementation of hepatitis A virus (HAV) vaccination, this disease caused large outbreaks,

**Table 2 Major causes of viral hepatitis in Aboriginal North Americans**

Aboriginal group	Study populations	Hepatitis A	Hepatitis B	Hepatitis C	Comments
Native Americans	South Dakota NA Navajo NA Urban and Veterans populations	Pre-vaccine incidence was 96/100000  Seroprevalence: 76%	N/A	Seroprevalence range: 3%-32%	Hepatitis A incidence now is comparable to general population: 1.0-2.0/100000
Alaska Natives	Random distribution of AN tribes	49% seroprevalance pre-vaccination	6% sAg+, 24% core IgG+	0.8% seroprevalance	Heavy burden of hepatitis a prior to vaccination. Hepatitis B endemic to AN with high rates of HCC in certain regions, vaccination has lead to decreased incidence
First Nations (Canada)	Manitoba FN British Columbia FN Inuit	Seroprevalence of 90% in those < 40 yr; 31/100000 incidence pre-vaccine	5% sAg+, 27% core IgG+ in circumpolar regions, much lower outside circumpolar areas	1.1% seroprevalence in Inuit, 2%-20% in non-Inuit	Similar patterns of hepatitis as seen in US.
Native Greenlanders (Inuit)	Inuit	54% seroprevalence	7%-12% sAg+, 42% core IgG+	< 1% seroprevalence	Hepatitis B may be spread sexually more frequently than other Aboriginal populations. HCC also less common

**Figure 2** Common causes of chronic liver disease in Aboriginal North Americans.

cycling every 5-7 years in many AI/AN communities. Although US national prevalence statistics have not

been published, incidence rates of hepatitis A in AI communities in South Dakota have been reported as 33

times higher than the non-Indian rates (92.6 compared to 2.8/100 000 persons per year)<sup>[10]</sup>. A study of Navajo schoolchildren found that 70% had antibodies to HAV, with higher rates in older children; this is one of the highest rates reported among US children<sup>[11]</sup>. Among ANs, almost 50% tested positive for antibody to HAV<sup>[12]</sup>. Past infection was strongly age-related, increasing from 7% in persons born since 1975 to 85% among persons born before 1945. The seroprevalence patterns indicated that village-wide outbreaks have been the norm and appear to be dependent on the presence of a young susceptible population. Finally, urban AI/ANs have a higher incidence of hepatitis A than their rural counterparts (151 vs 106/100 000)<sup>[13]</sup>.

Following the introduction of HAV vaccine in 1995, AI/AN communities witnessed remarkable reductions in incidence of this disease, declining to levels near the general US population<sup>[10,14,15]</sup>. In South Dakota, community wide immunization programs that provided hepatitis A immunizations to children aged 2-12 years halted outbreaks when 70% or more of these children were vaccinated<sup>[10]</sup>.

Because of frequent outbreaks and high levels of endemic transmission, the US Public Health Service Advisory Committee on Immunization Practices recommends routine HAV vaccination of all 2-year-old AI/AN children<sup>[10]</sup> and the US Public Health Service Preventive Services Task Force also recommends the vaccine for AI/AN adults. Widespread use of hepatitis A vaccine to maintain a high rate of seropositivity among young persons appears to be critical in preventing future epidemics in AI/AN communities.

Similar to the situation in the US, Canadian First Nations peoples had a much higher incidence and prevalence of hepatitis A than did the general population. In studies of Manitoba First Nations peoples, more than 90% of patients over age 40 had evidence of exposure to HAV<sup>[16]</sup>. British Columbian First Nations tribes studied from 1990 to 2001 had a crude incidence rate (31 per 100 000) twice the rate of the general population of British Columbia<sup>[17]</sup>.

Data from Greenland is sparse, but a cross-sectional study of Inuit Greenlanders found similarly high seroprevalence of 54%<sup>[18]</sup>. It is not known how the hepatitis A vaccine has changed the incidence in native Greenlanders.

## HEPATITIS B

A recent national study of hepatitis B in AI/ANs indicated that HBV accounted for just 11 deaths, a rate of 0.60/100 000 persons<sup>[4]</sup>. In contrast, hepatitis B is an important cause of CLD in AN living in the circumpolar region. Among AN, high rates of acute, icteric hepatitis B were first reported in the 1970's<sup>[19]</sup>. Subsequent studies found that 6.4% of ANs were surface antigen-positive and 24.2% were positive for hepatitis B core antibody<sup>[20]</sup>.

The vast majority of HBV infection in AN is acquired horizontally, usually before the age of five. A small pocket of AN with genotype C (Asian variant)

transmit HBV vertically (personal communication, Brian McMahon). As a result, most AN have an intermediate risk of becoming chronic carriers. Of those AN children exposed before the age of five, 28.8% will be chronic carriers<sup>[21]</sup>. Once chronically infected, AN carriers of HBV rarely clear the infection. A large study of HBsAg positive AI/AN patients showed an average annual seroconversion rate to anti-HBs of 0.3%; however, patients over 40 years had a much higher seroconversion rate (4.4%) than those under 40 years (1.7%)<sup>[22]</sup>.

Another survey of 1536 HBsAg positive AN people demonstrated that 42% were HBeAg positive at baseline<sup>[23]</sup>. Of those who were HBeAg positive, 73% cleared HBe-antigen within 10 years. After a median of 20.5 years of follow-up, 8% remained HBe antigen positive and 22% became anti-HBe positive but then reactivated to HBe antigen-positivity. Among those who were anti-HBe positive and HBe antigen negative at the beginning of the study, 86% remained anti-HBe positive throughout the period of observation<sup>[24]</sup>. The time to clearance was shorter for those who were HBeAg negative than for those who were HBeAg positive.

Several sequelae are common amongst hepatitis B carriers, most notably ESLD and hepatocellular carcinoma (HCC). These combined outcomes occur at a rate of 2.3 per 1000 carrier-years; the majority of events (82%) are HCC<sup>[23]</sup>. The link between chronic hepatitis B and HCC was first observed in the 1970s, when the incidence of HCC was reported to be five times greater in an Alaska Native population than the US white population<sup>[25]</sup>. Most of that excess risk was attributable to chronic hepatitis B carriage rather than alcohol consumption. In a study of 1400 HBsAg positive AI/ANs, 13 of the 60 deaths (22%) and 57% of all fatal neoplasms during the study were due to HCC<sup>[22]</sup>. The incidence of HCC is higher in AN men, 2.3 per 1000 carrier years, *versus* 1.2 per 1000 carrier years in AN women. The relative risk of developing HCC for an HBsAg carrier is 148 times greater compared to a non-carrier, as documented in a more recent study<sup>[22]</sup>.

Within HBsAg positive AI/AN populations, HCC is observed more frequently in older patients, those with reversion to HBeAg positivity, those of Yupik ethnicity, and carriers with genotype F<sup>[23,26]</sup>. AN living in the Yukon delta region of western Alaska develop HCC at very young ages, in the context of no cirrhosis<sup>[27]</sup>; however, more recent studies found that half (15/30) of the cases of HCC elsewhere in AN were found in cirrhotics<sup>[26]</sup>. It is possible that the genotype F variant seen in this part of Alaska confers a very high risk of developing HCC.

Among Canadian Inuit living in the Arctic region, 5% were surface antigen-positive and 27% had been exposed<sup>[28]</sup>. However, Canadian First Nations members living outside of the circumpolar region have similar chronic infection (0.3%-3%) and exposure rates (10%-22%) as compared to non-Aborigines engaging in similar activities<sup>[29]</sup>.

Among native Greenlanders, 7%-12% were surface antigen-positive carriers and 42% had evidence of past infection<sup>[18,30]</sup>. The peak incidence occurs in young adults,

suggesting that sexual acquisition is the predominant route of exposure<sup>[30]</sup>. This route of transmission differs from the perinatal or early horizontal transmission seen in AN<sup>[21]</sup>. Among native Greenlanders, the incidence of HCC is not higher than in the general Danish population (1.9 *vs* 2.2 cases per 100 000), despite the high prevalence of chronic hepatitis B<sup>[31]</sup>. There are several possible explanations for this discrepancy in HCC rates seen in the circumpolar regions. First, native Greenlanders acquire HBV later in life and more frequently clear the virus. Second, chronic carriers tend to have low viral loads (median: 40 000 copies/mL)<sup>[32]</sup>. Finally, the majority of native Greenlanders with chronic HBV appear to have a new genotype (Bj variant), which may be less carcinogenic than other genotypes.

Despite the heavy disease burden from chronic HBV in Aboriginal North Americans, there are still many unanswered questions. For example, it is unclear why the natural history, routes of transmission, and risk for HCC differ so dramatically within the circumpolar region. In addition, the route of transmission and natural history has not been thoroughly studied in AI/AN living in the lower 48 states of the US.

## HEPATITIS C

Hepatitis C is one of the most common and important causes of CLD in AI/AN (Table 1, Figure 1). However, seroprevalence studies have come to disparate conclusions regarding the prevalence of hepatitis C in AI/AN. For example, in the largest study of AI/AN, the overall seroprevalence was just 0.82%<sup>[33]</sup>, which is lower than the overall seroprevalence in the US (1.8%)<sup>[34]</sup>. In contrast, studies of pregnant AI women in the US Southwest, urban AI/AN in the US Midwest, and AI/AN in the Veterans Affairs system showed seroprevalence rates of 11.5%, 3%, and 32%, respectively<sup>[35–37]</sup>. Part of the discrepancy in findings may be attributable to selection bias; the study from Alaska was a true population-based study<sup>[33]</sup>, whereas the others were convenience samples.

The route of transmission appears to be similar to the general US population; namely, injection drug use and blood transfusions. In a study of HCV-positive Alaska Natives, 60.1% had a history of intravenous drug use and 14.0% had a history of blood transfusion<sup>[33]</sup>. Similarly, in a study from an urban US Midwest city, intravenous drug and cocaine use accounted for the majority of those who were HCV positive<sup>[36]</sup>. Other identified risk factors were tattoos > 5 years old and having a sexual partner with HCV.

In the US general population, genotype 1 accounts for the majority of chronic HCV (72%), followed by genotypes 2 (15%) and 3 (6%)<sup>[38]</sup>. In AI/AN, the more easily-treated genotypes are more common: 60% have genotype 1, 23% have genotype 2, and 14% have genotype 3<sup>[33]</sup>. In this Alaska cohort, 73% tested positive for HCV RNA; men were more likely to have higher levels of HCV RNA.

Studies of antiviral efficacy in AI/AN are limited

to one small study from Alaska. However, the results indicate that antiviral therapy may be markedly less efficacious than in American whites (35% *vs* 52%)<sup>[39,40]</sup>. The rate of sustained virologic response (SVR) was 7% (1/15) in patients with genotype 1, 54% (7/13) in genotype 2, and 50% (6/12) in genotype 3.

Studies of First Nations members in Canada show a similar pattern as those done in the US. In a study of Canadian Inuit, the seroprevalence of HCV was 1.1%<sup>[29]</sup>, but in areas outside of the circumpolar region, the seroprevalence ranged from 2.2% to 20%<sup>[16,41]</sup>. Among First Nations Canadians who used injection drugs, the seroprevalence was 33%<sup>[41]</sup>. Interestingly, Aboriginal North Americans may have an increased ability to clear the virus, a finding reported in both Canada and the US<sup>[16,42]</sup>. In native Greenlanders, the seroprevalence is similarly low, < 1%<sup>[31]</sup>.

## HEPATITIS D AND E

Hepatitis D (or delta) virus (HDV) is a very small RNA virus which is dependent on HBV for its life cycle; therefore, in humans it is only seen in the context of concurrent HBV infection. HBV-infected patients who become super-infected with HDV are more likely to develop acute fulminant liver failure than if they just had HBV<sup>[43]</sup>. Similarly, HBV/HDV co-infected patients progress more rapidly (1–2 years in some cases) and more frequently (70%) to cirrhosis<sup>[43]</sup>. HDV is believed to be uncommon in most Aboriginal North American populations, with the exception of Greenland, where 40% of those with HBV also have HDV<sup>[44]</sup>.

Hepatitis E virus (HEV) is also an RNA virus but is spread by the fecal-oral route, similar to HAV. It is predominantly seen in developing countries. However, a recent study showed that 3% of a population of Canadian Inuit had IgG antibodies to HEV (none were viremic). HEV infection has been linked to consumption of deer and caribou, although local caribou were all HEV-negative in this study<sup>[45]</sup>.

## ALCOHOLIC LIVER DISEASE

Alcohol has long been recognized as the most important cause of cirrhosis and liver-related death in AI/AN<sup>[46]</sup> and continues to be the most common cause of CLD (Figure 1). In one study, 65% of all deaths from CLD were attributable to alcoholic cirrhosis<sup>[4]</sup>. Moreover, the excess cirrhosis mortality observed in AI/AN men is independent of socioeconomic status<sup>[47]</sup>.

While binge alcohol use is common (up to 16% in a phone survey of AI/AN)<sup>[48]</sup>, there is also evidence that alcohol consumption in Native American populations is not significantly greater than consumption in other ethnic groups but rather that alcohol has more serious effects on AI/AN populations<sup>[49]</sup>. A study of alcohol consumption and subsequent health effects showed that Native Americans did not drink more alcohol per day or drink for longer periods of time than their Caucasian, Hispanic, and Afro-American counterparts, but

suffered significantly higher rates of alcoholic hepatitis and cirrhosis. In addition, AI/AN in this study had a much lower survival rate, although small sample sizes prevented statistical significance. Such results suggest that AI/AN populations have a genetic predisposition to alcohol liver injury, a finding that has been suggested by other studies<sup>[50]</sup>.

Data from Canada presents a more mixed picture regarding the influence of alcohol on CLD. One study found that the death rate from alcoholism and cirrhosis was three times greater in Canadian First Nations as compared to the general population<sup>[51]</sup>. However, a study reviewing indications for liver transplants in Aboriginal populations in British Columbia found that while alcoholic cirrhosis was the third leading indicator for transplant recipients in the general population, it was not an indicator for any of the fifteen Aboriginal patients who received a transplant. Instead, PBC and autoimmune hepatitis were the two most common reasons (13/15) for liver transplantation in Canadian First Nations<sup>[52]</sup>.

Native Greenlanders have a high per capita alcohol intake. However, when comparing patients in alcohol treatment programs in Greenland *vs* Denmark, the Greenlanders less frequently had abnormal liver function tests (42% *vs* 91%) and cases of cirrhosis or advanced fibrosis (0 *vs* 13)<sup>[53]</sup>. This study suggests that Greenland Inuit may be more resistant to the hepatotoxic effects of alcohol.

Prevention and treatment of alcohol abuse in Aboriginal populations has been an area of active research<sup>[54]</sup>.

## AUTOIMMUNE HEPATITIS

AI/AN have one of the highest rates of autoimmune hepatitis (AIH) in the world. A review of AI/AN in Alaska with AIH between 1984 and 2000 found a point prevalence of definite or probable AIH (using International Autoimmune Hepatitis Group criteria<sup>[55]</sup>) of 42.9/100 000<sup>[56]</sup>. Revised data from this cohort through 2005 showed a point prevalence of 61.7/100 000<sup>[57]</sup>. The only other population-based study of AIH was from Norway and reported a prevalence of 16.9/100 000<sup>[58]</sup>. Fortunately, AI/AN with AIH responded to treatment with systemic steroids with normalization of ALT more rapidly than in previously reported studies<sup>[59]</sup>.

AIH is similarly more common in First Nations peoples in British Columbia<sup>[52]</sup>. A study of indications for liver transplants in British Columbia showed that 7% of recipients the overall population received transplants with AIH as their primary diagnosis, whereas 27% of aboriginal recipients had AIH as their primary diagnosis. Autoimmune hepatitis was fourth leading indication for liver transplant overall, but was the second leading indication within the Aboriginal population. Among those referred for transplant evaluation with AIH, a statistically significant proportion, 12/68 (18%), were First Nations, when compared to their overall proportion in the general population (4.4%)<sup>[60]</sup>. Most cases appear to be type 1 AIH<sup>[61]</sup>.

There are no studies on AIH in native Greenlanders.

## PBC

PBC, like autoimmune hepatitis, is an autoimmune disease that has a strikingly high prevalence in Alaska Natives and British Columbia's First Nations population. A genetic predisposition and female gender are the primary risk factors for developing PBC.

In a study of AN persons with autoimmune liver disease, a combined prevalence rate of antimitochondrial antibody (AMA)-positive and AMA-negative persons with PBC was 21/100 000, including a prevalence of 71.5/100 000 in Southeast Alaska Indian persons<sup>[56]</sup>. Five of 23 persons (22%) had AMA-negative PBC and only 1/23 persons was male.

Outside of Alaska, PBC affects AI/AN populations less frequently than the general population. PBC accounted for 0.18 deaths per 100 000 in the general population in 1998, but a slightly lower rate of 0.11 deaths per 100 000 in the AI/AN population. The death rate from PBC in AI/AN women was 0.28 per 100 000. Furthermore, this rate remained static in the AI/AN population during the 1990's<sup>[4]</sup>.

Native populations in British Columbia, like AN, have a high PBC prevalence. For example, in British Columbia, First Nations people comprise 4% of the population, yet account for 25% of those needing liver transplants due to PBC<sup>[52]</sup>. From 1989 to 1998, PBC was the leading indication for a liver transplant in British Columbia First Nations patients<sup>[52]</sup>. First Nations patients were also referred for liver transplants at a much younger age than non-First Nations patients<sup>[62]</sup>. Of those First Nations patients referred for liver transplantation, most (33/34) are female.

Several factors indicate a strong genetic component to the occurrence of PBC. Within a population of British Columbia First Nations patients with PBC, 19/24 reported at least one other autoimmune condition and 33% had a family history of PBC<sup>[62]</sup>. First Nations patients were unusual in that 18% tested negative for AMA, which are normally present in 95% of PBC patients.

There are no studies of PBC in native Greenlanders.

## NAFLD

NAFLD is the deposition of fat in the liver, commonly as a consequence of obesity and diabetes mellitus type 2. This condition may progress to NASH, in which there is inflammation, sometimes with resulting fibrosis and cirrhosis. NAFLD was the fourth most common cause of CLD in a study from the Southwestern US, accounting for one-eighth of the total cases (Figure 1). Preliminary results from a retrospective study of abnormal ALT values in AI/AN indicate that NAFLD is the second most common etiology<sup>[7]</sup>.

It is not surprising that NAFLD accounts for a significant proportion of CLD, given the high prevalence of risk factors for NAFLD in AI/AN (obesity, hypertriglyceridemia and diabetes mellitus type 2). For example, the prevalence of obesity among AI/AN is 24%-40%, the highest rate in any American ethnic group<sup>[63]</sup>. Similarly, the prevalence of diabetes is higher

in AN/AI than any other ethnic group, ranging from 9.7%-19.7%<sup>[48,63]</sup>. Rates of these conditions are expected to rise in the general population, so NAFLD can likewise be expected to increase in the future.

There are no available studies of NAFLD in Canadian FN and native Greenlanders.

## GENETIC

Inherited causes of liver disease are an uncommon cause of cirrhosis in North American Aboriginal populations. One exception is in the Ojibway-Cree population in Northwestern Quebec where North American Indian childhood cirrhosis (NAIC) has been described<sup>[64]</sup>. This autosomal recessive trait is carried by 10% of the local population and 1/250 to 1/750 are believed to have the condition. A missense mutation on chromosome 16q22 causes a change in the secondary structure of cirrin, a protein crucial in embryonic liver development<sup>[65]</sup>. Intrahepatic cholestasis develops and manifests clinically as a sustained elevation in the alkaline phosphatase level. Children born with this condition have transient neonatal jaundice, with rapid progression to biliary cirrhosis and portal hypertension. In a case series of 30 children with NAIC, 14 (47%) died, mostly from complications of ESLD<sup>[64]</sup>. Liver transplantation is the only cure. A syndrome termed fatal familial cholestatic syndrome has been reported in Greenland Eskimo children<sup>[66]</sup>. This autosomal recessive disease is associated with dwarfism, osteodystrophy, jaundice, and malnutrition. Death in infancy is common and there is no specific treatment available.

Hemochromatosis, an autosomal recessive trait leading to iron overload and cirrhosis, is common in persons of northern European descent, affecting about 5 of every 1000 persons<sup>[67]</sup>. Initial studies screening patients from primary care clinics in Alabama suggested that patients with AI ancestry may have greater ferritin and lower mean transferrin levels, findings which are suggestive of hemochromatosis<sup>[68]</sup>. However, genetic testing of 80 patients with hemochromatosis did not show a difference in phenotype among those with and without AI ancestry<sup>[69]</sup>.

Familial clusters of HCC have been observed in AN, sometimes in childhood. Because p53 mutations have been observed in 29% of patients with HCC, researchers hypothesized that p53 mutations might account for the early onset and clustering<sup>[70]</sup>. However, they found no antibody, immunohistochemical or DNA evidence of p53 mutations in 14 AN patients with HCC. It is still possible, however, that a germline mutation in a tumor suppression gene exists.

There are no studies examining Wilson's disease in Aboriginal North Americans.

## CONCLUSION

CLD is an important cause of morbidity and mortality in Aboriginal North Americans. Alcohol abuse is the most common etiology of CLD in this population and incidence rates remain stable. However, the incidence of

CLD due to viral hepatitis, especially HCV, is rising. In addition, NAFLD is an understudied, but an increasingly more common, cause of CLD. Taken together, CLD in this population can be expected to increase in the coming decade. Accordingly, future studies should continue to monitor the incidence and etiology of CLD and should be geographically inclusive. In addition, future research should focus on the treatment of HCV and NAFLD.

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REVIEW

## Inflammatory bowel disease: Moving toward a stem cell-based therapy

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### INTRODUCTION

Despite a decrease in the occurrence of infective diseases, autoimmune and chronic inflammatory disorders are on the increase in developed countries. Many of these illnesses remain poorly understood due to the intricate network of interactions among genetic, cellular and environmental factors underlying pathogenesis.

Various theories have been developed as to the etiopathogenesis of inflammatory bowel disease (IBD), but so far none of them has led to a therapy with long-term efficacy and free of side effects. The advancement of our knowledge of the biological basis of pathogenesis, combined with recent findings on the regenerative, trophic and immunoregulatory potential of stem cells, have triggered research that could lead to a significant evolution, or revolution, in the treatment of IBD.

Mesenchymal and hematopoietic stem cells (MSCs and HSCs) are catalyzing the attention of IBD investigators, physicians and clinicians. After a number of case reports, and following initial steps within *in vitro* and *in vivo* models, cell-based approaches are now moving from the laboratory bench to the patient's bed. Stem cell transplantation may soon become a therapeutic option for IBD.

Crohn's disease (CD) and ulcerative colitis (UC), the two main forms of inflammatory bowel diseases, are chronic, relapsing and remitting diseases profoundly affecting the quality of life in an enlarging portion of the population: their incidence and prevalence are growing in Western countries<sup>[1]</sup>. CD may affect every tract of the digestive system-most commonly the ileal and colonic

### Abstract

The incidence and prevalence of Crohn's disease (CD) and ulcerative colitis (UC), the two major forms of inflammatory bowel diseases (IBD), are rising in western countries. The modern hygienic lifestyle is probably at the root of a disease where, in genetically susceptible hosts, the intestinal commensal flora triggers dysregulated immune and inflammatory responses. Current therapies ranging from anti-inflammatory drugs to immunosuppressive regimens, remain inadequate. Advances in our understanding of the cell populations involved in the pathogenetic processes and recent findings on the regenerative, trophic and immunoregulatory potential of stem cells open new paths in IBD therapy. Hematopoietic and mesenchymal stem cells are catalyzing the attention of IBD investigators. This review highlights the pivotal findings for stem cell-based approaches to IBD therapy and collects the encouraging results coming in from clinical trials.

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**Key words:** Inflammatory bowel disease; Stem cells;

tracts, and exhibits a histological pattern of transmural inflammation. UC only affects the colon starting from the rectum and moving backward, the inflammation being localized in the mucosal layer. In both CD and UC an alteration of the mucosa leads to the symptoms of the disease. CD can be complicated by the occurrence of fistulas, abscesses and stenosis.

Genetic, environmental and immunological factors contribute to the development of inflammatory bowel disease. The “hygiene hypothesis” proposes a scenario in which a radical change of lifestyle may have led to the shift from infective to chronic inflammatory diseases in Western countries. An immune system exposed to a low number of antigens and struggling against only a few challengers, may be lapsing into an uneducated state. Thus its efforts at eliminating offending agents could then be ineffective or misdirected. The vary environmental modifications that brought humans to this hygienic lifestyle may have contributed to the rise of immune-based disorders.

IBD is thought to be the result of an aberrant immune response to commensal bacteria and luminal antigens in a susceptible host. The “no bacteria, no colitis” paradigm summarizes the evidence obtained from animal models: experimental colitis can only be induced in a conventional, non-germ-free environment. The evidence supporting the existence of a classic infectious agent causing IBD is weak. The enteric flora may trigger an inappropriate, “loss of tolerance”-based immune response, further evolving to chronic inflammation and IBD.

The mucosal tolerogenic state is maintained by dynamic, strictly regulated, physical and immunochemical interactions in a complex crosstalk among the gut microbiome, gut luminal antigens, the intestinal epithelial barrier, lymphocytes, dendritic and mesenchymal cell populations<sup>[2]</sup>. The circuitry is coordinated so as to obtain a minimal persistent inflammatory state (physiological inflammation) able to control the enteric flora and to cope with non-self antigens. In normal conditions the innate and adaptive immune systems cooperate in order to create this controlled status of inflammation: in CD and UC the balance is lost.

## IMMUNE-BASED DISORDERS

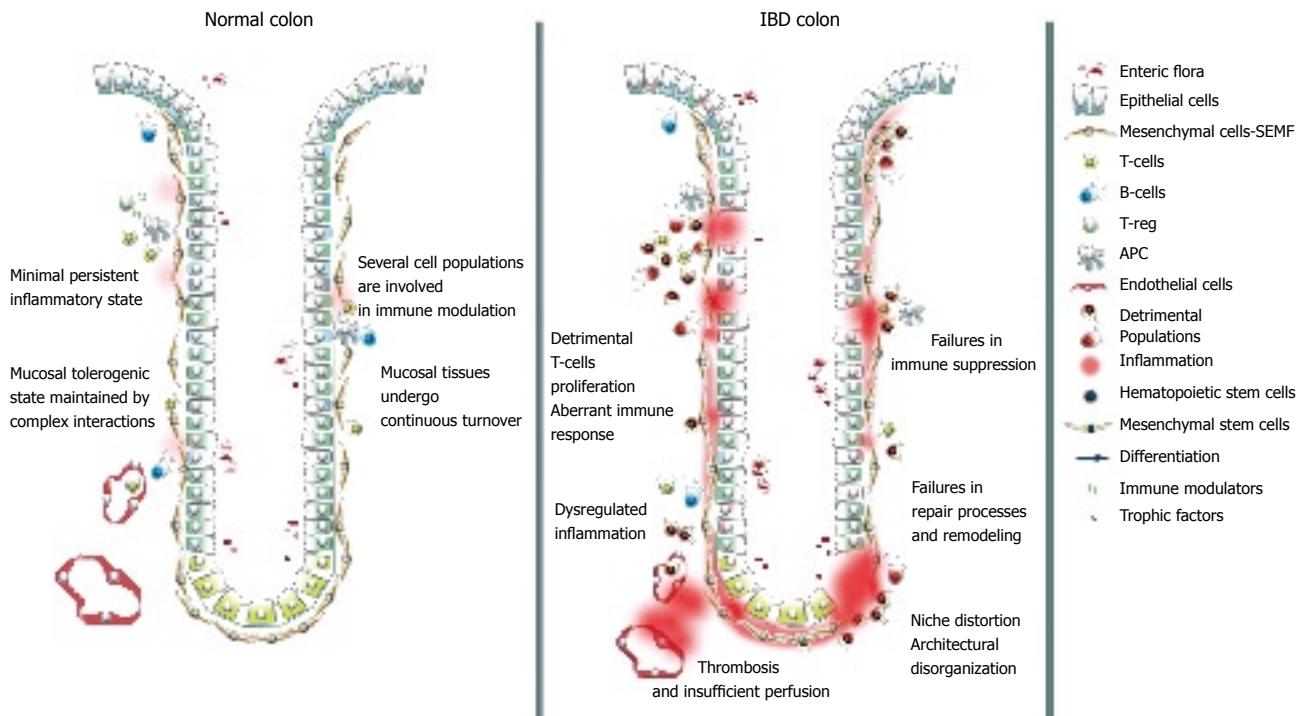
Analysis of genetics and immune responses in IBD patients and animal models sheds light on the biological function of the metabolic pathways and cell populations with a central role in the immune tolerance network. Most of the genetic factors that are so far known to contribute to susceptibility towards IBD act as key links in immune recognition and modulation. The existence of a genetic predisposition for IBD has been demonstrated. Genome-wide screening in familial clusters has identified 7 loci with a linkage to IBD (IBD 1-7)<sup>[3]</sup>. Most linkage regions are associated with both CD and UC: this suggests the existence of common genetic features and mechanisms. The genes residing in the IBD loci (e.g. NOD2/CARD15, TNF-

alpha) are implicated in immune function and intestinal permeability<sup>[4-7]</sup>. Initially, connections between innate immune impairments and IBD emerged. The association between CD and NOD2/CARD15 mutations showed that innate pattern recognition impairments and bacterial sensing may undermine the host/microbe interplay at a critical point. The NOD2/CARD15 protein recognizes peptidoglycans (bacterial muramyl dipeptide, MDP) and is expressed by macrophages and dendritic cells. The mutated gene product fails to bind MDP. The production of alfa-defensins by epithelial Paneth cells, expressing NOD2, is decreased in CD patients when NOD2 is mutated<sup>[8]</sup>. Innate and adaptive immunity are intimately interconnected, impaired mechanisms may simultaneously affect the two vast pathways of immune response.

Cell populations and mediators of adaptive immunity have been extensively investigated in IBD patients and murine models<sup>[9]</sup>. A dramatic increase in antibody production has been described both at the mucosal and at the systemic level in IBD patients. An alteration in the relative proportions of the immunoglobulin classes has been found<sup>[10]</sup>. Autoantibody production has been investigated, but confirmation of their existence is still needed<sup>[11]</sup>.

T lymphocytes are recognized as central effector cells. Their activation in IBD is accompanied multiple alterations in the production of cytokines and soluble mediators with proinflammatory and immunoregulatory significance. Consensus exists on the differing CD4 helper polarization tendency between the two major forms of IBD. In CD the IFN-gamma and IL-2 producing Th1 CD4+ phenotype is predominant, while UC seems to be dominated by atypical Th2 CD4+ T-cells, producing TGF beta and IL-5, and by IL-13 producing CD1d-restricted nonclassical Natural Killer T cells<sup>[12]</sup>. CD resembles experimental Th1-mediated colitis, whereas the features of UC are recapitulated in models of Th2-mediated colitis<sup>[13,14]</sup>. Observations in patients and murine models suggest that the suppressor activity of CD4+ CD25<sup>high</sup> T regulatory cells may be abrogated or insufficient to balance the chronic inflammation at the mucosal level<sup>[15]</sup>. Murine models confirm the ability of CD4+ CD25<sup>high</sup> T regulatory cells to prevent or ameliorate experimental colitis<sup>[16]</sup>, indicating the positive effect of anergy induction in the inflammatory environment. The suppressor activity of immunoregulatory populations, mediated by cytokines like IL-10 and TGF-beta, may fail because of a disruption of the signaling pathway in activated T cells: Smad7 overexpression antagonizes the TGF-beta dependent inhibition of T proliferation<sup>[17]</sup>.

The involvement of cell populations other than the classical immunological ones in immune modulation and IBD pathogenesis is becoming clear. Epithelial, endothelial, mesenchymal cells and platelets cooperate in modulating the immune response, and play a role in the establishment and protraction of inflammatory processes, in recovery and in tissue remodeling (Figure 1). Intestinal epithelial cells (IECs) have been claimed to



**Figure 1** Schematic representation of normal and IBD mucosal tissues. In normal conditions the innate and the adaptive immune systems cooperate in order to create a controlled status of inflammation; In IBD the balance is lost.

act as non professional antigen-presenting cells. In the inflamed mucosa, IECs inappropriately express HLA-DR class II and costimulators of the B7 family<sup>[18]</sup>. In healthy subjects antigen presentation by IECs results in activation of a CD8+ regulatory T cell subset through a non classical MHC class I pathway. IECs interact with the T compartment through members of the CEA family and CD1d, and in normal conditions expand a CD8+ VB5.1+ subset of T cells with regulatory functions. In IBD IECs a defective expression of CEA family members and CD1d occurs, leading to a failure in expansion of the regulatory subset and to a proliferation of CD4+ T cells with the production of inflammatory cytokines<sup>[19,20]</sup>. Moreover, UC IECs fail to express MHC I molecules and CD IECs express HLA-E, MICA and MICB, but not CD1d, failing to expand the regulatory T cell subset<sup>[21]</sup>.

Endothelial cells control the egression of migrating leukocytes in a complex process mediated by cytokines, chemokines and adhesion molecules. A deficit in nitric oxide synthase (NOS) production has been found in IBD intestinal microvascular endothelial cells. Impairment of NO-dependent vasodilation may result in decreased perfusion and poor wound healing<sup>[22]</sup>. Thrombosis is a frequent complication in IBD patients and platelets are implicated, but interest in these entities has risen following the definition of their immunological properties<sup>[23]</sup>. Platelets become activated in IBD patients and contribute to exacerbating inflammation through the release of CD40, which triggers a broad spectrum response in the intestinal microvasculature, stroma, and immune cells<sup>[24]</sup>.

The role of mesenchymal cells in mucosal homeostasis and microenvironmental modeling is far more critical than previously thought. Traditionally considered as

extracellular matrix (ECM) producers, fibroblasts were thought to be a passive cell population responsive to the chronic inflammatory environment and causing fibrotic complication<sup>[25]</sup>. Fibroblasts are the main source of matrix metalloproteinases (MMPs); proteolytic enzymes responsible for ECM degradation and ultimately for tissue destruction during inflammation. Interactions with activated T-cells potently stimulate fibroblast production of MMPs and result in tissue injury<sup>[26,27]</sup>.

## MESENCHYMAL CELLS AND RESTITUTIO AD INTEGRUM

Epithelial ulcerations caused by inflammatory process recover *via* tissue remodeling in the normal intestine. Resolution of inflammatory activity is associated with repair processes, and mesenchymal cells have a central role in coordinating these events to help remodeling. The repair processes in UC patients are usually able to restore a normal intestinal architecture, but in CD patients an excessive fibrosis frequently leads to the formation of strictures and obstructions. Fibrosis is one of the main complications and a recurrent finding in IBD patients: it is associated with mesenchymal cell persistence and hyperplasia, tissue disorganization and fibrillar collagen deposition.

Isolation and characterization of intestinal fibroblasts has allowed the determination of some peculiar biological features of IBD fibroblasts, for example they display significantly higher proliferation and collagen secretion rates than normal intestinal fibroblasts. Such evidence suggests that a fibroblast subset may be functionally activated in the IBD intestine<sup>[28,29]</sup>.

Recent findings suggest that mesenchymal cells derived from bone marrow stem cells may have an important role in repair processes and fibrosis<sup>[30]</sup>. Intestinal subepithelial myofibroblasts (ISEMFs) are located under the basement membrane, juxtaposed with the base of epithelial cells. These mesenchymal cells regulate a number of epithelial cell functions such as proliferation, differentiation and ECM metabolism, affecting the growth of the basement membrane<sup>[31]</sup>. They cooperate in pathogen sensing and actively participate in immune responses<sup>[32]</sup>. Moreover, they exert important functions in tolerance induction<sup>[33]</sup>. ISEMFs control mucosal repair processes by ECM manipulation, cytokine and growth factor release. Two separate mechanisms mediate the repair operations. Restitution is the first, a response to minor to moderate injury, when the basement membrane remains intact. Here, ISEMFs promote the proliferation and migration of residual epithelial cells over the denuded area by the release of TGF-beta, EGF, aFGF, bFGF and inflammatory cytokines. When the wound is deep and a reconstruction of the subepithelial tissues is needed, mesenchymal cells proliferate and form a new basement membrane, over which epithelial cells will then proliferate and migrate. ISEMFs are therefore responsible for a crucial process in mucosal repair: the maintenance and reconstitution of the basement membrane.

Alpha-SMA positive ISEMFs are increased in IBD mucosa as compared with normal mucosa, and the increase is very marked at the edges of UC ulcerations<sup>[34]</sup>. A higher ISEMF proliferation rate and growth factor secretion could account for CD fibrogenesis<sup>[28]</sup>. This cell population, actively participating in ECM metabolism and basement membrane turnover, is a major effector in the tissue remodeling process. Inflammatory cytokines and growth factors like TGF-beta, PDGF-BB, KGF, IGF-1 and EGF control MMP and their tissue inhibitor (TIMP) expression in ISEMFs, and it has been shown that the expression of MMPs and TIMPs is elevated in the inflamed mucosa of IBD patients<sup>[35]</sup>. ECM metabolism and inflammatory responses are regulated in close association: in colonic SEMFs TNF-alpha, IL-1 beta and IL-17 induce the expression of chemokines like monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) together with MMPs<sup>[36]</sup>. IL-11, a chemokine with antiinflammatory properties, is able to inhibit the production of IL-1 beta, TNF-alpha and other proinflammatory cytokines from LPS-stimulated macrophages. Data from animal models show that in the gastrointestinal tract IL-11 can prevent or improve acute and chronic inflammation<sup>[37]</sup>. ISEMFs secrete IL-11 in response to inflammatory cytokines like IL-1 beta, TGF-beta and IL-22. Activated T cells are the main source of IL-22, and this cytokine induces secretion of IL-11 by ISEMF. Mesenchymal cells thus display suppressive immunomodulatory properties<sup>[38]</sup>. ISEMFs provide support for epithelial cell proliferation and regeneration, having a role in the *restitutio ad integrum* in IBD. Evidence is mounting as to their role in forming the niche that accommodates epithelial stem cells and

in determining epithelial cell fate: pericryptal ISEMFs are the main source of morphogenic signaling<sup>[39]</sup>. Wnt signaling for example, has specific functions in the intestinal crypt stem cell region. Through its receptor, Fzd, expressed in both ISEMFs and crypt epithelium, Wnt triggers paracrine and autocrine responses. Nuclear localization of beta-catenin/T cell factor (TCF) is confined to epithelial cells at the bottom of the crypts, and upregulated by Wnt signaling. FoxF proteins are key mesenchymal factors that control Wnt expression and ECM deposition, affecting epithelial cell proliferation, polarization and differentiation<sup>[40]</sup>.

## MSCS AND HSCS: CONTRIBUTION TO INTESTINAL LINEAGES

Adult bone marrow HSCs possess well-known multipotent capacities and are able to restore the entire haemopoietic compartment after myeloablation. The hypothesis that certain adult stem cells might possess a different, greater potential first came from the observation that in human bone marrow transplants donor cells were subsequently found in several recipient tissues<sup>[41]</sup>. The attention of cell biologists moved to the bone marrow mesenchymal compartment and evidence regarding the stem potential of stromal cells soon appeared: the plastic-adhering fibroblast-like population displayed a self renewal capacity, high *in vitro* expansion potential and the ability to differentiate into multiple mesodermal lineages, e.g. osteoblasts, chondrocytes and adipocytes<sup>[42,43]</sup>. These cells, named MSCs and initially isolated from the bone marrow<sup>[44]</sup>, were found to reside in several other tissues. MSCs possess migratory capacity, may be diversely distributed *in vivo* and may occupy a ubiquitous stem cell niche<sup>[45,46]</sup>.

A remarkable amount of evidence exists regarding the multilineage differentiation potential of bone marrow stem cells. Marrow-derived cells have been shown to differentiate towards endodermal<sup>[47,48]</sup>, mesodermal<sup>[49,50]</sup> and ectodermal commitments<sup>[51]</sup>. A unitarian theory on the derivation of the intestinal epithelium from resident stem cells has been developed and widely accepted<sup>[52]</sup>, but a number of recent studies report a bone marrow contribution to gut epithelial and mesenchymal lineages<sup>[53-55]</sup>, even at the single cell level<sup>[56]</sup>. A repopulation of the gastrointestinal tissues occurs in bone marrow-transplanted patients and donor-derived cells engraft with a high efficiency in the areas of mucosal ulceration undergoing regeneration<sup>[57]</sup>. Bone marrow sex-mismatched transplants were performed in mice and a stable engraftment of alpha-SMA+, desmin-, F4/80-, CD34- pericryptal myofibroblasts of donor origin was documented through *in situ* hybridization for the Y chromosome. The analysis of intestinal biopsies from sex-mismatched BM-transplanted patients with GVHD gave similar results<sup>[50]</sup>. Inflammatory cytokines and signals may be strong stimuli for the recruitment of marrow stems in the areas of mucosal damage. The engraftment of BM-derived ISEMFs was investigated by using mouse models of chemo-induced acute colitis and IL-10-/ chronic

colitis. A significant increase in the number of donor-derived ISEMFs was found in the inflamed areas when compared with the normal adjacent mucosa<sup>[58]</sup>.

## MSCS: REGENERATION AND IMMUNE MODULATION

MSCs have been shown to differentiate into a wide range of cell types and to produce a number of growth factors and cytokines that are important for tissue repair and remodeling. Intense studies on the paracrine activity of these cells suggest they are able to participate in tissue healing and long-term repair as trophic mediators<sup>[59]</sup>. Of fundamental interest for the treatment of chronic inflammatory diseases is the fact that MSCs possess the ability to modulate the immune response and to persist at length in the tissues of allogeneic transplanted recipients<sup>[60]</sup>. Cells with mesenchymal stem potential can be isolated from several tissues, with differences in yield and in differentiation capacity<sup>[46,61,62]</sup>. The best characterized MSC population is the one found in the bone marrow where the *in vitro* expansion protocol is almost standardized<sup>[63]</sup> but due to the absence of characteristic markers it is still impossible to prospectively isolate MSCs from fresh samples. Differences among laboratories in expansion potential, differentiation capacity, gene expression and phenotype have been ascribed to slightly different *in vitro* culture and expansion conditions.

Several studies have demonstrated that MSCs possess valuable characteristics for tissue repair or regeneration: these cells have been shown to functionally integrate and remodel bone<sup>[64,65]</sup>, cartilage<sup>[66]</sup> and myocardial tissues<sup>[67-69]</sup>. MSCs display a further remarkable feature, the ability to migrate and home to the sites of injury<sup>[70-73]</sup>. Early clinical trials have shown the benefits of allogeneic MSC transplantation for the treatment of graft-versus-host disease (GVHD)<sup>[74]</sup>. A phase III clinical trial is currently enrolling patients to evaluate the efficacy of Prochymal™, an allogeneic bone marrow derived MSC preparation, for the treatment of steroid refractory acute GVHD<sup>[75]</sup>. Human MSCs express intermediate-low levels of HLA class I, low levels of HLA class II and do not activate allogeneic T cells. This ability to escape alloreactive recognition is probably due to a lack in the expression of costimulatory molecules like B7-1, B7-2, CD 40 and CD 40 ligand<sup>[76,77]</sup>. Moreover, MSCs suppress allogeneic T-cell proliferation and do not elicit an immune response after transplantation in immunocompetent recipients<sup>[69,76,78]</sup>. Several authors have reported the suppression of lymphocyte proliferation in primary mixed leukocyte reactions (MLR) and mitogen responses to phytohemagglutinin (PHA), concanavalin and tuberculin<sup>[79,80]</sup>. Again, MSCs inhibit the T-lymphocyte activation mediated by anti CD3 and CD28 antibodies at primary and *in vitro* expanded cultures<sup>[76,81]</sup>. The molecular mechanisms that enable MSC to abrogate lymphoproliferation are still unknown but several reports claim the importance of soluble factors: modifications in the cytokine balance, such as an increase in IL-2 and IL-10 levels, are

suggested to have lymphosuppressive ability<sup>[82-84]</sup>.

IL-10 is a pleiotropic anti-inflammatory cytokine which potently suppresses antigen presentation by the down-regulation of classical HLA-class I and II, and which inhibits the synthesis of pro-inflammatory cytokines like IFN-gamma, IL-2, IL-3, TNFalpha and GM-CSF<sup>[85]</sup>. Furthermore, IL-10 induces differentiation of regulatory T cells<sup>[86]</sup> and the secretion of soluble HLA-G molecules by activated CD14+ peripheral blood monocytes<sup>[87]</sup>. MSCs have been shown to improve the clinical outcome of autoimmune disease, as a result of suppressive modulation of the pathogenic T-cell autoimmune response<sup>[88]</sup>.

## ADVANCES IN CELL THERAPY: HSC AND MSC TRANSPLANTATION

The long-term management of inflammatory bowel disease depends on the intensity, location, endoscopic severity, clinical manifestations and complications of the disease. Many cellular and molecular pathological pathways have been identified as therapy targets<sup>[89]</sup>.

The common progression into an exacerbated form of IBD requires an escalation from antiinflammatory (e.g. 5-aminosalicylates, corticosteroids), to immunosuppressant (e.g. azathioprine, mercaptopurine, cyclosporin) regimens or to biological drugs (e.g. infliximab anti-TNFalpha, vizilizumab anti-CD3), usually with limited success. Non-responding IBD patients will frequently face the decision to undergo necessary invasive surgical procedures, although surgical intervention will not resolve the disease<sup>[89]</sup>. Since no curative options exist to date, a stem cell-based approach could drive a major change in disease management and treatment.

Impairment in the control of intestinal immune cell function and turnover appears to be of central importance for the dysregulated and protracted inflammatory response in IBD. A high-dose immune ablation regimen could allow detrimental T-lymphocyte repertoires to be eliminated and after HSC transplantation (HSCT) *de-novo* hematopoiesis would generate naive cells. Patients receiving an autologous HSCT are thought to be subject to an immune system reboot: the genetic defects would not be eliminated but remission could persist in the absence of deleterious environmental triggers. HSC allotransplanted patients probably experience a graft-versus-autoreactive (GVA) response and the immune system gets almost completely replaced. These concepts form the basis of a new therapeutic approach to IBD, and a number of case reports show long-lasting remission of IBD patients undergoing HSC or bone marrow transplantation.

In 1993 the first case of CD regression after autologous HSC transplantation for hematopoietic malignancy was reported<sup>[90]</sup>. More cases then started to be recorded: in 1996 one UC and two CD patients treated for coincidental malignancies experienced a remission from IBD after high dose chemotherapy and autologous HSCT<sup>[91]</sup>. In 1998 the case of a 9-year-old patient with CD was reported: after autologous BM transplantation for non-Hodgkin's

lymphoma, a clinical and laboratory CD remission occurred and lasted for at least 7 years<sup>[92]</sup>. In the same year the clinical outcome of six CD patients undergoing allogeneic transplantation for hematological malignancies was reported. In one patient the inactive CD remained inactive for at least 15 years without immunosuppression and in three patients with active CD the pathology became inactive<sup>[93]</sup>. In 2000 the case of a 30-year-old patient with a 10-year history of severe CD was reported: after developing Hodgkin's disease he received an autologous peripheral blood HSCT and remained in remission for both diseases for at least 3 years after transplant<sup>[94]</sup>. In 2001 a case report described the remission of a 57-year-old UC patient after an autologous peripheral blood HSCT performed for breast cancer chemotherapy<sup>[95]</sup>. In 2002 a report showed the case of a CD patient, treated with autologous HSCT for acute myeloid leukemia, who remained in clinical remission from both diseases for 5 years<sup>[96]</sup>. More recently a complete normalization of the Crohn's Disease Activity Index (CDAI) was reported after HSC transplantation in two patients with severe, non responsive, infliximab-resistant CD<sup>[97]</sup>. Two further cases were reported by the same group<sup>[98]</sup>. A phase I HSCT trial involving 12 patients with refractory CD showed evidence of beneficial effects from autologous HSCT<sup>[99]</sup>. Peripheral blood stem cells were mobilized with cyclophosphamide plus G-CSF and CD34+ enriched. Eleven of the 12 patients underwent remission with a significant reduction in the CDAI index. A recent phase I - II study investigated the safety and efficacy of autologous HSCT without CD34+ enrichment in patients with refractory CD: 3 out of 4 patients obtained and maintained clinical and endoscopic remission, despite withdrawal of all drugs<sup>[100]</sup>. All of these reports encourage further studies on HSC transplantation in IBD. A radical ablation of an aberrant immune system followed by autologous reconstitution may regenerate a naïve, non aberrant immune compartment. The European phase III "ASTIC" trial has been designed to investigate the potential clinical benefit of autologous HSCT after high-dose immune ablation in non-responding patients with severe CD.

Transplant conditioning calls for an aggressive immunosuppression regimen that may play a role in inducing remission, but patients with severely impaired mucosal barrier function undergoing such a regimen may worsen their plight and face even more severe consequences. Nevertheless, the management of CD and UC complications is a major goal of therapy. IBD patients develop non-healing, long-lasting ulcerations which are very resistant to common treatments and to advanced surgical cure. MSCs may allow a therapeutic approach targeting the site of injury, aimed at tissue regeneration and at local immune modulation.

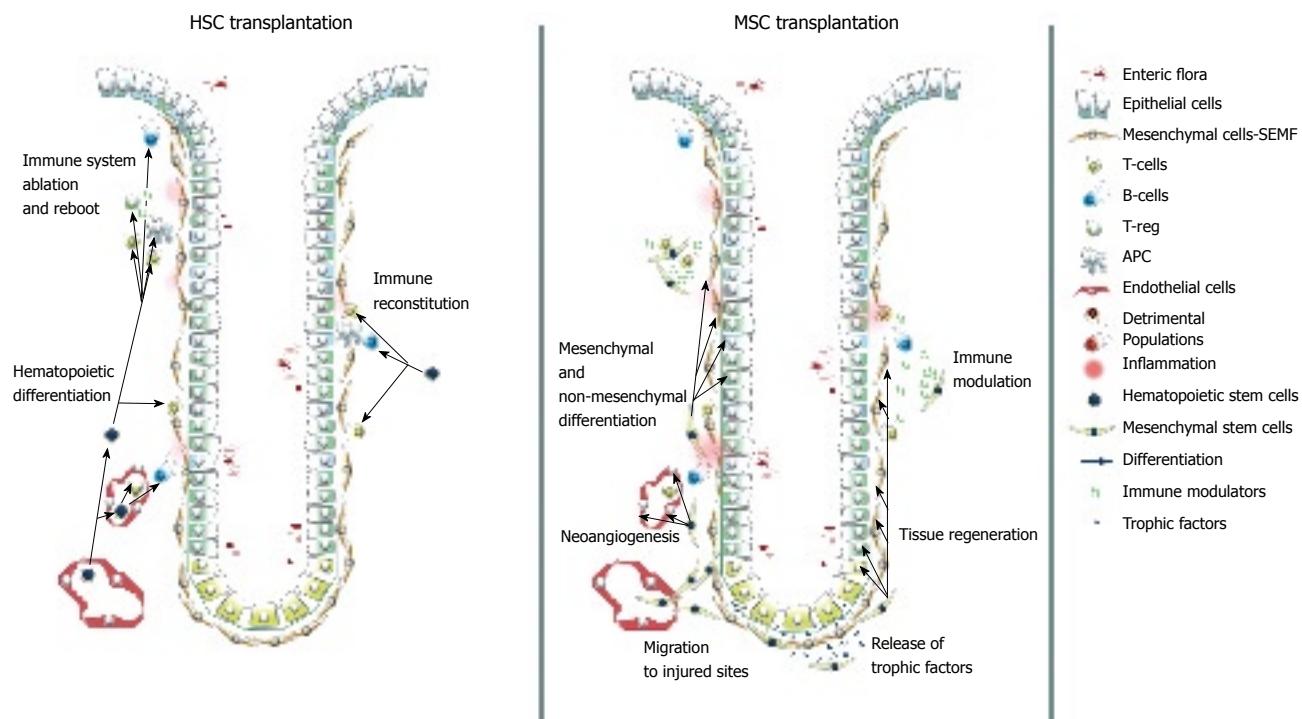
Adipose tissue-derived MSCs<sup>[101]</sup> have been shown to possess promising potential for ulceration healing in perianal manifestations. A young patient with CD and recurrent rectovaginal fistula was treated with autologous stem cell transplantation using lipoaspirate-derived MSCs. At the time of the stem cell therapy the patient had been treated with different surgical and medical procedures, including infliximab infusion, without effective manage-

ment of the manifestations. Autologous adipose tissue derived MSCs were isolated, expanded for three or fewer passages and injected into the rectal mucosa; the rectal opening approaching from the posterior vaginal wall was closed with absorbable stitches prior to cell injection. A complete closure of the wound was demonstrated one week after the injection without any adverse events<sup>[102]</sup>.

The group designed a prospective Phase I clinical trial involving 5 CD patients to study the safety and the efficacy of stem cell transplantation in perianal manifestations and complications using autologous adipose tissue-derived stem cells. Three  $\times 10^6$  to  $30 \times 10^6$  cells were injected directly under each lesion. Healing of the fistula was considered achieved when a total epithelialization of the external opening was evident. They observed complete healing in six of the eight procedures<sup>[103]</sup>. These reports describe an innovative approach to the local treatment of CD manifestations, based on *in situ* delivery of autologous MSCs. Whether MSCs contribute to tissue regeneration by direct differentiation, by trophic effect or by immunomodulatory functions has not been investigated yet and could become a matter of concern. Prochymal™, developed by Osiris Therapeutics Inc.<sup>[75]</sup>, is a preparation for intravenous infusion of bone marrow-derived MSCs obtained from healthy adult donors. The Prochymal™ phase II clinical trial was a prospective, randomized and open label trial. Patients with moderate to severe CD (CDAI > 220), who had previously failed treatment with steroids, infliximab and other immunosuppressive agents, were enrolled and received two infusions of the preparation. Every patient evaluated reported a reduction in CDAI, and a statistically significant decrease in mean CDAI scores, from 341 to 236, occurred by day 28 after the infusions. Improvement was rapidly obtained, with an average CDAI reduction of 62 points by day 7. One-third of the patients reported Inflammatory Bowel Disease Questionnaire (IBDQ) scores of at least 170, indicating they had achieved clinical remission of their disease. The Food and Drugs Administration recently allowed Prochymal™ to advance to a phase III double-blind placebo-controlled trial via Fast Track for the treatment of CD. After GvHD, CD is the second indication for which Osiris received Fast Track status to advance to Phase III. Osiris received orphan drug designation from the FDA and the European Medicine Agency (EMEA) for Prochymal™. This intravenous preparation of MSCs may have peripheral immunomodulatory functions, leading to abrogation of the pathological inflammation typical of CD. MSCs may contribute to tissue regeneration by direct differentiation toward intestinal lineages<sup>[104]</sup>, but they may also have trophic functions in the healing environment and their immunomodulatory ability may arrest disease protraction (Figure 2).

## CONCLUSION AND FUTURE PROSPECTS

IBD are thought to be the result of an abnormal immune response to commensal bacteria and luminal antigens in a susceptible host. The intermittent and



**Figure 2** Contribution of transplanted HSCs and MSCs to the immune compartment and to intestinal lineages. HSCs are able to restore the immune system to a naive state. MSCs contribute via direct differentiation to mesenchymal and non-mesenchymal lineages, exert trophic functions and modulate in a suppressive fashion the immune response.

aggressive presentation of the pathology profoundly affects the quality of life of the patients. The inadequacy of conventional therapies and the current understanding of IBD biology are motivating investigators to develop novel approaches to IBD treatment: the advancement in stem cell-based therapies could drive a major change.

Having to cope with exuberant commensal flora and abundant non-self antigens, a number of cell populations cooperate to control intestinal mucosa integrity and to maintain a status of persistent physiological inflammation. Impairments in immunological and regenerative functions at this level lead to IBD. The behavior of classical immune cells appears dysregulated in IBD patients. T-lymphocytes are considered central effector cells, responsible for the release of cytokines implicated in the onset and in the protraction of the inflammation. A lack of suppression is probably the cause of the abnormal and persistent activation of the T-cell compartment. Immunomodulatory activity, impaired in IBD, is carried out by populations of T regulators and by cell populations traditionally considered not involved in immunity. Aberrant epithelial and endothelial cell functions concur in the pathogenesis. Epithelial cells act as non-professional antigen presenting cells: defective expression of molecules implicated in the expansion of immunoregulatory T-cells may cause a failure in the immune inhibition. Deficits in cells of the microvasculature causing inadequate perfusion could account for ineffective ulceration healing. A set of intestinal subepithelial myofibroblasts (ISEMFs), a mesenchymal population juxtaposed to the mucosal epithelial layer exerting trophic and immunomodulatory effects, become activated

in IBD and impair correct tissue remodeling processes, so that the *restitutio ad integrum* ultimately fails (Figure 1). Stem cells residing in the bone marrow have been shown to contribute with their progeny to all of these intestinal lineages and may be of value and interest in clinical settings. Clinical improvements in IBD patients have been reported after allogeneic and autologous transplantation of hematopoietic and MSCs. After immune ablation and reconstitution via HSC transplantation, a reboot to a naive immune system could bring about long-lasting remission. In autologous transplantation genetic defects would persist, but the indulgence may last in the absence of environmental triggers. In allogeneic transplantation the immune replacement would be more radical and potentially more reliable, but the risks associated with graft rejection and complications are probably too high for application in IBD. MSC only received the attention of IBD investigators in recent times. Experimental and clinical data indicate that MSCs have great potential for those clinical applications that require tissue regeneration or repair promotion, owing to their plasticity and their immunomodulatory properties. The biology of this stem population is still largely obscure, but their impressive differentiation potential, combined with exceptional trophic and immunomodulatory capacities, could make MSC an outstanding tool in IBD treatment (Figure 2). Pioneering works have reported impressive results: in mouse models and in IBD patients treated for coincidental malignancies, bone marrow-derived cells of donor origin contribute to tissue repair by differentiating towards the epithelial, myofibroblastic, endothelial and pericytic lineages in the areas of inflammation. Bone

marrow transplantation from an unaffected donor is able to ameliorate pathology in a mouse model of chronic genetic-based colitis. Moreover, CD34- bone marrow- and peripheral blood-derived stem cells contributed to mucosal repair *via* neoangiogenesis in moderate-severe murine colitis and were effective in reducing the pathologic features associated with IBD<sup>[104]</sup>. Pivotal trials have demonstrated the efficacy of MSC transplantation in patients with CD, though the mechanisms of action underlying the clinical effects of MSCs still need clarifying and the follow-up periods need to be extended. Both HSC and MSC transplantation in IBD are currently being evaluated in Phase III clinical trials.

Ways of recognizing easily accessible and non-controversial new sources of pluripotent stem cells, such as term extraembryonal tissues<sup>[62,105]</sup> as well as improving methods for *ex vivo* isolation, expansion and delivery may become of central interest. Moreover, MSCs displaying pluripotent, immunomodulatory and trophic potential, and suitable for implanting without manipulation in an allogeneic setting, would have obvious implications and may be promising “off the shelf” therapeutic approaches to IBD. In the future, the possibility of banking cord-blood derived HSC and placenta-derived MSCs<sup>[106]</sup>, could enable these stem cells to be used both in autologous and in allogeneic transplantation settings.

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# Clinical role of <sup>18</sup>F-fluorodeoxyglucose positron emission tomography/computed tomography in post-operative follow up of gastric cancer: Initial results

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PET/CT, the final diagnosis was recurrence of mucinous adenocarcinoma in the anastomosis in one patient and abdominal wall metastasis in the other. Importantly, PET/CT revealed true-positive findings in 11 (47.8%) patients who had negative or no definite findings by CT. PET/CT revealed extra-abdominal metastases in 7 patients and additional esophageal carcinoma in one patient. Clinical treatment decisions were changed in 7 (30.4%) patients after introducing PET/CT into their conventional post-operative follow-up program.

**CONCLUSION:** Whole body <sup>18</sup>F-FDG PET/CT was highly effective in discriminating true recurrence in post-operative patients with gastric cancer and had important impacts on clinical decisions in a considerable portion of patients.

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**Key words:** <sup>18</sup>F-fluorodeoxyglucose; Positron emission tomography/computed tomography; Gastric cancer; Follow-up; Recurrence

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## Abstract

**AIM:** To evaluate the clinical role of <sup>18</sup>F-fluorodeoxyglucose positron emission and computed tomography (<sup>18</sup>F-FDG PET/CT) in detection of gastric cancer recurrence after initial surgical resection.

**METHODS:** In the period from January 2007 to May 2008, 23 patients who had previous surgical resection of histopathologically diagnosed gastric cancer underwent a total of 25 <sup>18</sup>F-FDG PET/CT scans as follow-up visits in our center. The standard of reference for tumor recurrence consisted of histopathologic confirmation or clinical follow-up information for at least 5 mo after PET/CT examinations.

**RESULTS:** PET/CT was positive in 14 patients (61%) and negative in 9 (39%). When correlated with final diagnosis, which was confirmed by histopathologic evidence of tumor recurrence in 8 of the 23 patients (35%) and by clinical follow-up in 15 (65%), PET/CT was true positive in 12 patients, false positive in 2, true negative in 8 and false negative in 2. Overall, the accuracy of PET/CT was 82.6%, the negative predictive value (NPV) was 77.7%, and the positive predictive value (PPV) was 85.7%. The 2 false positive PET/CT findings were actually chronic inflammatory tissue lesions. For the two patients with false negative

## INTRODUCTION

Gastric cancer is the second most common cause of cancer death worldwide<sup>[1]</sup>. Within the Asian region, high incidence areas include Japan, China and Korea. It is a major health burden in the Asia-Pacific region<sup>[2]</sup>. Complete surgical resection of gastric cancer is considered potentially curative, but its long-term survival is frequently reported as poor. In fact, despite successful surgery, the five-year survival rate is approximately 35%; and even with adjuvant chemoradiotherapy in selected patients, the survival rate is 40%<sup>[3]</sup>. After curative surgery,

about 80% of the patients die within a short period of time from locoregional recurrence (87%) and/or distant metastasis (30%)<sup>[4]</sup>.

Positron emission tomography (PET) and, particularly, positron emission tomography/computed tomography (PET/CT) are widely accepted imaging methods in the management of a variety of cancers<sup>[5]</sup>. Variable uptake of <sup>18</sup>F-fluorodeoxyglucose (<sup>18</sup>F-FDG) has been noticed in PET studies of gastric carcinoma patients, with low uptake occurring especially in some particular histological subtypes and early carcinomas. Larger or more advanced tumors with nodal involvement have a higher detection rate by PET. In the initial staging of gastric cancer, preoperative PET was useful for the detection of nodal involvement and distant metastasis<sup>[6,7]</sup>. The role of PET/CT in patients with gastric cancer after operation however, is not clear. In this study, we aimed to analyze the value of <sup>18</sup>F-FDG PET/CT scans in the follow up of post-operative patients with gastric cancer.

## MATERIALS AND METHODS

### **Patients**

A retrospective review of our electronic database of 23 post-operative patients with gastric cancer (15 males and 8 female; age range: 27-84 years; mean age: 55.4 years) imaged by <sup>18</sup>F-FDG PET/CT between January 2007 and May 2008 was performed to select and analyze PET/CT scan findings for patients with or without clinically and/or radiologically suspicious findings for tumor recurrence. Only the patients with correlative histopathological data were included. The standard of reference for tumor recurrence consisted of histopathologic confirmation ( $n = 8$ ) or clinical follow-up information ( $n = 15$ ) for at least 5 mo after PET/CT.

### **<sup>18</sup>FDG PET/CT technique**

The patients were asked to fast for at least 4 h before undergoing <sup>18</sup>F-FDG PET/CT. Their blood glucose level should be within the normal range (70-120 mg/dL) prior to intravenous injection of <sup>18</sup>F-FDG. The patients received an intravenous injection of 370-666 MBq (10-18 mCi) of <sup>18</sup>F-FDG. Data acquisition by an integrated PET/CT system (Discovery STE; GE Medical Systems, Milwaukee, WI, USA) was performed within 60 min after injection. The data acquisition procedure was as follows: CT scanning was first performed, from the head to the pelvic floor, with 110 kV, 110 mA, a tube rotation time of 0.5 s, and a 3.3-mm section thickness which was matched to the PET section thickness. Immediately after CT scanning, a PET emission scan that covered the identical transverse field of view was obtained. Acquisition time was 3 min per table position. PET image data sets were reconstructed iteratively by applying the CT data for attenuation correction, and coregistered images were displayed on a workstation.

### **Definitive diagnoses of positive and negative findings**

Reviewer 1 and Reviewer 2, who were aware of other clinical or imaging information, read the <sup>18</sup>F-FDG PET/

**Table 1 Patient characteristics**

Clinical characteristics	Data
Mean age (yr)	55.4
Gender	
Male	15
Female	8
Mean time after operation to PET/CT exam	2 mo-10 yr; mean 25 mo
Mean follow up time after PET/CT exam	5-18 mo; mean 9 mo

CT images on a high-resolution computer screen. The reviewers reached a consensus in cases of discrepancy. Reviewer 1 had 20 years of experience in both nuclear medicine and radiology, and Reviewer 2 had 5 years of experience in both nuclear medicine and radiology. <sup>18</sup>F-FDG PET/CT scan was considered positive or suspicious when abnormal non-physiologic metabolic activity was identified. Focal hypermetabolic activity within the liver which was greater than adjacent normal liver tissue was considered abnormal. Diffuse mild activity in the intestinal tract was considered normal physiologic uptake. Quantification of tumor metabolic activity was obtained using the Standardized Uptake Value (SUV) normalized to body weight. Mean  $\pm$  SD of maximum-pixel SUV (SUVmax) of the lesions were calculated.

## RESULTS

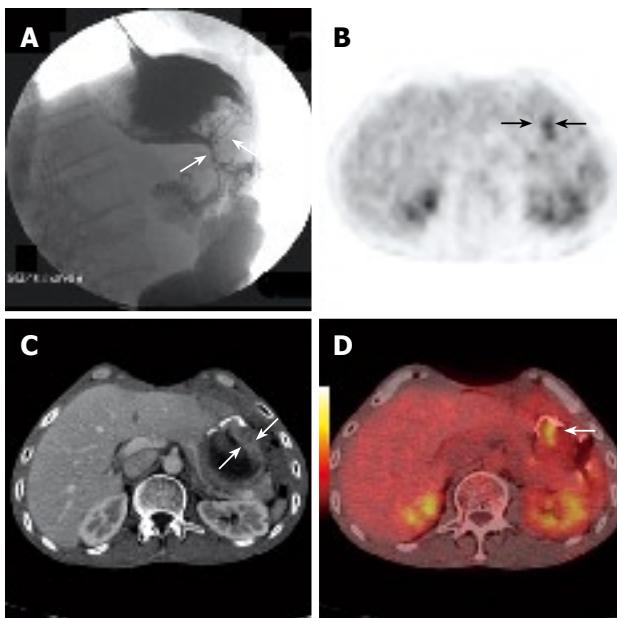
A total of 25 <sup>18</sup>F-FDG PET/CT studies in 23 patients after gastric cancer were reviewed. <sup>18</sup>F-FDG PET/CT was ordered in 12 patients due to suspected disease recurrence on conventional image examinations, on history and physical exam. The remaining 11 patients underwent <sup>18</sup>F-FDG PET/CT as part of routine post-operative follow-up. The characteristics of the patients are summarized in Table 1. At the time of recurrent gastric cancer being suspected, the mean patient age was 57 years with a tendency to male gender distribution (65%). All the patients had undergone surgical resection, with chemotherapy prior to or following the resection. Surgical exploration was undertaken within 1 mo after <sup>18</sup>F-FDG PET/CT scan in 5 patients.

<sup>18</sup>F-FDG PET/CT scan was considered negative in 9 patients (39%) and positive in 14 (61%, Table 2). Final diagnoses were confirmed by histopathologic evidence of tumor recurrence in 8 of the 23 patients (Figure 1) and by clinical follow-up in 15 patients (Figure 2). Overall, the accuracy of <sup>18</sup>F-FDG PET/CT diagnosis was 82.6%, negative predictive value (NPV) was 77.7%, and positive predictive value (PPV) was 85.7%. Of the 14 positive <sup>18</sup>F-FDG PET/CT scans, 12 were true positive and two were false positive. For the two patients with false positive <sup>18</sup>F-FDG PET/CT scans, the final diagnosis was anastomosis inflammation with high metabolism. Seven patients were found to have true negative <sup>18</sup>F-FDG PET/CT scans while two were false negative. For the patients with false negative <sup>18</sup>F-FDG PET/CT scans, the final diagnosis was recurrence of mucinous adenocarcinoma in the anastomosis in one patient and metastasis in the abdominal wall of another patient (Figure 3).

**Table 2** PET/CT findings in 14 patients with positive PET/CT scans

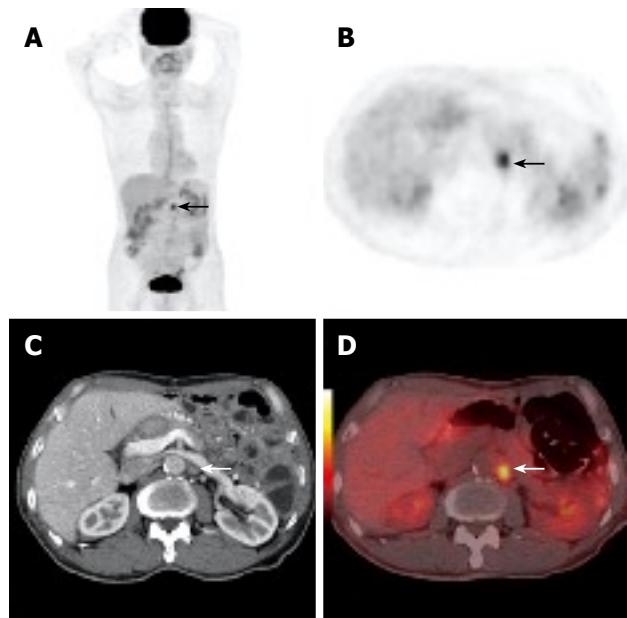
No.	PET/CT findings	SUV max	Pathology of resected GC	Interval time
1	Retroperitoneal lymph nodes	6.0	MPD tubular adeno ca and mucinous adeno Ca	12 mo
2	Retroperitoneal lymph nodes	5.2	PD adeno ca and signet-ring cell Ca	3 mo
	Supraclavicular lymph nodes	6.6		
	Osseous metastasis	3.8		
3	Esophageal carcinoma	14.2	MPD adeno Ca	10 yr
4	Anastomosis recurrence with pancreas involvement	5.6	PD adeno Ca	5 mo
5	Osseous metastasis	2.5	MPD adeno Ca	25 mo
6	Liver metastasis	7.2	MPD signet-ring cell Ca	15 mo
7	Greater omentum lymph nodes	1.3	PD signet-ring cell Ca	8 mo
8	Abdominal wall metastasis	2.5	PD signet-ring cell Ca	24 mo
	Ovarian metastasis			
9	Liver metastasis	5.3	PD adenosquamous Ca	3 mo
	Intraperitoneal lymph nodes	17.6		
10	Abdominal wall metastasis	3.2	MPD adeno Ca	24 mo
11	Intraperitoneal lymph nodes	7.7	MPD adeno Ca	36 mo
12	Brain metastasis	8.1	PD adeno Ca	6 mo
13	Osseous metastasis	12.6	MPD adeno Ca	20 mo
	Retroperitoneal lymph nodes	7.0		
14	Anastomosis recurrence	4.3	MPD adeno Ca	5 mo

MPD: Moderately to poorly differentiated; PD: Poorly differentiated; GC: Gastric cancer; Interval time: Time period from operation to PET/CT scan; Ca: Carcinoma.



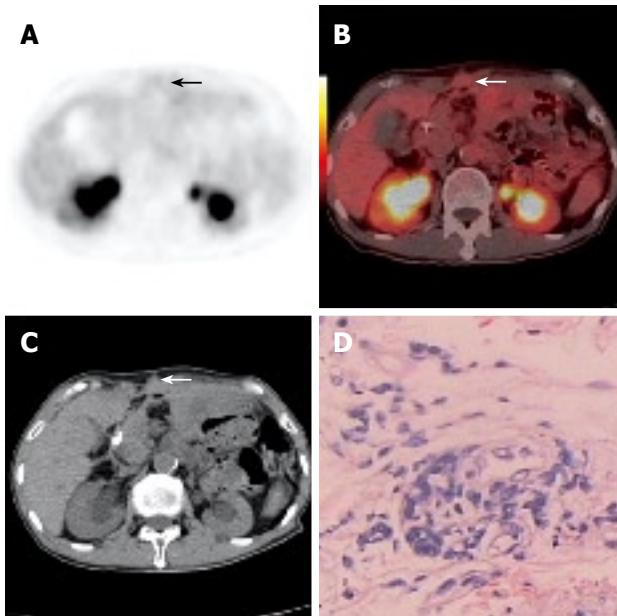
**Figure 1** A 56-year-men who had had gastric cancer resection 3 years previously underwent PET/CT because of suspected disease recurrence upon barium swallow examination (white arrows, A), Axial contrast CT demonstrated local thickened stomach wall at anastomosis (white arrows, C). Axial PET (black arrows, B) and PET/CT fusion images (white arrow, D) showed focal hypermetabolic activity in the remnant stomach, which was later verified as malignant by histopathology.

Importantly, tumor recurrence was revealed in 27.2% (3/11) of the patients who underwent PET/CT as part of routine post-operative surveillance; these patients were asymptomatic, with no evidence of disease. <sup>18</sup>F-FDG PET/CT revealed true-positive findings in 11 patients who had either negative or no definite CT findings. <sup>18</sup>F-FDG PET/CT demonstrated extra-abdominal



**Figure 2** A 75-year-old asymptomatic man who had gastric cancer resection 1 year previously underwent PET/CT as part of routine post-operative surveillance. Whole body PET projection image and axial PET image showed focal hypermetabolic activity in the abdomen (black arrow, A and B). Axial contrast CT detected a small lymph node at the same position (white arrow, C). PET/CT fusion images showed a focus of highly metabolic metastasis in retroperitoneal lymph node (white arrow, D). This was later verified by follow up. The case illustrated the value of early discovery by PET/CT in asymptomatic patients after surgery.

metastases in 7 patients (Figure 4) and new esophageal carcinoma in one patient. Clinical treatment decisions were changed in 7 (30.4%) patients after introducing <sup>18</sup>F-FDG PET/CT into their conventional post-operative follow up program.



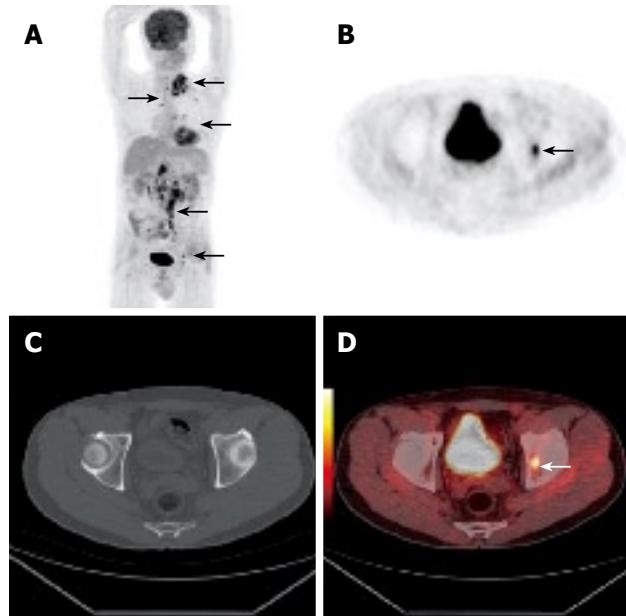
**Figure 3** A 71-year-old asymptomatic man underwent PET/CT as part of routine post-operative surveillance after gastric cancer resection was performed 2.5 years previously. Axial PET and PET/CT fusion images (arrows, **A** and **B**) showed no focal hypermetabolic activity in the abdominal wall. Axial contrast CT (white arrow, **C**) demonstrated local thickness in the abdominal wall. This was later verified as malignant by histopathological assessment of a CT guided core tissue biopsy (**D**).

In our study, 11 (84.5%) cases of recurrence after curative resection occurred within 25 mo and 2 (15.5%) occurred 3 years after the resection. A high percentage of first failures presented as regional nodal metastases and distant metastases. Only 4 (17.3%) patients with recurrence had increased serum tumor markers.

## DISCUSSION

At the time of diagnosis, gastric cancer is found to be localized and surgically resectable in approximately 50% of patients; however, regional nodal metastases or direct invasion into surrounding organs or structures are frequently encountered and preclude cure by surgery alone in many patients<sup>[8,9]</sup>. Analyses of patterns of relapse after complete surgical resection demonstrate that subsequent relapse of cancer is common in the tumor bed and nodal regions as well as all over the body<sup>[10,11]</sup>. The optimal method for assessing early recurrence in patients with gastric cancer is unknown<sup>[12]</sup>. Conventional imaging (ultrasonography, CT and magnetic resonance imaging) has represented the standard for staging and restaging of gastric cancer<sup>[13,14]</sup>. Conventional imaging is noninvasive and is the least costly of the available methods, although it has limited value in differentiating post-surgical changes from local tumor recurrence. Therefore, these techniques have limitations in terms of accurate assessment of re-staging results<sup>[15,16]</sup>.

Modern cancer care is critically dependent on imaging technologies, which are used to detect early tumors and guide their therapy or surgery<sup>[17,18]</sup>. Molecular imaging technologies provide information about the



**Figure 4** A 67-year-old man underwent PET/CT due to clinically suspected disease recurrence in supraclavicular lymph nodes after his gastric cancer resection which was performed 2 mo previously. Whole body PET projection image showed retroperitoneal and supraclavicular metastatic lymph nodes and bone metastasis. (arrows, **A**, **B** and **D**). Axial CT failed to reveal early bone metastasis (**C**).

functional or metabolic characteristics of malignancies, tumor stage and therapeutical response, and tumor recurrence; whereas conventional imaging technologies predominantly assess anatomical or morphologic features of the tumor including its size, density, shape, etc<sup>[19,20]</sup>. There are now various indications for <sup>18</sup>F-FDG PET and PET/CT imaging in gastrointestinal malignancies<sup>[21]</sup>. Detection and staging of recurrent colorectal cancer with <sup>18</sup>F-FDG PET has largely been documented<sup>[22,23]</sup>. However, there is a paucity of data concerning its role in patients with suspected gastric cancer recurrence. In our study, <sup>18</sup>F-FDG PET/CT could be applied reliably in such patients to allow for earlier detection of nodal involvement and distant metastasis in 3 asymptomatic patients. Because most metastatic gastric cancers are inoperable, they are usually treated with combination chemotherapy. Early detection and treatment of tumor recurrence may be the only hope to improve long-term survival and made the early management of recurrent gastric cancer possible in three patients in our study.

<sup>18</sup>F-FDG PET/CT is highly effective in discriminating true recurrence in patients with suspected recurrence, highly sensitive for detecting recurrence in asymptomatic patients, and has important impacts on clinical decisions in a considerable portion of patients<sup>[24]</sup>.

<sup>18</sup>F-FDG-PET is also of benefit in assessing response to neoadjuvant treatment<sup>[25]</sup>. <sup>18</sup>F-FDG PET/CT has been demonstrated as a useful molecular imaging modality to evaluate the biological response of advanced hepatic metastasis and peritoneal carcinomatosis to Cetuximab plus Endostar in patients after remnant gastric cancer resection<sup>[26]</sup>. In our study, there was a 55-year-old male who developed advanced hepatic metastasis and

peritoneal carcinomatosis after resection of remnant gastric cancer resection 3 mo previously. The patient received a treatment only by epidermal growth factor (EGF) receptor antibody (Cetuximab) plus recombinant human endostatin (Endostar). Anti-tumor activity was assessed by <sup>18</sup>F-FDG PET/CT at baseline and every 4 wk thereafter.

<sup>18</sup>F-FDG PET can reveal recurrent sites in the intraperitoneum, liver, lungs, bones and retroperitoneal lymph nodes<sup>[27,28]</sup>. However, the minimum tumor size detectable by PET depended on the sites of recurrence. In the two false-negative cases of intraperitoneal tumors suggested by CT images, <sup>18</sup>F-FDG PET/CT was able to detect a solitary small intraperitoneal mass, which was very difficult to reveal with CT alone<sup>[29]</sup>. <sup>18</sup>F-FDG PET/CT imaging was also able to detect normally sized metastatic lymph nodes in 4 patients with retroperitoneal metastasis. Our results demonstrated that the application of PET/CT imaging was useful for early detection of recurrent sites, especially for decision making in determining treatment strategy for patients with recurrent gastric cancer. Positive PET/CT findings did not affect the prognosis in 7 of the 14 recurrent patients; however, the remaining 7 patients consequently underwent combination therapy consisting of surgery and chemotherapy and survived for more than 10 mo after the positive <sup>18</sup>F-FDG PET/CT results.

Diagnostic contrast-enhanced multiphase CT as part of the combined <sup>18</sup>F-FDG PET/CT protocol has the potential to provide considerable augmented value in specific clinical conditions with resultant change of management in a substantial proportion of patients<sup>[30]</sup>. The greatest benefit of its diagnostic CT is in category localization of pathological FDG uptake and precise tumor delineation, making changes in <sup>18</sup>F-FDG PET/CT interpretation for some patients<sup>[31]</sup>. The reported increase in sensitivity of PET/CT over CT has been attributed to the ability of <sup>18</sup>F-FDG PET to detect metabolic abnormalities that precede the morphologic changes seen by CT. The global (from skull base to proximal thighs) nature of the <sup>18</sup>F-FDG PET/CT study also contributes to the increased sensitivity through detection of distant metastatic lesions. In our study, <sup>18</sup>F-FDG PET/CT demonstrated extra-abdominal metastases (metastases in lymph nodes, the abdominal wall, bones, and the brain) in 7 patients and in one gastric cancer patient who had the comorbidity of a new esophageal carcinoma, which was confirmed by post-operative pathology.

Our study had several limitations. The first was our small sample size which may have limited the robustness of our study in terms of statistics. The second was the retrospective nature of our study. Because of this nature, we were unable to obtain baseline clinical and laboratory data in some of the patients, except for one patient who accepted three PET/CT scans during his treatment. Additionally, there are some disadvantages associated with PET/CT imaging. For example, small, early-stage tumors may go undetected because partial-volume effects result in a falsely low measurement of true <sup>18</sup>F-FDG activity, as was indicated in our

previous report<sup>[32]</sup>. Another drawback of PET/CT is that <sup>18</sup>F-FDG frequently accumulates in areas of inflammation<sup>[33,34]</sup>. In our study, two patients had false positive PET/CT scan results, and the final diagnosis was anastomosis inflammation with high metabolism. Low <sup>18</sup>F-FDG uptake occurred especially in some particular histological subtypes or at special locations. For the two patients with false negative PET/CT findings, the final diagnosis was recurrence of mucinous adenocarcinoma in the anastomosis in one patient and abdominal wall metastasis in the other.

In conclusion, whole body <sup>18</sup>F-FDG PET/CT was highly effective in discriminating true recurrence in gastric cancer patients with suspected recurrence and highly sensitive in detecting recurrence in asymptomatic patients. This had important impacts on clinical decisions in a considerable portion of these patients. Any positive finding in post-operative patients with gastric cancer that could lead to a clinically significant change in patient management should be confirmed by subsequent histopathologic examination because of the risk of false-positive results.

## COMMENTS

### Background

After curative surgery, about 80% of patients die within a short period of time from locoregional recurrence and/or distant metastasis. The optimal method for assessing early recurrence in patients with gastric cancer is not clear. This study aimed to analyze the value of <sup>18</sup>F-fluorodeoxyglucose positron emission tomography/computed tomography (<sup>18</sup>F-FDG PET/CT) in the follow up of post-operative patients with gastric cancer.

### Research frontiers

<sup>18</sup>F-FDG PET and, particularly, <sup>18</sup>F-FDG PET/CT are widely accepted imaging methods in the management of a wide variety of cancers. However, the role of PET/CT in patients with gastric cancer after surgery is not clear.

### Innovations and breakthroughs

Whole body <sup>18</sup>F-FDG PET/CT was highly effective in discriminating true recurrence in gastric cancer patients with suspected recurrence and highly sensitive in detecting recurrence in asymptomatic patients. This had important impacts on clinical decisions in a considerable portion of these patients.

### Applications

The findings could be helpful for early discrimination of true recurrence in gastric cancer patients with suspected recurrence and highly sensitive at detecting recurrence in asymptomatic patients, especially when recurrence can not be accurately diagnosed by conventional imaging.

### Peer review

In this study, the authors reported the clinical significance of <sup>18</sup>F-FDG PET/CT for early discrimination of true recurrence in gastric cancer patients. This manuscript arouses interest for readers and provides an important clue to assess whole body, early recurrence in gastric cancer patients. The paper is scientific and innovative and is for rapid communication part of journal.

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# Lentivirus mediated shRNA interference targeting MAT2B induces growth-inhibition and apoptosis in hepatocellular carcinoma

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## Abstract

**AIM:** To investigate the effects of lentivirus vector mediated short hairpin RNA interference targeting methionine adenosyltransferase 2 $\beta$  gene (LV-shMAT2B) on hepatocellular carcinoma (HCC) cells.

**METHODS:** We constructed four plasmids of RNA interference targeting the MAT2B gene. After LV-shMAT2B was transfected with L-02 cells and two kinds of HCC cells, cell viability and proliferation were measured with MTT and [<sup>3</sup>H]thymidine assays respectively. Flow cytometry was used to assess cell apoptosis. The level of S-adenosyl methionine (SAMe) in HepG2 cells was evaluated. The expressions of cyclin D1, cyclin D2, bcl-x<sub>L</sub> and bcl-x<sub>S</sub> were detected with western blot.

**RESULTS:** We constructed LV-shMAT2B successfully. LV-shMAT2B was safe for human normal liver cells. LV-shMAT2B caused dramatic reduction in proliferation compared with controls in HCC cells Bel-7402 ( $P = 0.054$ ) and HepG2 ( $P = 0.031$ ). Flow cytometry analysis showed that cell apoptosis caused by LV-shMAT2B was greater in HCC cells Bel-7402 and HepG2 than in control induced by scrambled siRNA ( $P = 0.047$ ), but apoptosis rates in L-02 induced by LV-shMAT2B and scrambled siRNA respectively had no significant difference. Moreover, LV-shMAT2B significantly suppressed expression of MAT2B leading to growth-inhibition effect on HCC cells by down-regulating cyclin D1. Apoptosis induced by LV-shMAT2B was involved in

down-regulating bcl-x<sub>L</sub> and up-regulating bcl-x<sub>S</sub>.

**CONCLUSION:** LV-shMAT2B can induce cell apoptosis and growth-inhibition in HCC cells. MAT2B may be a therapy target in HCC in the future.

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**Key words:** Lentivirus; Methionine adenosyltransferase 2 $\beta$  gene; Growth inhibition; Apoptosis; Hepatocellular carcinoma

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## INTRODUCTION

In mammals, methionine adenosyltransferase 1A (MAT1A) and MAT2A encode two homologous MAT catalytic subunits, MAT $\alpha_1$  and MAT $\alpha_2$  respectively. MAT1A is expressed mostly in liver and encodes MAT $\alpha_1$  found in two native MAT isozymes, which are either a dimer (MATIII) or tetramer (MAT I) of this single subunit. MAT2A encodes MAT $\alpha_2$  found in a native MAT isozyme (MAT II) which is associated with a catalytically inactive regulatory subunit ( $\beta$ ) in lymphocytes encoded by the MAT2B gene. MAT2A predominates in the fetal liver and is progressively replaced by MAT1A during liver development<sup>[1,2]</sup>. It has been demonstrated that a switch in MAT expression in liver cancer (from MAT1A to MAT2A) played an important pathogenetic role in facilitating the tumorigenesis of liver cancer<sup>[3]</sup>. The switch in MAT expression of liver cancer is important, because it offers cancerous cells a growth advantage as well as a decrease in intracellular S-adenosyl methionine (SAMe) content. The function of the  $\beta$  subunit is to regulate MATII activity by lowering its  $K_m$  for L-methionine and by increasing the sensitivity of the enzyme to feedback inhibition by SAMe<sup>[4]</sup>. Therefore, regulation

of the expression of the  $\beta$  subunit may be a mechanism to regulate the intracellular content of SAMe. The importance of MAT expression on SAMe level and liver phenotype has been confirmed in the MAT1A knockout mouse model. In this research, the replacement of MAT1A with MAT2A has resulted in chronic hepatic SAMe depletion and eventual development of hepatocellular carcinoma (HCC)<sup>[5,6]</sup>. SAMe has also been found to be antiapoptotic in cultured rat hepatocytes but proapoptotic in human hepatoma cells<sup>[7]</sup>.  $\beta$  subunit has been proved to be associated with cirrhosis and cancer by providing a proliferative advantage in hepatoma cells through its interaction with MAT II  $\alpha_2$  and down-regulation of SAMe levels<sup>[8]</sup>. Since the decreased content of intracellular SAMe has been proved to be related with the increased expression of the MAT2B gene, we wanted to know whether cancerous cell growth advantage or SAMe depletion could be prevented by knockout of MAT2B. So we constructed lentivirus vector mediating RNA interference targeting MAT2B (LV-shMAT2B). The efficacy of siMAT2B plasmids in interference with MAT2B was confirmed by two different ways. We found that LV-shMAT2B promoted growth-inhibition and apoptosis in human hepatocellular cancer cells by markedly increasing the intracellular content of SAMe and the expression of bcl-x<sub>S</sub> and decreasing the expressions of cyclin D1 and bcl-x<sub>L</sub>. But it had no effect on normal liver cells.

## MATERIALS AND METHODS

### **Construction and production of Lentivirus vectors**

The construction and production of Lentivirus vectors were previously described<sup>[9]</sup>. We designed and cloned a short hairpin siRNA template into a lentivirus vector. A third generation of self-inactivating lentivirus vector containing a CMV driven GFP reporter and a U6 promoter upstream of cloning restriction sites (Age I and EcoR II) to allow the introduction of oligonucleotides encoding shRNAs (Figure 1A) was as described previously<sup>[10]</sup>. We constructed the scrambled siRNA (as a control) and four siMAT2B lentivirus vectors, namely LV-shiMAT2B-1, LV-shMAT2B-2, LV-shMAT2B-3 and LV-shMAT2B-4, targeting human MAT2B from corresponding siMAT2B plasmids (pGCL-GFP-siMAT2B). Each hairpin consisted of a 20-21 nt sense sequence, a short spacer (CTCGAG), the antisense sequence, 5Ts (a stop signal for RNA polymerase III), and an Age I site<sup>[10,11]</sup>. The oligos were annealed and inserted between the Age I and EcoR II sites of the plasmid. Some mutations were introduced in the sense sequence of the hairpin structure to facilitate sequence and avoid destruction by bacteria during amplification in bacterial host<sup>[12]</sup>. Correct insertions of shRNA cassettes were confirmed by restriction mapping and direct DNA sequencing. Recombinant lentivirus vectors were produced by co-transfected 293T cells with the lentivirus expression plasmid and packaging plasmids (pHelper 1.0 including gag/pol and pHelper 2.0 including VSVG) using the calcium phosphate

method<sup>[13-15]</sup>. Infectious lentivirus vectors were harvested at 48 and 72 h post-transfection, centrifuged to get rid of cell debris, and then filtered through 0.22 cellulose acetate filters<sup>[16]</sup>. The infectious titer was determined by fluorescence-activated cell sorting analysis of GFP positive in 293 cells. The virus titers were at the range of  $10^9$  transducing units/mL medium.

### **Cell culture and transfection**

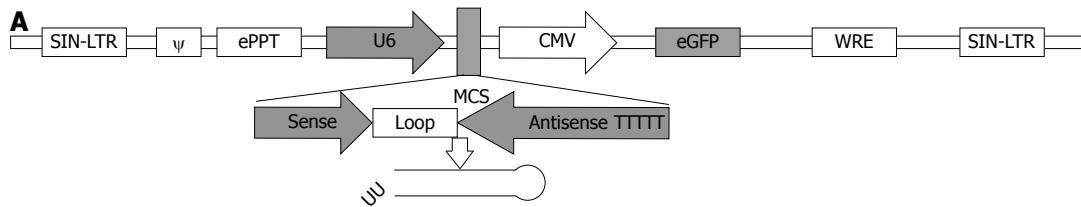
Human hepatocellular cancer HepG2 and Bel-7402 cell lines and normal liver cell L-02 were purchased from Classic Specimen Culture and Storage Centre at Wuhan University. Cells were grown in 5% CO<sub>2</sub> saturated humidity, at 37°C and cultured as monolayers in RPMI 1640 supplemented with penicillin/streptomycin, 2 mmol/L glutamine and 10% FBS. Cells were subcultured at  $1 \times 10^5$  cells per well into six-well tissue culture plates. After 72 h of culture, the cells were transfected with LV-shMAT2Bs (0.5  $\mu$ L per well) formulated into liposomes according to the manufacturer's instructions (TransMessenger™, Qiagen, Valencia, CA; Figure 1B). The final volume of culture medium was 2 mL per well.

### **Knockdown of MAT2B in HepG2 cells by real time-PCR analysis**

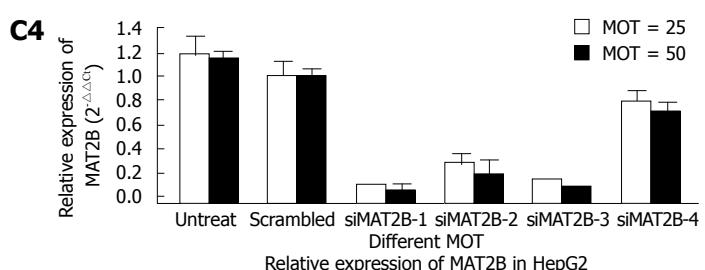
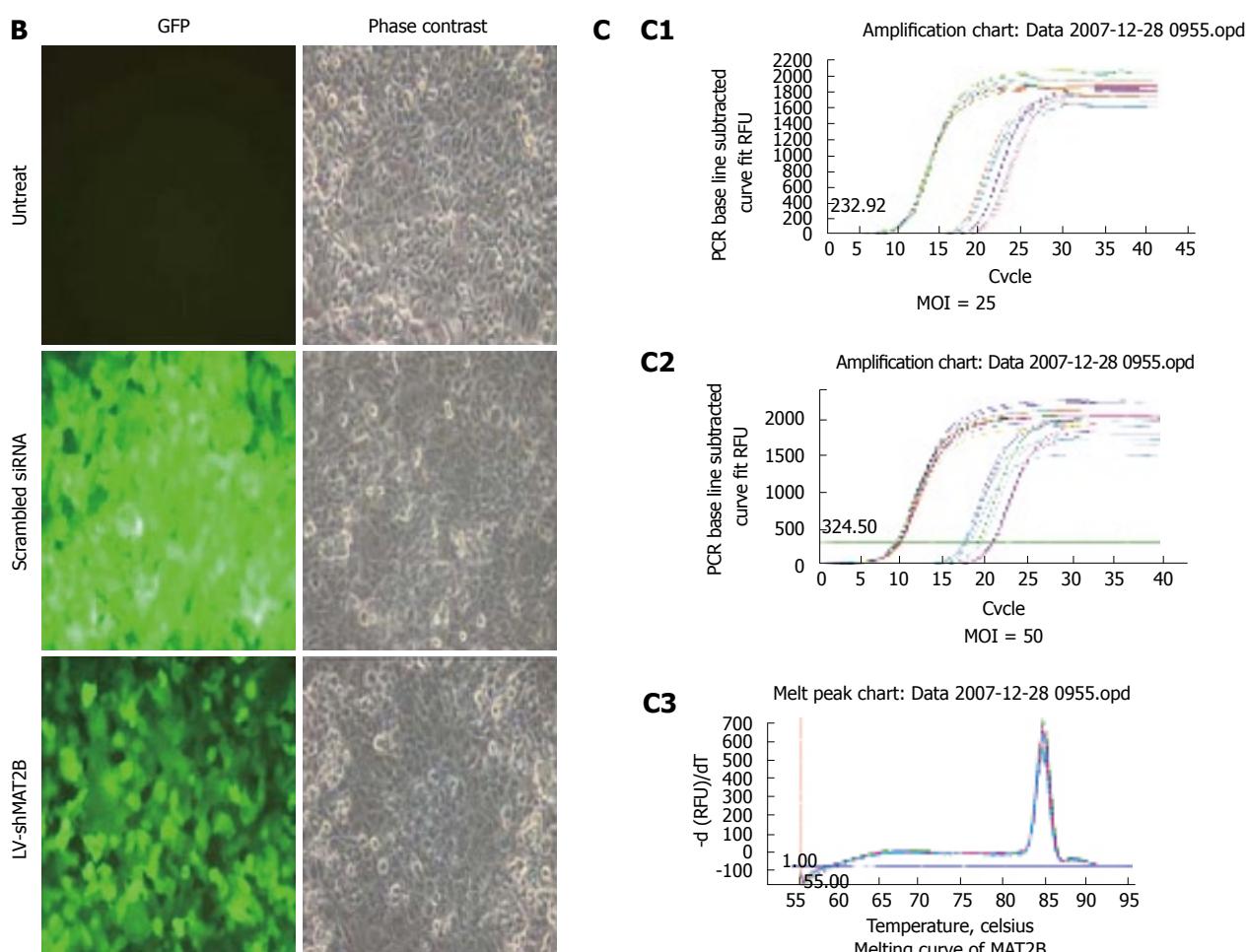
Total RNA was subjected to reverse transcription (RT) with Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA). Cells were then respectively prepared with scrambled siRNA, and lentivirus vectors such as LV-shMAT2B-1, LV-shMAT2B-2, LV-shMAT2B-3 and LV-shMAT2B-4. Three days after virus infection at the multiplicity of infection (MOI) 25 and 50, the RT products were subjected to quantitative real-time PCR analysis. The primers and TaqMan probes for human MAT2B and universal PCR master mix were purchased from Promega Company. GAPDH was used as a control housekeeping gene. The thermal profile consisted of 1 cycle at 95°C for 15 min followed by 45 cycles at 95°C for 15 s and at 60°C for 30 s. The expression of MAT2B was checked by normalization of the cycle threshold (C<sub>t</sub>) of these genes to that of the control housekeeping gene (GAPDH). The delta C<sub>t</sub> ( $\Delta C_t$ ) = (C<sub>t</sub> of MAT2B) - (C<sub>t</sub> of GAPDH in each group).  $\Delta\Delta C_t$  obtained was used to find the relative expression of MAT2B gene according to the following formula: Relative expression =  $2^{-\Delta\Delta C_t}$ ;  $\Delta\Delta C_t$  = (mean  $\Delta C_t$  of MAT2B genes in siRNAs groups) - ( $\Delta C_t$  of MAT2B genes in different treatment groups). Melting curve of MAT2B was drafted.

### **Western-blot analysis**

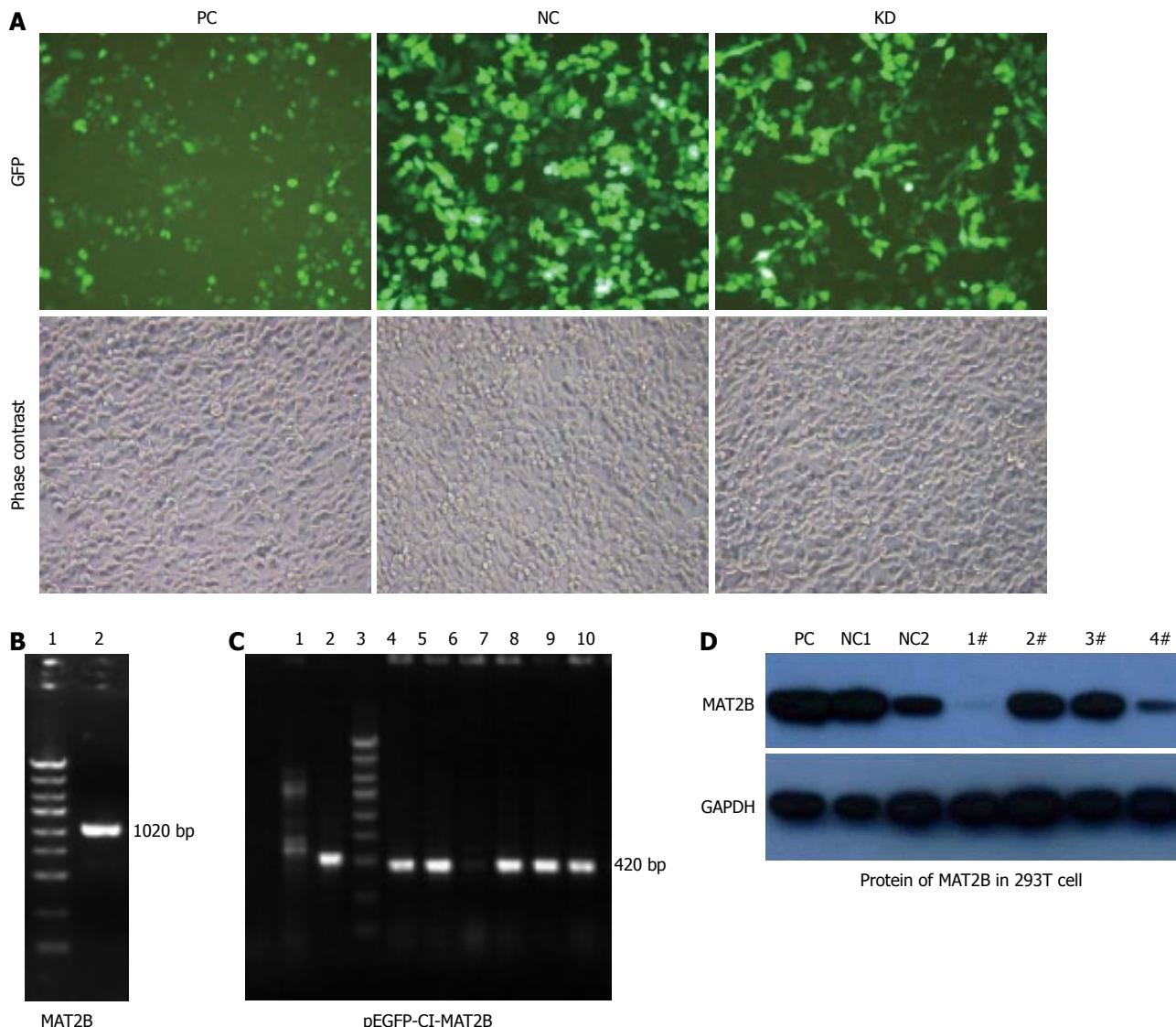
pEGFP-C1-MAT2B was eukaryotic expression plasmid of the MAT2B gene (Figure 2C). Four siMAT2B plasmids (pGCL-GFP-siMAT2B) were co-transfected with pEGFP-C1-MAT2B in 293T cells, respectively. Forty-eight hours after transfection, cells were collected, rinsed in ice-cold PBS 15 min, and lysed in lysis buffer containing 50 mmol/L HEPES (pH 7.9), 0.4 mol/L



	5'	STEMP	Loop	STEMP
siMAT2B-1	CCGG	GCAGTTCATCACATCATTAT	CTCGAG	ATGAATGATGTGATGAACTGC
	AATTAAAAAA	GCAGTTCATCACATCATTAT	CTCGAG	ATGAATGATGTGATGAACTGC
siMAT2B-2	CCGG	CCTTACAGAGAGGAAGACATA	CTCGAG	TATGTCTTCCTCTGTAAAGG
	AATTAAAAAA	CCTTACAGAGAGGAAGACATA	CTCGAG	TATGTCTTCCTCTGTAAAGG
siMAT2B-3	CCGG	GCTGTGACTGTTATGTTTGT	CTCGAG	ATCAAACATAACACTCACAGC
	AATTAAAAAA	GCTGTGACTGTTATGTTTGT	CTCGAG	ATCAAACATAACACTCACAGC
siMAT2B-4	CCGG	GCCTCTCAACTTAATGTGGA	CTCGAG	ATCCACATTAAGTTGAGAGGC
	AATTAAAAAA	GCCTCTCAACTTAATGTGGA	CTCGAG	ATCCACATTAAGTTGAGAGGC
siRNAs	T	TTCTCCGAACGTGTCACGT	TTCAAGAGA	ACGTGACACGTTGGAGAA
	TCGAGAAAAAA	TTCTCCGAACGTGTCACGT	TCTCTGAA	ACGTGACACGTTGGAGAA



**Figure 1** A: Each consist of a hairpin as was demonstrated; B: HepG2 cells were without treatment (untreated), and infected with control lentivirus (scrambled siRNA) or LV-shMAT2B. The cells were infected (MOI = 25), GFP expression and the phase contrast images of the same areas were taken after 72 h. C: mRNA level of MAT2B after HepG2 cell was treated with LV-shMAT2B in different MOI (C1 and C2) detected by real-time PCR (C4), melting curve of MAT2B (C3).



**Figure 2** MAT2B gene was cloned from the cDNA library with production of 1020 bp by PCR (**B**), pEGFP-C1-MAT2B was constructed after restricted enzyme cutting and connection with production of 420 bp by PCR as showed in Figure 2C. Plasmids containing siMAT2B (pGCL-GFP-siMAT2B) were co-transfected with pEGFP-C1-MAT2B in 293T cell, phase contrast and GFP expression under a fluorescent microscope was taken after 72 h (**A**). Protein level of MAT2B in 293T cells was detected by western blot (**D**). pGCL-GFP-siMAT2B-1 and -4 can significantly knock down expression of MAT2B at the protein level (**D**). PC, pcDNA3.1; NC, non-silence control vector. Lanes 1-4: pGCL-GFP-siMAT2B-1, -2, -3 and -4, respectively.

NaCl, 1 mmol/L EDTA, 2 µg/mL leupeptin, 2 µg/mL aprotinin, 5 µg/mL benzamidine, 0.5 mmol/L phenylmethylsulfonyl fluoride and 1% NP40. The lysates were centrifuged at 14 000 r/min to remove any cellular debris. Protein concentrations of the lysates were determined by the Bio-Rad Dc protein assay system which was 2 µg/µL. An equal amount of protein was separated by 12% SDS-PAGE, transferred to PVDF membrane, and blocked with 5% nonfat dry milk in TBS/Tween 20 (0.05% v/v) for 1 h at room temperature. The membrane was incubated with primary antibody overnight. Anti-cyclin D1, anti-cyclin D2 and anti-GAPDH antibodies were obtained from Santa Cruz Biotech Anti-bcl-x<sub>L</sub> and Anti-bcl-x<sub>S</sub> antibodies were obtained from Dako Biotech. Anti-MAT2B was purchased from Abnova Company (Taiwan). After washing, the membrane was incubated with secondary antibody (1:4000, Amersham Pharmacia Biotech

Arlington Heights, IL) for 1 h. After several washes, the blots were developed by enhanced chemiluminescence (Amersham Arlington Heights, IL). Each experiment was repeated at least twice with similar results.

#### Viability assay

Equal numbers of cells per well were seeded into 24-well plates and incubated until 60% confluent and treated with LV-shMAT2B (0.5 µL) or DMSO for 72 h. 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma, St. Louis, MO), 10% weight/volume in phosphate buffered saline was added to each well. After appropriate incubation, cells were washed, lysed with isopropanol and the solubilized product spectrophotometrically quantified at 490 nm.

#### Proliferation assay

Proliferation assay with [<sup>3</sup>H]thymidine. Equal numbers

of cells per well were plated onto 24-well plates and cultured until 60% confluent. Cells were treated with LV-shMAT2B (0.5  $\mu$ L) or DMSO as described above. [ $^3$ H]thymidine was added to the medium for 72 h at 2.5 Ci/mL. Cells were fixed with 0.5 $\mu$ g LV-shMAT2B, rinsed, dried and 1 mL of 0.33 mol/L sodium hydroxide added. [ $^3$ H]thymidine incorporation was measured in a liquid scintillation counting system (Beckman Coulter, Fullerton, CA).

### Flow cytometry

The inducing apoptosis effects of LV-shMAT2B on human hepatocellular cancer cells and normal liver cell (L-02) were analyzed by Flow cytometry. Briefly,  $1 \times 10^5$  cells were trypsinized, then washed with PBS, centrifuged at 800 r/min, fixed with 70% precooling alcohol to the second day, alcohol was extracted by trypsinization at 1000 r/min, the cells were incubated in the solution mixture with PI (100  $\mu$ g), Triton-X100 (0.5%) and RNase (2000  $\mu$ g/mL) 15 min then analyzed by FCM.

### Assay of MATII enzyme activity

Enzyme activity was measured as described previously<sup>[17]</sup>. Protein extracts were obtained from transfected cells by sonicating and then centrifuged at 13000 g for 15 min. Two hundred and fifty  $\mu$ g of protein as determined by the method of Bradford<sup>[18]</sup> was added to the reaction mixture containing 80 mmol/L Tris-HCl (pH 7.4), 50 mmol/L KCl, 40 mmol/L MgCl<sub>2</sub>, 5 mmol/L ATP, 10 mmol/L dithiothreitol, 0.5 mmol/L EDTA, 50  $\mu$ mol/L methionine and 0.3  $\mu$ Ci L-[Methyl-3H] methionine. The mixture was applied to a phosphocellulose paper disc (HA 0.45  $\mu$ m, Millipore) and placed on a filtering system for washing. The disc was added to 10 mL of ScintiVerse E for scintillation counting using a Beckman model LS6000TA Liquid Scintillation Counter (Beckman Instruments, Fullerton, CA, USA). MAT II activity was reported as nmol SAMe formed per mg protein per 40 min.

### Assay of hepatic SAMe levels

Hepatic SAMe levels were measured using a method described previously<sup>[19]</sup> with slight modifications. Liver specimens were homogenized in phosphate-buffered saline, and an aliquot was saved for protein assay. The rest was treated with 100  $\mu$ L of 1 mol/L perchloric acid (PCA) on ice for 5 min and centrifuged at 1000 g for 15 min at 4°C. The aqueous layer was quantitatively removed, neutralized with 3 mol/L KOH and centrifuged at 3000 g for 10 min at 4°C. SAMe levels were determined in the neutralized PCA extracts by HPLC (LC-10ATVP pump, SCL-10AVP system control) with a SPD-10AVP UV detector and a SIL-10ADVP autosampler (Shimadzu) using a Partisil SCX 10  $\mu$ m column (25 cm  $\times$  0.44 cm i.d.; Whatman Chem-Sep, Maidstone, Cleveland). SAMe was eluted isocratically at 1 mL/min with 0.19 mol/L NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> adjusted to pH 2.6 with 2 mol/L H<sub>3</sub>PO<sub>4</sub>. SAMe levels were calculated

using a standard curve of SAMe prepared at the same time as the samples and are reported as nmol/mg protein.

### Statistical analysis

Data are expressed as mean  $\pm$  SD, and statistical analyses of the data were performed using ANOVA Post Hoc Dunnett *t* test. Values of *P* < 0.05 were considered to be statistically significant.

## RESULTS

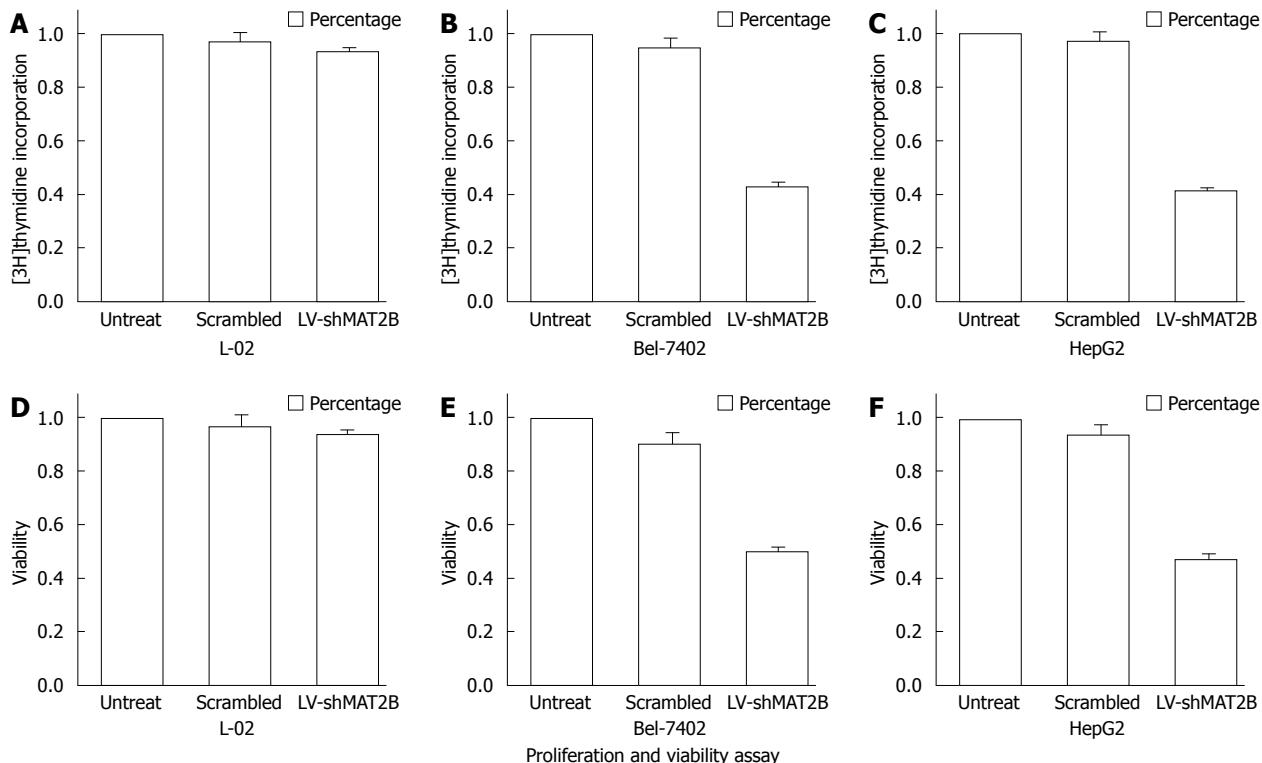
### Expression of MAT2B suppressed by LV-shMAT2B in HCC HepG2 cells *in vitro*

In this research, GFP expression was observed under a fluorescent microscope in HepG2 cells 72 h after infection with LV-shMAT2B at MOI 25 (Figure 1B). Then we performed real time-PCR<sup>[8]</sup> to determine the mRNA level of MAT2B in HCC HepG2 cells *in vitro*. We observed significant suppression of MAT2B by LV-shMAT2B-1 and LV-shMAT2B-3 at two different MOI 25 and 50 (Figure 1C).

Phase contrast and GFP expression was observed (Figure 1B), 72 h after 4 plasmids containing siMAT2B (pGCL-GFP-siMAT2B) co-transfected with pEGFP-C1-MAT2B in 293T cell respectively. Results of a quantitative RT-PCR assay showed that the expression of MAT2B mRNA was reduced by 90% (Figure 2C) when siMAT2B-1 or siMAT2B-3 cotransfected with pEGFP-C1-MAT2B in 293T cells. Results of western-blot assay showed that pGCL-GFP-siMAT2B-1 and pGCL-GFP-siMAT2B-4 could significantly suppress the expression of MAT2B at the protein level in 293T cells (Figure 2D). According to the results of the quantitative RT-PCR and western-blot assays, siMAT2B-1 was the most ideal siMAT2B of the four siMAT2B. Thus LV-shMAT2B-1 corresponding to siMAT2B-1 was used as LV-shMAT2B in the following research.

### Cell viability and proliferation significantly inhibited by LV-shMAT2B in HCC cells

To assess the potential effects of Lentivirus RNAi-mediated MAT2B silencing, we investigated cell proliferation and viability 72 h after L-02 and HCC cells were transfected with LV-shMAT2B. We then examined thymidine incorporation as a measure of DNA synthesis and cell proliferation. MTT assay was used to evaluate cell viability. Value of absorption at 490 nm was obtained. Percentage of cell viability treated with LV-shMAT2B compared with controls was counted. As a result we found that scrambled siRNA had no effect on cell proliferation and viability in all cells (Figure 3), LV-shMAT2B caused dramatic reduction in proliferation (Figure 3B, C) compared with controls in HCC cells Bel-7402 (*P* = 0.054) and HepG2 (*P* = 0.031), but it had no effect on normal liver cells L-02 (Figure 3D). In all HCC cells, significant decrease in viability was observed by MTT assay (Figure 3E, F). We discovered that the growth-inhibition caused by LV-shMAT2B was involved in down-regulation of cyclin D1 by western-blot assay.



**Figure 3** Proliferation of L-02 cell and HCC cells Bel-7402, HepG2 were shown in Figure 3A-C. The relative value of [ $^3\text{H}$ ]thymidine incorporation was measured after cells were treated with LV-shMAT2B. Percentage cell viability of L-02 cells and HCC cells Bel-7402, HepG2 were counted with MTT after cells were treated with LV-shMAT2B Figure 2D-F. It was seen that LV-shMAT2B can inhibit cell viability and proliferation of HCC cells but had no effect on normal liver cells.

#### Apoptosis induced by LV-shMAT2B in HCC cells

To investigate the effects of LV-shMAT2B on cell death, apoptosis rate was evaluated with flow cytometry analysis. Seventy-two hours after hepatoma cells were transfected with LV-shMAT2B, the sub-G1 population of apoptotic cells was determined. Results from analysis of L-02, Bel-7402 and HepG2 cells showed that apoptosis rates were  $10.1\% \pm 1.9\%$ ,  $26.3\% \pm 2.1\%$  and  $32.6\% \pm 3.7\%$ , in the presence of LV-shMAT2B, respectively. Flow cytometry analysis also showed cell apoptosis caused by LV-shMAT2B was greater in HCC cells Bel-7402 and HepG2 than in control cells induced by scrambled siRNA ( $P = 0.047$ ), but apoptosis rates in L-02 induced by LV-shMAT2B and scrambled siRNA, respectively, had no significant difference (Figure 4). Results of western-blot indicated that LV-shMAT2B caused the apoptosis in HCC cells by decreasing the expression of  $\text{bcl-x}_L$  and increasing the expression of  $\text{bcl-x}_S$ .

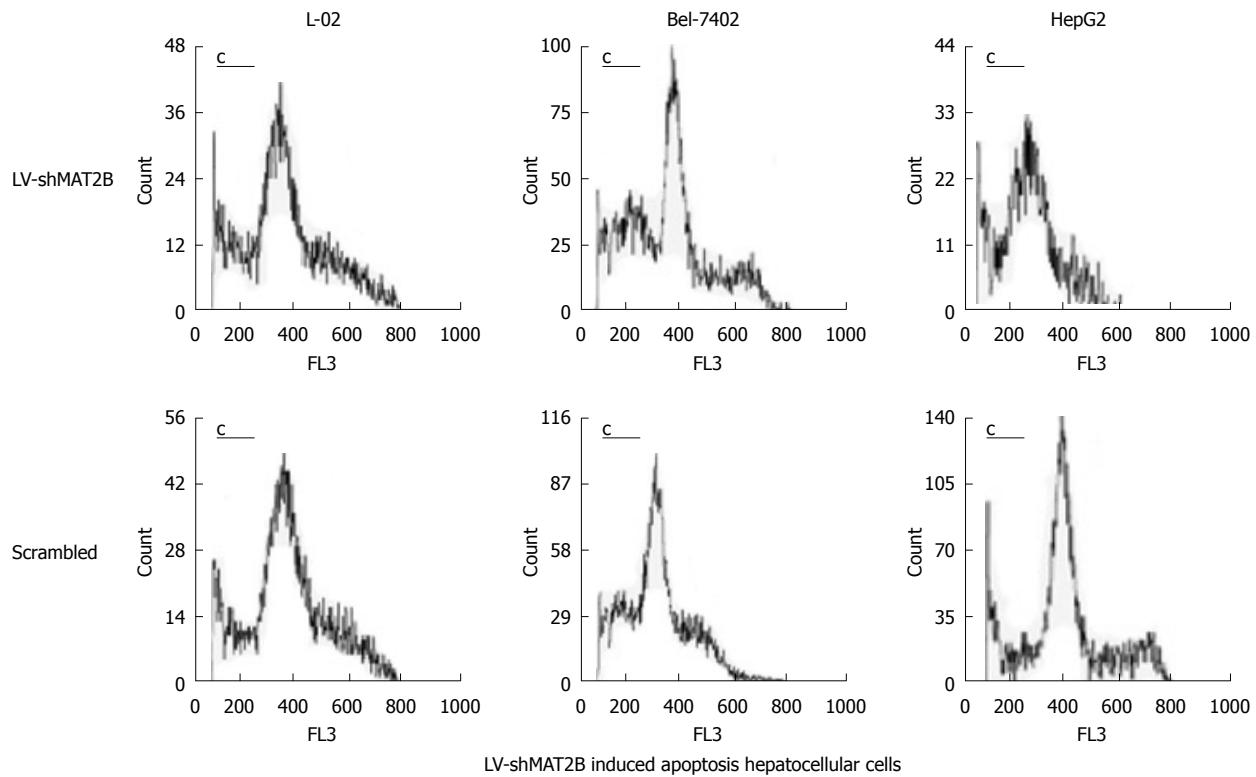
#### Decreased MAT II enzyme activity and increased intracellular SAMe content caused by LV-shMAT2B in HCC cells

To determine the effect of LV-shMAT2B on enzymatic activity of MAT II, HepG2 and Bel-7402 cells were transfected with LV-shMAT2B. At different time points after transfection, protein extracts were prepared, enzyme activity assays were performed. Results from Liquid Scintillation Counter analysis indicated that MAT II enzyme activity was gradually decreased from

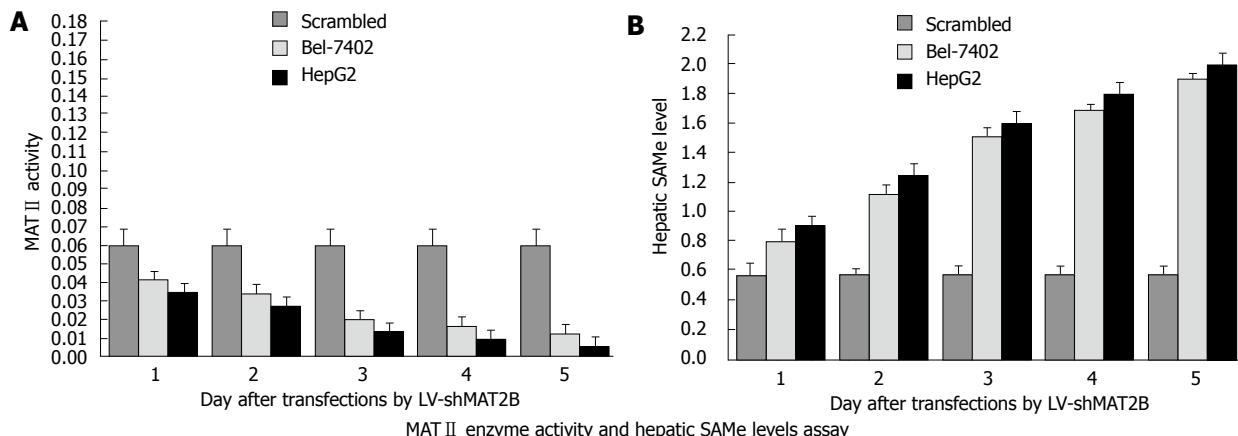
1 d to 5 d in a time-dependent manner, but remained relatively unchanged in cells treated with scrambled siRNA (Figure 5A). These data demonstrated that LV-shMAT2B significantly inhibited MAT II enzyme activity in Bel-7402 and HepG2 cells. The changes in MAT gene's expression were likely to cause changes in the production of SAMe. To analyze the effect of LV-shMAT2B on SAMe production, we measured the contents of SAMe in HCC cells. Results from reverse-phase high performance liquid chromatography analysis showed that the intracellular content of SAMe in cells transfected with scrambled siRNAs was low at  $0.58 \pm 0.05$  (nmol/mg protein), and increased to  $1.89 \pm 0.13$  in HepG2 and  $1.81 \pm 0.17$  in Bel-7402 after they were treated with LV-shMAT2B in a time-dependent manner ( $P = 0.0071$ , Figure 5B).

#### Down-regulation of cyclin D1 and $\text{bcl-x}_L$ by LV-shMAT2B in HCC cells

It has been indicated that the  $\beta$  subunit provided a proliferative advantage in HCC cells and this effect could be mediated through its interaction with MAT II  $\alpha_2$  and down-regulating SAMe levels. SAMe inhibited HGF-dependent induction of cyclin D1 and cyclin D2 expression without affecting the activation of extracellular signal-regulated protein kinase (ERK)<sup>[20]</sup> and SAMe is proapoptotic in HCC cells *in vitro* by promoting the expressions of  $\text{bcl-x}_L$  and  $\text{bcl-x}_S$ <sup>[21]</sup>. Our results showed that LV-shMAT2B could down-regulate the protein levels of cyclin D1 (Figure 6B) and  $\text{bcl-x}_L$



**Figure 4** Cell apoptosis caused by LV-shMAT2B was increased in human hepatocellular cancer cells Bel-7402 and HepG2 compared to controls induced by siRNAs, but they all had no impact on the normal hepatic cell (L-02) which does not express MAT2B.



**Figure 5** MAT II enzyme activity was gradually decreased from 1 d to 5 d in a time-dependent manner after transfected with LV-shMAT2B, but remained relatively unchanged in cells treated with siRNAs. In human cancer cells Bel-7402 and HepG2 (A), Hepatic SAMe level in the two HCC cells were increased by LV-shMAT2B in a time-dependent manner, but remained relatively unchanged in cells treated with siRNAs (B).

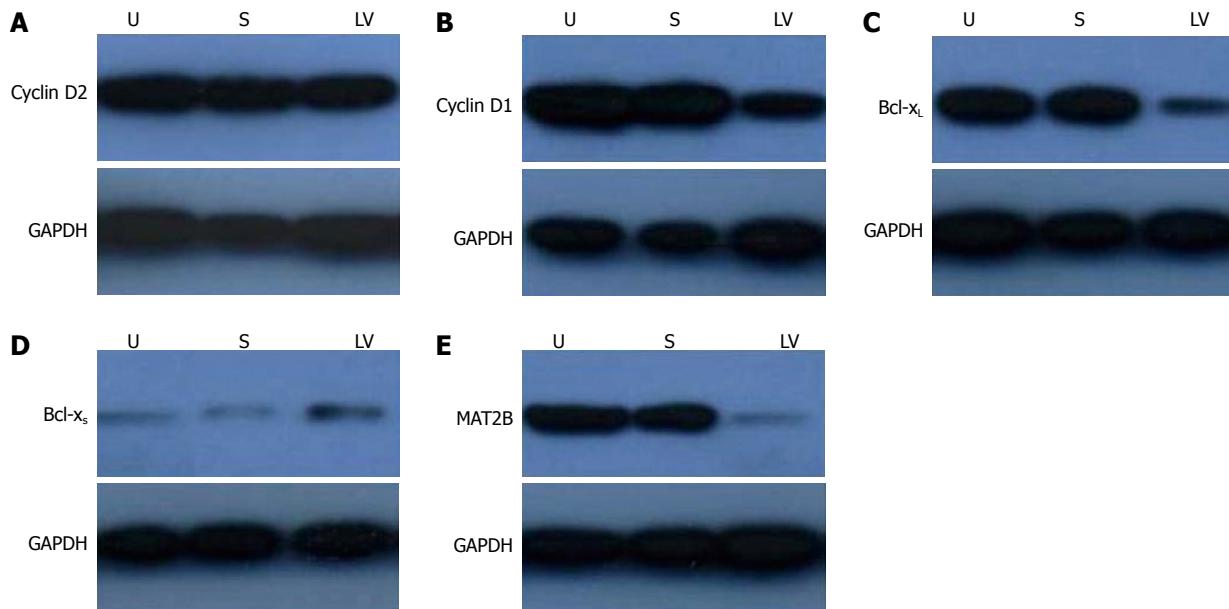
(Figure 6C) in human liver cancer cells. LV-shMAT2B increased the expression of bcl-x<sub>S</sub> (Figure 6D) but had no effect on cyclin D2 (Figure 6A).

#### Expression of MAT2B suppressed by LV-shMAT2B in protein level in HepG2 cells

To investigate the effect of LV-shMAT2B on HCC cells, we also detected the protein level of MAT2B in HepG2 cells. There was no expression of MAT2B after it was treated with LV-shMAT2B (Figure 6). This demonstrated that expression of MAT2B was suppressed by LV-shMAT2B.

## DISCUSSION

It was demonstrated that a switch in MAT expression in liver cancer was accompanied with increasing expression of MAT2B which encoded the  $\beta$  subunit. The  $\beta$  subunit is associated with MAT II and MAT2B is not expressed in normal liver. The expression of MAT2B is increased in liver cirrhosis and hepatocellular carcinoma, and chronic hepatic SAMe deficiency leads to malignant degeneration<sup>[22]</sup>. Recently there was also a report that hepatocyte growth factor (HGF) could promote proliferation of hepatoma cells by up-regulating the expression of MAT2B<sup>[23]</sup>. Leptin used to be taken as an



**Figure 6** After HepG2 cells were transfected with LV-shMAT2B for 72 h, western blot was used to detect the protein levels of cyclin D1 and cyclin D2, bcl-x<sub>L</sub> and bcl-x<sub>S</sub>, results show the expressions of cyclin D1 (B) and Bcl-x<sub>L</sub> (C) were decreased compared to controls and untreated cells. The expression of bcl-x<sub>S</sub> (D) was increased but that of cyclin D2 did not change (A). The expression of MAT2B was suppressed by LV-shMAT2B significantly (E). U, untreated HepG2 cell; S, HepG2 cell treated with scrambled siRNA; LV, HepG2 cells treated with LV-shMAT2B. The protein level of MAT2B was measured by western blot.

adipokine shown to be mitogenic in human liver cancer cell lines HepG2 and Huh7, and its mitogenic effect was also related with the expression of MAT2B.

In order to find the role played by MAT2B in HCC, we suppressed MAT2B in HCC cells using the RNAi method. The inhibitory potency and the method of transferring siRNA into HCC cells were two critical factors for successful application of RNAi method. shRNAs were proved to provide long-lasting silencing and maximal inhibition of gene expression at lower concentration<sup>[24,25]</sup>. Inhibitory potency of shRNA was related to specificity of the targeting sequence, so we used two different methods to confirm the efficacy of siMAT2B in HepG2 cells and in 293T cells. Then we selected siMAT2B-1 which could knock out the expression of MAT2B in both methods. Thus we decided to use LV-siMAT2B-1 as LV-shMAT2B in following research. In this research we found that LV-shMAT2B could suppress the expression of MAT2B at the level of both mRNA and protein in HCC. LV-shMAT2B could dramatically decrease cell proliferation and viability as well as increase cell apoptosis in Bel-7402 and HepG2 cells, but it had no effect on normal liver cell L-02 which had no expression of MAT2B. We concluded that LV-shMAT2B had a high specificity for MAT2B.

In this research we chose lentivirus vector as our shRNAs delivery vehicle because lentivirus could transfect both dividing and nondividing cells at a high efficiency and sustain long-term gene expression by integrating into the host genome. It was shown that the suppression of MAT2B caused by LV-siMAT2B was in a MOI-dependent manner. Significant inhibitory effects were found at MOI of 25 and 50, where

the expression of MAT2B was all reduced by 90%. Lentivirus vector is also safe for humans. Lentivirus vector encoding antisense targeting HIV envelope sequence has been used for HIV treatment in clinical trials with no obvious side effects<sup>[26,27]</sup>. Most recently, lentivirus vector containing beta-globin gene has been approved in phase I / II clinical trials for human beta-thalassemia and sickle cell anemia gene therapy<sup>[28]</sup>. The MAT2B's expression is very important because it not only can provide a proliferative advantage in hepatoma cells but can also decrease intracellular SAMe content. It was discovered that transfection with  $\beta$  subunit reduced the cellular content of SAMe in HuH7 cells, on the contrary we suppressed the expression of MAT2B by LV-shMAT2B, which resulted in decreased activity of MATII and increased content of SAMe. We found that their changes were all time-dependent. SAMe was antiapoptotic in cultured rat hepatocytes but proapoptotic in human hepatoma cells<sup>[7]</sup>. LV-shMAT2B could induce apoptosis of HCC cells but had no impact on normal liver cells, the reason might be that there was no expression of the MAT2B gene in normal liver cells. It was demonstrated that induction mRNA of bcl-x<sub>S</sub> by SAMe in HepG2 cells resulted in apoptosis, but SAMe had no effect on expression of bcl-x<sub>L</sub><sup>[29]</sup>. We discovered that the protein level of bcl-x<sub>S</sub> was increased and the expression of bcl-x<sub>L</sub> was down-regulated by LV-shMAT2B. We supposed that it might be related to cellular stress brought on by LV-shMAT2B, bcl-x<sub>L</sub> was able to induce apoptosis in response to cellular stress<sup>[30]</sup>. We found that LV-shMAT2B prevented proliferation in HepG2 cells, Leptin could induce proliferation in human hepatocarcinoma cells by up-regulating cyclin D1<sup>[31]</sup> but Komal<sup>[30]</sup> found that Leptin could increase SAMe levels

in HepG2 cells. On the contrary, SAMe was believed to inhibit the expressions of cyclin D1 and cyclin D2<sup>[20]</sup>. It was indicated in our research that the expression of cyclin D1 was decreased, so we considered that the down-regulation of cyclin D1 may be caused by knocking down of MAT2B, but not by increasing SAMe. We also found that the decreased activity of MAT II was caused by LV-shMAT2B. The  $\beta$  subunit associated with cirrhosis and cancer could provide a proliferative advantage in hepatoma cells through its interaction with MAT II  $\alpha_2$  and down-regulate SAMe level<sup>[8]</sup>. When the MAT2B gene was knocked out, the interaction between  $\beta$  and  $\alpha_2$  subunit was interrupted. As a result, the activity of MAT II decreased and SAMe level increased time-dependently, the proliferative advantage induced by MAT2B was prevented by LV-shMAT2B.

On the whole, we found that the suppression of MAT2B with LV-shMAT2B could lead to growth-inhibition and apoptosis in HCC cells. We concluded that the MAT2B gene could be used as a therapy target for hepatoma in the future.

## COMMENTS

### Background

It was demonstrated that a switch in methionine adenosyltransferase (MAT) expression in liver cancer played an important pathogenetic role by facilitating liver cancer growth. MAT2A encodes a catalytic subunit ( $\alpha_2$ ) found in a native MAT isozyme (MAT II) which is associated with a catalytically inactive regulatory subunit ( $\beta$ ) encoded by MAT2B. It has been proved that  $\beta$  subunit is associated with cirrhosis and cancer providing a proliferative advantage in hepatoma cells through its interaction with MAT II  $\alpha_2$  and down-regulation of S-adenosyl methionine (SAMe) levels. We supposed that suppression of MAT2B may result in growth-inhibition and increasing of SAMe which could induce hepatoma cells into apoptosis and we wanted to find the mechanism.

### Research frontiers

We found that expression of MAT2B in hepatic cancer HepG2 cells were decreased by 90%. Suppression of MAT2B in hepatocellular carcinoma (HCC) cells significantly induced their growth-inhibition by down-regulation of cyclin D1. Apoptosis was induced by LV-shMAT2B involving down regulation of bcl-x<sub>S</sub> and up-regulation of bcl-x<sub>L</sub>.

### Innovations and breakthroughs

LV-shMAT2B could induce HCC cells into apoptosis but had no impact on normal liver cells, which may occur because there is no expression of the MAT2B gene in the normal liver cell. It has been demonstrated that induction mRNA of bcl-x<sub>S</sub> by SAMe in HepG2 cells resulted in apoptosis but SAMe had no effects on expression of bcl-x<sub>L</sub>. We discovered that protein level of bcl-x<sub>S</sub> was increased and the expression of bcl-x<sub>L</sub> was down-regulated by LV-shMAT2B. We considered that the down-regulation of cyclin D1 may be caused by knocking down MAT2B but not because of increasing of SAMe.

### Applications

Lentivirus vector is also safe for humans. Lentivirus vector encoding the antisense targeting HIV envelope sequence has been used for HIV treatment in clinical trials with no obvious side effects. Most recently, lentivirus vector containing beta-globin gene has been approved in phase I / II clinical trials for human beta-thalassemia and sickle cell anemia gene therapy. What is, more LV-shMAT2B could induce HCC cells into apoptosis but had no impact on normal liver cells.

### Peer review

The manuscript written by Wang et al reports that knockdown of MAT2B by transfection of lentivirus vector carrying siRNAs inhibits the growth of human hepatocellular cell lines but not that of human normal liver cells. The data are interesting and provide potentially important new clues for the future treatment of HCC. However, there are some points which need to be addressed.

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CLINICAL RESEARCH

## Replication of *interleukin 23 receptor* and *autophagy-related 16-like 1* association in adult- and pediatric-onset inflammatory bowel disease in Italy

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19 years), 749 healthy controls, and 546 healthy parents (273 trios) were included in the study. The rs2241880 [*autophagy-related 16-like 1 (ATG16L1)*], rs11209026 and rs7517847 [*interleukin 23 receptor (IL23R)*], rs2066844, rs2066845, rs2066847 (*CARD15*), rs1050152 (*OCTN1*), and rs2631367 (*OCTN2*) gene variants were genotyped.

**RESULTS:** The frequency of G allele of *ATG16L1* SNP (Ala197Thr) was increased in patients with CD compared with controls (59% vs 54% respectively) (OR = 1.25, CI = 1.08-1.45, P = 0.003), but not in UC (55%). The frequency of A and G (minor) alleles of Arg381Gln, rs11209026 and rs7517847 variants of *IL23R* were reduced significantly in CD (4%, OR = 0.62, CI = 0.45-0.87, P = 0.005; 28%, OR = 0.64, CI = 0.55-0.75, P < 0.01), compared with controls (6% and 38%, respectively). The A allele (but not G) was also reduced significantly in UC (4%, OR = 0.69, CI = 0.5-0.94, P = 0.019). No association was demonstrated with sub-phenotypes and interaction with *CARD15*, and *OCTN1/2* genes, although both gene variants were associated with pediatric-onset disease.

**CONCLUSION:** The present study confirms the association of *IL23R* polymorphisms with IBD, and *ATG16L1* with CD, in both adult- and pediatric-onset subsets in our study population.

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**Key words:** Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Genetic predisposition; *Autophagy-related 16-like 1*; *Interleukin 23 receptor*; Genome-wide association study; Pediatric inflammatory bowel disease

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### Abstract

**AIM:** To investigate gene variants in a large Italian inflammatory bowel disease (IBD) cohort, and to analyze the correlation of sub-phenotypes (including age at diagnosis) and epistatic interaction with other IBD genes.

**METHODS:** Total of 763 patients with Crohn's disease (CD, 189 diagnosed at age < 19 years), 843 with ulcerative colitis (UC, 179 diagnosed <

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## INTRODUCTION

Inflammatory bowel disease (IBD) is a polygenic trait that includes two similar, yet distinct conditions, namely Crohn's disease (CD) and ulcerative colitis (UC)<sup>[1]</sup>. It is widely accepted that both diseases result from an inappropriate response of a defective mucosal immune system to indigenous flora and other luminal agents in a genetically susceptible host<sup>[2]</sup>.

Eleven IBD genome-wide linkage analyses in families with multiple IBD affected members, as well as two different meta-analyses<sup>[3,4]</sup> have identified several linkage regions<sup>[5]</sup>. Following the identification of *NOD2* (or *CARD15*), the first gene contributing to CD susceptibility (IBD1 locus), further fine mapping studies have identified a risk haplotype (IBD5 locus) on chromosome 5q<sup>[6]</sup>, along with two polymorphisms in the solute carrier family 22A4/22A5 (*SLC22A4/A5*) coding for *OCTN1* and *OCTN2*, suggested as candidate genes<sup>[7]</sup>, and another polymorphism on the *disk large homolog 5 (DLG5)* gene<sup>[8]</sup>. However, these risk-associated variants and several others reported<sup>[9-13]</sup> with conflicting results, explain only a minor component of the genetic risk in IBD.

Whole genome association (GWA) studies in IBD have rapidly led to the identification of novel susceptibility loci associated with CD<sup>[14]</sup>, such as *interleukin-23 receptor (IL23R)* and *autophagy-related 16-like 1 gene (ATG16L1)*. An uncommon coding mutation (rs11209026) in the *IL23R* gene<sup>[15]</sup> on chromosome 1 (1p32.1-p31.2), a G-to-A transition at nucleotide 1142 (Arg381Gln), has been identified to confer strong protection against CD in case-control ( $P = 5.05 \times 10^{-9}$ ) and family-based studies of Caucasian and Jewish cohorts with ileal CD. Further, GWA<sup>[16-19]</sup> and replication studies<sup>[1,2]</sup> have consistently confirmed strong association between variations at *IL23R* and CD. The intronic rs7517847 SNP (single nucleotide polymorphism) gave the most significant signal ( $P = 3.36 \times 10^{-13}$ )<sup>[15]</sup>. The association of this variant appeared statistically independent from Arg381Gln and not in linkage disequilibrium ( $r^2 = 0.03$ )<sup>[15]</sup>. A large number of replications in adult<sup>[20-24]</sup> and pediatric-onset<sup>[25-29]</sup> cohorts have been reported, but no significant association with specific CD sub-phenotype has been identified. Moreover, an association with UC has also been observed<sup>[23,24,28,30,31]</sup>.

In a recent German GWA scan<sup>[32]</sup>, the non-synonymous rs2241880 (Ala197Thr) variant of *ATG16L1* on chromosome 2p37.1 was found to be associated with CD, and appeared to account for all of the disease risk conferred by this locus. This association has been consistently demonstrated in a number of independent studies in adult<sup>[24,33-36]</sup> but with conflicting data in pediatric<sup>[34,37,38]</sup> IBD cohorts.

The aims of the present study were to investigate the association between variants of two candidate genes

*IL23R* and *ATG16L1* and IBD in an Italian cohort. In addition, we examined the genotype-phenotype correlation with specific disease subtypes, including the early onset of disease, and the possible genetic interactions with *OCTN1/2*, and *CARD15* genes.

## MATERIALS AND METHODS

### Materials

The study population included 763 CD and 843 UC unrelated patients recruited from four referral centers for adult IBD: the "Casa Sollievo della Sofferenza" Hospital of San Giovanni Rotondo, and the University Hospitals of Padua, Naples and Milan. In addition, 368 patients with pediatric onset of disease (age at diagnosis < 19 years) were included in the study because of a multi-center effort of the Italian Society of Pediatric Gastroenterology, Hepatology and Nutrition (SIGENP). This study cohort has been reported elsewhere<sup>[39]</sup>. Two hundred and seventy-three IBD patients also had both parents available for the purpose of a family-based analysis. The 749 healthy controls (415 male, mean age  $43 \pm 11$  years, range 22-75) were randomly recruited from three sites: San Giovanni Rotondo (Southern Italy,  $n = 451$ ), Rome (Central Italy,  $n = 114$ ) and Milano (Northern Italy,  $n = 184$ ), in order to minimize potential geographic heterogeneity. These subjects comprised of unrelated, asymptomatic individuals (blood donors, students, and staff members), all Caucasians with no Jewish descent. The study protocol was approved by the local Ethics Committees, and a written informed consent was obtained from each subject (or parents).

Demographic and sub-phenotype data of IBD patients are presented on Table 1. The diagnosis of CD and UC was established according to accepted clinical, endoscopic, radiological, and histological criteria<sup>[1]</sup>. The Montreal<sup>[40]</sup> classification was used for CD, based on the age at diagnosis (A), location (L), and disease behavior (B). In patients with UC, the disease location was categorized according to the Montreal classification, by distinguishing ulcerative proctitis (E1), left-side colitis (E2), and extensive colitis (E3). Patients with indeterminate colitis were excluded from the study. In all patients, the following clinical features were recorded: family history, age at diagnosis, duration of follow-up, presence of perianal fistulae, extraintestinal manifestations (presence or absence of any extraintestinal manifestation), previous abdominal surgery (either colectomy in UC or bowel resection in CD), and smoking habit (at least 1 cigarette/d). To account for the known modification of clinical characteristics during the disease course, only patients with at least two years of follow-up from the time of a confirmed diagnosis were included in the genotype/phenotype analysis.

### Methods

Genotyping of rs11209026 (*IL23R*), rs7517847 (*IL23R*), rs2241880 (*ATG16L1*), rs10150152 (*OCTN1*) and rs2631367 (*OCTN2*)<sup>[41]</sup> SNPs was performed using Applied Biosystems 7700 TaqMan assay

**Table 1 Demographic and clinical features of CD and UC according to the Montreal classification<sup>[39]</sup>**

	<b>CD (n = 763)</b>	<b>UC (n = 843)</b>
Sex (M/F)	435/328	492/351
Duration of follow-up, mean ± SD (range)	8 ± 7 (1-37)	9 ± 7 (1-41)
Age at diagnosis (yr), mean ± SD (range)	29 ± 15 (1-79)	32 ± 16 (1-76)
≤ 16 (A1)	173 (22%)	155 (18%)
17-40 (A2)	431 (57%)	458 (55%)
> 40 (A3)	159 (21%)	230 (27%)
Disease localization CD, n (%)		
Ileum (L1 ± L4)	241 (31%)	
Colon (L2 ± L4)	200 (26%)	
Ileo-colon (L3 ± L4)	315 (41%)	
Upper GI (L4)	7 (2%)	
Disease extent UC, n (%)		
Rectum (E1)		102 (13%)
Left colon (E2)		416 (49%)
Pancolitis (E3)		325 (38%)
Disease behavior CD, n (%)		
Inflammatory (B1 ± p)	346 (46%)	
Stricture (B2 ± p)	177 (23%)	
Penetrating (B3 ± p)	240 (31%)	
Perianal disease y/n (%)	137/626 (18%)	17/826 (2%)
Smoking history		
Yes	242 (33%)	119 (15%)
No	423 (55%)	510 (60%)
Ex	98 (12%)	214 (25%)
Family history of IBD y/n (%)	74/689 (10%)	53/790 (6%)
Surgery y/n, n (%)	237/526 (31%)	95/748 (13%)
Extra-intestinal manifestations y/n (%)	304/459 (40%)	182/661 (21%)

(Applied Biosystems, Foster City, CA). The *CARD15* variants were detected by means of Denaturing High Performance Liquid Chromatography (DHPLC) (Wave System, Transgenomic Ltd, UK) (R702W, L1007fsinsC) and RFLP (G908R), respectively, as described previously<sup>[39]</sup>. PCR reactions were carried out in 96-well plates on ABI 9700 Thermocyclers (Applied Biosystems, Foster City, CA). All samples were genotyped at the Molecular Laboratory of Gastroenterology Unit in San Giovanni Rotondo Hospital, Italy.

For the purpose of statistical analysis, after genotyping the markers, the Hardy-Weinberg equilibrium was tested by comparing the expected and observed genotypes in  $2 \times 3 \chi^2$  tables. All markers showed no deviation from the Hardy-Weinberg equilibrium in controls ( $P > 0.05$ ). Allele-genotype frequencies and genotype/phenotype association were analysed by  $\chi^2$  and Fisher exact tests, when appropriate, by the SPSS software ver 1.5. The Hardy-Weinberg equilibrium and marker linkage disequilibrium analysis were also performed by the Arlequin software ver 2.0. Pairwise SNP linkage disequilibrium (LD) coefficients, haplotype frequencies, and Transmission Disequilibrium Test (TDT) were estimated using Haplovview<sup>[42]</sup>. Logistic regression analysis was used to assess the conditional independence between genotypes and IBD phenotypes and to test for gene-gene interactions. The frequencies and odds ratios of individual *CARD15* genotypes were stratified by *ATG16L1* (rs2241880) and *IL23R* (rs1209026); and *IL23R* genotypes were stratified by *ATG16L1* genotypes. An interaction was considered significant at  $P < 0.05$ .

## RESULTS

### Patients and controls

A total of 2901 subjects were investigated. These comprised of 763 patients with CD (435 males and 328 females), with a mean age at diagnosis of 29 years (range 1-79), and 843 patients with UC (492 males, 351 female), with a mean age at diagnosis of 32 years (range 1-76 years). In addition, 749 healthy controls and 546 healthy parents were also evaluated. Of note, 368 patients (180 male) were diagnosed at the age of 18 years or less.

### Genotyping of *ATG16L1* variant in IBD patients

The evaluation of the T300A polymorphism (rs2241880) was available in 667 CD and 668 UC patients: the frequency of the G allele was increased in CD patients compared with controls (59% vs 54%) ( $P = 0.003$ , OR = 1.25, CI = 1.08-1.45) (Table 2). Accordingly, by comparing genotype frequencies (Table 2), a significant increase in carriers of G risk allele was found in CD patients compared to controls (84% vs 79%) ( $P = 0.008$ , OR = 1.44, CI = 1.10-1.89). After stratifying the CD cohorts on the basis of age at diagnosis (adult  $\geq 19$  years; pediatric  $< 19$  years), the allele/genotype difference remained significant only in the adult subgroup ( $P = 0.004$ ), perhaps due to the small sample size of the pediatric subgroup, although the frequencies were similar.

There was no significant difference in the allele and genotype frequencies between UC patients and controls for the groups as a whole and after stratifying on the basis of pediatric and adult age at diagnosis.

### Genotyping of *IL23R* variants in IBD patients

A total of 723 CD, 804 UC, and 716 controls were genotyped for the rs7517847 polymorphism. The minor allele (G) frequency was significantly reduced (28%) in CD cases compared with controls (38%) ( $P < 0.01$ , OR = 0.64, CI = 0.55-0.75), with a significant reduction in carriers (49% vs 61% in controls) ( $P < 0.01$ , OR = 0.63, CI = 0.51-0.77) (Table 2). These differences remained significant after stratification of the patients on the basis of age at diagnosis. In contrast, no significant difference in allele and genotype frequencies was found in UC patients (Table 2).

A total of 735 CD and 823 UC patients, and 726 healthy controls were genotyped for rs1209026 polymorphism. The minor allele frequency (A) in CD patients was 4%, compared with 6% in controls, yielding a protective OR of 0.62 (CI = 0.45-0.87;  $P = 0.005$ ) (Table 2). Compared with controls (87%), the frequency of the risk genotype was significantly increased in CD patients (92%) ( $P = 0.005$ , OR = 0.61, CI = 0.43-0.86). These differences resulted in similar statistical significance after stratifying CD patients on the basis of age at diagnosis.

In addition, a high significant association was also found in UC patients, with a similar MAF frequency (4%) leading to a protective OR of 0.69 (CI = 0.50-0.94;  $P = 0.019$ ). Accordingly, the frequency of GG genotype was also significantly increased in UC patients (91%,  $P = 0.015$ )

**Table 2** Genotypes and alleles distribution for *ATG16L1* and *IL23R* SNPs in CD, UC and controls

	Genotypes						Alleles			
	AA	Aa	aa	Total	P	OR (95% CI)	Freq	P	OR (95% CI)	
<i>ATG16L1</i>										
rs2241880										
CD Total	227 (34%)	335 (50%)	105 (16%)	667	0.008	1.44 (1.10-1.89)	G	0.59	0.003	1.25 (1.08-1.45)
CD adult	165 (34%)	254 (52%)	72 (15%)	491	0.004	1.57 (1.16-2.13)		0.59	0.004	1.27 (1.08-1.49)
CD pediatric	62 (35%)	81 (46%)	33 (19%)	176	0.466	1.17 (0.77-1.77)		0.58	0.122	1.20 (0.95-1.52)
UC Total	212 (32%)	315 (47%)	141 (21%)	668	0.956	1.01 (0.78-1.30)		0.55	0.381	1.07 (0.92-1.24)
UC adult	157 (31%)	244 (48%)	105 (21%)	506	0.839	1.03 (0.78-1.36)		0.55	0.469	1.06 (0.90-1.25)
UC pediatric	55 (34%)	71 (44%)	36 (22%)	162	0.779	0.94 (0.63-1.42)		0.56	0.473	1.09 (0.86-1.39)
Controls	214 (29%)	376 (50%)	159 (21%)	749	-			0.54		
<i>IL23R</i>										
rs7517847										
CD Total	366 (51%)	305 (42%)	52 (7%)	723	< 0.01	0.63 (0.51-0.77)	G	0.28	< 0.01	0.64 (0.55-0.75)
CD adult	273 (51%)	225 (42%)	42 (8%)	540	< 0.01	0.63 (0.50-0.79)		0.29	< 0.01	0.65 (0.55-0.78)
CD pediatric	93 (51%)	80 (44%)	10 (5%)	183	< 0.01	0.62 (0.45-0.86)		0.27	< 0.01	0.61 (0.48-0.79)
UC Total	326 (41%)	390 (49%)	88 (11%)	804	0.567	0.94 (0.77-1.16)		0.35	0.111	0.89 (0.76-1.03)
UC adult	259 (41%)	301 (48%)	71 (11%)	631	0.468	0.92 (0.74-1.15)		0.35	0.121	0.88 (0.75-1.03)
UC pediatric	67 (39%)	89 (51%)	17 (10%)	173	0.927	1.02 (0.72-1.43)		0.36	0.400	0.90 (0.70-1.15)
Controls	280 (39%)	328 (46%)	108 (15%)	716	-			0.38		
rs11209026										
CD Total	675 (92%)	60 (8%)	0 (0%)	735	0.005	0.61 (0.43-0.86)	A	0.04	0.005	0.62 (0.45-0.87)
CD adult	502 (91%)	50 (9%)	0 (0%)	552	0.042	0.69 (0.48-0.99)		0.05	0.041	0.69 (0.49-0.99)
CD pediatric	173 (95%)	10 (5%)	0 (0%)	183	0.006	0.40 (0.20-0.78)		0.03	0.007	0.41 (0.21-0.80)
UC Total	750 (91%)	72 (9%)	1 (0%)	823	0.015	0.67 (0.48 - 0.93)		0.04	0.019	0.69 (0.50-0.94)
UC adult	589 (91%)	58 (9%)	1(0%)	648	0.035	0.69 (0.49-0.98)		0.05	0.043	0.71 (0.51-0.99)
UC pediatric	161 (92%)	14 (8%)	0 (0%)	175	0.085	0.60 (0.33-1.08)		0.04	0.087	0.61 (0.34-1.08)
Controls	634 (87%)	91 (13%)	1 (0%)	726	-			0.06		

"A" refers to wild type and "a" to mutated allele. Adult and pediatric subgroups are divided on the basis of the age at diagnosis ( $\geq 19$  years and  $< 19$  years, respectively).

**Table 3** Haplotypes frequency of the two *IL23R* variants in IBD patients and controls

Haplotype		IBD		CD		UC	
rs7517847	rs11209026	Cases/Controls (%) Haplotype	P	Cases/Controls (%) Haplotype	P	Cases/Controls (%) Haplotype	P
T	G	68/62	0.0032	71/62	< 0.01	65/62	0.2166
G	G	28/32	0.0548	25/32	< 0.01	31/32	0.7398
G	A	4/6	0.0121	4/6	0.0088	4/6	0.0578

(Table 2). These statistical differences remained after stratifying the cohort on the basis of age at diagnosis.

Haplotype association analysis of two *IL23R* variants, rs7517847 and rs11209026, was also estimated (Table 3). An increase in TG haplotype frequency was observed in IBD (68%,  $P = 0.0032$ ) and CD patients (71%,  $P \leq 0.01$ ), compared with controls (62%).

### Family-based analysis

Given the lack of a significant association of allele/genotype frequencies of the *ATG16L1* gene in the pediatric subset, and the availability of 273 pediatric IBD Trios (138 CD, and 135 UC), the T300A polymorphism was tested by transmission disequilibrium test; a significant over-transmission of the G allele was found in CD patients ( $T:U = 90:62$ ;  $P = 0.023$ ), but not in UC (Table 4). With respect to *IL23R*, a significant over-transmission of the T allele of rs7517847 ( $T:U = 137:101$ ), and the G allele of rs11209026 ( $T:U = 34:19$ ) SNPs was observed in the whole IBD cohort ( $P = 0.0196$ ,  $P = 0.0394$ ; respectively), and more specifically in CD patients ( $P = 0.0015$ ,  $P = 0.0833$ , respectively). However,

the results in the UC subset did not reach statistical significance. An association between the rs7517847-rs11209026 (TG) haplotype and IBD, CD, and UC was also tested using the TDT. Consistent evidence of over-transmission of this haplotype in the whole IBD cohorts ( $T:U = 141:100$ ,  $P = 0.0084$ ) was maintained (Table 4).

### Genotype/phenotype correlation

Analysis of the allele and genotype frequencies of the rs2241880 SNP of the *ATG16L1* gene, showed no association with disease location, behaviour, and age at diagnosis based on the Montreal classification of CD cases (Table 5). More specifically, we observed the lowest frequency of the G allele in isolated colonic disease (55%), increasing to 60% in ileal and ileo-colonic disease, without a clear difference in adult and pediatric subsets (colonic disease: adult 55%, pediatric 54%). Similarly, there was no association with gender, smoking history, perianal fistulae, and presence of extra-intestinal manifestations. Moreover, no association was found with any specific sub-phenotypes of UC (Table 6).

Similarly, there was no correlation between risk

**Table 4** Transmission disequilibrium test (TDT) for rs7517847 and rs11209026 *IL23R* SNPs for IBD, CD, and UC

Marker	Over-trans	Haplotype	IBD (n = 273)			CD (n = 138)			UC (n = 135)		
			T	U	P	T	U	P	T	U	P
rs7517847	T		137	101	0.0196	74	40	0.0015	63	61	0.8575
rs11209026	G		34	19	0.0394	18	9	0.0833	16	10	0.2393
		TG	141	100	0.0084	52	60	0.0012	45	34	0.5947
		GG	97	123	0.0790	50	39	0.0098	28	28	0.9940
		GA	14	24	0.1088	12	20	0.0585	9	13	0.6555
		TA	5	10	0.1831	13	8	0.7037	-	-	-
rs2241880	G		-	-	-	90	62	0.0231	-	-	-

TDT haplotypes are also included. Data of over-transmitted allele are given, with counts of transmitted (T) and un-transmitted (U) alleles.

**Table 5** Genotype and allele frequency distribution of *ATG16L1* and *IL23R* SNPs in CD cases stratified by phenotypic subgroups, (n)

SNP/gene genotype	rs2241880 <i>ATG16L1</i>					rs7517847 <i>IL23R</i>					rs11209026 <i>IL23R</i>				
	AA (105)	AG (335)	GG (227)	Total (667)	Freq (G) 0.501	GG (52)	GT (305)	TT (366)	Total (723)	Freq (G) 0.283	AA (0)	AG (60)	GG (675)	Total (735)	Freq (A) 0.041
Sex															
Male	54	193	126	373	0.597	28	169	212	409	0.275	0	27	390	417	0.032
Female	51	142	101	294	0.585	24	136	154	314	0.293	0	33	285	318	0.052
Age at diagnosis (yr)															
≤ 16 (A1)	28	72	53	153	0.582	9	76	74	159	0.296	0	9	150	159	0.028
17-40 (A2)	56	180	123	359	0.593	24	160	209	393	0.265	0	29	378	407	0.036
> 40 (A3)	20	71	40	131	0.576	14	57	73	144	0.295	0	19	123	142	0.067
Disease localization															
Ileum (L1 ± L4)	28	97	67	192	0.602	12	86	118	216	0.255	0	21	200	221	0.048
Colon (L2 ± L4)	31	92	48	171	0.550	10	76	104	190	0.253	0	18	170	188	0.048
Ileo-colon (L3 ± L4)	43	131	101	275	0.605	25	131	129	285	0.318	0	19	276	295	0.032
Upper GI (L4)	2	4	1	7	0.429	0	2	4	6	0.167	0	0	6	6	0.000
Disease behavior															
Inflammatory (B1)	44	154	98	296	0.591	21	133	162	316	0.277	0	25	294	319	0.039
Stricturing (B2)	22	79	46	147	0.582	10	64	82	156	0.269	0	16	145	161	0.050
Penetrating (B3)	38	85	70	193	0.583	17	93	103	213	0.298	0	17	201	218	0.039
Perianal disease															
Yes	14	52	37	103	0.612	8	54	59	121	0.289	0	11	113	124	0.044
No	89	269	175	533	0.581	40	233	293	566	0.277	0	46	529	575	0.040
Smoking history															
No	57	168	116	341	0.587	24	149	191	364	0.271	0	29	339	368	0.039
Yes	32	105	71	208	0.594	20	100	107	227	0.308	0	20	213	233	0.043
Ex	14	39	30	83	0.596	3	41	49	93	0.253	0	8	88	96	0.042
Surgery															
Yes	34	91	68	193	0.588	15	83	113	211	0.268	0	16	202	218	0.037
No	70	231	149	450	0.588	32	211	241	484	0.284	0	42	447	489	0.043

Only patients with at least 2 years of follow-up from diagnosis were included.

alleles and genotypes of the *IL23R* genes with specific sub-phenotypes of CD (Table 5) and UC (Table 6). Of note, the frequency of A allele of rs11209026 was the lowest in patients diagnosed at < 16 years of age (2.8%), increasing to 3.6% in the age group of 17-40 years, and 6.7% in those over 40 years, but the differences did not reach statistical significance (Table 5). More specifically, despite the large number of pediatric onset IBD patients investigated, genotype/phenotype frequencies were similar after further stratifying IBD population into adult and pediatric age of onset of the disease (data not shown).

#### Interaction between *ATG16L1*, *IL23R*, *CARD15*, and *OCTN1/2* genes

Logistic regression analysis was used to evaluate the individual contributions of *CARD15* (at least 1 variant against wild type), *ATG16L1* (GG/AG vs AA), *IL23R*

rs11209026 (AG/AA vs GG) and *OCTN* (diplotype TTCC against the rest) and CD risk, and to test for statistical interaction. The analysis included 763 CD cases and 749 controls. Table 7 shows the results of the logistic regression model with the individual contributions of *CARD15*, *ATG16L1*, *IL23R*, and *OCTN* to disease risk, their contribution after adjustment for *CARD15* genotype, and the contribution of interactions with *CARD15*. The individual contribution of all the predisposing genes was confirmed. By contrast, there was no evidence of a statistical interaction between all loci ( $P = 0.428$ ), and *ATG16L1*, *IL23R*, and *OCTN* by pairs and triplets (data not showed).

## DISCUSSION

With the recent introduction of the GWA technology,

**Table 6** Genotype and allele frequencies of *ATG16L1* and *IL23R* SNPs in UC cases stratified by phenotypic subgroups, (n)

SNP/gene genotype	rs2241880 <i>ATG16L1</i>					rs7517847 <i>IL23R</i>					rs11209026 <i>IL23R</i>				
	AA (141)	AG (315)	GG (212)	Total (668)	Freq (G) 0.553	GG (88)	GT (390)	TT (326)	Total (804)	Freq (G) 0.352	AA (1)	AG (72)	GG (750)	Total (823)	Freq (A) 0.045
Sex															
Male	77	183	112	372	0.547	41	236	185	462	0.344	0	45	432	477	0.047
Female	64	132	100	296	0.561	47	154	141	342	0.363	1	27	318	346	0.042
Age at diagnosis (yr)															
≤ 16 (A1)	30	58	48	136	0.566	15	76	49	140	0.379	0	11	129	140	0.039
17-40 (A2)	79	159	102	340	0.534	40	213	170	423	0.346	0	33	401	434	0.038
> 40 (A3)	24	84	55	163	0.595	28	86	89	203	0.350	1	25	184	210	0.064
Disease localization															
Rectum	18	41	15	74	0.480	13	38	39	90	0.356	1	8	80	89	0.056
Rectum-sigmoid	20	55	39	114	0.583	20	82	46	148	0.412	0	21	134	155	0.068
Left colon	41	101	64	206	0.556	27	104	100	231	0.342	0	19	215	234	0.041
Colon	54	104	86	244	0.566	24	148	124	296	0.331	0	22	283	305	0.036
Perianal disease															
Yes	3	3	6	12	0.625	0	6	8	14	0.214	0	2	13	15	0.067
No	129	298	198	625	0.555	82	367	301	750	0.354	1	67	699	767	0.045
Smoking history															
No	81	173	125	379	0.558	51	203	185	439	0.347	1	38	408	447	0.045
Yes	25	54	25	104	0.500	10	65	41	116	0.366	0	12	104	116	0.052
Ex	25	72	51	148	0.588	20	105	77	202	0.359	0	18	191	209	0.043
Surgery															
Yes	11	33	26	70	0.607	9	37	35	81	0.340	0	9	74	83	0.054
No	122	268	178	568	0.549	74	337	274	685	0.354	1	61	638	700	0.045

Only patients with at least 2 years of follow-up from diagnosis were included.

**Table 7** Logistic regression analysis

Genes	P	OR	95% CI
CARD15	2.34E-14	2.908	2.211-3.825
ATG	0.008	1.442	1.099-1.894
ATG (adj CARD15)	0.014	1.439	1.077-1.924
ATG* CARD15	0.692	0.851	0.382-1.894
IL23	0.005	0.613	0.435-0.863
IL23 (adj CARD15)	0.029	0.629	0.414-0.954
IL23* CARD15	0.981	1.010	0.432-2.360
OCTN	0.028	1.434	1.039-1.980
OCTN (adj CARD15)	0.021	1.479	1.061-2.061
TTCC* CARD15	0.632	0.821	0.367-1.839
ATG*IL23*OCTN*CARD15	0.428	0.651	0.226-1.880

Individual contribution of *CARD15* (at least 1 variant), *ATG16L1* (GG/AG vs AA), *IL23R* rs11209026 (AG/AA vs GG), and *OCTN* dyplotype (TTCC) to risk CD. adj CARD15: Contribution from the term after adjustment for *CARD15* genotype.

several novel genes and loci involved in the pathogenesis of IBD have been uncovered, as well as the successful identification and replication of multiple susceptibility genes for CD<sup>[11,15,18,20,21,32]</sup>, such as *IL23R* and *ATG16L1*. However, these genetic variations account for only a small portion of the overall genetic susceptibility to CD, and their contribution to the pathogenesis of UC is even lower.

The present study confirms the reported association between *IL23R* and *ATG16L1* variants and susceptibility to CD, both in the case-control and family-based analysis, and in both adult- and pediatric-onset disease. Moreover, we also replicated the significant association between the rs11209026 variant of *IL23R* and UC ( $P = 0.018$ ), suggesting that this gene may also have a role in the genetic susceptibility to UC. Although the size of the cohort was sufficiently large, consisting

of more than 1600 IBD patients, we were unable to detect an association between *IL23R* and *ATG16L1* variants and any sub-phenotypes for both diseases, as described in the majority of published studies, thus suggesting that the overall effect of these variants on certain CD subtypes<sup>[22,23,31,34-36,38]</sup> is much weaker than that observed in *CARD15*. Moreover, we found no statistical evidence for epistatic interaction between *IL23R*, *ATG16L1*, *OCTN1/2*, and *CARD15* genes. Since our cohort also included a large number of pediatric cases (368 diagnosed at age < 19 years), this study confirmed that the rs2241880 polymorphism in the *ATG16L1*<sup>[34,37]</sup> gene and the rs7517847, rs11209026 polymorphisms in the *IL23R* gene<sup>[22,25,27-30]</sup> also influence susceptibility to CD in pediatric-onset patients, at allele and genotype frequencies comparable to that seen in adults.

Recent studies have reported genotype-phenotype associations in adult-pediatric onset CD and *ATG16L1* Ala197Thr variant. Specifically, Prescott *et al*<sup>[34]</sup> demonstrated an association with ileal form of CD with or without colonic involvement (61.7%) but not with isolated colonic disease (52.2%), as well as with diagnosis at an earlier age (≤ 16 years at diagnosis: 63.8%). Subsequently, van Limbergen *et al*<sup>[38]</sup> confirmed the association of isolated ileal disease with rs2241880G-allele ( $P = 0.02$ ) in a combined genotype-phenotype analysis of early and adult onset CD, although after stratifying CD cases in early- and adult-onset the association did not reach statistical significance ( $P = 0.28$  and  $P = 0.08$ , respectively), probably due to the reduced power of the sample sizes.

We observed rs2144880G variant allele frequency of 55% in colonic disease (L2 ± L4), which increased to 60% in pure ileal disease (L1 ± L4), without significant

association with any sub-phenotypes. To definitively answer this question, studies with a larger cohort of CD are needed to increase the statistical power, particularly when considering SNPs that only show a modest increase in the odds ratios for susceptibility. Moreover, different ethnic populations and true heterogeneity may also explain these differences.

IL-23 is a heterodimeric cytokine, composed of the IL-12p40 and p19 subunits. Human and mouse IL-23 share structural homologies with IL-12, and exhibit similar activities, but differ in their capacity to stimulate populations of specific memory T cells, activated to produce the proinflammatory mediators IL17 and IL16<sup>[43]</sup>. This so-called TH17 T-cell subset expresses the master transcription factor POp $\gamma$ t and mediates chronic inflammatory and autoimmune diseases in animal models<sup>[44]</sup>. IL-23 binds with a complex consisting of IL-23R and IL-12R $\beta$ . IL-23R associates constitutively with Jak2 and in a ligand-dependent manner with stat3. The human IL-23R gene is on human chromosome 1 within 150 kb of IL-12R $\beta$ 2<sup>[45]</sup>. It appears that IL-23 plays a unique role in the initiation and perpetuation of innate and T cell-mediated forms of IBD, and variations in the early IL-12 and IL-23 dependent regulatory mechanisms may impact the subsequent inflammatory response. The strong effect of the protective allele, first identified by Duerr *et al*<sup>[15]</sup>, could potentially be exploited to define functional outcomes and targeted therapy that to date remain as possibilities.

*ATG16L1* is a member of a large family of genes involved in autophagy, a mechanism by which cells recycle redundant organelles, an essential process in the resistance to pathogens, targeted for immune evasion by viruses and bacteria<sup>[46]</sup>. The exact functional impact of the rs2241880 variant is currently unknown, although the *ATG16L1* protein is a key component of autophagy and the T300A substitution occurs in an evolutionarily conserved domain<sup>[32]</sup>. The gene is expressed in intestinal epithelial cell lines and functional knockdown of this gene abrogates autophagy of *Salmonella typhimurium*<sup>[16]</sup>. The important role of autophagy as predisposing factor to CD has recently been highlighted with the identification of variants in immunity-related guanosine triphosphatase (IRGM) gene, involved in elimination of intracellular bacteria, and susceptibility to CD<sup>[47]</sup>. How this process is implicated in the pathogenesis of CD remains unclear, although it further supports the concept of inflammatory barrier disorder<sup>[48]</sup>.

Our results provide an independent confirmation of the association between the candidate genetic variations in *IL23R* and *ATG16L1* genes and CD, and reinforce the role of these new polymorphisms as genetic determinants in IBD. Further research is needed to understand how *IL23R* and *ATG16L1* variants contribute to disease susceptibility in IBD, and whether they have therapeutic implications.

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## COMMENTS

### Background

It is widely accepted that ulcerative colitis (UC) and Crohn's disease (CD) result from an inappropriate response of a defective mucosal immune system to indigenous flora and other luminal agents in a genetically susceptible host. Eleven inflammatory bowel disease (IBD) genome-wide linkage analyses in families with multiple IBD affected members, and two meta-analyses have identified several linkage regions. Following the identification of *NOD2* (or *CARD15*), the first gene contributing to CD susceptibility (IBD1 locus), further fine mapping studies have identified a risk haplotype (IBD5 locus) on chromosome 5q, and another polymorphism on the *disk large homolog 5* (*DLG5*) gene. However, these risk-associated variants and many others reported with conflicting results, explain only a minority of the genetic risk in IBD. Whole genome association (GWA) studies have rapidly led to the identification of novel susceptibility loci such as *interleukin-23 receptor* (*IL23R*) and *autophagy-related 16-like 1 gene* (*ATG16L1*).

### Research frontiers

An uncommon coding mutation (Arg381Gln) in the *IL23R* gene has been identified as conferring strong protection against CD with ileal involvement. Further GWA and replication studies have consistently confirmed strong association between variations at *IL23R* and CD, with a large number of replications in adult- and pediatric-onset cohorts, without significant association with specific CD sub-phenotype. Moreover, an association with UC has also been observed. The non-synonymous (Ala197Thr) variant of *ATG16L1* was found to be associated with CD, but not UC, and has been consistently demonstrated in a number of independent studies in adult subjects, but with conflicting data in pediatric IBD cohorts.

### Innovations and breakthroughs

The present study included a large population of Italian IBD patients (763 CD and 843 UC), including 368 patients with pediatric onset of disease (age at diagnosis < 19 years), 546 healthy parents and 749 healthy controls. All patients were accurately phenotyped. Two SNPs of *IL23R*, one of the *ATG16L1*, and three of the *CARD15* genes were genotyped, and were also evaluated for interaction with specific clinical features among genes.

### Applications

The present study confirms the reported association between *IL23R* and *ATG16L1* variants and susceptibility to CD, both in the case-control and family-based analysis, and both in adult- and pediatric-onset disease. Moreover, we also replicated the significant association of the rs11209026 variant of *IL23R* with UC, suggesting that this gene may also have a role in genetic susceptibility to UC. Although the size of the cohort was large, consisting of more than 1600 IBD patients, we were unable to detect an association between *IL23R* and *ATG16L1* variants and any sub-phenotypes for both diseases.

## Terminology

GWA is the Genome Wide Association which is examined through a chip platform evaluating several thousand SNPs. SNP is Polymorphism of Single Nucleotide evaluated to investigate variants of gene probably modifying the gene function.

## Peer review

This is a nice follow-up study which confirms the association between the candidate genetic variations in *IL23R/ATG16L1* and IBD, in both pediatric- and adult-onset population.

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CLINICAL RESEARCH

## Single nucleotide polymorphism in the tumor necrosis factor-alpha gene affects inflammatory bowel diseases risk

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### Abstract

**AIM:** To investigate the role that single nucleotide polymorphisms (SNPs) in the promoter of the tumour necrosis factor-alpha (TNF- $\alpha$ ) gene play in the risk of inflammatory bowel diseases (IBDs) in a New Zealand population, in the context of international studies.

**METHODS:** DNA samples from 388 patients with Crohn's disease (CD), 405 ulcerative colitis (UC), 27 indeterminate colitis (IC) and 201 randomly selected controls, from Canterbury, New Zealand were screened for 3 common polymorphisms in the TNF- $\alpha$  receptor: -238 G→A, -308 G→A and -857C→T, using a Taqman<sup>®</sup> assay. A meta-analysis was performed on the data obtained on these polymorphisms combined with that from other published studies.

**RESULTS:** Individuals carrying the -308 G/A allele had a significantly (OR = 1.91,  $\chi^2 = 17.36$ ,  $P < 0.0001$ ) increased risk of pancolitis, and a 1.57-fold increased risk (OR = 1.57,  $\chi^2 = 4.34$ ,  $P = 0.037$ ) of requiring a bowel resection in UC. Carrying the -857 C/T variant decreased the risk of ileocolonic CD (OR = 0.56,  $\chi^2 =$

4.32,  $P = 0.037$ ), and the need for a bowel resection (OR = 0.59,  $\chi^2 = 4.85$ ,  $P = 0.028$ ). The risk of UC was reduced in individuals who were smokers at diagnosis, (OR = 0.48,  $\chi^2 = 4.86$ ,  $P = 0.028$ ).

**CONCLUSION:** TNF- $\alpha$  is a key cytokine known to play a role in inflammatory response, and the locus for the gene is found in the IBD3 region on chromosome 6p21, known to be associated with an increased risk for IBD. The -308 G/A SNP in the TNF- $\alpha$  promoter is functional, and may account in part for the increased UC risk associated with the IBD3 genomic region. The -857 C/T SNP may decrease IBD risk in certain groups. Pharmacogenomic approaches may be desirable for individuals with such affected genotypes.

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**Key words:** Tumour necrosis factor alpha; Single nucleotide polymorphisms; Inflammatory bowel diseases; Crohn's disease; Ulcerative colitis

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### INTRODUCTION

Inflammatory bowel diseases (IBDs) are chronic multifactorial disorders, commonly classified as autoimmune diseases, and comprise of Crohn's disease (CD) and ulcerative colitis (UC). Up to 10%-15% of reported cases are described as "indeterminate colitis" (IC), where an unequivocal classification is not possible using established diagnostic criteria. Twin studies and segregation analysis strongly support IBD, especially CD, as complex genetic traits<sup>[1]</sup>, whose etiology also involves immunological and environmental factors, including diet. Several IBD-susceptibility chromosomal regions have been replicated

in a number of studies, and in some cases causative genes have been found. For example, the key gene in the IBD1 region on chromosome 16q12, has been identified as Nucleotide Oligomerization Domain 2 (*NOD2*)<sup>[2]</sup>. Three polymorphisms of this gene (*R702W*, *G908R*, and *1007fs*) increase the susceptibility to IBD, especially CD.

The IBD3 region on chromosome 6p was initially described in a large European cohort<sup>[3]</sup> by Hampe and coworkers, who suggested that this observation was consistent with single nucleotide polymorphism (SNPs) in either the Human Leukocyte Antigen (HLA) and/or tumor necrosis factor (TNF) genes as being risk factors for IBD. Rioux *et al*<sup>[4]</sup> confirmed this association in a genome-wide search of 158 Canadian sib-pair families, a finding also replicated by Dechairo *et al*<sup>[5]</sup> in 284 IBD-affected sib pairs from 234 Caucasian families in the UK, showing an almost equal contribution to the linkage with CD and UC. Fisher *et al*<sup>[6]</sup> investigated 353 northern European sib pairs, and suggested that the strongest evidence for linkage in both CD and UC occurred at the IBD3 region on chromosome 6p21.3 (OMIM 604519). This effect appeared to be male-specific. It should be noted that the human TNF-alpha (TNF- $\alpha$ ) gene is located on chromosome 6p21.3, spanning approximately 3 kb<sup>[7]</sup>.

TNF- $\alpha$  is an important pro-inflammatory cytokine that is involved in systemic inflammation, and stimulates acute phase reactions<sup>[8,9]</sup>. TNF- $\alpha$ /TNF receptor interactions not only play a pivotal role in the pathogenesis of the inflammatory response, but also cause programmed cell death (apoptosis), cell proliferation and differentiation<sup>[10]</sup>. Ligand binding to TNF receptors causes a conformational change in the receptor, leading to several downstream effects including activation of the heterodimeric transcription factor and nuclear factor (NF)- $\kappa$ B. Through translocation to the nucleus, this mediates the transcription of a variety of proteins involved in cell survival and proliferation, as well as inflammatory response. Alterations in the regulation of TNF- $\alpha$ , especially TNF- $\alpha$  overproduction, have been implicated in a variety of symptoms associated with autoimmune disorders, including IBD, and especially CD. Inflammation, anorexia, and weight loss are all associated with increased levels of circulating TNF- $\alpha$  that is seen in CD. Indeed, a common treatment of CD is the use of TNF- $\alpha$  inhibitors such as the monoclonal antibody, infliximab (IFX).

Several SNPs in the TNF- $\alpha$  promoter region are known to affect the level of gene expression. For example, two SNPs located at nucleotides -238 and -308 represent substitutions of adenine for guanine in the TNF transcriptional start site<sup>[11]</sup>. Such variations in the TNF- $\alpha$  promoter region have previously been associated with susceptibility to a range of autoimmune disorders, including asthma<sup>[12,13]</sup>, psoriasis<sup>[14]</sup> and rheumatoid arthritis<sup>[15]</sup>. The G $\rightarrow$ A polymorphism at position -238 in the TNF gene is associated with lower production of TNF- $\alpha$  in patients with UC<sup>[16]</sup>. Conversely, the -308A polymorphism is associated with enhanced TNF- $\alpha$  production in cells *in vitro* and in CD patients *in vivo*<sup>[16-18]</sup>. The -857 C $\rightarrow$ T SNP is functional through binding to the transcription factor octamer transcription factor-1 (OCT-1). Carriers of the -857C allele show higher levels

of circulating TNF- $\alpha$ , and van Heel *et al*<sup>[19]</sup> suggested that the TNF -857C/T SNP increased the susceptibility to IBD in a UK population through its effects on the interaction between the OCT-1 gene and the NF- $\kappa$ B transcription factor.

The present study was designed to determine whether SNPs in the TNF- $\alpha$  promoter region confer susceptibility to CD or UC, and whether they are associated with the clinical phenotype, in a New Zealand Caucasian population.

## MATERIALS AND METHODS

### Study participants

The Canterbury IBD Project is a population-based study of genetic and environmental determinants of the aetiology IBD, which has been described in detail elsewhere<sup>[20]</sup>. The participants consented to the collection of peripheral blood for DNA extraction and genotyping. The subjects included in the present study were a random subset of the Caucasian participants of the Canterbury IBD Project. Both CD and UC were defined using standard diagnostic criteria<sup>[21]</sup>. The subjects were phenotyped according to the Montreal Classification systems, allowing genotype-phenotype analysis to be performed<sup>[22]</sup>.

A total of 388 CD participants, 405 UC participants, and 27 IC participants were genotyped for this study. All participants self-reported European ancestry, and patients who self-reported having any Maori or other non-Caucasian ancestry are not included in the data set. Clinical and demographic characteristics of the Caucasian IBD cohort for this study are shown in Table 1.

The New Zealand Caucasian controls used in this study were selected at random from the electoral roll, comprising 93% of the population over eighteen years of age in Canterbury, New Zealand<sup>[23]</sup>.

### Criteria for SNP selection

The TNF- $\alpha$  region is complex, and a considerable number of SNPs are necessary to haplotype tag this region<sup>[24]</sup>. Therefore, we elected to study 3 SNPs in the promoter region for which functionality has been shown previously<sup>[10,25]</sup>. The SNPs studied were: -238 G $\rightarrow$ A (rs361525), -308 G $\rightarrow$ A (rs1800629) and -857C $\rightarrow$ T (rs1799724).

### Applied biosystems TaqMan® SNP genotyping assay for TNF- $\alpha$ variants

The three alleles were genotyped using the ABI TaqMan MGB dallellic discrimination system. A custom made, quality controlled and functionally tested genotyping assay (Assay-by-Design online service) for all three variants was obtained from Applied Biosystems (Melbourne, Australia) (Table 2). The reactions were prepared by using 2  $\times$  TaqMan Universal Master Mix, 20  $\times$  SNP Genotyping Assay Mix, DNase-free water, 10 ng genomic DNA in a final volume of 5 l per reaction. The PCR amplification was performed using the ABI Prism 7900 HT sequence-detector machine under the following conditions: 10 min 95°C enzyme activation followed by 40 cycles at 92°C for 15 s and 60°C for 1 min.

**Table 1 Summary of clinical and demographic data on Caucasian IBD patients genotyped for at least one TNF- $\alpha$  polymorphism n (%)**

	<b>CD</b>	<b>UC</b>	<b>IC</b>
Gender	388	405	27
Female	249 (64.2)	214 (52.8)	15 (55.6)
Male	139 (35.8)	191 (47.2)	12 (44.4)
Age at first diagnosis			
Below 17	39 (10.0)	26 (6.4)	0
Between 17 and 40	199 (51.3)	184 (45.4)	15 (55.6)
Above 40	150 (38.7)	195 (48.2)	12 (44.4)
CD location			
Ileal	125 (32.2)		
Colonic	169 (43.6)		
Ileocolonic	90 (23.2)		
Unknown (U + UN)	4 (1.0)		
UC location			
Proctitis		140 (34.6)	3 (11.1)
Left colon		107 (26.4)	5 (18.5)
Pancolitis		154 (38.0)	19 (70.4)
Unknown		4 (1.0)	0
Behaviour			
Non-structuring, non-penetrating perianal disease: With	47 (21.5)		
Without	172 (78.5)		
Structuring perianal disease: With	46 (38.0)		
Without	75 (62.0)		
Penetrating perianal disease: With	17 (35.4)		
Without	31 (64.6)		
Any relative with IBD: Yes (n = 143)	74 (19.1)	65 (16.1)	5 (18.5)
Bowel resection: Yes (n = 214)	142 (36.6)	70 (17.3)	2 (7.4)
Smoker at diagnosis: Yes (n = 147)	97 (25.7)	49 (12.3)	2 (7.7)
Ever used immunomodulators: Yes (n = 296)	203 (52.3)	86 (21.2)	8 (29.6)
Extra intestinal manifestations: Yes (n = 142)	75 (19.3)	64 (15.8)	3 (11.1)

### Statistical analysis

The allelic trend test<sup>[26]</sup> and Fisher's exact genotypic test were used to compare patients and control allele frequencies. An exact test was used to test for departures from Hardy-Weinberg equilibrium (HWE) in the patient and the control samples<sup>[27]</sup>. Allelic odds ratios were calculated and confidence intervals for the allelic odds ratio were calculated under the assumption of HWE in the patient and the control groups. Within the region of TNF- $\alpha$ , HAPLO. SCORE in R was used to estimate haplotypes and to test for association of these haplotypes within the patient-control population. Meta-analysis was carried out to obtain an overall index of the effect magnitude of the studied relationship. These analyses were carried out using R and SAS (V9.1 SAS Institute., Cary, NC, USA). We also performed an exploratory analysis of allele frequency differences between controls and patient subgroups, using the clinical characteristics shown in Table 1.

## RESULTS

For each of the polymorphisms studied, the risks of carrying the variant was compared between CD, UC

**Table 2 Primer and probe sequences for custom made TaqMan SNP genotyping assay for TNF- $\alpha$  -857C/T, TNF- $\alpha$  -308G/A and TNF- $\alpha$  -238G/A**

<b>TaqMan SNP genotyping assay</b>	<b>DNA sequence</b>
TNF- $\alpha$ -857C/T forward primer	5'-GGGCTATGGAAGTCGAGTATGG-3'
TNF- $\alpha$ -857C/T reverse primer	5'-GTCCTGGAGGCTCTTCACT-3'
TNF- $\alpha$ -857C/T VIC probe	5'-CCCTGTCTCGTTAAGG-3'
TNF- $\alpha$ -857C/T FAM probe	5'-CCTGCTCTCATTAAGG-3'
TNF- $\alpha$ -308G/A forward primer	5'-CCAAAAGAAATGGAGGCAATAGGTT-3'
TNF- $\alpha$ -308G/A reverse primer	5'-GGACCCCTGGAGGCTGAAC-3'
TNF- $\alpha$ -308G/A VIC probe	5'-CCCGTCCCCATGCC-3'
TNF- $\alpha$ -308G/A FAM probe	5'-CCCGTCCCTCATGCC-3'
TNF- $\alpha$ -238G/A forward primer	5'-CAGTCAGTGGCCCAGAAC-3'
TNF- $\alpha$ -238G/A reverse primer	5'-CCCTCACACTCCCCATCCT-3'
TNF- $\alpha$ -238G/A VIC probe	5'-CTCGGAATCGGAGCAG-3'
TNF- $\alpha$ -238G/A FAM probe	5'-CTCGGAATCAGAGCAG-3'

and control groups, as shown in Tables 3 and 4. Those individuals carrying the variant TNF- $\alpha$  -238A allele showed no statistically significant effect on the patterns of disease risk. There were no significant differences between males and females in the risk for CD or UC (Table 5).

Carrying the TNF- $\alpha$  -308 G/A allele again showed no statistically significant effect on patterns of disease risk, and there was no significant difference between males and females in the risk for CD or UC (Table 5). Having the A allele increased the probability of the risk for pancolitis in UC, from 0.17 (for controls) to 0.28, giving an odds ratio of 1.91 (OR = 1.91, CI = 1.41-2.60, P < 0.0001). Carrying this polymorphism, significantly increased the necessity for bowel resection in UC (OR = 1.57, CI = 1.02-2.41, P = 0.037).

Carrying the TNF- $\alpha$  -857 C/T variant did not result in any statistically significant changes in the overall disease risk, or a significant male-female imbalance. However, in individuals carrying the variant allele, the risk of having CD in an ileocolonic location was reduced compared to control subjects (OR = 0.56, CI = 0.32-0.97, P = 0.037), and the risk of requiring a bowel resection was also significantly reduced to 0.59 (CI = 0.37-0.95, P = 0.028) (Table 4). Only in individuals who were smokers at the time of diagnosis, did this allele have a significant impact on UC (OR = 0.48, CI = 0.25-0.94, P = 0.028).

### Haplotype analysis

Haplotype analysis of two-SNPs was performed using HAPLO.SCORE. The results are summarized in Table 6. Haplotype frequencies were estimated, and association analyses were performed with respect to CD and

**Table 3** Genotype and allele counts for TNF- $\alpha$  variants in New Zealand IBD patients and Caucasians

SNP	Controls	CD	UC	CD + UC	IC
TNF- $\alpha$ -238 G/A					
AA	0	2 (0.5)	2 (0.5)	4 (0.5)	0
AG	50 (12.1)	47 (12.1)	39 (9.6)	86 (10.9)	2 (7.4)
GG	365 (87.9)	338 (87.3)	364 (89.9)	702 (88.6)	25 (92.6)
Genotype P		0.51	0.19	0.37	
HWE P	0.38	0.68	0.31	0.35	1
A	50 (6.0)	51 (6.6)	43 (5.3)	94 (5.9)	2 (3.7)
G	780 (94.0)	723 (93.4)	767 (94.7)	1490 (94.1)	52 (96.3)
OR (95% CI)		1.10 (0.72, 1.68)	0.87 (0.56, 1.36)	0.98 (0.68, 1.42)	
Allelic P		0.64	0.53	0.93	
TNF- $\alpha$ -308 G/A					
AA	10 (2.4)	16 (4.1)	23 (5.7)	39 (4.9)	1 (4.0)
AG	123 (29.7)	112 (28.9)	122 (30.3)	234 (29.6)	4 (16.0)
GG	282 (67.9)	260 (67.0)	258 (64.0)	518 (65.5)	20 (80.0)
Genotype P		0.41	0.049	0.1	
HWE P	0.49	0.4	0.1	0.07	0.29
A	143 (17.2)	144 (18.6)	168 (20.8)	312 (19.7)	6 (12.0)
G	687 (82.8)	632 (81.4)	638 (79.2)	1270 (80.3)	44 (88.0)
OR (95% CI)		1.09 (0.84, 1.42)	1.27 (0.98, 1.63)	1.18 (0.94, 1.48)	
Allelic P		0.49	0.06	0.14	
TNF- $\alpha$ -857C/T					
CC	359 (88.2)	332 (86.0)	358 (88.4)	690 (87.2)	21 (77.8)
CT	45 (11.1)	51 (13.2)	41 (10.1)	92 (11.6)	6 (22.2)
TT	3 (0.7)	3 (0.8)	6 (1.5)	9 (1.1)	0
Genotype P		0.61	0.57	0.85	
HWE P	0.2	0.45	0.004	0.01	1
C	763 (93.7)	715 (92.6)	757 (93.5)	1472 (93.0)	48 (88.9)
T	51 (6.3)	57 (7.4)	53 (6.5)	110 (7.0)	6 (11.1)
OR (95% CI)		0.84 (0.56, 1.26)	0.95 (0.63, 1.45)	0.89 (0.63, 1.28)	
Allelic P		0.38	0.82	0.52	

UC and Caucasian controls. A score for each haplotype (Hap-score) was calculated and *P*-value was obtained for the significance of each Hap-score. A positive hap-score implied that the haplotype occurred more frequently in the CD or UC patient group. A global *P*-value indicated the overall association between haplotypes and the response. The results showed that there was no significant association between these haplotypes for either CD or UC patients as compared with control subjects, *P* = 0.82 and *P* = 0.18 respectively (Table 6).

In the analysis of the two SNPs (rs1800629, rs361525) in CD, the haplotypes in patient and control subjects occurred at equal frequencies (*P* > 0.05, Table 6). Frequency haplotype AA was uncommon in both CD and UC subjects, 0.3% and 0% respectively and 0% for control subjects.

### Meta-analysis

Forest plots in Figure 1 show the results of the meta-analysis of three common polymorphisms in the TNF- $\alpha$  receptor: -238, -308, and -857. Four to sixteen studies satisfied, depending on the TNF- $\alpha$  receptor, the inclusion criteria. All studies provided description of the participants, CD and UC status, and compared the control group with CD or UC subjects. We included our own data along with the other studies. Cochran's *Q* test and *I*<sup>2</sup> index were performed in order to examine the heterogeneity of the outcomes of the studies.

**TNF- $\alpha$  -238:** The forest plot in Figure 1A shows the risk

of CD in each study. These seven studies appeared to be homogeneous in results (*Q* statistic = 4.10, df = 6, *P* = 0.66) and *I*<sup>2</sup> index was negative (*I*<sup>2</sup> < 0). The pooled odds ratio for these studies was 1.15 (95% CI = 0.88-1.49), indicating that carrying the variant TNF- $\alpha$  -238G/A allele did not significantly affect the overall risk of CD. The forest plot of six studies on UC subjects with controls is shown in Figure 1B. These studies were reasonably homogeneous (*Q* statistic = 2.24, df = 5, *P* = 0.82, *I*<sup>2</sup> < 0) and the pooled odds ratio was 1.04 (95% CI = 0.77-1.42), indicating that carrying the variant TNF- $\alpha$  -238G/A allele was not significantly associated with the overall risk of UC.

**TNF- $\alpha$  -308:** The forest plot of sixteen studies assessing the effect of carrying the variant allele on risk of CD is shown in Figure 1C. The combined estimate of odds ratio for these studies was 0.99 (95% CI = 0.84-1.15) by the random effect model with no homogeneity (*Q* statistic = 17.4, df = 15, *P* = 0.30, *I*<sup>2</sup> = 0.14). This finding indicates that carrying the variant TNF- $\alpha$  -308G/A allele was not significantly associated with the risk of CD. In addition, Figure 1D provides a graphical summary of the risk of carrying the variant TNF- $\alpha$  -308G/A allele in UC. The results indicate that this variant was not significantly associated with the overall risk of UC. The pooled odds ratio for the thirteen studies was 1.14 (95% CI = 0.84-1.54) by the random effect model (*Q* statistic = 9.5, df = 12, *P* = 0.08, *I*<sup>2</sup> = 0.39).

**TNF- $\alpha$  -857:** The forest plot Figure 1E shows the risk

**Table 4** Allelic odds ratios and 95% confidence intervals for comparison of TNF- $\alpha$  variants with IBD status in New Zealand IBD patients and Caucasians

	TNF- $\alpha$ 361525 (-238 G/A)				TNF- $\alpha$ 1799724 (-857C/T)				TNF- $\alpha$ 1800629 (-308 G/A)			
	CD		UC		CD		UC		Crohn's		UC	
Female	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
	1.00	0.59-1.69	0.64	0.35-1.18	1.06	0.65-1.74	1.07	0.64-1.79	1.01	0.72-1.41	1.19	0.85-1.67
Male	1.29	0.69-2.41	1.15	0.63-2.08	1.08	0.62-1.89	1.46	0.84-2.53	1.26	0.85-1.87	1.34	0.93-1.93
Age at first diagnosis												
0-16 yr	1.30	0.54-3.14	0.31	0.04-2.29	1.67	0.51-5.48	0.8	0.28-2.31	1.66	0.97-2.85	1.60	0.83-3.07
17-40 yr	0.92	0.55-1.54	0.8	0.46-1.39	0.85	0.53-1.36	0.75	0.47-1.20	1.01	0.74-1.39	1.23	0.90-1.68
> 40 yr	1.30	0.78-2.17	1.02	0.62-1.69	0.72	0.44-1.18	1.31	0.76-2.25	1.08	0.77-1.52	1.26	0.93-1.71
CD location												
Ileal	0.93	0.51-1.71			0.98	0.55-1.75			1.00	0.69-1.45		
Colonic	1.09	0.65-1.83			1.00	0.59-1.69			1.25	0.91-1.72		
Ileocolonic	1.44	0.79-2.63			0.56	0.32-0.97			0.92	0.59-1.42		
UC location												
Proctitis			1.07	0.61-1.87			0.68	0.41-1.12			0.90	0.62-1.30
Left colon			0.76	0.38-1.52			1.36	0.68-2.73			0.97	0.65-1.45
Pancolitis			0.74	0.40-1.36			1.08	0.62-1.88			1.91	1.41-2.60
CD Behaviour												
Inflammatory	1.27	0.81-2.00			1.05	0.64-1.71			1.19	0.89-1.60		
Structuring	1.19	0.67-2.10			0.66	0.39-1.11			1.01	0.69-1.47		
Penetrating	0.16	0.02-1.17			0.65	0.31-1.37			0.89	0.50-1.59		
Ileal/Structuring	1.34	0.70-2.58			0.95	0.47-1.92			1.04	0.66-1.64		
Colonic/Inflammatory	1.29	0.77-2.17			1.06	0.60-1.87			1.29	0.92-1.80		
	TNF- $\alpha$ 361525 (-238 G/A)				TNF- $\alpha$ 1799724 (-857C/T)				TNF- $\alpha$ 1800629 (-308 G/A)			
Any relative with IBD	1.15	0.57-2.32	0.89	0.39-2.01	1.02	0.49-2.12	1.02	0.47-2.20	1.49	0.98-2.27	0.98	0.60-1.60
Bowel resection	1.12	0.65-1.93	0.7	0.29-1.66	0.59	0.37-0.95	0.97	0.47-2.02	0.93	0.65-1.34	1.57	1.02-2.41
Smoker at diagnosis	1.04	0.54-1.99	0.66	0.23-1.87	1.00	0.52-1.91	0.48	0.25-0.94	0.84	0.54-1.30	1.08	0.63-1.86
Ever used immunomodulators	1.07	0.66-1.75	0.66	0.29-1.48	0.93	0.57-1.51	0.75	0.41-1.39	1.09	0.80-1.48	1.46	0.98-2.17
Any EIMs	1.11	0.55-2.24	0.5	0.18-1.41	1.60	0.67-3.80	2.07	0.74-5.83	1.46	0.96-2.22	1.28	0.81-2.03

**Table 5** Comparison of TNF- $\alpha$  variants with IBD status between Males and Females in New Zealand IBD patients and Caucasians

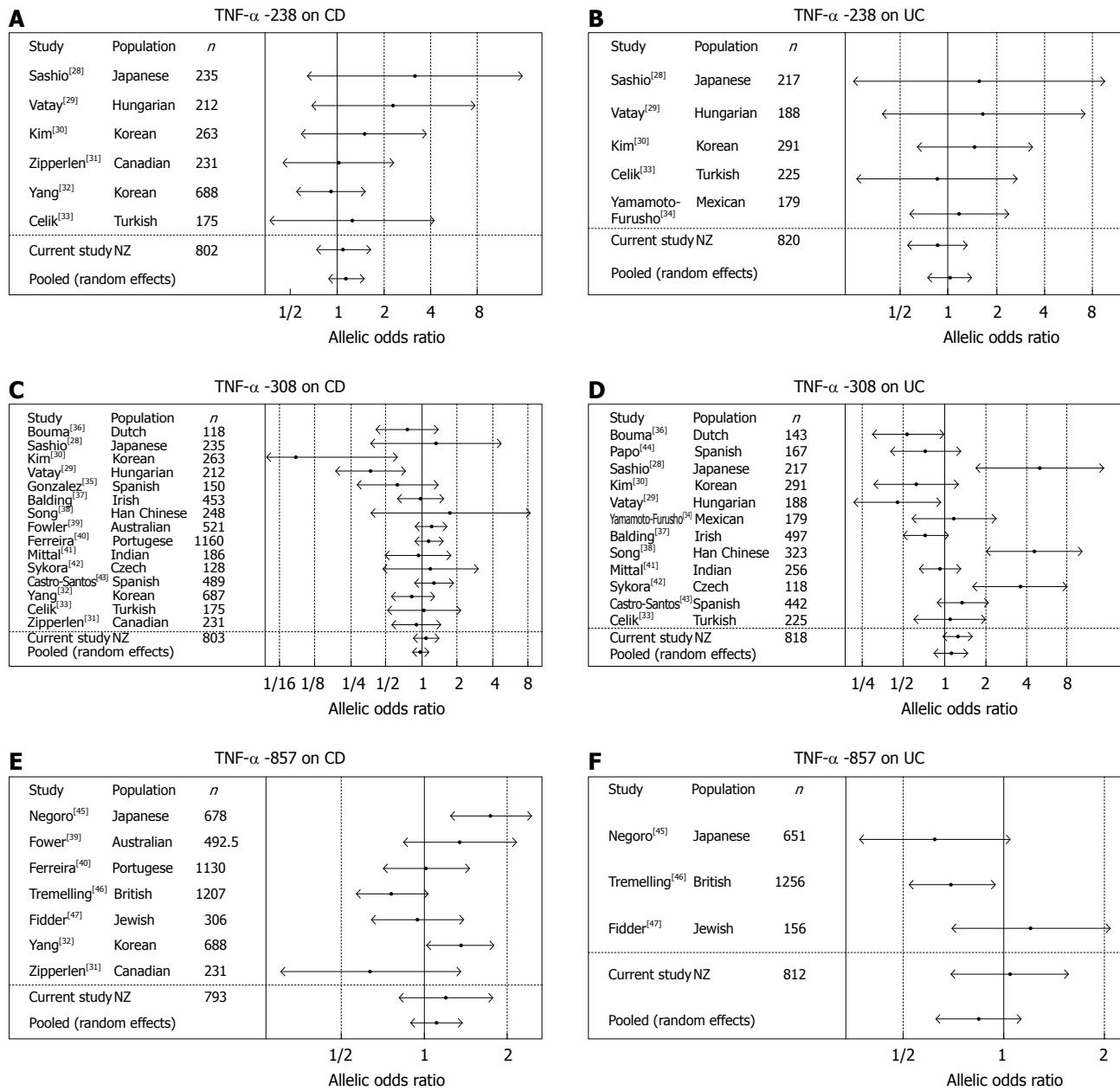
Gender	<i>n</i>	TNF- $\alpha$ -238		TNF- $\alpha$ -857		TNF- $\alpha$ -308	
		OR (95% CI)	<i>P</i>	OR(95% CI)	<i>P</i>	OR (95% CI)	<i>P</i>
CD vs Control	F	248/239	1.31 (0.99-1.75)	0.06	1.30 (0.98-1.73)	0.07	1.32 (0.99-1.75)
	M	139/176	1.00		1.00		1.00
UC vs Control	F	214/239	0.83 (0.63-1.09)	0.17	0.82 (0.62-1.08)	0.16	0.83 (0.63-1.09)
	M	191/176	1.00		1.00		1.00

**Table 6** Haplotype analysis of two-SNP TNF- $\alpha$  haplotype in IBD status in New Zealand IBD patients and Caucasians

	Haplotype	Case subject frequency (%)	Control subject frequency (%)	Hap-score	Haplotype-specific scores <i>P</i>	Global score statistics
CD	Two SNPs TNF- $\alpha$ region [-308 (rs1800629), -238 (rs361525)]					
	GG	75.3	76.7	-0.76	0.45	$\chi^2 = 0.85, df = 3, P = 0.82$
	GA	6.2	6.0	0.32	0.75	
UC	AG	18.2	17.3	0.59	0.56	
	Two SNPs TNF- $\alpha$ region [-308 (rs1800629), -238 (rs361525)]					
	GG	73.9	76.4	-1.31	0.19	$\chi^2 = 3.37, df = 2, P = 0.18$
	GA	5.3	6.0	-0.63	0.53	
	AG	20.8	17.5	1.79	0.07	

of CD in each study of carrying the variant TNF- $\alpha$  -857C/T allele. The overall estimate of odds ratio for the eight studies was 1.11 (95% CI = 0.89-1.38) by the random effect model with no homogeneity (*Q* statistic

= 6.73, *df* = 7, *P* = 0.46,  $I^2 < 0$ ). Carrying the variant TNF- $\alpha$  -857C/T allele was not significantly associated with the risk of CD. In addition, the random effect model gave a pooled odds ratio of 0.84 (95% CI = 0.63-1.13)



**Figure 1** Tree plots for meta-analysis. **A:** TNF- $\alpha$  -238 on CD; **B:** TNF- $\alpha$  -238 on UC; **C:** TNF- $\alpha$  -308 on CD; **D:** TNF- $\alpha$  -308 on UC; **E:** TNF- $\alpha$  -857 on CD; **F:** TNF- $\alpha$  -857 on UC.

with no homogeneity ( $Q$  statistic = 3.1,  $df = 3$ ,  $P = 0.38$ ,  $I^2 = 0.03$ ) for carrying the variant TNF- $\alpha$  -857C/T allele in UC (Figure 1F).

The present meta-analysis shows that carrying the TNF- $\alpha$  receptor SNPs -238, -308, or -857 was not significantly associated with the risk of IBD, both in the New Zealand population as well as in the international studies. However, the present analysis does not provide any information on male-female differences or site specificity.

## DISCUSSION

The present study adds to the international database available for all 3 alleles. In both males and females, carrying the variant TNF- $\alpha$  -238 G→A allele had no

significant effect in our study population. Previous reports suggesting that this allele was a risk factor for IBD, especially CD, have generally used small sample sizes, and the data were not consistent. The initial publications reported positive data in Japanese, Hungarian and Korean groups, albeit with small numbers of patients. Kim *et al*<sup>[30]</sup> in a study on Korean patients observed a significantly higher frequency of -238A allele of TNF- $\alpha$  in CD patients with perianal lesions compared with patients without perianal disease. Vatay *et al*<sup>[29]</sup> also showed an increased risk in their study on Hungarian subjects. However, several negative studies have been published, including Levine *et al*<sup>[48]</sup> who examined a pediatric Jewish population, and Sashio *et al*<sup>[28]</sup> in a study on Japanese patients. In particular, the latter authors found a difference in the carrier frequency for haplotype AG (-308 A, -238

G) between UC patients and controls, and suggested that one of the genes responsible for UC may be the TNF gene. Our own haplotype analysis failed to confirm these findings. However, we observed that this SNP led to lower production of TNF- $\alpha$  in IBD patients<sup>[16]</sup>.

The overall risk of either UC or CD was not significantly affected by carrying the TNF- $\alpha$  -308 G→A allele in our study. The meta-analysis shows 8 positive and 8 negative studies, leading to an overall allelic odds ratio of almost exactly zero. The published data are somewhat skewed by the strong negative association with CD found by Kim *et al* in the Korean population<sup>[30]</sup>. The data for UC are also somewhat dominated by the strong positive association reported by Sashio (Japanese)<sup>[28]</sup>, Song (Han Chinese)<sup>[38]</sup> and Sykora (Czech)<sup>[42]</sup>. Our own data is close to the overall figure based on the meta-analysis on UC patients.

Subgroup analysis may provide greater information on this allele. The present study shows that individuals carrying the variant TNF- $\alpha$  -308 G→A allele have a significantly greater risk of pancolitis in UC, and are also more likely to require bowel resection. The probable need for bowel resection in these individuals is consistent with other reports. Cucchiara *et al*<sup>[49]</sup> reported that Italian CD patients carrying this allele were more frequently resistant to steroids compared with non-carriers, and were more likely to require surgical resection. They suggested a causal relationship between these two events. This is in contrast to the findings of Louis *et al*<sup>[50]</sup> who found that the presence of this variant favoured steroid-dependent disease and to a lesser extent fistulizing and colonic disease. Both authors suggested that this genotype indicated a more intense TNF- $\alpha$ -driven inflammatory reaction at the mucosal level.

The increased need for surgery in our subjects carrying the variant TNF- $\alpha$  -308 G→A allele is consistent with the observations of other authors, including Cucchiara *et al*<sup>[49]</sup> in an Italian pediatric cohort and Sykora *et al*<sup>[42]</sup>, in their studies on Czech subjects. The latter group not only genotyped the TNF- $\alpha$  308 G→A polymorphism, but also measured the serum levels of inflammatory C-reactive protein (CRP), in relation to disease activity. Both UC and CD patients carrying the TNF- $\alpha$  -308 A variant showed a significant increase in CRP. The authors concluded that the TNF- $\alpha$  308 A polymorphism may play a role in modifying the IBD phenotype, influencing disease activity and leading to a more intense inflammatory activity. Other authors have also associated this genotype with inflammation, as measured by CRP or other measures<sup>[29,35,51]</sup>.

Carrying the TNF- $\alpha$  -857C→T variant failed to modify the overall risk in our study. The tree diagram shows that the international database provides a good number of Japanese and Australian individuals with an increased risk for CD, whereas a small study on Canadian subjects suggests a strong protective effect. Although the overall risk of CD is somewhat increased, there appears to be a comparable decreased risk for UC associated with carrying the variant allele. However, the TNF- $\alpha$  -857C→T variant significantly decreased the

risk of specific types and locations of CD. The risk of disease in an ileocolonic location decreased to 0.56 (limits 0.32-0.97), and the risk of requiring a bowel resection reduced to 0.59 (0.37-0.95). Only in individuals who were smokers at diagnosis, did this allele have an impact on UC [OR 0.48 (0.25-0.94)].

Fowler *et al*<sup>[39]</sup> identified a possible association of TNF- $\alpha$  -857 variant with an increased CD risk, especially in patients with structuring disease, in their case-control study on Australian subjects. Additionally, the TNF- $\alpha$  -857 CC genotype was independently associated with familial CD. The CD risk in their study was the strongest when individuals carried polymorphisms in both 308G/A and -857C/T. However, in a study in the UK, van Heel *et al*<sup>[25]</sup> showed an association of TNF-857C with IBD overall and with sub-phenotypes in either UC or CD, only in patients not carrying other common mutations. The transcription factor OCT1 binds TNF-857T but not TNF-857C, and interacts *in vitro* and *in vivo* with the pro-inflammatory NF- $\kappa$ B p65 subunit RELA at an adjacent binding site. van Heel *et al*<sup>[25]</sup> hypothesized that interaction of these transcription factors with specific alleles of TNF- $\alpha$  in gut tissue may be relevant in the pathogenesis of IBD.

Infliximab (IFX) is a monoclonal antibody against TNF- $\alpha$  that is commonly used as an IBD treatment. Ozeki *et al*<sup>[52]</sup> found that the -857T allele was in linkage with a lymphotoxin alpha (LTA) haplotype. They suggested that differences in therapeutic effects of IFX among patients with CD may be explained in part by the induction ability of TNF- $\alpha$  via the -857C/T polymorphism.

Each of the alleles in the present study appeared to enhance the risk in males as compared to females. Unfortunately, such gender stratification was not carried out in many of the other published studies. Therefore, it is not possible to determine whether this is true in other populations. However, it should be noted that such a sex imbalance is characteristic of the risks that have previously been associated with the IBD3 locus<sup>[6]</sup>. The TNF- $\alpha$  gene is closely linked to HLA-B<sup>[53]</sup>, and both Satsangi *et al*<sup>[54]</sup> and Ruuls and Sedgewick<sup>[55]</sup> pointed to the difficulties of distinguishing the effect of TNF genes from that of other genes of the Major Histocompatibility Complex, including HLA, to susceptibility and disease phenotype in IBD. It is possible that genes in this region interact with one another to influence IBD risk, as has been suggested in cardiovascular disease<sup>[56]</sup>. We propose that our data is consistent with TNF- $\alpha$  itself being the key factor in the increased IBD risk that has so consistently been associated with the IBD3 region.

The analysis of SNPs in the TNF- $\alpha$  promoter may suggest both pharmacogenomic and nutrigenomic approaches to overcoming the pathway defect. Stio *et al*<sup>[57]</sup> observed that TX 527 [19-nor-14,20-bisepi-23-yne-1,25(OH)(2)D(3)], a Vitamin D analogue, could exert an immunosuppressive effect on TNF- $\alpha$  production in CD patients, and that this effect was mediated by NF- $\kappa$ B down-regulation. The activation of NF- $\kappa$ B leads to its migration into the nucleus where it binds to the DNA

response elements in gene promoter regions. TX 527 inhibited the activation of NF- $\kappa$ B through the activation of the Vitamin D receptor (VDR). Thus, natural or synthetic vitamin D analogues may provide a basis for nutrigenomic or pharmacogenomic interventions that could be especially beneficial in individuals carrying variant alleles in the TNF- $\alpha$  promoter.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

There is international recognition that the IBD3 region, on chromosome 6p21, *IBD3* (MIM604519), confers susceptibility to inflammatory bowel disease (IBD). However, the nature of the relevant gene has been debated. Tumor necrosis factor-alpha (TNF- $\alpha$ ) is a strong possibility, and variant single nucleotide polymorphisms (SNPs) in the TNF- $\alpha$  promoter have previously been associated in some studies with increased risk of IBD, while other studies have produced conflicting results. The present study provides data based on a substantial New Zealand cohort, and a meta-analysis, that suggests increased IBD risk in individuals carrying any of three functional SNPs in the TNF- $\alpha$  promoter.

### Research frontiers

While an overall association with risk was not found, the carriers of either the -308 or -857 variants showed modified risk of specific IBD phenotypes. In particular, the study provides strong evidence that those individuals carrying the -308 G/A variant allele had an increased risk of pancolitis, and bowel resection in ulcerative colitis (UC). In contrast, carrying the -857 C/T variant decreased the risk of Crohn's disease (CD) in an ileocolonic location, and for the need for a bowel resection.

### Innovations and breakthroughs

The present study reveals that specific variant polymorphisms either increase the risk of certain forms of IBD, while others actually decrease the risk. Until now, there has been an assumption that all variants may be detrimental, but the present data suggests that this is not the case.

### Applications

There may be some justification for genotyping IBD patients for the presence of these polymorphisms, since this may indicate the need for surgery (or not) at an early stage. Further studies may usefully stratify patients according to gender and disease characteristics.

### Terminology

An "allele" is one of several alternative forms of a gene occupying a given locus on a chromosome. "SNP" describes a polymorphism (variation in sequence between individuals) caused by a change in a single nucleotide. This is responsible for most of the genetic variation between individuals.

### Peer review

This is a well written paper. The analysis is especially welcome considering the different conclusions from different researchers. This is an interesting paper which indicated that SNPs in the promoter of TNF- $\alpha$  gene may play a role in the risk of IBD in a New Zealand population.

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RAPID COMMUNICATION

# Incidence and risk factors for infantile colic in Iranian infants

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## Abstract

**AIM:** To assess the incidence of infantile colic and its association with variable predictors in infants born in a community maternity hospital, Tehran, Iran.

**METHODS:** In this prospective cohort study, mothers who gave birth to live newborns between February 21 and March 20, 2003 at the hospital were invited to join to the study. For every infant-mother dyad data were collected on infant gender, type of delivery, gestational age at birth, birth weight, birth order, and mother's reproductive history. Then mothers were given a diary to document the duration of crying/fussiness behaviors of their infants for the next 12 wk. We scheduled home visits at the time the infants were 3 mo of age to collect the completed diaries and obtain additional information on infants' nutritional sources and identify if medications were used for colic relief. Cases of colic were identified by applying Wessel criteria to recorded data. Chi-square and Mann-whitney *U* tests were used to compare proportions for non-parametric and parametric variables, respectively.

**RESULTS:** From 413 infants, follow-up was completed for 321 infants. In total, 65 infants (20.24%) satisfied the Wessel criteria for infantile colic. No statistical significance was found between colicky and non-colicky infants according to gender, gestational age at birth, birth weight, type of delivery, and, infant's feeding pattern. However, firstborn infants had higher rate for developing colic ( $P = 0.03$ ).

**CONCLUSION:** Colic incidence was 20% in this popu-

lation of Iranian infants. Except for birth order status, no other variable was significantly associated with infantile colic.

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**Key words:** Infantile colic; Incidence; Iran; Risk factors

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## INTRODUCTION

Infantile colic refers to a behavioral syndrome occurring during the first 3 mo of life. Crying is the core symptom, but clinicians differ on what other behaviors constitute the syndrome. By far the most widely used quantitative definition of colic is the one proposed by Wessel & his colleagues known as the "rule of threes"<sup>[1]</sup>.

Infants are considered to have colic if they cry for more than 3 h a day, for more than 3 d a week, and for more than 3 wk. It is usually self-limiting, without long term adverse consequences, but caring for an infant with colic can be distressing and frustrating for parents<sup>[2]</sup>. Estimates of cumulative incidence have varied from 10% to 40%<sup>[3-7]</sup>. This wide range may reflect differences in definitions, methods of data gathering, and study design<sup>[3,8]</sup> but also it may be related to a true difference in the occurrence rate of infantile colic among different communities. Notably, most studies are from western societies and many reviews only use data from selected populations<sup>[9-13]</sup>. While, due to the possible contribution of psychosocial factors to colic incidence<sup>[14-17]</sup>, it is unclear whether the data from developed countries can be applied to others, there are scarce reports on colic incidence from developing countries. On December 1, 2007 we performed a Medline search (1966-2007) using "colic"

as a text free word. The search was limited to "humans" and "infants (age up to 23 mo)". No other limitation was made in order to contain the search sensitivity. The Medline search revealed 795 citations. No study was found to evaluate the occurrence rate of infantile colic in the Middle East countries. We conducted this study in an effort to contribute new data from Iran as well as to identify risk factors for developing infantile colic.

## MATERIALS AND METHODS

### **Study setting and subjects**

This prospective cohort study was performed in Shahid Akbar Abadi maternity hospital, a high volume maternity hospital that serves as a major facility for a non-selected population of pregnant women in the southern part of Tehran, Iran; the majority of them having no medical problem. Anticipating the prevalence of colic in infants less than 3 mo to be 25% and absolute precision to be 5% ( $d = 0.05$ ), the chance of which should be at least 95%; a sample size of 289 was required. Considering the average hospital census of about 500 live births per month, and predicting a drop-out rate of up to 40% for nonparticipation or follow-up losses, we chose to consecutively include all live births at the hospital throughout a month period. Accordingly, between February 21 and March 20, 2003, all mothers of newborn babies at the hospital were invited to join the study.

### **Data collection**

Upon agreement to enter to the study, for every infant-mother dyad data were extracted from hospital files on infant gender, type of delivery, gestational age at birth, birth weight, birth order, and mother's reproductive history. Whenever the data were obscure or insufficient in charts, clarification was made by direct interviews with mothers. Within 3 d of delivery and before leaving the hospital, participating mothers received a 12 sheet diary leaflet, for the coming 12 wk. The diary format was based on a design introduced by Canivet *et al* elsewhere<sup>[18]</sup>. Briefly, it included a detailed form with squares for every 5 min period of 24 h for one predetermined day (Wednesday) of each week. Parents noted "crying" and "fussiness" separately and recorded them at their earliest convenience with letter symbols in relevant blocks on Wednesdays. The selection of 1 d out of a week, was a compromise to improve parents compliance in filling the forms and, therefore, to improve the diagnostic quality of estimating crying duration. As to the other 6 d of the week, parents made an estimation of crying and fussing time. Mothers were told not to register episodes of crying that were without any doubt related to hunger, fatigue or diaper changing. The crying thus had to cease instantly when the baby was fed, had fallen asleep, or when the diaper change was completed, and in no case continue for more than 5 min.

### **Follow-up**

When the children assessed in the perinatal study were 3 mo old, a home visit was made in order to obtain ad-

ditional information on whether medication had been required to manage crying/fussing lasted 1 wk or more, whether feeding formula had been added to or substituted for breast milk by the age of 2 mo and finally to collect the diaries. Arrangement was made to set the date of home visit not to exceed a time frame of  $\pm 3$  d from the exact age of 3 mo for any individual infant. Cases of colic were identified by applying Wessel criteria to recorded data. Crying and fussiness both were accounted for colic identification. We also accepted colic when crying/fussing continued for 3 h per day on 3 d for at least 1 wk and needed drug intervention to be controlled. This has been known as Wessel medication subcriterion<sup>[18]</sup>. We excluded cases if the diary questionnaire was returned incomplete or could not be reached, or if the infant was not alive by the age of 3 mo.

### **Statistical analysis**

The statistical analysis was performed using SPSS 11 for Windows. Chi-square was used for comparison of proportions. For reporting of results only variables with a *P* value less than or equal to 0.05 was considered significant.

## RESULTS

### **Baseline and follow-up data**

During the enrollment period a total of 442 infants were born from 437 mothers; 29 infants were not included in the study as their mothers chose not to be enrolled ( $n = 18$ ) or could not reliably fill in the diaries as they were illiterate ( $n = 11$ ). As a result 413 infants were initially entered to this study. At the follow-up visit 90 infants were excluded from the study-as parents could not be reached at home either due to nonattendance or moving to another address ( $n = 49$ ) or their diaries returned incomplete ( $n = 41$ ). We also excluded 2 premature infants who died early in their neonatal period. The rate of overall drop-out after enrolling to the study was 22%. Of 316 successful home visits, we gathered complete data for 321 infants (312 were singletons, 3 twins, and 1 triplet). Subsequent analyses were performed on these 321 infants who completed the study protocol. Of them, 168 cases (52.3%) were male and 153 (47.7%) were female. The range of birth orders was from 1 to 8; with the most frequent one was the first order which comprised 169 infants (52.6%). According to gestational age at birth, 229 infants (71.3%) were born full-term, 85 (26.5%) pre-term and 7 (2.2%) post-term. Type of delivery was vaginal delivery in 198 cases (61.7%) and cesarean section in 123 cases (38.3%). The mean  $\pm$  SD of birth weights was  $3072 \pm 580$  g (range: 1200-5000 g). In total, 57 infants (17.7%) had a low birth weight (< 2500 g). By 2 mo of age, the frequencies of exclusive breast-fed, exclusive formula-fed, and complementary-fed infants were 273 (85%), 17 (5.3%), and 31 (9.7%), respectively.

### **Colic incidence and risk factors**

In total, 65 infants fulfilled the Wessel criteria for infantile colic; all had cry/fuss behaviors of at least 3 h a day on

**Table 1 Comparison of potential predictors of infantile colic in colicky and non-colicky subgroups**

Factor	Colicky group (n = 65)	Non-colicky group (n = 256)	P
Gender			
Male	31	137	
Female	34	119	
Ratio	0.91	1.15	0.40
Mode of delivery			
Vaginal	42	156	
Cesarean	23	100	
Ratio	1.82	1.56	0.58
Gestational age at birth			
≥ 37 wk	45	191	
< 37 wk	20	65	
Ratio	2.25	2.93	0.38
Birth weight			
≥ 2500 g	50	214	
< 2500 g	15	42	
Ratio	3.33	5.09	0.21
Birth order			
First born	42	127	
Later born	23	129	
Ratio	1.82	92	0.03
Mode of feeding			
Exclusive breastfed	54	219	
Non-exclusive breastfed	11	37	
Ratio	4.90	4.76	0.24

3 d per week, lasting for 3 wk or more. No infant met the Wessel medication subcriterion i.e. their symptoms resolved after 1 wk by taking medication. Overall, the cumulative incidence rate of colic was found to be 20% in this study.

The frequency of selected potential risk factors for infantile colic in colicky and non-colicky subgroups has been demonstrated in Table 1.

Being the firstborn infant was more probable in colicky population ( $P = 0.03$ ), but no significant difference for other variables including gender, mode of delivery, gestational age at birth, birth weight, and mode of feeding was found between the two subgroups.

## DISCUSSION

The cumulative incidence rate of colic in the first 3 mo of infancy was 20% in this study. This rate is in concert with most reports from developed countries<sup>[3]</sup>. We enrolled all the infants whose mothers consented to participate in the study if they were literate enough to reliably fill in the diary. We did not exclude the infants born prematurely and/or needed special care at neonatal care unit. The only two infants who died over the study period (both within 2 wk after enrollment) were excluded from the study, as there was no chance to detect colic.

It has been claimed that surveys recruiting cases from well baby clinics reported lower occurrence rates compared to recruitment from birth-registered hospitals<sup>[3]</sup>. Hence, according to the non-selective design of our inclusion criteria and the site of the study which was a maternity hospital, the rate of 20% for infantile colic may be considered an overestimate if the figure

is to be extrapolated to the reference community. Nonetheless, in urban areas in Iran, the vast majority of all births occur in hospitals. In such settings, the results of prevalence studies on newborns in hospital settings could be considered representative of general population.

It has been suggested that the vast majority of infants with colic are presented by the age of 6 wk. Collection of cry/fuss data at this time point was expected to capture most cases of colic because the sixth week of life represents the peak of infant crying<sup>[19]</sup>. Colic is widely believed to remit by 3 mo of age<sup>[1,2]</sup>. Therefore it would be improbable that we missed significant number of colics by following the infants for 90 d.

We defined the occurrence of colic using the criteria proposed by Wessel, which are the criteria for diagnosis of colic in infants, which has gained most acceptance<sup>[19]</sup>. Although we also adopted Wessel medication subcriterion for colic definition, not even a single case required to be enrolled only by this definition. The popularity of colic definition in this study would make our results more comparable with the counterpart studies from western societies.

The reported occurrence rates of infantile colic vary within a wide range up to 40%. There is a tendency to attribute the different results to inconsistencies in case definitions and study protocols<sup>[3]</sup>. Little attention has been paid to the possibility of true epidemiological differences, probably due to paucity of qualified data from non-Western societies. Different diet and care taking activities, both of potential relevance to colic incidence<sup>[3]</sup> between developed and developing countries make comparative studies more plausible. Two retrospective studies published from India and Brazil revealed colic prevalence of 16% and 16.3%, respectively<sup>[12,20]</sup>. In a prospective study of 160 Korean infants, no case of infantile colic was found<sup>[21]</sup>. Interestingly, the latter survey was adequate according to the quality criteria in this study as the researchers use a 24 h diary and a definition of infantile colic that included a time criteria<sup>[3]</sup>.

The only variable showed to be predictive of colic development in this study was the birth order. Other factors including gender, weight at birth, type of delivery, mode of feeding and prematurity were not associated with the development of colic. The literature is very controversial in defining the risk factors for colic. Some methodological issues have been blamed to be responsible. Those include inconsistent definitions for colic, the failure to control covariates, and potentially biased assessments of exposure and/or outcome variables because of non-prospective study designs<sup>[19]</sup>.

Our study showing no difference regarding gender for colic presentation is in accordance with most other reports<sup>[3,22-25]</sup>. In a systematic review only one study reported a significantly higher proportion of boys crying more than 3 h per day<sup>[3,22]</sup>. Mode of delivery was not associated with colic in our study, which is in agreement with a report by Hogdall *et al*<sup>[24]</sup>. Like our study, no association between colic and low birth weight was

found in Lucassen's review<sup>[3]</sup>. That is contradicting with Crowcroft's study<sup>[2]</sup>.

We were unable to demonstrate a protective role of exclusive breastfeeding on the development of colic. Among surveys compared breast fed and formula fed infants: four found no difference<sup>[17,18,26,27]</sup>, in three studies the occurrence rates among breast fed infants were slightly higher<sup>[2,28,29]</sup>, and in one it was slightly lower<sup>[22]</sup>. When analyzing the presence of association between breastfeeding and the development of colic, reverse causality is an important distorting factor, since the mother of crying children may stop breastfeeding because they relate the condition to feeding or believe that their milk is insufficient or weak. In order to prevent this deviation we used the same definition used by Saavedra *et al* in their cohort study, i.e. children were considered exclusively breastfed if they were receiving only their mother's milk at the onset of colic<sup>[28]</sup>.

Despite observing some trend toward less colic in breast fed subgroup we were not able to reproduce the results of the latter study. Our smaller sample size might have caused the inability to detect meaningful differences in subgroups, but methodological differences could be the reason. Data were collected retrospectively in that study applying a questionnaire by the time the infants were 3 mo of age. In our study data gathering was performed prospectively by using diaries. It seems further prospective studies are needed to resolve this issue.

Being first borne was the single predicting factor for colic in our study. Surprisingly, this association has been evaluated in very few studies. Crowcroft *et al* published similar result but Lucassen *et al* and Saavedra *et al* reported contradicting finding<sup>[2,3,28]</sup>, the latter although benefited from a large sample size, was a retrospective study.

To evaluate the incidence and risk factors of infantile colic, we took the primary advantage of cohort studies for being not susceptible to reverse causality distortions<sup>[28]</sup>. Furthermore, we used diaries to collect data on cry/fuss behaviors. The prospective design of the study yields more reliable estimates of occurrence rates than retrospective studies, as the latter are prone to recall bias<sup>[3]</sup>.

Our study had two main limitations. Firstly, this was a single-center study and accordingly prone to selection bias distorting the application of the results to the reference community. Admittedly, because of differences in referral pattern in community and private hospitals, we can not exclude this potential source for selection bias. Nevertheless, the site in which our study was performed is located in the southern part of Tehran, where a minority of births occurs in private hospitals. Secondly, we lost 22% of cases initially enrolled to the study. This may raise the possibility of selection bias in the results. Higher rates for drop-out, however, are usual in other prospective studies. Moreover, the part of case loss which might be more important to selection bias was the subgroup who excluded for having incomplete

diaries. This subgroup consisted of 41 infants (10%) out of 331 infants initially recruited, a figure which was reasonably low.

In summary, this was a rare prospective cohort study conducted in a developing country to detect the occurrence rate of colic. The cumulative incidence rate for colic was defined 20% in the study group. The only significant predictor for developing colic was being the first live-born infant. According to this study, similar to Western societies, infantile colic is a common condition in Iranian infants.

## COMMENTS

### Background

Published data from developing countries on the incidence and/or risk factors of infantile colic are scarce. The possible contribution of cultural, nutritional, and socioeconomical factors to colic development make it prudent to conduct more studies in developing countries.

### Research frontiers

There is consensus in the literature on the complexity of the nature and heterogeneity of the risk factors associated with infantile colic. Most data on this issue has been obtained from the studies conducted in Western societies, especially in North America and Scandinavian countries. Those populations differ in the frequency of many infantile colic risk factors from developing countries.

### Innovations and breakthroughs

Our study is one of the rare prospective studies which have ever performed in developing countries to define the infantile colic incidence and its associated risk factors. During 3 mo follow-up of a population of Iranian infants, around 20% met the Wessel criteria for infantile colic which is in concert with most reports from developed countries. We also found firstborn infants were at higher risk to develop colic.

### Applications

Despite difference in many demographical, social and cultural factors between developed and developing countries, we demonstrated a comparable incidence of infantile colic in our study population. Although further studies are needed to define colic incidence in developing countries, our results may suggest colic occurrence is not tightly related to social or cultural variables.

### Peer review

It's a well-written paper. The study showed that colic incidence was 20% in this population of Iranian infants. Except for birth order status, no other variable was significantly associated with infantile colic.

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RAPID COMMUNICATION

# Lack of nitrate tolerance in isosorbide dinitrate- and sodium nitroprusside-induced relaxation of rabbit internal anal sphincter

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## INTRODUCTION

The internal anal sphincter (IAS) is the specialized continuation of the circular smooth muscle layer of the rectum. It is innervated by autonomic nerve system and plays an important role in anorectal physiology. Prominent nonadrenergic noncholinergic (NANC) innervations of IAS have been demonstrated<sup>[1-3]</sup>. *In vivo* and *in vitro* studies showed that the NANC neurons caused relaxation of the IAS<sup>[4-6]</sup>. The tonic resting pressure of IAS is called “anal resting pressure”. The IAS pressure has been found to display an overshoot phenomenon in the patients with chronic anal fissure<sup>[7,8]</sup>.

A number of drugs and chemicals were studied to decrease basal and precontracted tonus of IAS *in vitro*<sup>[9-14]</sup>. It has been shown that some drugs such as glyceryl trinitrate (GTN) and isosorbide dinitrate (ISDN) as nitric oxide (NO) donor might be used in treatment of chronic anal fissure<sup>[15,16]</sup>. However, prolonged exposure to high levels of nitroglycerine and other organic nitroesters is known to induce tolerance in the cardiovascular system drug both in humans and in experimental animals<sup>[17-20]</sup>. The same type of tolerance was also shown in rabbit penile tissue against relaxant effect of ISDN<sup>[21,22]</sup>.

It would be reasonable to expect that a similar type of tolerance may develop in internal anal sphincter smooth muscle against the relaxant effect of NO donating drugs. The present study is conducted to test this hypothesis, in rabbit IAS strips smooth muscle using ISDN as a tolerance-inducing agent.

## Abstract

**AIM:** To investigate the tolerance development against the relaxant effect of nitric oxide donating drug isosorbide dinitrate (ISDN) and sodium nitroprusside (SNP) in internal anal sphincter (IAS) smooth muscle.

**METHODS:** Relaxation responses of ISDN, and electrical field stimulation (EFS) were obtained before and after tolerance induction by ISDN incubation.

**RESULTS:** ISDN ( $10^{-7}$ - $10^{-4}$  mol/L) and SNP ( $10^{-8}$ - $10^{-4}$  mol/L) caused a concentration-dependent relaxation on the basal tonus of the isolated rabbit IAS strips. After a period of 2 h incubation of the  $6 \times 10^{-4}$  mol/L ISDN the relaxation effects of ISDN and SNP did not change compared to control strips. EFS evoked frequency-dependent relaxation in internal anal sphincter smooth muscle and  $E_{max}$  obtained from control strips were not changed in ISDN tolerance-inducing condition. In this study nitrate tolerance was not observed in rabbit IAS smooth muscle.

**CONCLUSION:** This result shows that nitric oxide donating drugs relaxes the internal anal sphincter of the rabbits without the development of tolerance.

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**Key words:** Internal anal sphincter; Nitrate tolerance;

## MATERIALS AND METHODS

### **Isolation of rabbit internal anal sphincter**

Twelve white New Zealand rabbit weighing between 2.5-3 kg were used in this study. Rabbits were sacrificed by cervical dislocation after sodium pentobarbital (40 mg/kg per body weight) anesthesia. The entire anal canal was isolated in continuation with a part of the rectum and transferred to oxygenated (95% O<sub>2</sub> and 5% CO<sub>2</sub>) Krebs' solution of following composition (mmol/L): NaCl, 118; KCl, 4.7; CaCl<sub>2</sub>, 2.5; NaHCO<sub>3</sub>, 25; MgSO<sub>4</sub>, 1.2; KH<sub>2</sub>PO<sub>4</sub>, 1.2, glucose, 11. The anal canal was carefully freed of all extraneous tissues. It was opened along its anterior wall, rinsed quickly, and pinned on a wax block containing oxygenated Krebs' solution, at its *in vivo* length as mucosa facing up. The mucosa was removed by sharp dissection under magnification. Distal 2 mm of the anal canal was used for preparation of 2 mm width and 1 cm long IAS smooth muscle strips. Each end of the IAS strips was tied with fine silk ligatures.

### **Measurement of isometric tension**

IAS strips transferred to 10 mL tissue baths containing oxygenated (95% O<sub>2</sub> and 5% CO<sub>2</sub>) Krebs' solution maintained at 37°C. One end of each strip was anchored to the bottom of the tissue bath, and other end was anchored to force transducer (model FT03 Grass Instruments Co., Quincy, MA) under an initial load of 2 g for the measurement of isometric tension on a pen polygraph recorder (model 79E Grass Instruments Co., Quincy, MA). After equilibration, relaxation responds of ISDN, SNP and Papaverine were obtained in different concentrations by adding these agents to the bath in a cumulative manner. Maximum relaxations induced by ISDN and SNP were expressed as the percentage of relaxation induced by papaverine.

### **Induction of ISDN tolerance**

After obtaining pre-incubation concentration-response curves with relaxant agents, IAS muscle strips were incubated by ISDN at a concentration of  $6 \times 10^{-4}$  mol/L for 2 h in different experiments in order to induce the tolerance. After exposure to the tolerance-inducing condition, strips were washed repeatedly with Krebs' solution for 15 min and then exposed cumulative concentrations of SNP, ISDN and papaverine again and post-incubation-response curves were obtained.

### **Electrical field stimulation (EFS)**

In another series of experiments, rabbit IAS muscle strips were electrically stimulated. Stimulation was provided *via* two parallel platinum electrodes. It was conducted at sequential frequencies of 2 Hz, 4 Hz, 8 Hz, 16 Hz as square-wave pulses of 50 V (0.8 ms) delivered by a current amplifier and a stimulator (S 88, Grass). Muscle strips were allowed to return to the base-line of normal basal tonus before each new frequency was delivered during EFS. In all studies, the duration of the electrical stimulation was 10 s. In order to eliminate adrenergic

and cholinergic components of nerve stimulation and to study relaxation responses to the non adrenergic, non-cholinergic nerves all experiments were performed in presence of atropine ( $10^{-5}$  mol/L) (muscarinic nerve blocker) and guanethidine ( $10^{-6}$  mol/L) (adrenergic nerve blocker). The application of the nitric oxide synthase inhibitor L-NAME [N (G)-nitro-L-arginine of methyl ester], a potent inhibitor of NO synthase, inhibited the relaxation responses, which were restored by application of L-arginine, and it has been concluded that the relaxation responses elicited by EFS were nitrergic-mediated activation. After the EFS relaxation was obtained, the same procedure was repeated in the presence of ISDN tolerance-induced conditions.

### **Drugs**

Guanethidine sulphate, atropine sulphate, sodium nitroprusside, L Name, L arginine, and papaverine hydrochloride were dissolved in distilled water. ISDN was dissolved in dimethylsulphoxide and dilutions were made in distilled water. All drugs were purchased from Sigma Chemical Company.

### **Statistical analysis**

All data are expressed as mean  $\pm$  SE. Tissue relaxant responses were expressed as percentage of papaverine-induced relaxation. In order to evaluate the effect of agonists; maximum responses (E<sub>max</sub>) and pD<sub>2</sub> values were calculated. The concentration-response data obtained in each individual experiment were plotted as the response/concentration (y) against the response (x). This produced a straight-line relationship in each experiment as predicted from the Scathard equation for drug-receptor interaction: Response/Concentration = -1/EC<sub>50</sub> × Response + Maximum response/EC<sub>50</sub>.

The pD<sub>2</sub> value was expressed as the negative logarithm of the EC<sub>50</sub> value. Inter-group differences were tested by Wilcoxon test. P value of less than 0.05 was considered to be statistically significant.

## RESULTS

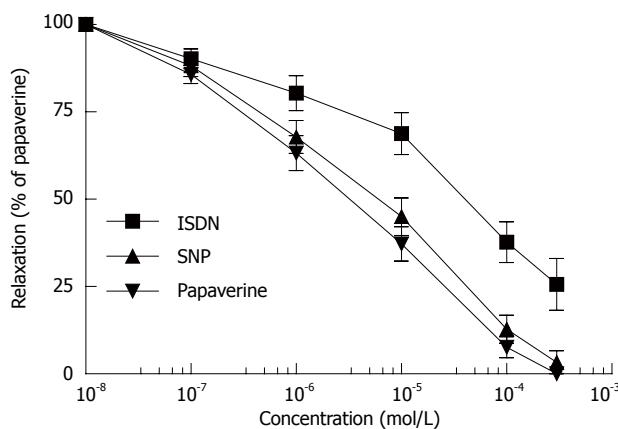
The IAS muscle strips developed spontaneous basal tonus several minutes after equilibration. ISDN ( $10^{-7}$ - $10^{-4}$  mol/L) caused a concentration-dependent relaxation on the basal tonus of the isolated rabbit IAS muscle strips. The maximum relaxation caused by ISDN was  $74.4 \pm 7.2$  percent of the relaxation caused by papaverine (Table 1, Figure 1). pD<sub>2</sub> value (negative logarithm of the EC<sub>50</sub> value) was  $5.48 \pm 0.05$  (Table 1). After a period of 2 h incubation by  $6 \times 10^{-4}$  mol/L ISDN, the relaxation effect induced by ISDN was not change compared to control strips (Figure 2A). pD<sub>2</sub> value of ISDN was not changed either by the attempt to induce tolerance with ISDN incubation. When we compared the concentration-response curves of the strips incubating by ISDN and control, there was no statistically significant difference in E<sub>max</sub> values between groups (Table 1).

SNP ( $10^{-8}$ - $10^{-4}$  mol/L) caused a concentration-dependent relaxation on the basal tonus of the isolated

**Table 1 Maximum relaxation responses ( $E_{max}$ ) and pD2 values to agonists in strips of internal anal sphincter smooth muscle from two groups of rabbits ( $n = 6$  each experiments)**

	Control	ISDN-induced tolerance
ISDN	$E_{max}$ 5.48 ± 0.05	74.4 ± 7.2 5.40 ± 0.06
SNP	$E_{max}$ 5.48 ± 0.05	96.7 ± 3.3 5.57 ± 0.05
EFS	$E_{max}$	72.5 ± 7.4 78.1 ± 6.3

$E_{max}$ : The maximum relaxation response (percentage of papaverine induced relaxations); pD2: Negative logarithm of the  $EC_{50}$  value.



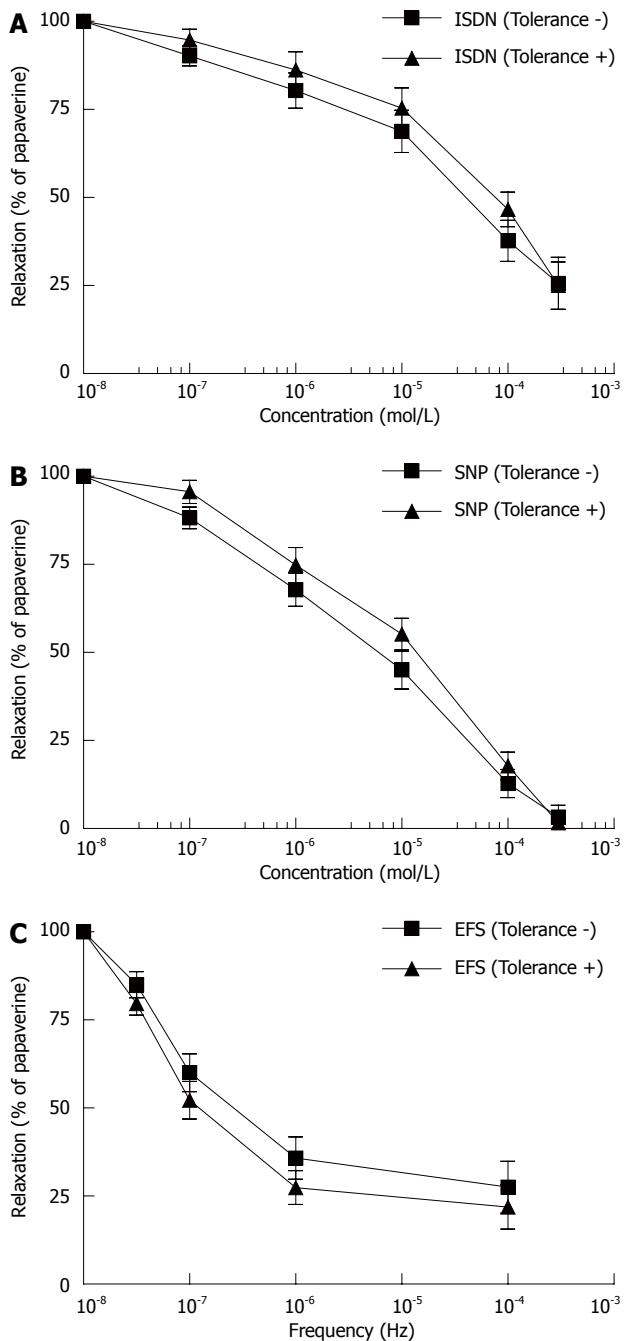
**Figure 1** The relaxant effects of ISDN, SNP and papaverine, in isolated rabbit internal anal sphincter smooth muscle strips. Papaverine induced relaxation was accepted as 100%. Maximum relaxations of ISDN and SNP were expressed as percentage of relaxation induced by papaverine. Each curve represents the mean ± SE for 6 experiments.

rabbit IAS muscle strips the maximum relaxation induced by SNP was  $96.7 \pm 3.3$  percent of the relaxation induced by papaverine (Figure 1). The attempt to induce tolerance with ISDN incubation had no significant effect on these responses and there was no change in  $E_{max}$  values of drug (Table 1, Figure 2B). pD2 values of SNP in basal tonus and ISDN-induced condition were  $5.60 \pm 0.04$  and  $5.57 \pm 0.05$ , respectively.

EFS evoked frequency-dependent relaxation of IAS smooth muscle strips in the presence of  $5 \times 10^{-5}$  mol/L guanethidine and  $10^{-6}$  mol/L atropine.  $E_{max}$  ( $72.5 \pm 7.4$ ) obtained from vehicle incubated control strips was not significantly changed in ISDN tolerance-inducing condition ( $78.1 \pm 6.3$ , Table 1, Figure 2C).

## DISCUSSION

Traditionally, lateral internal sphincterotomy was the gold standard treatment for chronic anal fissures. However this procedure is associated with a risk of incontinence to some degree in 30% of patients<sup>[23]</sup>. Recent studies have suggested that NO release as an important mediator from enteric inhibitory neurons and causes the relaxation of the IAS muscle<sup>[24,25]</sup>. In a similar way, NO donating agents lead to IAS relaxation via a non-adrenergic non-cholinergic pathway<sup>[26]</sup>. This effect of NO donating agents such as



**Figure 2** The influence of *in vitro* isosorbide dinitrate (ISDN)-induced tolerance on relaxant effects of ISDN (A), relaxant effects of sodium nitroprusside (SNP) (B) and relaxant effects of electric field stimulation (C) compared with control values in isolated strips of internal anal sphincter smooth muscle. Each curve represents the mean ± SE for 6 experiments.

ISDN and GTN can produce beneficial clinical effects in the healing of anal fissures by relaxing of IAS. Topical application of GTN and ISDN seems to promote the healing process of chronic anal fissures<sup>[16,27-30]</sup>.

In this study, the organic nitrates ISDN and SNP caused concentration-dependent relaxation in rabbit IAS muscle strips. The maximum relaxation effects induced by ISDN and SNP in basal tonus were 74% and approximately 100% respectively as compared to papaverine response.

Organic nitrates have long been used in treating

cardiovascular diseases due to their potent relaxation effects on the vascular smooth muscle. This relaxant effect is generated by *in vivo* metabolic conversion of these compounds to nitric oxide (NO), which then stimulates vascular relaxation by producing of cyclic GMP<sup>[31,32]</sup>.

One of the major drawbacks of NO donating agent therapy in cardiovascular diseases is the rapid development of pharmacological tolerance. It develops shortly after the beginning of treatment and occurs both *in vitro* and *in vivo* conditions<sup>[32,33]</sup>. Both human and experimental studies showed that prolonged exposure to high levels of nitroglycerin and other organic nitroesters induce tolerance against the cardiovascular effects of the drugs<sup>[17-20]</sup>.

So far, only one study has been reported about NO donating drug tolerance in anorectal smooth muscle<sup>[34]</sup>. Wang *et al* showed that GTN caused significant and sustained relaxation of anorectal smooth muscle in the anaesthetized rat without evidence of tolerance development<sup>[34]</sup>. In this study the relaxation response induced by NTG was not diminished in the anorectum in conditions that produced vascular tolerance.

The mechanisms of nitrate tolerance are complex and multi-factorial, including processes such as decreased tissue sulphhydryl availability, reduced bioactivation of the parent drug, physiological compensation and tissue oxidative stress<sup>[32,33,35]</sup>. Some investigators suggested that tolerance may arise from one or more steps involving the biotransformation process of organic nitrates prior to the formation of NO<sup>[21,22]</sup>. The action of nitrates is dependent on the liberation of from their molecule which subsequently activating guanilate cyclase<sup>[36]</sup>. The production of cGMP (the alleged mediator of nitrate-induced vascular smooth muscle relaxation) is reduced in tissues, which develop tolerance<sup>[37]</sup>. cGMP accumulation, was significantly decreased after NTG pre-incubation in the rat aorta but not in the rat anorectal smooth muscle and anal sphincter<sup>[34]</sup>.

Prolonged exposure of rabbit IAS muscle strips to high concentration of ISDN did not change the relaxant effect of the NO donating agents. As far as we know this result is the first *in vitro* evidence showing that rabbit IAS smooth muscle is spared from the tolerance induced by organic nitrates.

In conclusion, the main finding of the current study is that nitrate tolerance is not developing in isolated rabbit internal anal sphincter smooth muscle.

## COMMENTS

### Background

The internal anal sphincter pressure has been found to display an overshoot phenomenon in the patients with chronic anal fissure. It has been showed that some drugs such as glyceril trinitrate (GTN) and isosorbide dinitrate (ISDN) as nitric oxide (NO) donor might be used in treatment of chronic anal fissure. Prolonged exposure to high levels of nitroglycerin and other organic nitroesters is known to induce tolerance in the cardiovascular system and penile tissue both in humans and in experimental animals.

### Research frontiers

In this study, the organic nitrates ISDN and SNP caused concentration-

dependent relaxation in rabbit IAS muscle strips. The main finding is that nitrate tolerance is not developing in isolated rabbit internal anal sphincter smooth muscle.

### Innovations and breakthroughs

Our study is the first *in vitro* evidence showing that rabbit IAS smooth muscle is spared from the tolerance induced by organic nitrates. Only one *in vivo* study has been reported about NO donating drug tolerance in anorectal smooth muscle before.

### Applications

Although animal findings cannot be assumed to apply to human beings and *in vitro* experimentation should not lead straight to clinic conclusions, topical use of NO donating drugs should be continued for the treatment of anal fissure disease without a fear of tolerance development. In the future, this result may be verified in human studies.

### Peer review

The paper deals nicely with an interest subject. The main finding of the current study is that nitrate tolerance is not developing in isolated rabbit internal anal sphincter smooth muscle. Topical use of NO donating drugs should be continued for the treatment of anal fissure disease without a fear of tolerance development.

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RAPID COMMUNICATION

## Genotype phenotype correlation in Wilson's disease within families-a report on four south Indian families

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**CONCLUSION:** We report concordance between ATP7B mutation and WD phenotype within each family with > 1 member affected with WD. Homozygous ATP7B mutation was present in 3 of the 4 families studied. Our report supports allelic dominance as a determinant of WD phenotype. However, in one family with compound heterozygous mutation, there was a similar WD phenotype which suggests that there may be other factors determining the phenotype.

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**Key words:** Wilson's disease; Genotype phenotype correlation

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### Abstract

**AIM:** To study the genotype phenotype correlation in Wilson's disease (WD) patients within families.

**METHODS:** We report four unrelated families from South India with nine members affected with WD. Phenotype was classified as per international consensus phenotypic classification of WD. DNA was extracted from peripheral blood and 21 exons of ATP7B gene and flanking introns were amplified by polymerase chain reaction (PCR). The PCR products were screened for mutations and the aberrant products noted on screening were sequenced.

**RESULTS:** Four separate ATP7B mutations were found in the four families. ATP7B mutations were identical amongst affected members within each family. Three families had homozygous mutations of ATP7B gene while one family had compound heterozygous mutation, of which only one mutation was identified. We noted concordance between ATP7B gene mutation and Wilson's disease phenotype amongst members within each family. The age of onset of symptoms or of detection of asymptomatic disease, baseline serum ceruloplasmin and baseline urinary copper levels were also similar in affected members of each family. Minor differences in phenotype and baseline serum ceruloplasmin level were noted in one family.

### INTRODUCTION

Wilson's disease (WD) is an autosomal recessive disorder characterized by excess hepatic copper accumulation and impaired biliary copper excretion. In 1993, the gene encoding WD protein (ATP7B) was cloned and was found to encode a copper transporting P-type ATPase required for biliary copper excretion. However, although the characterization of the molecular genetic basis of this disease has provided insight into the mechanisms of copper homeostasis, clinical studies of specific patients have not been useful in elucidating the mechanism of hepatic copper metabolism<sup>[1]</sup>. One intriguing question in WD is how closely the genotype determines the phenotype. While over 300 mutations of ATP7B gene have been reported in WD, over one-half of these occur rarely in any given population. Most patients are compound heterozygotes, possessing alleles with two different mutations. This degree of allelic heterogeneity has been a major hindrance in studying genotype phenotype correlation in WD<sup>[2,3]</sup>.

In such a situation, one approach is to study the

phenotypes of a single predominant genotype in a population. The results of this approach have shown poor genotype phenotype correlation in the different populations studied<sup>[4-6]</sup>. Another approach is to compare genotype and phenotype in WD occurring within members of a family. In a report of two Japanese families, the affected family members had similar compound heterozygous mutations of ATP7B gene but had different phenotypes and different ages of onset of disease suggesting allelic dominance as a factor determining the phenotype<sup>[7]</sup>. We report four families wherein the affected members within each family had identical mutations of ATP7B. We compared the phenotypes within these families to see if the phenotypes were true to the index case.

## MATERIALS AND METHODS

### Subjects

We report four unrelated families from the states of Tamil Nadu and Andhra Pradesh in South India with nine documented cases of WD. The basis of the diagnosis of WD was the presence of Kayser Fleischer rings in the cornea on slit lamp examination and the presence of a low serum ceruloplasmin level (measured by copper oxidase method). Further investigations were carried out as clinically indicated. WD phenotype was classified as per the international consensus classification<sup>[8]</sup>. Neurological involvement was determined by clinical examination, while liver involvement was assessed by liver function tests, ultrasonography and liver biopsy, if indicated, in addition to clinical examination.

### Genetic studies

Peripheral blood was collected from the patients after obtaining an informed consent. The genomic DNA was extracted from white blood cells by standard phenol-chloroform method. The 21 exons of ATP7B gene and their flanking intronic sequences were amplified by polymerase chain reaction (PCR) using primers described earlier<sup>[5]</sup>. PCR amplified products were subjected to conformation sensitive gel electrophoresis (CSGE) for mutation screening<sup>[9]</sup>; those exhibiting aberrant patterns in CSGE were sequenced using automated sequencer (ABI 310, Applied Biosystems, CA).

### Genotype phenotype correlation

Mutations of ATP7B gene and WD phenotype in each of the affected family member was compared with other affected members within that family. Similarly, other indices like age of onset of symptoms/age of detection of asymptomatic disease, baseline serum ceruloplasmin and baseline urinary copper levels were also compared within symptomatic members of each family.

## RESULTS

All the nine patients had Kayser Fleischer rings and low serum ceruloplasmin levels. Three of the nine patients

were asymptomatic and were detected to have WD on family screening, after the index patient was diagnosed within the family. All nine patients had abnormal liver function tests and/or abnormal liver on ultrasound. Only one patient (family 4, Table 1) underwent liver biopsy, which showed cirrhosis of liver. The affected members in families 1 to 3 had identical homozygous mutations of ATP7B gene within each family (Table 1). These three mutations of ATP7B gene are novel mutations. The ATP7B gene mutations identified were A2623G (Arg-Gly) in family 1, G4021A intronic mutation in family 2, and a point mutation A3029G (Lys-Arg) in family 3. The two siblings in family 4 were compound heterozygotes, in whom the only mutation identified, G3282A (Phe1094Leu), was identical in both the siblings. This mutation has been recently reported from Brazil<sup>[10]</sup>.

In addition to the mutations, 2-5 polymorphisms of ATP7B gene were also identified in each of the nine patients studied. Similar to the mutations, the polymorphisms of ATP7B gene were also identical in members within the same family (Table 1). In Family 1, 4 of 6 children born to partners in a consanguineous marriage, had WD. DNA was available for mutation analysis from three of the four affected children. While all three had hepatic involvement, the elder brother also had arthritis involving the right knee (Table 1). In Family 2, of the 4 children-products of a consanguineous marriage-one son and one daughter had neurological and neuro-psychiatric involvement respectively. In Family 3, (non-consanguineous marriage), the father presented at age 45 years with WD accompanied with a metabolic bone disorder and renal tubular acidosis. On screening the family, his son was detected to have WD (diagnosed on the basis of low serum ceruloplasmin level and KF rings on slit lamp examination). He also had elevated urinary copper level. However, his liver function tests were normal and the liver appeared normal on ultrasonography. A liver biopsy was refused. Since this subject did not have neurological involvement, it was assumed that he had sub-clinical liver disease. Unlike the father, the son did not have renal tubular acidosis. The mother and daughter did not have WD. In family 4, four children (4 sons) were born to parents in a consanguineous marriage. One son died of liver disease (age of onset of symptoms: 13 years), however he had not been evaluated for WD. Another son aged 17 years, who was healthy, had no clinical, biochemical, and radiological signs of liver disease. Of the two affected sons who were alive, one had overt and the other had asymptomatic hepatic involvement.

The WD phenotype, age of onset of symptoms/age of detection of WD, baseline serum ceruloplasmin and baseline urinary copper levels were similar among members of a family, except for some differences in family 3 (Table 1). In family 3, there were marked differences in the age of onset of symptoms/age of detection of WD and baseline serum ceruloplasmin levels (difference of 50 U/L) between the father and

**Table 1** Detailed genotype and phenotype in four families with Wilson's disease

Family	WD patient	Age at onset (yr)	ATP7B mutation	ATP7B gene polymorphisms	Clinical phenotype	Baseline serum ceruloplasmin <sup>5</sup> (U/L)	Baseline urinary copper <sup>6</sup> ( $\mu\text{g}/24\text{ h}$ )
1	Brother	12	Ex <sup>7</sup> 11: A2623G	Ex <sup>7</sup> 2: T1216G	H2 <sup>1</sup>	8	Not available
	Sister <sup>4</sup>	14		Ex <sup>7</sup> 3: G1366C			
	Brother	16		Ex <sup>7</sup> 12: G2855A Ex <sup>7</sup> 13: G3009A		4	430
2	Brother	14	In <sup>8</sup> 19: G4021A	Ex <sup>7</sup> 2: T1216G	NI <sup>3</sup>	6.2	166
	Sister	12		Ex <sup>7</sup> 3: G1366C Ex <sup>7</sup> 10: T2495C Ex <sup>7</sup> 12: G2855A Ex <sup>7</sup> 13: G3009A		1.9	112
3	Father	45	Ex <sup>7</sup> 13: A3029G	Ex <sup>7</sup> 2: T1216G	H2 <sup>1</sup> , O <sup>2</sup>	6	340
	Son <sup>4</sup>	16		Ex <sup>7</sup> 10: C2495A		56	983
4	Brother	11	Ex <sup>7</sup> 15: G3282A/?	Ex <sup>7</sup> 10: T2495C	H2 <sup>1</sup>	32	1067
	Brother <sup>4</sup>	10		Ex <sup>7</sup> 12: G2855A			
	Brother <sup>9</sup>					26	630

H2<sup>1</sup>: Hepatic; O<sup>2</sup>: Other; NI<sup>3</sup>: Neurological phenotypes of Wilson's disease; <sup>4</sup>Asymptomatic subject, WD detected on family screening; <sup>5</sup>Serum ceruloplasmin: (normal range) 62-140 U/L; <sup>6</sup>Urine copper: normal upto 150  $\mu\text{g}/24\text{ h}$ ; <sup>7</sup>Ex: denotes Exon; In<sup>8</sup>: Denoted Intron; <sup>9</sup>Died of liver disease (age of onset of symptoms: 13 years), was not tested for Wilson's disease.

son. In addition, renal tubular acidosis was present only in the father.

## DISCUSSION

The present study, examined the genotype phenotype correlation in WD occurring within members of each family, whereas previous studies examined the question with respect to a single common mutation in a given population. While the previous studies gave answers related to that particular mutation, we investigated the impact of four different mutations in members of four unrelated families with more than one member affected with WD. Although the number of subjects studied was small, our report highlights some aspects of interest in genotype phenotype correlation in WD. In the present report, the affected members of each family shared identical mutations of ATP7B gene within each family (homozygous mutations in three families and compound heterozygous mutation in one family). We found strong genotype phenotype concordance in members of each family. The two minor differences in phenotype within members of a family were the following: in the first family, one sibling had monoarticular arthritis; a known association with WD, which was not present in the other two siblings, while in family number 3, the father had renal tubular acidosis and metabolic bone disease where as the son did not have these abnormalities.

The three additional phenotypic indices studied (age of onset of symptoms or age at detection of asymptomatic disease, baseline serum ceruloplasmin and baseline urine copper levels) were similar in members of each family, except for family 3 (Table 1). While the father in family 3 became symptomatic with disabling metabolic bone disease and liver involvement only at the age of 45, WD was detected at an early asymptomatic stage by screening in the son at the age

of 16. It is possible that some of the differences in phenotype between father and son in this family could be due to longstanding untreated copper overload state in the father. Defining disease onset accurately in WD is difficult and this could be a confounding factor in genotype phenotype correlation in WD<sup>[11]</sup>.

In summary, we found genotype phenotype concordance in four families with different types of ATP7B mutations in each family: homozygous exonic mutation in two families, homozygous intronic mutation (Family 3) and compound heterozygous mutation (Family 4). It is of interest that such a concordance of genotype and phenotype was seen in different phenotypic manifestations, including neurological involvement in childhood in family 2, an uncommon presentation of WD.

The occurrence of identical homozygous mutations within members of a family could reflect genetic inbreeding in the population perpetuated by consanguineous marriages. Consanguineous marriages are common in South India (28% in one study)<sup>[12]</sup>. Analysis of data from the 1992-1993 National Family Health Survey to assess trends in consanguinity in the South Indian states of Andhra Pradesh, Karnataka, Kerala and Tamil Nadu showed that in Kerala the frequency of consanguineous marriages was very low and one type of preferred marriage of the Dravidian marriage system-uncle niece marriage-was conspicuously absent, whereas in the other states of South India, consanguinity and the coefficient of inbreeding were high<sup>[13]</sup>. In a study of 407 infants and children in Karnataka, of a total of 35 genetic diseases detected, autosomal recessive disorders formed the largest single disease category diagnosed<sup>[14]</sup>.

Differences in phenotypic classification used in different studies could affect the interpretation of results of genotype phenotype correlation in WD. Although

the Japanese used a phenotypic classification different from the international consensus classification which we have applied in our report, the distinction between hepatic versus neuropsychiatric phenotypes in both the classification systems appears to be similar<sup>[7]</sup>. While the Japanese study of two families showed that within each family, members with different mutations of ATP7B gene had different WD phenotypes; we report four families wherein affected members of each family had identical mutations of ATP7B gene and similar phenotypes. Thus, both these studies suggest that within families, a particular ATP7B genotype is associated with a particular WD phenotype thereby supporting the concept of allelic dominance as a significant determinant of WD phenotype.

In a previous report, we did not find symptomatic WD in >1 member in each family. On studying the phenotype of presumed WD in deceased siblings of index patients with WD, we found concordance of WD phenotype within each family in 6 of 8 families studied. The WD phenotype in these 6 families with concordant phenotype within each family was hepatic (4 families), hepatic and neurological (1 family) and neuro-psychiatric (1 family)<sup>[15]</sup>.

By contrast, studies on WD patients with a common mutation in a given population have not shown this degree of concordance between genotype and phenotype. In a study on patients mainly from Austria, neurological presentation was significantly more common than hepatic presentation in H1069Q homozygotes compared to H1069Q compound heterozygotes or H1069Q negative patients<sup>[11]</sup>. However, these findings were not borne out by studies from other parts of Europe and North America<sup>[4,16]</sup>. Some studies have reported later age of onset of symptoms in H1069Q homozygotes compared to H1069Q compound heterozygotes<sup>[4,17]</sup>.

Apolipoprotein E genotype, intestinal metallothionein inducibility and the individual's capacity to withstand "copper stress" by utilizing glutathione, superoxide dismutase and heat shock proteins are some epigenetic factors that may affect the WD phenotype<sup>[11,18]</sup>. It is possible that common epigenetic or environmental factors within families accentuate the genotype phenotype concordance seen in family members affected with WD.

Further studies of WD within families in different populations are needed to assess whether the concept of allelic dominance determining the phenotype holds true. It would also be important to see if the phenotypes associated with a particular mutation of ATP7B gene in WD within families are the same as in WD patients with the same mutations, but in a different family or a different population<sup>[19]</sup>. Application of a standard phenotypic classification like the international consensus phenotypic classification for WD<sup>[8]</sup> in future studies will enable valid comparison of the results.

In conclusion, we report 4 families with WD wherein affected members had identical mutations of the ATP7B gene and showed genotype phenotype concordance amongst members within each family. This finding

supports allelic dominance as a determinant of WD phenotype and is at variance with previous studies of WD conducted in a given population. Further studies of genotype phenotype correlation in WD occurring in families are needed.

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S- Editor Pan BR L- Editor Anand BS E- Editor Zhang WB



RAPID COMMUNICATION

# Blood F<sub>2</sub>-isoprostanes are significantly associated with abnormalities of lipid status in rats with steatosis

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oxidative damage precedes that of systemic oxidative imbalance. Predominant metabolic features of the increased lipid peroxidation further suggest a close association of the oxidative imbalance and the dyslipidemia with functional deterioration of the steatotic liver. The findings need to be further evaluated, especially in human studies.

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**Key words:** Isoprostanes; Oxidative stress; Lipid peroxidation; Steatosis; Non-alcoholic fatty liver disease

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Zhu MJ, Sun LJ, Liu YQ, Feng YL, Tong HT, Hu YH, Zhao Z. Blood F<sub>2</sub>-isoprostanes are significantly associated with abnormalities of lipid status in rats with steatosis. *World J Gastroenterol* 2008; 14(29): 4677-4683 Available from: URL: <http://www.wjnet.com/1007-9327/14/4677.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4677>

## Abstract

**AIM:** To investigate oxidative stress and lipid peroxidation in hepatic steatosis and the underlying implications in pathological mechanisms of non-alcoholic fatty liver disease (NAFLD).

**METHODS:** F<sub>2</sub>-isoprostanes (iPF<sub>2α</sub>-III) in blood and liver samples from steatotic ( $n = 9$ ) and control ( $n = 7$ ) rats were measured as *in vivo* marker of lipid peroxidation by a mass spectrometric approach. The lipid profile and endogenous antioxidant status (SOD and CAT) in the rats were also analyzed.

**RESULTS:** Significantly higher levels of iPF<sub>2α</sub>-III (mean 3.47 vs 2.40 pmol/mg tissue,  $P = 0.004$ ) and lower activities of SOD (mean 1.26 U vs 1.40 U,  $P < 0.001$ ) and CAT (mean 1026.36 U/mg vs 1149.68 U/mg protein, without significance) were observed in the livers of steatotic rats. Plasma total iPF<sub>2α</sub>-III was significantly correlated with the abnormalities of blood lipids as well as alanine aminotransferase (ALT) levels in the rats with simple steatosis, whereas no similar tendencies were observed in the control rats.

**CONCLUSION:** Enhancement of hepatic oxidative imbalance occurring at the steatotic stage of NAFLD suggests a possibility that manifestation of the local

## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), in the absence of alcohol abuse, represents a pathological spectrum of fatty liver disorders including simple steatosis and non-alcoholic steatohepatitis (NASH)<sup>[1]</sup>. The cause of the disease progression remains elusive. The “two hits” hypothesis addresses a requirement of one or more “second hits”, in addition to the excessive hepatic fat deposition (the “first hit”), for presumed transition of simple steatosis to NASH<sup>[1,2]</sup>. Despite the involvement of many other proinflammatory mediators, lipid peroxidation is one hypothesis as the “second hit” to explain the development of the disorder in humans<sup>[1,3-8]</sup>.

Lipid peroxidation as an important mechanism of NAFLD has been a field of intense research but is not yet fully understood. Majority of previous researches were conducted mainly in NASH in both human subjects and animals. Only a few studies have focused on the effects of lipid peroxidation in simple steatosis, an earlier stage of NAFLD<sup>[9,10]</sup>. Given that ectopic lipids

accumulation in the liver is primarily linked to hepatic mitochondrial dysfunction and insulin resistance<sup>[1]</sup>, the steatosis stage of the disease might be of real importance in evaluating the cause-effect association of lipid peroxidation with the disease progress, thus providing clues for earlier pharmacological interventions against the development of NASH. Furthermore, malondialdehyde (MDA), a widely used index of lipid peroxidation, has been considered neither the sole end product of fatty peroxide formation and decomposition nor a substance generated exclusively through lipid peroxidation<sup>[11]</sup>. It remains uncertain if MDA in blood or tissues reflexes oxidative stress status associated with biological changes observed.

$\text{F}_2$ -isoprostanes, also referred to as iPF<sub>2 $\alpha$</sub> -III, are a group of prostaglandin-like isomers produced by free radical-catalyzed peroxidation of arachidonic acid<sup>[12]</sup>. Results from the Biomarkers of Oxidative Stress Study (BOSS) suggested that the most accurate marker to assess lipid peroxidation *in vivo* is the plasma or urinary iPF<sub>2 $\alpha$</sub> -III<sup>[13]</sup>. Elevated plasma or urinary levels of iPF<sub>2 $\alpha$</sub> -III have been reported in patients<sup>[14,15]</sup> and in animals with NASH<sup>[16]</sup>. Information in the literatures with regard to iPF<sub>2 $\alpha$</sub> -III formation related to hepatic steatosis, however, is limited. Tong *et al*<sup>[17]</sup> found elevated iPF<sub>2 $\alpha$</sub> -III in plasma and liver of the rats with valproic acid-triggered liver injury.

The present study was conducted to investigate the oxidative imbalance by measuring local and circulating iPF<sub>2 $\alpha$</sub> -III and enzymatic antioxidant status in the rats with high-fat diet induced hepatic steatosis. The measures of the lipid peroxidation were then correlated with metabolic risk factors such as lipid profile, which are believed to be greatly involved in the pathogenesis of NAFLD among the steatotic rats.

## MATERIALS AND METHODS

### Animals

Adult male Sprague-Dawley rats were obtained from the Slac Animal Center (Shanghai, China), and maintained with a 12 h light/dark cycle under constant temperature and humidity. The rats were chosen according to their lipid levels and then randomly assigned into a defined lard-based high-fat (HF) diet ( $n = 9$ ) and standard laboratory chow ( $n = 7$ ) groups (45% *vs* 10% kcal as fat, respectively). Both diets were made based on the ingredients of products D12451 and D12450B (Research Diets, New Brunswick, NJ, USA), respectively, and supplied by Double Lion Ltd. (Suzhou, China). Animals were fed ad libitum water and their corresponding diet for up to 16 wk. At the end of the study, two rats from each group were chosen randomly for the identification of hepatic steatosis. All rats were anesthetized and killed. Their fasting plasma samples were kept at -70°C, and the liver samples were thoroughly rinsed with ice-cold saline and frozen in liquid nitrogen and then stored at -70°C. The study was approved by the Animal Ethics Committee and all procedures complied

**Table 1 Body weight and biochemical parameters of HF rats and control**

Parameter	HF ( $n = 9$ )	Control ( $n = 7$ )	P
Body weight (g)	560.76 ± 5.59	482.37 ± 10.19	< 0.001
Liver			
TC (mg/dL)	0.42 ± 0.04	0.46 ± 0.02	
TG (mg/dL)	0.89 ± 0.07	0.64 ± 0.07	0.023
Plasma			
GLU (mg/dL)	9.18 ± 0.32	7.84 ± 0.15	0.004
TG (mg/dL)	0.98 ± 0.12	0.36 ± 0.16	< 0.001
TC (mg/dL)	1.72 ± 0.11	1.39 ± 0.11	
LDL-C (mg/dL)	0.35 ± 0.03	0.18 ± 0.03	0.001
HDL-C (mg/dL)	0.96 ± 0.06	0.90 ± 0.05	
ALT (U/L)	58.11 ± 4.09	43.71 ± 3.17	0.019
AST (U/L)	70.22 ± 2.31	70.86 ± 4.76	

with international standards of humane care in animal experimentation.

### Biochemical measurements

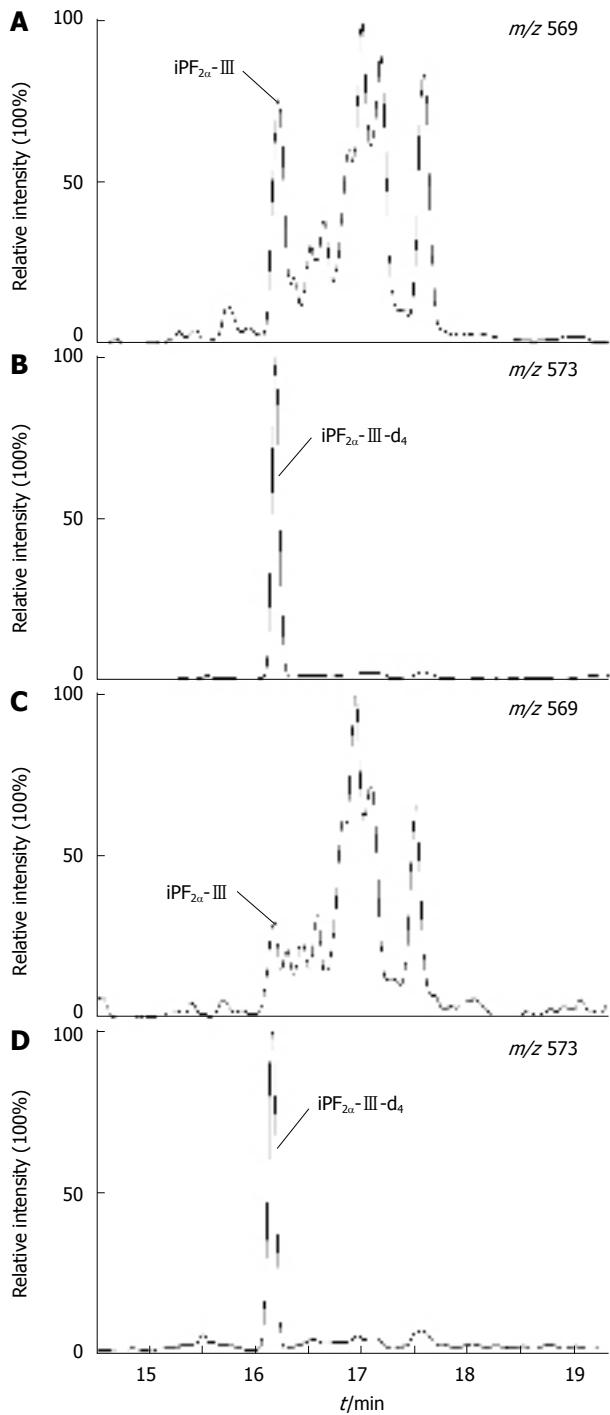
Fasting plasma glucose (GLU), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using a 7020 biochemical analyzer (Hitachi, Japan). The hepatic TC and TG were also determined in the same manner. The biochemical data for the control and HF rats are listed in Table 1.

### Histological examinations

Fragments of liver tissues were fixed in 10% formalin, embedded in paraffin, and sectioned (5 mm in thickness). The sections were mounted on the charged microscopic slides (Okando, USA). Hematoxylin-eosin (HE) staining was performed on all slides. The slides were then examined and pictured using a DM4000B fluorescent microscopy (Leica, Switzerland).

### Hydrolysis and solid-phase extraction of liver and plasma iPF<sub>2 $\alpha$</sub> -III

The purification of iPF<sub>2 $\alpha$</sub> -III from the liver and plasma were performed as described<sup>[18,19]</sup> with modifications. Briefly, the tissue sample (50–100 mg) was homogenized in 4 mL ice-cold chloroform:methanol (2:1, v/v). For plasma, the aliquots (500  $\mu$ L) were transferred to Eppendorf tubes. The internal standard iPF<sub>2 $\alpha$</sub> -III-d<sub>4</sub> (50 ng for liver and 5 ng for plasma) was added. For the liver sample, the homogenate was mixed at 4°C for 1 h, 1.6 mL of 0.9% NaCl was added to the sample followed by centrifuging at 2000  $\times g$  for 10 min. The residual was dried under nitrogen. One mol/L KOH (1 mL for the liver residual and 500  $\mu$ L for the plasma) was added to the tubes containing the samples. Both liver and plasma samples were hydrolyzed at 40°C for 45 min. One mol/L HCl (1 mL for the liver sample and 500  $\mu$ L for the plasma sample) and 1 mL of 10 mmol/L formate buffer (pH 3.0) were then



**Figure 1** GC-NICI-MS SIM chromatograms of (A) plasma total iPF<sub>2α</sub>-III and (C) hepatic iPF<sub>2α</sub>-III isolated from a rat with simple steatosis, and (B) and (D) the internal standard iPF<sub>2α</sub>-III-d<sub>4</sub> in the plasma and tissue samples, respectively.

added. The sample was centrifuged at 12 000 × g for 20 min; the supernatant was removed and applied to an Oasis HLB extraction cartridge<sup>[19]</sup>. The extraction steps were programmed into an ASPEC XL SPE System (Gilson S.A.S., France) and run automatically.

#### Derivatization and GC-MS analysis of liver and plasma iPF<sub>2α</sub>-III

The iPF<sub>2α</sub>-III extracts were analyzed using gas chromatography-negative ion chemical ionization-mass spectrometry (GC-NICI-MS) as described<sup>[19]</sup>. After

derivatizations with pentafluorobenzyl bromide (PFB) and N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA), the PFB-TMS derivatives of iPF<sub>2α</sub>-III were analyzed on a TRACE DSQ gas chromatograph-mass spectrometer (ThermoFisher Scientific, USA). Selected ion monitoring (SIM) was performed to monitor the carboxylate anion (M-181) at *m/z* 569 for the iPF<sub>2α</sub>-III and *m/z* 573 for iPF<sub>2α</sub>-III-d<sub>4</sub>. The representative GC-NICI-MS chromatograms of hepatic and plasma F<sub>2</sub>-isoprostanes are shown in Figure 1.

#### Determination of activities of liver and erythrocyte superoxide dismutase (SOD) and blood catalase (CAT)

Liver and erythrocyte SOD activities were measured with a SOD Assay Kit-WST (Dojindo Laboratories, Japan), and liver and blood CAT activities and the protein levels of the samples were determined using the Catalase Assay Kit and the BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Jiangsu, China), using the protocols provided by the manufacturers, respectively.

#### Statistical analysis

Data were expressed as the mean ± SE. The differences between the mean values of two groups were determined by paired *t* test. Associations between the different variables were examined by Pearson correlation analysis. Statistical significance was set at *P* < 0.05.

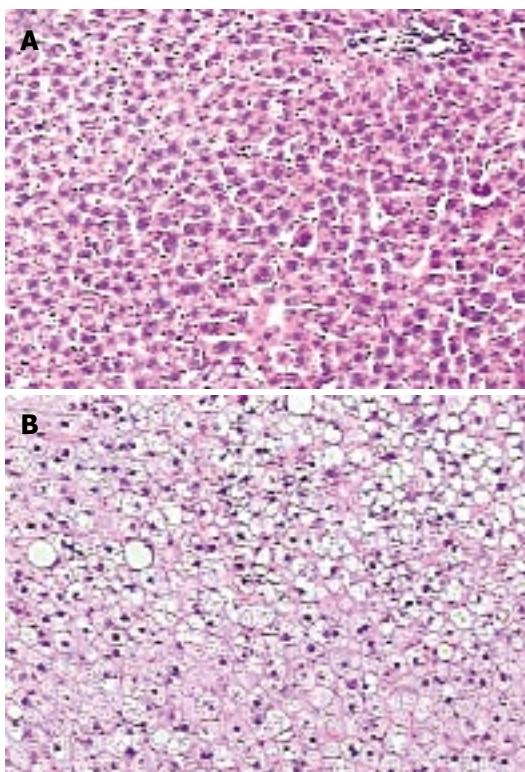
## RESULTS

#### Effects of high-fat diet on body weight, metabolic indices and occurrence of hepatic steatosis

Prolonged feeding with the high-fat diet led to significant increases in body weight (*P* < 0.001) and fasting plasma glucose levels (*P* < 0.005) in HF rats compared to the control rats. Plasma LDL-C (*P* = 0.001) and TG (*P* < 0.001) were significantly higher in HF rats than those in the controls, whereas no difference in TC and HDL-C levels was found between the groups. Notably, feeding with high-fat diet increased the hepatic TG levels (*P* = 0.023), and typically resulted in steatosis in approximately 40% of hepatic lobules in the liver section examined (Figure 2). The hepatic steatosis was further supported by a significant increase in plasma ALT in HF rats (*P* = 0.019) (Table 1). No development of NASH was found in the rats.

#### Liver and plasma iPF<sub>2α</sub>-III as indices of oxidative imbalance in vivo in rats with hepatic steatosis

Hepatic and plasma total iPF<sub>2α</sub>-III levels and the activities of antioxidant enzymes were examined (Figure 3). While no significant difference appeared in circulating total iPF<sub>2α</sub>-III, an elevated hepatic iPF<sub>2α</sub>-III was observed in HF rats as compared to the control rats (mean 3.47 pmol/mg vs 2.40 pmol/mg tissue, *P* = 0.004) (Figure 3). Analysis of the endogenous antioxidant capacity revealed that the SOD activities in the liver, but not in circulation, were significantly reduced in the rats with steatosis as compared with that in the control (mean 1.26 U vs 1.40 U, *P* < 0.001) (Figure 3). Similar changes were also found



**Figure 2** Enhanced lipid accumulation in the liver of rat by high-fat diet. (A) Control, and (B) Steatotic liver (HE, magnification  $\times 100$ ).

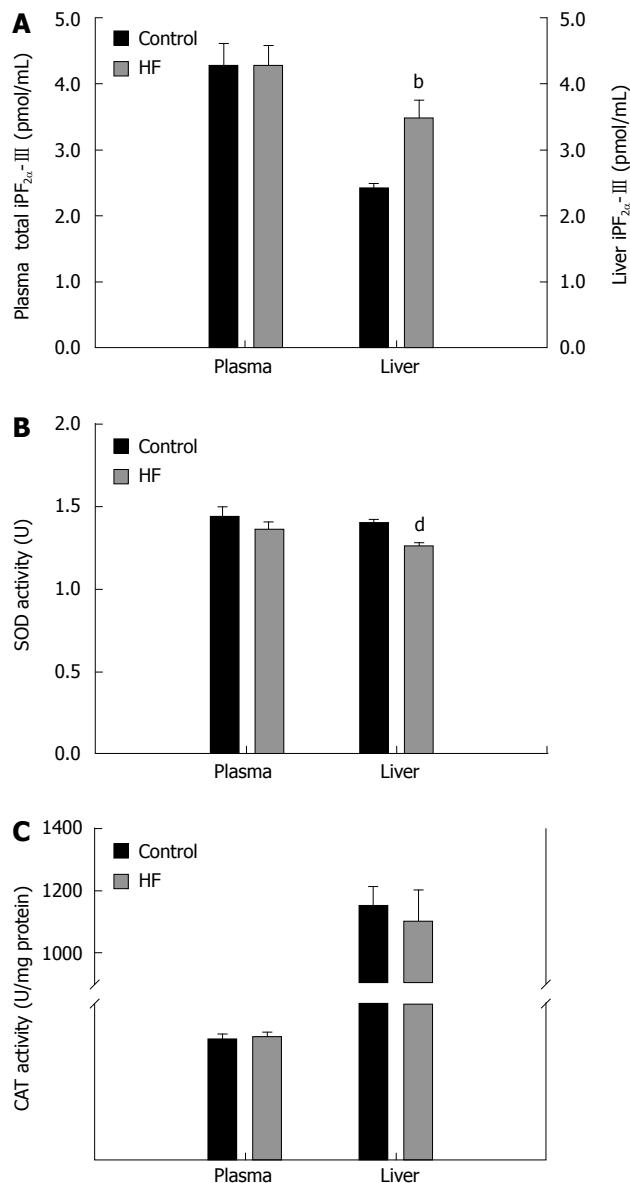
for the liver CAT activities, however, the difference did not reach statistical significance.

#### **Relationship between oxidative imbalance and metabolic risk factors in rats with hepatic steatosis**

Pearson correlation analysis was performed between iPF<sub>2α</sub>-III and the metabolic risk factors, e.g. the abnormal lipid status, in HF rats. There were no significant associations of plasma total iPF<sub>2α</sub>-III with either erythrocyte SOD and blood CAT activities or fasting plasma glucose among HF rats. The significant associations were found between the plasma iPF<sub>2α</sub>-III concentrations and the plasma TC ( $r = 0.799$ ,  $P = 0.005$ ) as well as TG ( $r = 0.624$ ,  $P = 0.036$ ) in the HF rats (Figure 4A and B). A similar correlation but without significance was also found in the plasma LDL-C levels ( $r = 0.578$ ,  $P = 0.052$ ). Furthermore, there was a strong relationship between the iPF<sub>2α</sub>-III levels and the plasma ALT concentrations ( $r = 0.629$ ,  $P = 0.035$ ) (Figure 4C). In the liver, however, there were no relationships of iPF<sub>2α</sub>-III with either TC and TG or SOD and CAT in HF rats.

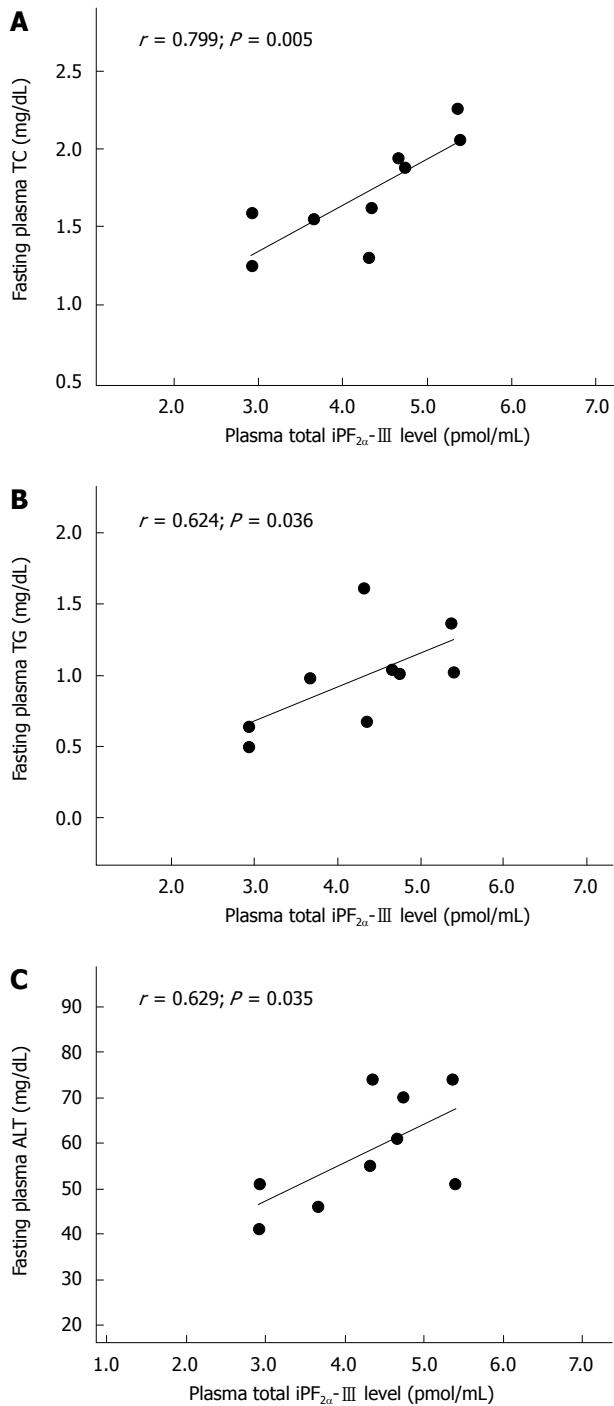
## **DISCUSSION**

In the present study, a Sprague-Dawley rat model with simple steatosis was established by feeding the rats for up to 16 wk with a high-fat diet. The purpose of using such dietary regimen in the normal rats was, by resembling a common fat-rich diet in humans (60% kcal of fat) without toxin ingestion and alimentary deficiency,



**Figure 3** Plasma and hepatic levels of iPF<sub>2α</sub>-III and antioxidant enzymes in rats. (A) Significant differences in iPF<sub>2α</sub>-III levels were found in the liver, but not in plasma between HF rats and control; (B) Significant decreases in SOD activity were found in the liver, but not in erythrocytes of HF rats compared to that in control, and (C) Reducing tendency of CAT activities was observed in liver, but not in blood of HF rats. <sup>b</sup> $P < 0.01$  and <sup>d</sup> $P < 0.001$ .

to naturally reproduce typical features of metabolic abnormalities at the simple steatotic stage of NAFLD seen in humans<sup>[20,21]</sup>. As shown in Table 1, hepatic TG in the HF rats were elevated significantly, indicating an occurrence of lipotoxicity due to the imbalance between TG synthesis and degradation in the liver. The enhanced plasma ALT activity also suggested the lipotoxicity-induced impairment in the liver function. A recent cross-sectional and observational study in 257 Italians also showed that elevation of TG and ALT levels is the most predictive condition for hepatic steatosis<sup>[22]</sup>. In the present study, the presence of hepatic steatosis in HF rats was further confirmed histologically, showing mild to moderate macrovesicular fat accumulation in the liver samples observed (Figure 2). Despite of being



**Figure 4** The formation of plasma total iPF<sub>2α</sub>-III was significantly correlated with the levels of fasting plasma TC (A), TG (B) and ALT (C) in HF rats.

multifactorial in nature in the onset of steatosis<sup>[23]</sup>, these data clearly demonstrate that prolonged treatment with the high-fat diet can successfully cause liver steatosis in the experimental rats. Our results are supportive to the previous publications<sup>[20,24]</sup>. Given that NAFLD is an important hepatic manifestation of metabolic syndrome<sup>[25]</sup>, as expected, the steatotic rats in the present study were also featured with a spectrum of metabolic risk factors (Table 1), suggesting a close association of hepatic steatosis with obesity and insulin resistance, as commonly seen in humans<sup>[26]</sup>.

With the rat model established, we investigated

the oxidative imbalance status specifically at the earlier steatotic stage of NAFLD. The hepatic and blood iPF<sub>2α</sub>-III and enzymatic antioxidants (SOD and CAT) were measured to characterize the effects of oxidative imbalance on the underlying pathogenesis of NAFLD. The main finding is that hepatic iPF<sub>2α</sub>-III concentrations were significantly higher in HF rats than that in the control rats ( $P = 0.004$ ). Associated with this marked elevation in iPF<sub>2α</sub>-III, hepatic activities of SOD ( $P < 0.001$ ) and CAT ( $P = 0.068$ ) of the HF rats were declined as compared to the control, though the decrease of CAT activity did not show statistical significance. In contrast, circulating levels of iPF<sub>2α</sub>-III as well as the activities of SOD and CAT were not different between two groups of rats (Figure 3). The results demonstrate that there was a local but possibly not systemic enhancement of oxidative imbalance in the rats with steatosis. It has been postulated that abnormal hepatic accumulation of TG has implication as the “first hit” in the formation of steatosis<sup>[2]</sup>. This accumulation is believed to be mediated, not exclusively, by alteration in intracellular fatty acid trafficking and the decrease in mitochondrial fatty acid β-oxidation<sup>[27]</sup>. All of these mediators can trigger oxidative imbalance by excessive production of free oxygen radicals capable of inducing lipid peroxidation of hepatocyte membranes. In this regard, while being consistent with those postulations, increased iPF<sub>2α</sub>-III in the steatotic livers observed in the present study is also compatible with the F<sub>2</sub>-isoprostanes results<sup>[17]</sup> and the MDA results<sup>[9]</sup> derived from the animals with simple steatosis. Taking together with these observations, our data also demonstrate enhanced oxidative imbalance mechanisms especially at the steatotic stage of NAFLD. The discrepancy in circulating status of oxidative stress exists between our study and others in steatotic animal models<sup>[17]</sup>. One explanation for the disagreement could be the way to induce steatosis used in the present study is different from that of Tong *et al*<sup>[17]</sup>, possibly resulting in two distinct pathways towards different biochemical and physiological manifestations of systemic oxidative stress. In addition, in the study by Tong *et al*, the local as well as systemic antioxidant status was not characterized as to whether the valproic acid treatment would also parallelly lead to significant damages in antioxidative capacities of the rats with liver injury is unclear. Given that in the present study the circulating SOD and CAT activities were not changed among the experimental rats, it should make sense to postulate that the absence of increase in plasma iPF<sub>2α</sub>-III in HF rats may simply reflect an apparent systemic balance between oxidative stress and antioxidant defense existing temporally in simple steatosis before NASH develops. Indeed, as in the NASH mice fed with a choline-deficient diet, Yoshida *et al*<sup>[16]</sup> reported a more prominent elevation of iPF<sub>2α</sub>-III in the liver than that in plasma. Machado *et al*<sup>[28]</sup> also observed that there was no significant difference in plasma concentrations of 8-OHdG, a DNA oxidation marker, and 4-HNE, a toxic lipid peroxidation product, between patients with NASH and the control. These findings from both animals and human studies suggest a

possibility that manifestation of oxidative imbalance in the local might precede that in circulation in the steatotic stage of NAFLD. This possibility certainly needs to be validated further.

In agreement with the present observation, there have also been increasingly concerns as to whether circulating oxidative stress status can accurately be a reflection of hepatic oxidative damage in NAFLD<sup>[29]</sup>. Despite of the absence of increased circulating iPF<sub>2α</sub>-III in HF rats, the present study found that plasma iPF<sub>2α</sub>-III concentrations were strongly correlated not only with plasma TC and TG (and LDL, but without significance) but also with plasma ALT in rats with steatosis (Figure 4), whereas such a correlation was not found in the control rats. Thus, the abnormal lipid profile could be, at least in part, important contributors to oxidation imbalance related to the high-fat induced steatosis, whereas systemic relationship of lipid peroxidation with lipid status could be an important reflection of the functional decline of steatotic liver. While it might be expected that enhanced lipid peroxidation in NAFLD would be dependent, at least partially, on some lipidemic measures, there was little published support for such a correlation to date. Konishi *et al*<sup>[30]</sup> found no significant associations of plasma iPF<sub>2α</sub>-III with TC and TG in patients with NAFLD. In addition, Madan *et al*<sup>[31]</sup> failed to find relationship between oxidant stress and ALT levels in either NAFLD patients or diseased controls. Nevertheless, the present data suggest that oxidative imbalance and lipid peroxidation play a significant role in pathogenesis of NAFLD, especially at the steatotic stage. The results may indicate a potential value of measuring circulating total iPF<sub>2α</sub>-III to assess the extent of hepatic lipotoxicity during *in vivo* oxidative imbalance and its relevance to the progression of simple steatosis to NASH. This might further provide the rationale for interventions to slow the steatosis in human studies.

In summary, the present study demonstrates an enhanced oxidative imbalance at the steatotic stage of NAFLD. Such oxidative imbalance was not observed in the circulation, suggesting a possibility that manifestation of the local oxidative damage precedes that of systemic oxidative imbalance. The association analysis shows predominant metabolic features of the increased lipid peroxidation and also suggests a close association of the oxidative imbalance and the dyslipidemia with functional deterioration of the steatotic liver. Although there is no confirmatory conclusion in terms of the cause-effect relationship between oxidative stress and occurrence of steatosis from the present study, the results suggest that the steatotic stage of NAFLD might be a valuable model for evaluating the roles of oxidative imbalance in pathogenesis of NAFLD and for providing clues for earlier interventions against the development of NASH in human studies.

## COMMENTS

### Background

Oxidative imbalance as an important mechanism of non-alcoholic fatty liver

disease (NAFLD) has been a field of intense research but is not yet fully understood. While majority of previous researches were conducted mainly in NASH, only a few studies have focused on the effects of lipid peroxidation in simple steatosis, an earlier stage of NAFLD. As ectopic lipids accumulation in the liver is primarily linked to hepatic mitochondrial dysfunction and insulin resistance, it is reasonable to postulate that the steatotic stage of NAFLD might be of real importance in evaluating the cause-effect association of lipid peroxidation with the disease progression, thus providing clues for earlier pharmacological interventions against the development of NASH.

### Research frontiers

This study utilized the quantitative assay of F<sub>2</sub>-isoprostanes (i.e. iPF<sub>2α</sub>-III), which has been considered to be the most accurate *in vivo* lipid peroxidation marker, along with endogenous enzymatic antioxidants SOD and CAT to characterize hepatic as well as peripheral oxidative imbalance and its metabolic relevance in a rat model with simple steatosis.

### Innovations and breakthroughs

F<sub>2</sub>-isoprostanes, also referred to as iPF<sub>2α</sub>-III, are a group of prostaglandin F<sub>2</sub>-like isomers produced by free radical-catalyzed peroxidation of arachidonic acid. Increased plasma or urinary levels of iPF<sub>2α</sub>-III have been reported both in humans and animal models with NASH. Information in the literatures with regard to iPF<sub>2α</sub>-III production related to hepatic steatosis, however, is limited. The basic findings of the study are (1) significantly higher levels of iPF<sub>2α</sub>-III and lower activities of SOD and CAT were observed in the steatotic livers as compared with the control rats, while no such oxidative imbalance was found in the circulation; and (2) the formation of iPF<sub>2α</sub>-III in the circulation was significantly correlated with the abnormalities of blood lipids as well as ALT levels in the rats with simple steatosis, whereas no similar tendencies were evident in the control rats.

### Applications

The findings of the present study suggest an enhanced oxidative imbalance at the steatotic stage of NAFLD prior to the appearance of NASH, and it may further suggest a possibility that manifestation of the local oxidative damage precedes that of systemic oxidative imbalance. Furthermore, the results reveal not only a predominant metabolic feature of the increased lipid peroxidation, but also a close association of the oxidative imbalance and the dyslipidemia with functional deterioration of the steatotic liver in the rats with simple steatosis. We believe that these findings do provide supports to the postulation mentioned above.

### Peer review

This study performed a fine measurement of iPF-III using GC-MS to show a strong evidence for oxidative imbalance in the steatotic liver. It demonstrated in this paper that F<sub>2</sub>-isoprostanes (iPF<sub>2</sub>-III) in the liver increased when steatosis was induced in rats by treatment of high fat diet. This result together with decreased in SOD and catalase levels led to the conclusion that oxidative imbalance proceeds in early stages of NAFLD.

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RAPID COMMUNICATION

## Inhibition of hepatitis B virus gene expression and replication by artificial microRNA

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### Abstract

**AIM:** To investigate the inhibitory effects of hepatitis B virus (HBV) replication and expression by transfecting artificial microRNA (amiRNA) into HepG2.2.15 cells.

**METHODS:** Three amiRNA-HBV plasmids were constructed and transfected into HepG2.2.15 cells. HBV antigen secretion was detected in the cells with transient and stable transfection by time-resolved fluoroimmunoassays (TRFIA). HBV DNA replication was examined by fluorescence quantitative PCR, and the level of HBV S mRNA was measured by semi-quantitative RT-PCR.

**RESULTS:** The efficiency of transient transfection of the vectors into 2.2.15 cells was 55%-60%. All the vectors had significant inhibition effects on HBsAg and HBeAg at 72 h and 96 h after transfection ( $P < 0.01$  for all). The secretion of HBsAg and HBeAg into the supernatant was inhibited by  $49.8\% \pm 4.7\%$  and  $39.9\% \pm 6.7\%$ , respectively, at 72 h in amiRNA-HBV-S608 plasmid transfection group. The copy of HBV DNA within culture supernatant was also significantly decreased at 72 h and 96 h after transfection ( $P <$

0.01 for all). In the cells with stable transfection, the secretion of HBsAg and HBeAg into the supernatant was significantly inhibited in all three transfection groups ( $P < 0.01$  for all, vs negative control). The copies of HBV DNA were inhibited by  $33.4\% \pm 3.0\%$ ,  $60.8\% \pm 2.3\%$  and  $70.1\% \pm 3.3\%$ , respectively.

**CONCLUSION:** In HepG2.2.15 cells, HBV replication and expression could be inhibited by artificial microRNA targeting the HBV S coding region. Vector-based artificial microRNA could be a promising therapeutic approach for chronic HBV infection.

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**Key words:** Hepatitis B virus; RNA interference; Artificial microRNA; HepG2.2.15 cell

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### INTRODUCTION

RNA interference (RNAi) has become a new promising approach to develop effective antiviral drugs in recent years, including hepatitis C virus (HCV), human immunodeficiency virus (HIV), poliovirus and hepatitis B virus (HBV)<sup>[1-3]</sup>. For HBV, RNAi was shown to have impressive inhibitory effects against viral gene transcription and expression<sup>[4-7]</sup>. One recent study further demonstrated the viral clearance from the liver of transgenic mice by RNAi targeted HBV<sup>[8]</sup>, which sheds light on the use of RNAi in HBV gene therapy.

HBV replication and gene expression can be strongly inhibited with virus specific siRNA treatment. However, the high sequence specificity of siRNAs, combined with prolonged treatment, promoted the emergence of siRNA-resistant virus variants. Selection of RNAi

escape mutants has been reported *in vitro* for HIV, HCV and HBV<sup>[9-11]</sup>. These findings indicate that the antiviral properties of specific siRNAs targeted virus are not as effective as expected.

RNAi can be triggered by small RNA molecules such as siRNA and microRNAs (miRNAs). MiRNAs endogenously expressed small ssRNA sequences of about 22 nucleotides in length, which naturally direct gene silencing through components shared with the RNAi pathway<sup>[12]</sup>. The mature miRNAs regulate gene expression by mRNA cleavage or translational repression<sup>[13,14]</sup>. Compared with siRNA, miRNA still can play a translational repression role when miRNAs partially complement with the target gene. Because of the flexibility of miRNA in binding with partially complementary mRNA targets, miRNA can serve as an anti-virus drug or vaccine to achieve a breakthrough in the treatment of virus mutation. Moreover, since miRNAs are single-stranded molecules insensitive to interferon systems, the utilization of this Pol-II-mediated miRNA generation can be safe both *in vitro* and *in vivo* without the cytotoxic effects of dsRNAs and siRNAs<sup>[15]</sup>. These findings indicate that miRNA mediated RNAi can be used as a tool for gene-specific therapeutics against viral infections.

In this study, we applied the recently developed artificial miRNA (amiRNA) expression vector based on the murine miR-155 sequence<sup>[16]</sup>, to observe whether amiRNA could efficiently suppress the expression and replication of HBV *in vitro*.

## MATERIALS AND METHODS

### Design of amiRNA and plasmid construction

The pcDNA™6.2-GW/± EmGFP-miR plasmid (Invitrogen, Carlsbad, CA, USA), driving the expression of amiRNA with polymerase II and containing a spectinomycin resistance gene, was used in this study. The engineered pre-miRNA sequence structure is based on the murine miR-155. According to the sequences of conserved region in HBV genome, we designed three target sequences against the S region of the HBV genome using Invitrogen's RNAi design algorithm at <https://rnaidesigner.invitrogen.com/rnaiexpress/>. The BLAST algorithm was also used to ensure the designed sequences would not target other gene transcripts to avoid off-target effects. These oligonucleotides (Table 1) were annealed and ligated into pcDNA6.2-GW/EmGFP-miR vector. The pcDNA6.2-GW/EmGFP-miR-negative control plasmid contains an insert that can form a hairpin structure which is processed into mature miRNA, but is predicted not to target any known vertebrate gene. Thus, this plasmid serves as a suitable negative control. Control cells were mock transfected with Lipofectamine 2000 alone.

### Cell culture and transfection

The HepG2.2.15 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Gibco), 380 mg/L

antibiotic G-418 sulfate (Promega, USA), and 100 IU/mL penicillin and streptomycin, and 1% L-glutamine, at 37°C in the atmosphere of 5% CO<sub>2</sub>. Transfections were performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Briefly, cells were trypsinized and plated in 12-well plates at a density of 5.0 × 10<sup>4</sup> cells/mL (1 mL/well) for 24 h before transfection. Four μL of lipofectamine was diluted dropwise into Opti-MEM I (Gibco, USA) for a final volume of 100 μL and incubated at room temperature for 5 min. Then 1.6 μg of amiRNA expression vector was added to the diluted lipofectamine and incubated for another 20 min. The normal HepG 2.2.15 cells and the cell transfection with negative control plasmid containing scrambled miRNA were used as the negative control. Each plasmid was repeated in three wells. After transfection, the medium was partly removed for analysis every 24 h and the cells were replenished with fresh medium. The cell culture supernatant was collected for detection of HBsAg, HBeAg and HBV-DNA.

Stable transfection was carried out at 24 h after transfection, the cells were passaged at a 1:10 dilution into fresh growth medium and selection was performed with 10 mg/L Blasticidin 30 h later. The Blasticidin resistant colonies were picked with 200 μL pipet tip and cultured with medium containing 10 mg/L Blasticidin to establish individual clone lines. Successfully transfected cellclones were obtained by a long-term culture in a selected medium containing 6 mg/L Blasticidin.

### Detection of HBV-DNA in cell culture medium by real-time PCR

After transfection, 50 μL of the supernatant were mixed with an equal volume of the DNA extractant. Samples were incubated at 94°C for 10 min and then centrifuged at 10000 × g for 5 min. The supernatant was used as template for real-time PCR. The forward primer of HBV-DNA is 5'-GGAGTATGGATTTCGCACTCCTC-3', the reverse primer is 5'-TTGTTGTTGTAGGGGACCTG CCT-3', the fluorescent probe 5'-ACTTCCGGAAAC TACTGTTAGACGA-3', and the quenching probe 5'-GTAGTTCCCGAAGT-3'. PCR amplification and analysis were performed using the ABI 7500 real-time PCR detector (ABI). Assays were repeated in triplicate and average threshold cycle values were used to determine the concentration of HBV-DNA. The inhibitory rate was calculated using the follow formula: inhibitory rate (%) = (C control-C tester)/C control × 100%. Control represents HBV-DNA copies in HepG2.2.15 cells transfected with Lipofectamine alone, C tester represents HBV-DNA copies in cells transfected with amiRNA.

### HBsAg and HBeAg assay

To assess the effect of amiRNAs on HBV at the protein level, the viral proteins of hepatitis B surface antigen (HBsAg) and e antigen (HBeAg) of the culture supernatant from transfected cells at various times were measured with time-resolved fluoroimmunoassays kit (TRFIA) according to the supplier's instructions. The inhibitory rates were calculated according to the

**Table 1** Three pairs of two single-stranded DNA oligonucleotides

Plasmid	Top oligo strand	Bottom oligo strand
amiRNA-HBV-S89 (89-109 nt)	5'-TGCTGTCCCCACCACGAGTCTAGACTCTGTTGGCC ACTGACTGACAGAGTCACTCGTGGTGA -3'	5'-CCTGTCCACCACGAGTAGACTCTGTCAGTCAGT GGCCAAAACAGAGTCTAGACTCGTGGTGA -3'
amiRNA-HBV-S367 (367-387nt)	5'-TGCTGTGAGCAGTAGTCATGCAGGTGTTGGCC ACTGACTGACACCTGCATCTACTGCTCAA -3'	5'-CCTGTGAGCAGTAGATGCAGGTGTCAGTCAGT GGCCAAAACACCTGCATGACTACTGCTCAAC -3'
amiRNA-HBV-S608 (608-628nt)	5'-TGCTGTCAGATGCTGTACAGACTTGGTGGCC ACTGACTGACCAAGTCTGCAGCATCTGAC -3'	5'-CCTGTCAAGATGCTGTCAGACTTGGTCAGTCAGT GGCCAAAACCAAGTCTGTACAGCATCTGAC -3'

following formula: inhibitory rate (%) = (C control-C tester)/C control × 100%. Assays were performed in triplicate, and the average inhibitory rate was expressed as a mean ± SD.

#### Semiquantitative RT-PCR analysis of HBS mRNA

Total RNA was isolated by Trizol Reagent (Invitrogen) following the manufacturer's instructions. The quantity of total RNA was first determined by  $A_{260}$  measurement, and the quality of total RNA was estimated by 1.5% agarose gel electrophoresis. cDNA was synthesized from total RNA using the Reverse Transcription System (Promega) according to the manufacturer's protocol. The first-strand cDNA product was used for semi-quantitative PCR. PCR reaction was performed in a single reaction of 25  $\mu$ L volume. The forward (fp) and reverse (rp) primers used were: HBVS-FP: 5'-TAGACTCGTG-GTGGACTTC-3', HBVS-RP: 5'-ATTGGTAACAGC-GGTAAA-3', GAPDH-FP: 5'-ACCACAGTCCATGC-CATCAC-3', GAPDH-RP: 5'-TCCACCACCTGTT-GCTGTA-3'. The schedule consisted of incubation for 5 min at 94°C followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, with a final cycle for 5 min at 72°C. PCR products were run on a 1% agarose gel and visualized by ethidium bromide staining, and the intensities were then measured by scanning the gel with JIEDA 801 (JIEDA, Nanjing, China). The products were quantified by densitometry and normalized with respect to GAPDH as internal control.

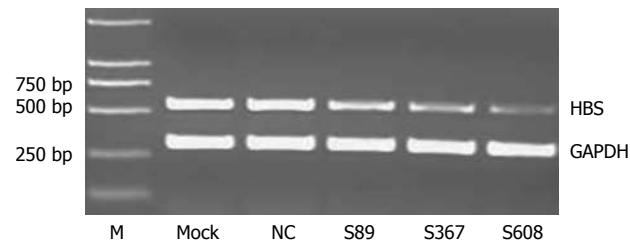
#### Statistical analysis

The data were expressed as mean ± SD. Statistical analysis was performed using Student's *t* test, with *P* value less than 0.05 being considered statistically significant. All statistical analyses were performed using SPSS software.

## RESULTS

#### Identification of recombinant plasmid amiRNA-HBV

RNAi was performed using the BLOCK-iT™ Pol II RNAi expression vector kit as recommended by the manufacturer (Invitrogen). Three plasmids containing pre-miRNA sequences were constructed and designated as amiRNA-HBV-S89, amiRNA-HBV-S367, and amiRNA-HBV-S608, respectively. In order to identify successful construction of recombinant plasmids, the miRNA forward sequencing primer 5'-GGCATGGACGAGCTGTACAA-3' and miRNA reverse sequencing primer 5'-CTCTAGATCAACCA



**Figure 1** Effect of amiRNA-HBV with stable transfection on HBV S mRNA levels, compared with negative control, all three plasmids had significant inhibitory effect on HBV S mRNA (*P* = 0.00094, 0.000047 and 0.000011, respectively).

CTTGTG-3', (Invitrogen) were used to perform PCR. And the PCR products, which contained the miRNA insert fragments, were verified by DNA sequencing. The mutant in these inserted oligonucleotides was excluded from this experiment.

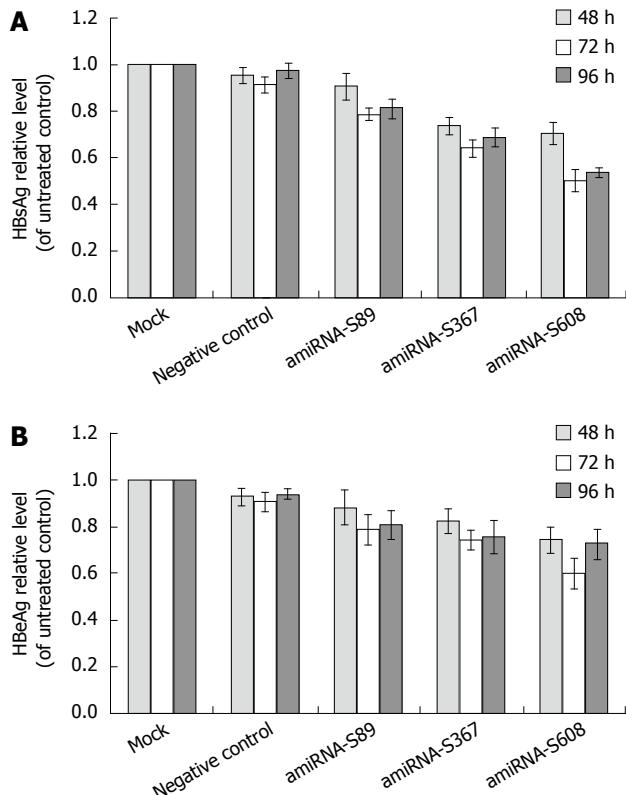
#### Inhibition of HBS mRNA expression by amiRNA

Once the recombinant plasmids were transfected into HepG2.2.15 cells, the expression of Emerald green fluorescent protein (EmGFP) could be detected directly under fluorescence microscopy. By this way, we found that the transfection efficiency of these vectors into HepG2.2.15 cells was 55%-60% in the current study. At 72 h after transfection, compared with mock and negative control vector, amiRNA-mediated RNAi resulted in a significantly reduced level of HBS mRNA in three plasmids transfected cells (*P* = 0.008, 0.0015 and 0.00074, respectively). Among them, amiR-HBV-S367 and amiRNA-HBV-S608 were more efficient with 38.7% and 47.4% average inhibitory rate in viral mRNA (Figure not shown).

For cell clones with stable transfection, amiRNA-mediated RNAi resulted in a higher decrease degree of HBS mRNA in three plasmid transfected groups than the cells with transient transfection. Among them, compared with negative control vector, the inhibitory rate of HBV S mRNA was 40.6% ± 3.8% in amiRNA-HBV-S89 group (*P* = 0.00094). amiR-HBV-S367 and amiRNA-HBV-S608 was much more efficient with an inhibitory rate of 63.4% ± 2.5% and 83.0% ± 4.3% in HBV S mRNA (*P* = 0.000047 and 0.000011, respectively; Figure 1).

#### Inhibition of HBeAg and HBsAg secretion by amiRNA

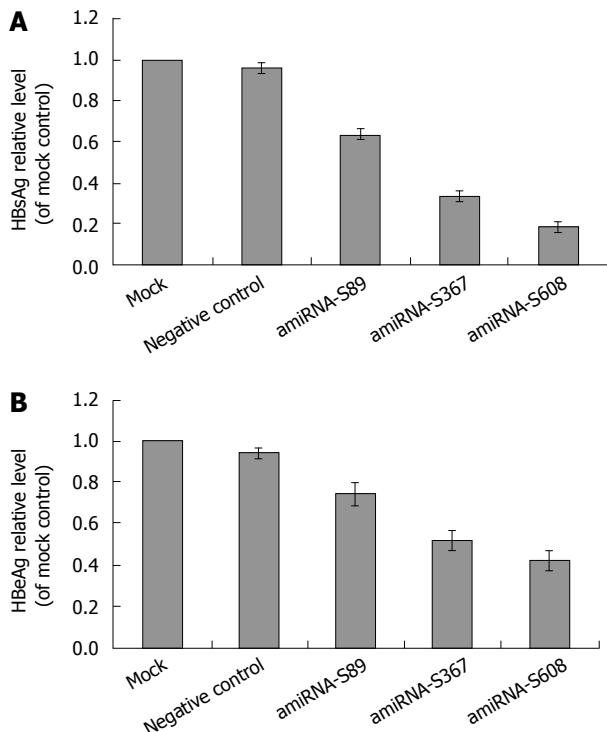
The concentrations of HBsAg and HBeAg in the culture supernatant were measured at 48 h, 72 h and 96 h, which were stable after transfection using TRFIA. As shown in Figure 2, our results demonstrated that three amiRNAs



**Figure 2.** A: Effect of amiRNA-HBV on HBsAg levels; B: Effect of amiRNA-HBV on HBeAg levels. HBsAg and HBeAg levels are expressed as mean  $\pm$  SD. Compared with negative control, all three plasmids had significant inhibitory effect on HBsAg and HBeAg at 72 h and 96 h after transfection (HBsAg:  $P = 0.00039$  and  $0.0012$  for S89 group,  $P = 0.000011$  and  $0.00021$  for S367 group,  $P = 0.000016$  and  $0.000056$  for S608 group; HBeAg:  $P = 0.02$  and  $0.035$  for S89 group,  $P = 0.0093$  and  $0.0032$  for S367 group,  $P = 0.007$  and  $0.0025$  for S608 group).

had distinct inhibition effects on HBsAg and HBeAg at 72 h and 96 h after transfection (HBsAg:  $P = 0.00039$  and  $0.0012$  for S89 group,  $P = 0.000011$  and  $0.00021$  for S367 group,  $P = 0.000016$  and  $0.000056$  for S608 group; HBeAg:  $P = 0.02$  and  $0.035$  for S89 group,  $P = 0.0093$  and  $0.0032$  for S367 group,  $P = 0.007$  and  $0.0025$  for S608 group). No significant reduction was measured when transfected with negative control vector, compared with mock control ( $P > 0.05$ ). Transfection with amiRNA-HBV-S608 vector had the greatest reduction of HBsAg and HBeAg compared with the negative vector control. At 48 h, 72 h and 96 h after amiRNA-HBV-S608 transfection compared with negative control, the inhibitory rates of HBsAg secretion were  $29.5\% \pm 5.0\%$ ,  $49.8\% \pm 4.7\%$  and  $39.9\% \pm 6.7\%$ , and the inhibitory rates of HBeAg secretion were  $25.5\% \pm 5.6\%$ ,  $39.9\% \pm 6.7\%$  and  $37.4\% \pm 6.7\%$ , respectively.

HBsAg and HBeAg in the culture medium of stably transfected cells were also assayed. The HBsAg and HBeAg levels of all the three cell lines integrated with the pCDNA6.2-HBV-amiRNA vector were significantly reduced compared with negative control of transfected HepG2.2.15 cells, (HBsAg:  $P = 0.00019$ ,  $0.000035$  and  $0.000012$ , respectively; HBeAg:  $P = 0.003$ ,  $0.0002$  and  $0.000024$ , respectively). The greatest reduction of HBsAg in amiRNA-S608 was  $81.5\% \pm 2.2\%$  ( $P = 0.000012$ ,

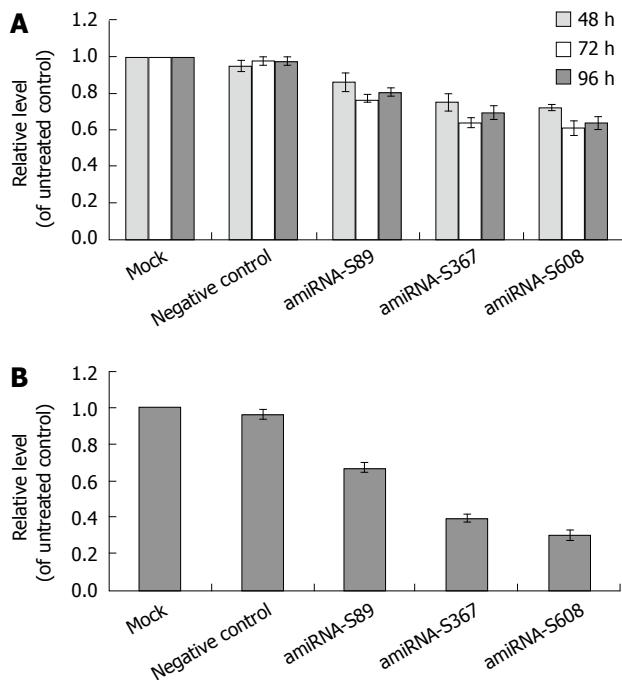


**Figure 3.** A: Effect of amiRNA-HBV with stable transfection on HBsAg levels; B: Effect of amiRNA-HBV with stable transfection on HBeAg levels. HBsAg and HBeAg levels are expressed as mean  $\pm$  SD. Compared with negative control, all three plasmids had significant inhibitory effect on HBsAg and HBeAg (HBsAg:  $P = 0.00019$ ,  $0.000035$  and  $0.000012$ , respectively; HBeAg:  $P = 0.003$ ,  $0.0002$  and  $0.000024$ , respectively).

Figure 3A), while the greatest reduction of HBeAg in amiRNA-S608 was  $58.1\% \pm 5.2\%$  ( $P = 0.000024$ , Figure 3B). No significant reduction was measured on the cells stably transfected with negative control plasmid compared with HepG2.2.15 cells ( $P > 0.05$ ).

#### Inhibition of HBV DNA replication by amiRNA

Real-time fluorescence quantitative PCR was performed to determine whether transfection with amiRNA-HBV-S vector would result in reduction of HBV DNA level. Quantitative assay revealed that HBV DNA levels of all three plasmid transfection groups decreased at 48 h, 72 h and 96 h after transfection, compared with negative control ( $P = 0.049$ ,  $0.000021$  and  $0.0011$  for S89 group;  $P = 0.0002$ ,  $0.000016$  and  $0.0012$  for S367 group;  $P = 0.0003$ ,  $0.00006$  and  $0.00016$  for S608 group). The greatest reduction was found in the amiRNA-HBV-S608 transfected group. The copies of HBV DNA in cells treated with amiRNA-HBV-S608 were reduced by  $39.0\% \pm 4.5\%$  at 72 h ( $P = 0.00006$ , vs negative control; Figure 4A). In the cell clones with stable transfection, compared with negative control vector, amiRNA-mediated RNAi resulted in a higher reduction level of HBV DNA in three plasmids transfected cells than those with transient transfection (Figure 4B). Among them, the HBV DNA level of amiRNA-HBV-S89 group decreased by  $33.4\% \pm 3.0\%$  ( $P = 0.00009$ ); amiRNA-HBV-S367 and amiRNA-HBV-S608 was much more efficient with inhibitory rates of  $60.8\% \pm 2.3\%$  and  $70.1\% \pm 3.3\%$ , respectively ( $P = 0.000007$  and  $0.000006$ , respectively).



**Figure 4.** A: Effect of amiRNA-HBV with transient transfection on HBV DNA levels; B: Effect of amiRNA-HBV with stable transfection on HBV DNA levels. The amounts of HBV DNA are expressed as mean  $\pm$  SD. Compared with negative control, all three plasmids had significant inhibitory effect on HBsAg and HBeAg at 48 h, 72 h and 96 h after transfection ( $P = 0.049$ ,  $0.000021$  and  $0.0011$  for S89 group;  $P = 0.0002$ ,  $0.000016$  and  $0.0012$  for S367 group;  $P = 0.0003$ ,  $0.00006$  and  $0.00016$  for S608 group). All three plasmids had significant inhibitory effect on HBV DNA in stably transfected groups, ( $P = 0.00009$ ,  $0.000007$  and  $0.000006$ , respectively).

## DISCUSSION

MicroRNA-induced RNA silencing has become a commonly used tool for the analysis of gene function<sup>[17,18]</sup>. The artificial microRNAs (amiRNAs) technology exploits endogenous miRNA precursors to generate sRNAs that direct gene silencing in either plants or animals<sup>[15,18-20]</sup>. AmiRNAs were first generated and used in human cell lines<sup>[15]</sup> and later in *Arabidopsis*<sup>[18]</sup>, where they were shown to effectively interfere with reporter gene expression. Subsequently, it was demonstrated that not only reporter genes but also endogenous genes can be targeted with amiRNAs (also called synthetic miRNAs). A few tumor genes have been successfully targeted with artificial synthetic miRNAs<sup>[21-23]</sup>.

In this paper, microRNA-induced RNA silencing was utilized to suppress HBV replication and expression. Three different miR-155 based vectors targeting HBV S gene were constructed. The three different plasmids all proved effective, but were not to the same extent at all three times. Among the three amiRNAs, amiRNA-HBV-S608 is the most potent in inhibiting HBV replication and expression in the transient transfection group. We found that HBsAg and HBeAg in the supernatant were inhibited by  $49.8\% \pm 4.7\%$  and  $39.9\% \pm 6.7\%$  at 72 h after transfection with amiRNA-HBV-S608 plasmid. The HBV DNA levels were also found to be decreased similarly. In view of transfection efficiency of 55%-60%, we concluded that actual antigen

inhibition rate of amiRNA-HBV-S608 vector was above 80%. To further confirm the effect of amiRNA-HBV, we performed stable transfection of amiRNA-HBV plasmid to exclude the effect of transfection efficiency. In the stably transfected cells with amiRNA-S608 plasmid, amiRNA-mediated RNAi resulted in a higher reduction level of both HBV antigen and DNA than with transiently transfected cells. The greatest reduction of HBsAg and HBeAg in stably transfected cells with amiRNA-HBV-S608 was  $81.5\% \pm 2.2\%$  and  $58.1\% \pm 5.2\%$ , respectively in stably transfected cells. The copies of HBV DNA in cells with stable transfection of amiRNA-HBV-S608 were reduced by  $70.1\% \pm 3.3\%$  compared to negative controls. These data provided a strong indication that continuous expression of amiRNA could provoke stable and sequence-specific silencing of target genes of HBV.

The HepG2.2.15 cells can produce replicative viral DNA intermediates, mature Dane particles and high level of viral antigens constitutively, which may be similar to the behavior in HBV-infected human being. But the inhibitory effect of amiRNA-mediated RNAi in HepG2.2.15 cells is lower than that in other hepatocytes and mice using co-transfection method<sup>[24,25]</sup>. We believed that all the HepG2.2.15 cells contained HBV genome and could secrete HBV proteins and DNA, but only approximately 55%-60% of the cells received the amiRNA-HBV vector. So the low transfection efficiency on HepG2.2.15 cells resulted in the reduction of inhibition effect. When stable transfection was performed in our experiment with the same vectors, all of these vectors acted with a similar effect as against other cotransfection methods.

Our results showed that artificial miRNA-mediated RNAi could inhibit HBV protein expression and HBV DNA replication in HepG2.2.15 cells *in vitro*. Similar to the siRNA mediated RNAi, microRNA mediated RNAi was sufficient to disrupt the viral life cycle and inhibited HBV DNA replication. The inhibition was sequence-specific, because the transfection with the negative control had no such effects. This is the first time to our knowledge that a marked reduction of HBV replication induced by artificial miRNA has been noted.

In summary, we constructed three artificially expressed miRNA plasmids and used them as a tool to inhibit HBV replication. We systematically evaluated the effects of amiRNA-based RNAi on HBV expression and replication in HepG2.2.15 cells. The data from our experiments suggested that amiRNA mediated RNAi might represent an alternative approach for the treatment of chronic HBV infection, which can enhance the anti-HBV efficacy and overcome the drawbacks of current therapies.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

MicroRNAs (miRNAs) endogenously expressed small ssRNA sequences with

about 22 nucleotide, which naturally direct gene silencing through components shared with the RNAi pathway.

### Research frontiers

Recently, it has been described how artificial miRNAs (amiRNAs) designed to target one or several genes of interest could provide a new and highly specific approach for effective post-transcriptional gene silencing.

### Innovations and breakthroughs

The results of our study suggest that amiRNA-expressing vectors can be used as RNAi-based anti-HBV therapeutics. amiRNA mediated RNAi is more advantageous for treating chronic HBV infection, which is easy to mutate *in vivo*.

### Applications

Vector-based amiRNA could be a promising approach for the treatment of chronic HBV infection.

### Peer review

AmiRNA is a hot topic and the papers dealing with it is scarce. The importance of this strategy as a therapeutic tool of HBV infection could be outstanding.

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CASE REPORT

## Asymptomatic ileal adenocarcinoma in the setting of undiagnosed Crohn's disease

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## INTRODUCTION

The incidence of inflammatory bowel disease (IBD) is increasing with rates of around 6/100 000 for Crohn's disease with a marked rise in the age group between twenty to forty years<sup>[1]</sup>. Small bowel carcinomas are uncommon representing less than 5% of all gastrointestinal malignancies<sup>[2-7]</sup>. Prognosis is unfavorable with 1 and 2 year survival of 30%-60%, depending on the stage of the cancer<sup>[8-12]</sup>. Crohn's disease has been associated with an elevated risk for the development of small bowel adenocarcinoma<sup>[9,13-20]</sup> and chronic inflammation is implicated in this neoplastic progression<sup>[21]</sup>. Although most adenocarcinomas arise in the duodenum, those associated with Crohn's disease generally occur in the ileum 20 years or more after the onset of Crohn's disease<sup>[9,11,12]</sup>. In most cases, detection of small bowel carcinoma associated with Crohn's disease is at operation for other reasons or due to obstructive symptoms<sup>[22-25]</sup>.

Neither the risk factors nor screening for early diagnosis of small bowel adenocarcinoma in patients with Crohn's disease have been established. Here we report on an asymptomatic patient who on routine screening colonoscopy was diagnosed with adenocarcinoma of the terminal ileum which eventually led to the establishment of his Crohn's disease.

## CASE REPORT

A 53-year old man presented for evaluation of a small bowel mass found on a screening colonoscopy. He denied any episodes of abdominal pain or diarrhea. He noted that his father had been diagnosed with Crohn's disease at the time of his death. He subsequently underwent a computed tomography (CT) examination

## Abstract

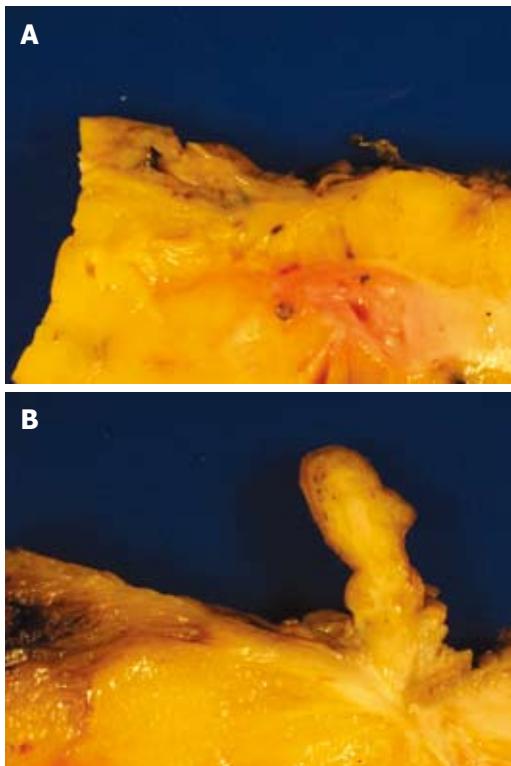
A 53-year old previously healthy male underwent a screening colonoscopy for detection of a potential colorectal neoplasm. The terminal ileum was intubated and a mass was noted. Examination of the colon was normal. The biopsy of the ileal mass was consistent with an adenocarcinoma arising from the terminal ileum. His father who had never been previously ill from gastrointestinal disease died of natural causes, but was found to have Crohn's disease postmortem. The patient underwent exploratory laparotomy and a right hemicolectomy with a 30 cm section of terminal ileum in continuity. Findings were consistent with ileal adenocarcinoma in the setting of Crohn's disease. The patient made an uneventful recovery. The pathology was stage 1 adenocarcinoma. This is a unique case in that on a screening colonoscopy, a favorable ileal adenocarcinoma was discovered in the setting of asymptomatic, undiagnosed ileal Crohn's disease in a patient whose father had Crohn's disease diagnosed postmortem.

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**Key words:** Ileal adenocarcinoma; Crohn's disease; Colonoscopy



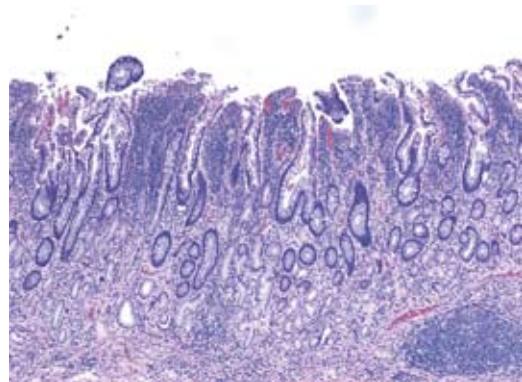
**Figure 1** CT scan of the abdomen showing thickened and spiculated ileal wall.



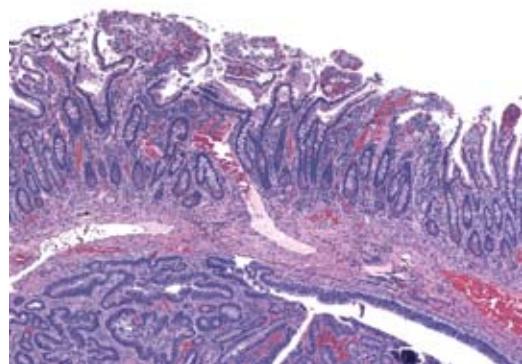
**Figure 2** A: Stricture adjacent to the ileocecal valve; B: 3 cm polyp, sections of which revealed a white spiculated area extending into the underlying fat.

of his abdomen and pelvis with both oral and intravenous contrast which revealed a thickened and spiculated wall of the terminal ileum with fistulization from the terminal ileum to the cecum (Figure 1). Given these findings, he was taken to the operating room for exploration.

On exploration, he was found to have a dense inflammatory mass in the right lower quadrant with multiple adhesions to the abdominal wall and bladder. Further exploration revealed a mass in the terminal ileum with dense adhesions involving the terminal ileum and cecum. Given these findings, a right hemicolectomy with a concomitant terminal ileal resection was performed. The surgical specimen consisted of a 20 cm segment of the cecum and a 53 cm segment of the terminal ileum with pericolic fat averaging 9 cm in diameter.



**Figure 3** Crohn's changes in the terminal ileum (HE, x 400).



**Figure 4** Adenocarcinoma invading the muscularis propria (HE, x 400).

Macroscopic examination revealed Crohn's disease involving the distal most segment of the terminal ileum with a stricture adjacent to the ileocecal valve (Figure 2A). Adjacent to the stricture was a 3 cm polyp, sections of which revealed a white spiculated area that extended into the underlying fat (Figure 2B).

Histopathological examination in the area of the stricture revealed areas of chronic inflammatory change with marked architectural distortion and extensive pseudo-pyloric metaplasia in the mucosa. Extensive transmural lymphoid aggregates, muscular hypertrophy, neural hyperplasia, and submucosal fibrosis, compatible with Crohn's disease were also noted (Figure 3). In many areas, the mucosa was sitting directly in contact with the muscularis propria without any intervening submucosa. In the background of this inflammation, areas of low and high grade dysplasia were identified. Scattered glands were seen infiltrating into the markedly hypertrophic and disorganized muscularis mucosa. Isolated tumor glands were also seen in the submucosa and as discussed above, in some areas, there were hardly any intervening submucosa with the tumor glands infiltrating the muscularis propria (Figure 4). The size of the tumor was difficult to estimate, as grossly no obvious mass was identified. However, from microscopic sections, it was estimated that the intramucosal carcinoma extended over a 2.0 cm area. The staging of this tumor was also difficult due to complex architecture and histological changes in the bowel wall. However, due to the presence

of occasional tumor glands in the muscularis propria, it was staged as pT2. Twenty-nine lymph nodes were harvested and all were negative for carcinoma. The patient recovered well from surgery.

## DISCUSSION

A thorough search of the worldwide literature has revealed that the case presented here is the first ever of a patient with ileal adenocarcinoma as the first manifestation of Crohn's disease in an otherwise asymptomatic patient. There are, however, other reports of ileal adenocarcinoma as the first manifestation of Crohn's disease in patients with vague abdominal complaints<sup>[26,27]</sup>.

In 1956, Ginzburg *et al* first described carcinoma as a rare complication of small bowel Crohn's disease<sup>[28]</sup>. To date, several cases of small bowel carcinoma in Crohn's disease have been reported<sup>[29]</sup>. The most common small bowel carcinoma is adenocarcinoma, and risk factors include long-standing disease, surgically bypassed loops, male sex, onset of disease before the age of 30 years, and associated chronic active disease with strictures and fistulas<sup>[11,30-32]</sup>.

Munkholm *et al* reported an incidence of 0.54% of small bowel cancer in Crohn's disease compared to an expected rate of 0.04% ( $P = 0.0001$ )<sup>[30]</sup>. In a later study, the same group reported a more than 60-fold increased risk of small bowel adenocarcinoma independent of age and sex in patients with Crohn's disease<sup>[33]</sup>. The lifetime prevalence of small bowel adenocarcinoma in patients with Crohn's disease is 1%-3%<sup>[34]</sup>. The combination of genetic susceptibility, mechanical irritation and surgery has been suggested as possible etiologies for this elevated risk in Crohn's disease. Small bowel carcinomas are also mostly localized to strictures<sup>[22,35,36]</sup>. Immunosuppression has also been implicated<sup>[32]</sup>.

These occult carcinomas pose a challenge to conventional diagnostic investigations such as upper or lower gastrointestinal endoscopy and small bowel series. CT has now emerged as the imaging modality of choice<sup>[37-40]</sup>. Magnetic resonance imaging (MRI)<sup>[41]</sup>, double-contrast enteroclysis<sup>[42]</sup>, and video wireless capsule endoscopy<sup>[43,44]</sup> have also been promising.

Survival rates for small bowel malignancies are much worse than for large bowel cancers with a mean survival of 6 mo as compared to 65 mo for the latter<sup>[9]</sup>. Mortality rates range from 30%-60% depending on the stage of the carcinoma<sup>[8-12]</sup>. Poor prognostic factors include positive resection margins, extramural venous spread, lymph node metastases, poor tumor differentiation, depth of tumor, and a history of Crohn's disease<sup>[45]</sup>.

Only small studies have evaluated adjuvant therapy for small bowel adenocarcinoma<sup>[46,47]</sup>. These studies are not in patients with Crohn's disease and randomized controlled trials are definitely needed.

We report a patient with ileal adenocarcinoma as the first manifestation of Crohn's disease. The patient had no symptoms of Crohn's disease and had only a family history of Crohn's disease. This case report, as well as others discussed above, highlight the need for a higher

index of suspicion in screening for Crohn's disease and initiating a regular follow-up with ileocolonoscopy.

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CASE REPORT

## Lymphoepithelioma-like hepatocellular carcinoma: A case report and a review of the literature

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### Abstract

Lymphoepithelioma is a particular form of undifferentiated carcinoma, characterized by a prominent lymphoid stroma, originally described in the nasopharynx. Lymphoid stroma-rich carcinomas arising in other organs have been termed lymphoepithelioma-like carcinoma (LELC). In the liver, primary LELCs are very rare, and the majority has been identified as cholangiocarcinomas. Here a rare case of lymphoepithelioma-like hepatocellular carcinoma (HCC) is described. A 47-year old woman presented with abdominal pain. Ultrasoundography revealed a liver nodule, 2.2 cm in diameter, localized in the right lobe, adjacent to the gallbladder. Viral markers for hepatic B virus (HBV), hepatic C virus (HCV) and Epstein-Barr virus (EBV) were negative. The nodule was hypoechoic. The patient underwent surgery, with resection of the nodule. Histology showed hepatocellular carcinoma, characterized by a prominent lymphoid infiltrate. At immunocytochemistry, tumor cells were reactive for Hep Par1 and glypican 3. Immunophenotyping of tumor infiltrating lymphocytes evidenced the predominance of CD8+ cytotoxic suppressor T cells. The postoperative clinical outcome was favorable and the patient was recurrence-free 15 mo after resection. This case, to the best of our knowl-

edge, is the first reported non EBV and non cirrhosis-associated lymphoepithelioma-like hepatocellular carcinoma. The association between the lack of EBV infection, the absence of cirrhosis, a "cytotoxic profile" of the inflammatory infiltrate and a good prognosis could identify a variant of lymphoepithelioma-like HCC with a favorable clinical outcome.

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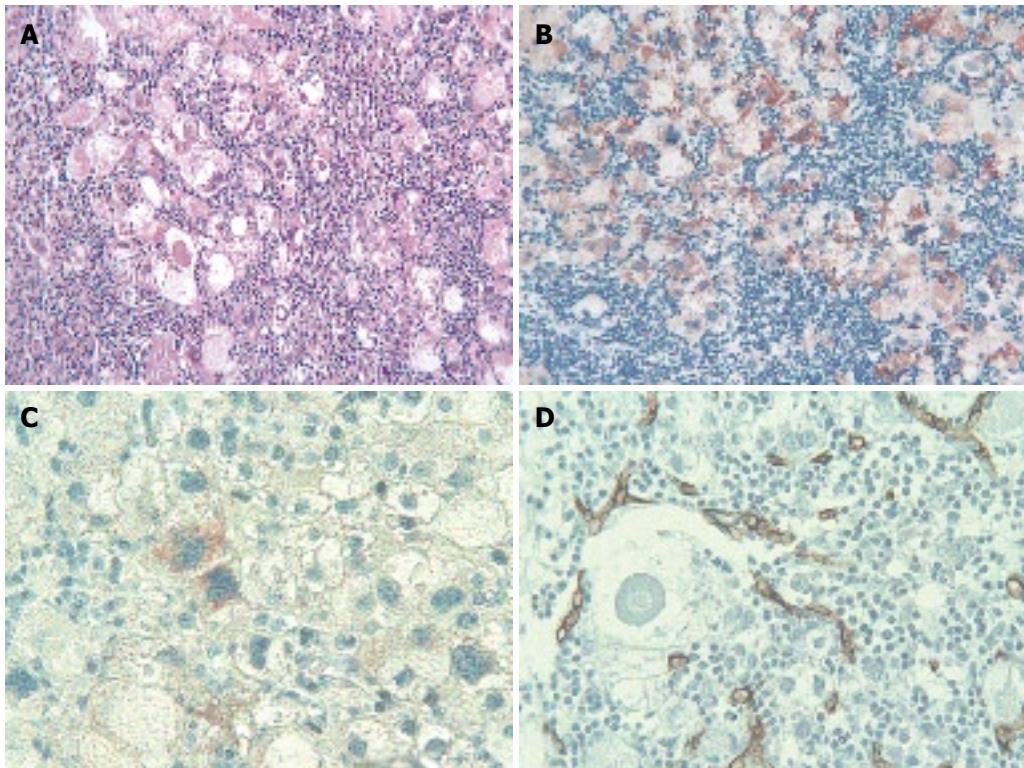
**Key words:** Liver; Hepatocellular carcinoma; Tumor infiltrating lymphocytes; Primary liver tumors; Liver lymphoepithelioma

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### INTRODUCTION

Lymphoepithelioma is a particular form of undifferentiated carcinoma, characterized by a prominent lymphoid stroma, primarily described in the 1920's by Regaud and Schminke in the head and neck region of Asian subjects<sup>[1]</sup>. Subsequently, histologically similar carcinomas have been described in other organs, such as salivary glands<sup>[2]</sup>, lung<sup>[3]</sup>, stomach<sup>[4]</sup>, colon<sup>[5]</sup>, thymus<sup>[6]</sup>, uterus<sup>[7]</sup>, bladder<sup>[8,9]</sup> and urinary tract<sup>[10]</sup>. Lymphoid stroma-rich carcinomas in these locations have been termed Lymphoepithelioma-like carcinoma (LELC). The similarities among these neoplasms arising in diverse sites are not limited to the histological picture, i.e. to the presence of a heavy lymphocytic intratumoral infiltrate. The majority of these tumors, particularly those originating from the nasopharynx<sup>[11]</sup>, salivary glands<sup>[2]</sup>, thymus<sup>[12]</sup>, lung and stomach<sup>[13]</sup>, show relevant pathogenetic similarities: a close etiopathogenetic linkage with Epstein-Barr virus (EBV) infection. At the immunohistochemical level, EBV expression in these tumors has been associated



**Figure 1** Histological picture characterized by a strong lymphoid intratumoral infiltrate (HE, x 250) (A), vast majority of neoplastic cells showing intense granular cytoplasmic reactivity for Hep Par 1 (x 250) (B), scattered atypical tumor cells showing cytoplasmic immunoreactivity for Glycocalyx 3 (x 400) (C), and a large atypical tumor cell surrounded by CD34-positive newly formed capillaries (x 400) (D).

with p53 over expression in the nuclei of EBV-infected tumor cells<sup>[14]</sup> and hyperreactivity of tumor cells for bcl-2 and proliferating cell nuclear antigen (PCNA)<sup>[15]</sup>.

Although it has been suggested by many authors that lymphoepithelioma-like carcinomas have a better prognosis than conventional carcinomas with a less marked lymphocytic infiltrate<sup>[10]</sup>, given the few studies on the clinical course of lymphoepithelioma-like carcinomas in the literature to date, this topic needs further investigation.

In the liver, lymphoepithelioma-like primitive carcinomas are extremely rare. To the best of our knowledge, only nine cases of cholangiocarcinomas have been reported, and the majority was associated with EBV infection<sup>[16]</sup>. Here we report a rare case of hepatocellular carcinoma (HCC) characterized by a prominent lymphoid stroma, indistinguishable on morphological grounds from tumors called lymphoepithelioma-like carcinomas with a discussion on their histological, immunohistochemical and clinical findings.

## CASE REPORT

A 47-year-old woman presented with acute abdominal pain, localized in the right liver lobe. Ultrasonography evidenced a hypoechoic liver mass, 2.2 cm in diameter, adjacent to the gallbladder. At CT-scan, the nodule showed signs of a recent intranodular hemorrhage. Laboratory tests showed negativity for HBV (serum HBsAg, HbeAg, anti-HbcAg and HBV DNA), HCV, and EBV markers. Molecular analyses for detecting EBV DNA, performed in tumor samples by PCR and Southern blot hybridization, were negative. The patient underwent surgery, with resection of the liver nodule. Histological examination

showed proliferation of atypical large cells, characterized by an eosinophilic cytoplasm, with large nuclei and prominent nucleoli. Epithelial cells were surrounded by a dense lymphoid stroma, extending inside the tumor (Figure 1A). At immunocytochemistry, tumor cells were diffusely Hep Par1-positive (Figure 1B) and focally immunoreactive for glycocalyx 3 (Figure 1C). No reactivity for cytokeratins 7, 19 and 20 was observed. CD34 showed diffuse capillarization of intratumoral sinusoids (Figure 1D). The clinical outcome was favorable and the patient was recurrence-free after a 15-mo follow-up.

## DISCUSSION

The case reported here is, to the best of our knowledge, the fourth reported case of Lymphoepithelioma-like HCC. The first case described in 2000 by Emile *et al* and defined as HCC with lymphoid stroma<sup>[17]</sup>, was characterized by a good prognosis after liver transplant and negativity for EBV infection<sup>[18]</sup>. Subsequently, in a letter to the Journal, Szekely E. argued that the tumor was indistinguishable from LELC described in other organs, suggesting the final diagnosis of lymphoepithelioma-like HCC<sup>[19]</sup>. The second case of hepatocellular LELC, described in 2004 by Si MW and coworkers<sup>[20]</sup>, showed some similarities and marked differences as compared with the first one. Both patients affected by end stage chronic liver disease underwent liver transplant. The patient described by Si was younger and infected with HCV and EBV, which was detected in tumor samples. The clinical course was precipitous, multiple recurrences appeared 3 mo after liver transplant and the patient expired within a few weeks. The third case of hepatocellular LELC was recently reported by Chen *et al*<sup>[21]</sup> in a 56-year old male patient with cirrhosis related

to HCV infection and negative EBV markers. The case reported here shows some peculiarities as compared with the previous 3 cases. It is the first case of hepatocellular LELC arising in a non cirrhotic liver. Moreover, the risk factor for Lymphoepithelioma-like HCC in our case was unknown since EBV, HBV and HCV markers were negative. The absence of EBV infection was associated with a favorable clinical outcome, as in two previously reported cases of EBV-negative lymphoepithelioma-like HCC<sup>[17,21]</sup>, contrasting with the aggressive course of one case of EBV-positive hepatocellular LELC<sup>[20]</sup>. These data show that, even in LELCs arising in the liver, and in spite of a striking similarity of the histological picture of all tumors, the clinical aggressivity of HCC in a single case may be extremely variable. In our case, we observed the predominance of CD8+ cytotoxic T cells, in the inflammatory intratumoral infiltrate, suggestive of an effective immune response to the tumor<sup>[22]</sup>.

Finally, this case is the first case of non EBV and non cirrhosis-associated lymphoepithelioma-like HCC. The association between the lack of EBV infection, the absence of cirrhosis, a CD8+/cytotoxic profile of the inflammatory infiltrate and a good prognosis could identify a variant of lymphoepithelioma-like HCC with a favorable clinical course.

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CASE REPORT

# Vanishing bile duct and Stevens-Johnson syndrome associated with ciprofloxacin treated with tacrolimus

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## INTRODUCTION

Stevens-Johnson syndrome (SJS), a rare but severe dermatological condition<sup>[1]</sup>, is considered a severe type of erythema exsudativum multiforme<sup>[2]</sup>, characterized by erythema with bullous and eroded lesions of skin and mucous membranes. It typically occurs after ingestion of medications such as nonsteroidal drugs, antibiotics, and anticonvulsants<sup>[1,2]</sup>. Extracutaneous manifestations of the syndrome may involve the conjunctiva, trachea, buccal mucosa, gastrointestinal tract, and genitourinary tract<sup>[2,3]</sup>. Cholestatic liver disease, which may precede the cutaneous manifestations of SJS, occurs in a very limited number of patients with SJS. In the present case, SJS and acute vanishing bile duct syndrome (VBDS), the most severe cholestatic liver diseases, were identified.

## CASE REPORT

A 26-year-old woman presented to the emergency department of our hospital, complaining of dysphagia, dysuria, fever and rashes on her body. Two weeks prior to eruption, 500 mg ciprofloxacin, twice daily, was started for acute bronchitis by a pulmonologist. Her medical history was unremarkable. Physical examination revealed macular erythematous eruption on her face, back, arms and erosions on the lips, buccal and genital mucous membranes. She was febrile (39°C), her conjunctivae were icteric and injected. Laboratory investigations showed 4.07 mg/dL total bilirubin (< 1.2 mg/dL), 3.58 mg/dL conjugated bilirubin (< 0.2 mg/dL), 326 IU/L alanine transaminase (ALT, < 31 IU/L), 280 IU/L gamma-glutamyl-transpeptidase (GGT, < 36 IU/L), 229 IU/L alkaline phosphatase (ALP, < 104 IU/L), and 107 mm/h erythrocyte sedimentation rate (ESR). Serologic tests were negative for viral hepatitis A-C, E, Epstein Barr virus, cytomegalovirus, Herpes simplex virus, Mycobacteria and Mycoplasma pneumonia. Abdominal ultrasound showed that liver

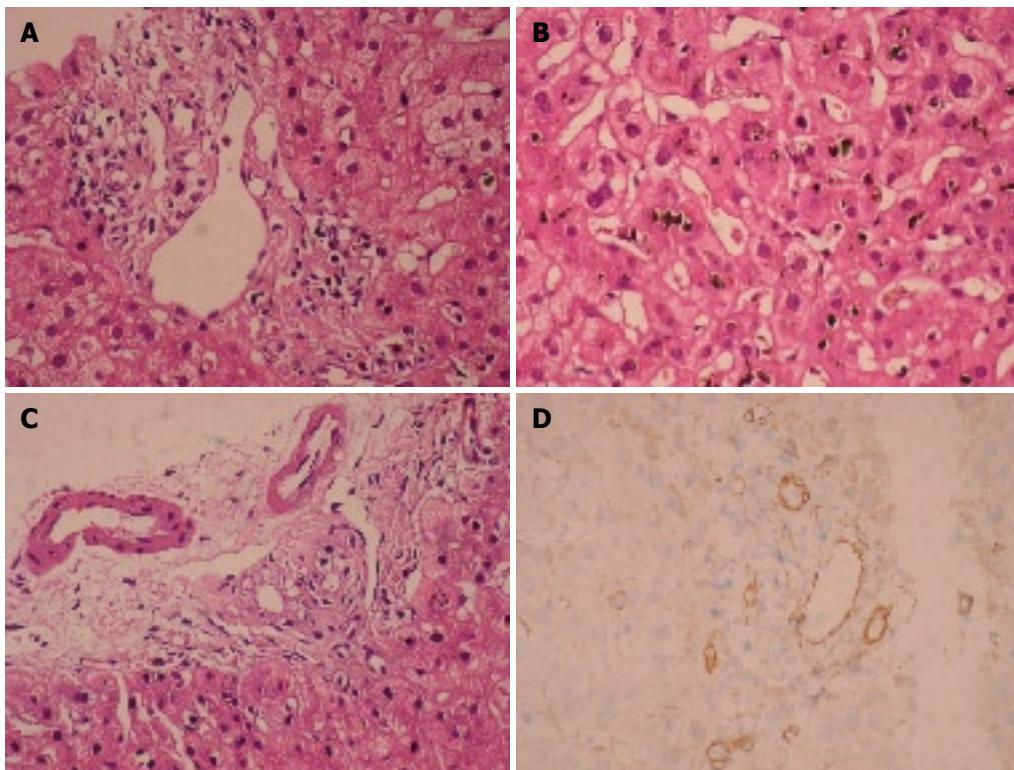
## Abstract

Stevens-Johnson syndrome (SJS) is a serious and potentially life-threatening disease. Vanishing bile duct syndrome (VBDS) is a rare cause of progressive cholestasis. Both syndromes are mostly related with drugs. We report a case of a patient with ciprofloxacin-induced SJS and acute onset of VBDS, and reviewed the related literature. It is the first case of ciprofloxacin-induced VBDS successfully treated with tacrolimus. This case reminds physicians of the importance of drug reactions, their severity, techniques for diagnosis and methods of management.

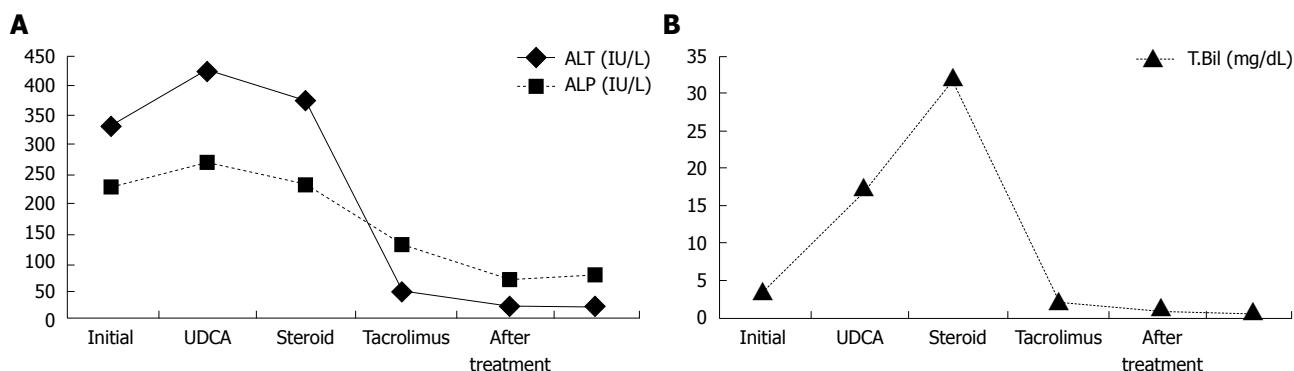
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**Key words:** Stevens-Johnson syndrome; Vanishing bile duct syndrome; Ciprofloxacin; Tacrolimus

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**Figure 1** Moderate predominantly lymphocytic mixed portal infiltration and absence of interlobular bile duct destruction (HE,  $\times 400$ ) (A), intralobular severe canalicular cholestasis (HE,  $\times 400$ ) (B), marked cytoplasmic vacuolization in cholangiolar epithelium (HE,  $\times 400$ ) (C), and absence of bile ducts by cytokeratin 19 immunohistochemical staining (IHC,  $\times 400$ ) (D), observed in our patient.



**Figure 2** ALT and ALP (A) and total bilirubin (B) levels during and after treatment in our case.

had homogeneous texture with normal bile ducts and gallbladder. Skin biopsy from the neck showed subepidermal vesiculation and epidermal necrosis, which was consistent with SJS. Skin eruptions resolved after supportive care. However, cholestatic picture persisted. Ursodeoxycholic acid treatment (15 mg/kg per day) did not alleviate the symptoms. Antinuclear antibody, anti-neutrophil cytoplasmic antibody, anti-liver/kidney microsomal antibody and antismooth muscle antibody titers turned out to be negative. Magnetic resonance cholangiopancreatography showed no abnormality in bile ducts. Percutaneous liver biopsy 30 d after the diagnosis showed cholestasis, along with a decreased number of bile ducts and mild portal inflammation, which was consistent with VBDS (Figure 1). Prednisone (40 mg/d) was added to the treatment with ursodeoxycholic acid

and discontinued after 4 wk because of lack of efficacy and iatrogenic Cushing's syndrome. Cholestasis persisted and bilirubin level exceeded 30 mg/dL (Figure 2). After long medical discussions and repetitive literature reviews, she was put on treatment with tacrolimus (0.15 mg/kg per day in two divided doses). Significant clinical and biochemical improvements were observed 3 mo after treatment. She took tacrolimus for 3 mo, ursodeoxycholic acid (15 mg/kg per day) for 7 mo. She had a normal physical status with normal liver synthetic functions and was instructed to avoid ciprofloxacin in the future.

## DISCUSSION

SJS is a serious and potentially life-threatening disease,

mainly caused by drugs. More than 100 different medications have been implicated<sup>[1]</sup>. The syndrome usually begins within 1-14 d of ingestion of the offending agent but may not manifest itself for up to three to 6 wk after ingestion<sup>[3]</sup>. In our case, the interval was 3 wk. Anticonvulsants, sulfonamides, penicillins, allopurinol, nonsteroidal anti-inflammatory drugs are the most common culprit medications while ciprofloxacin and cephalosporin are rarely reported as the causative agents of SJS<sup>[1,3,4]</sup>.

SJS is a well-recognized immune complex-mediated hypersensitivity reaction that affects all age groups<sup>[5-7]</sup>. It has classic systemic, mucosal and dermatologic manifestations<sup>[5]</sup>. VBDS, a rare cause of progressive cholestasis, is mostly related with drugs. Drugs act as a hapten and produce autoantibodies against cytokeratin which is in the bile duct, skin, conjunctival epithelium and orogenital mucosa<sup>[5]</sup>. Autoantibodies destroy biliary apparatus and as a result, with resultant disappearance of intrahepatic bile duct<sup>[8]</sup>.

The mechanism of biliary epithelial cell injury and interlobular duct loss in the VBDS has not been fully understood yet<sup>[5]</sup>. Toxic, idiosyncratic, metabolic, and immune etiologies have been suggested<sup>[5,7]</sup>. The latest evidence supports the importance of the immune system in the pathogenesis and suggests mechanisms common to both SJS and VBDS<sup>[5]</sup>. In SJS, there are immune complex formation and deposition, followed by a cytokine- and cell-mediated response<sup>[5,7]</sup>. Many drugs associated with VBDS are also associated with SJS like antibiotics, non-steroidal anti-inflammatory drugs and carbamazepine.

In the literature, there are less than ten reported cases of SJS and VBDS co-occurrence<sup>[1,5,7]</sup>. So it is great deal to have a standardized approach to these patients. There is no proven effective therapy for VBDS and SJS, but authors mostly agree that treatment modalities for both VBDS and SJS include withdrawal of the offending agent, supportive care, and usage of immunosuppressants<sup>[9,10]</sup>. Steroids, choleretic agents and immunosuppressants have been tried<sup>[1,2,10]</sup>. Acute onset of VBDS may be unresponsive to these modalities and progress to biliary cirrhosis, thus liver transplantation may be needed<sup>[5]</sup>. Because of the paucity of cases, there are no available success and failure rates, frequency of complication and percentage of liver transplantation need. Case-based literature just gives us some clinical clues for treatment approaches.

In our case, VBDS was refractory to steroid and ursodeoxycholic acid therapies. The clinical and biochemical improvements were obtained by immune suppression with tacrolimus therapy in our patient. In the literature, it is the first case of ciprofloxacin-induced VBDS successfully treated with tacrolimus.

Tumor necrosis factor-alpha (TNF- $\alpha$ ) has been shown to be strongly expressed in SJS lesions by an Italian group<sup>[11]</sup>. Authors suggest that IFN-gamma may play an important role in SJS. IL-2, IL-5 and IL-13 may contribute to the cutaneous immunoinflammation in this disease. Since immune complex-mediated reaction is very

important in these drug-induced syndromes, anti-TNF- $\alpha$  antibodies may be a promising therapy in the future<sup>[12,13]</sup>. However, it is not available in clinical practice, and there is no reported SJS or VBDS case treated with anti-TNF- $\alpha$  yet in literature.

Several recent studies have reported strong genetic associations between HLA alleles and susceptibility to drug hypersensitivity<sup>[14]</sup>. The genetic associations can be drug specific, such as HLA-B1502 being associated with carbamazepine-induced SJS, HLA-B5701 with abacavir hypersensitivity and HLA-B5801 with allopurinol-induced severe cutaneous adverse reactions<sup>[15,16]</sup>. The high sensitivity and specificity of some markers provide a plausible basis for developing tests to identify individuals at risk for drug hypersensitivity. Drug specific genetic screening tests may prevent those catastrophic diseases. Prescribing medication, according to history and genetic tests, will decrease the prevalence of both diseases in the future.

Our case reminds physicians of the importance of drug reactions, their severity, techniques for diagnosis and ways of management. Prescribing any drug with ultimate care, early diagnosis and treatment of both syndromes will improve the outcome.

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CASE REPORT

# An autopsy case of a primary aortoenteric fistula: A pitfall of the endoscopic diagnosis

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## INTRODUCTION

A primary aortoenteric fistula (PAEF) is a rare but often life-threatening cause of massive gastrointestinal bleeding<sup>[1-5]</sup>. PAEFs have a mortality rate of nearly 100% in the absence of surgical intervention, and diagnosis is not established preoperatively<sup>[2-5]</sup>. Diagnostic procedures are often non confirmatory and can sometimes impede urgently needed surgical intervention<sup>[3,4,6]</sup>. Although it is infrequent, gastrointestinal endoscopy may be a double-edged sword when PAEFs coexist with multiple bleeding sites<sup>[7]</sup>. Here, we report such a case in which the cause of death was massive gastrointestinal bleeding due to a PAEF after leaving the hospital.

## CASE REPORT

A 68-year-old man went to a hospital after suffering from melena for several days. He was conscious but looked pale. He had a blood pressure of 120/60 mmHg. His hemoglobin level was 5.7 g/dL and his hematocrit was 19.8%. An abdominal examination appeared normal, and no abdominal mass was observed. An emergency gastrointestinal endoscopy revealed a bleeding ulcer in the second part of the duodenum (Figure 1A). Other potential sources of acute bleeding were excluded. The patient was diagnosed with a probable duodenal ulcer, which was the presumed source of his bleeding. Endoscopic hemoclipping was performed (Figure 1B), and the patient received a blood transfusion and hemostatic agents. Following these procedures, the patient had no signs of hemorrhaging and recovered from anemia. After a follow-up endoscopy on the 13th d (Figure 1C), the patient was discharged from the hospital. However, the following day, the patient complained of lumbago and abdominal distension. In the middle of the night, he suddenly entered into a state of shock, experienced a cardiac arrest, and was taken to the hospital. In the emergency room, a nasogastric tube was inserted and

## Abstract

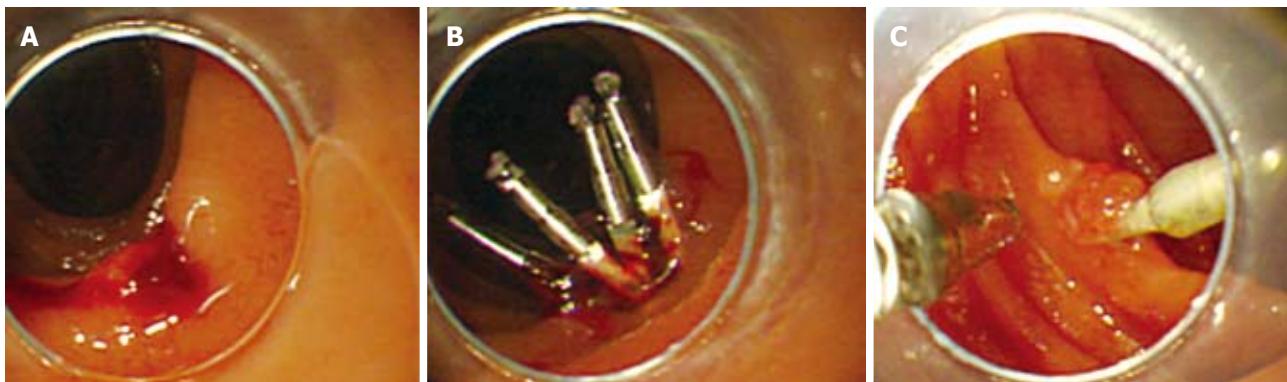
A primary aortoenteric fistula (PAEF), defined as a communication between the native aorta and the gastrointestinal tract, is a rare cause of gastrointestinal bleeding. The preoperative diagnosis of PAEF is extremely difficult. Consequently, PAEF may cause sudden and unexpected death. We present an autopsy case of a 68-year-old man who died of massive gastrointestinal bleeding due to a PAEF. Autopsy revealed a pinhole rupture located on the third part of the duodenal mucosa and fistulized into the adjacent abdominal aortic aneurysm (AAA). Our case indicates that the aortoenteric fistula can result in fatal gastrointestinal bleeding. Consequently, a PAEF should be included in the differential diagnosis of gastrointestinal bleeding.

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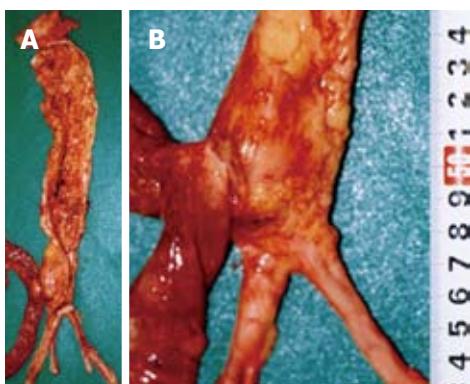
**Key words:** Primary aortoenteric fistula; Gastrointestinal bleeding; Herald bleeding; Misleading; Medico-legal autopsy

**Peer reviewer:** William Dickey, Altnagelvin Hospital, Londonderry, Northern Ireland BT47 6SB, United Kingdom

Ihama Y, Miyazaki T, Fuke C, Ihama Y, Matayoshi R, Kohatsu



**Figure 1** Endoscopic image showing a bleeding ulcer in the second part of the duodenum (A), the endoscopic clipping procedure revealing no other bleeding site detected by upper and lower gastrointestinal endoscopy (B), and follow-up gastrointestinal endoscopic view 12 d after hemostatic procedure (C). Only one hemoclip remained on the scar of the duodenal ulcer.



**Figure 2** The presence of a firm adhesion between the AAA and the digestive duodenal wall located on the third part of duodenum (A), and magnification of the firm adhesion between the anterior site of the abdominal aorta and the posterior serosa of the duodenum (B).

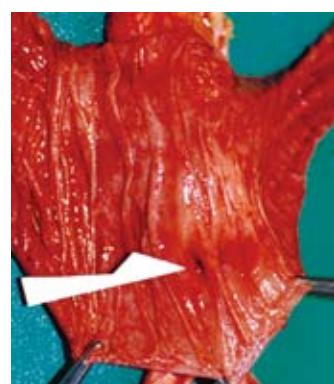
nasogastric aspiration was performed producing 880 mL of blood.

#### Autopsy findings

The patient measured 162 cm in height and weighed 58 kg. Postmortem hypostasis on the back was very slight. No petechiae were observed in the palpebral conjunctivae. External examination revealed no injuries except for those caused by clinical procedures performed in the emergency room. Internal examination revealed that the gastrointestinal tract was dilated and contained 2200 mL of clotted blood between the stomach and jejunum. No abdominal organ injury and blood were found in the abdominal cavity. An abdominal aortic aneurysm (AAA) was located above the bifurcation of the aorta and resembled a fusiform swelling, 4 cm in diameter and 5 cm in length. An AAA with atherosclerosis thrust forward, adhering firmly to the digestive duodenal wall (Figure 2). The inferior mesenteric artery was hardly identified due to fibrous adhesion. The internal surface of the AAA had highly calcified atheromatous ulcers covered with mural thrombus (Figure 3). A pinhole rupture was located on the third part of the duodenal mucosa (Figure 4) and fistulized into the adjacent AAA. A scarred ulcer with a hemoclip was observed on the second part of duodenal mucosa



**Figure 3** The AAA has severe atherosclerosis with calcification and clotted blood on the aneurysm inside the aortic lumen.

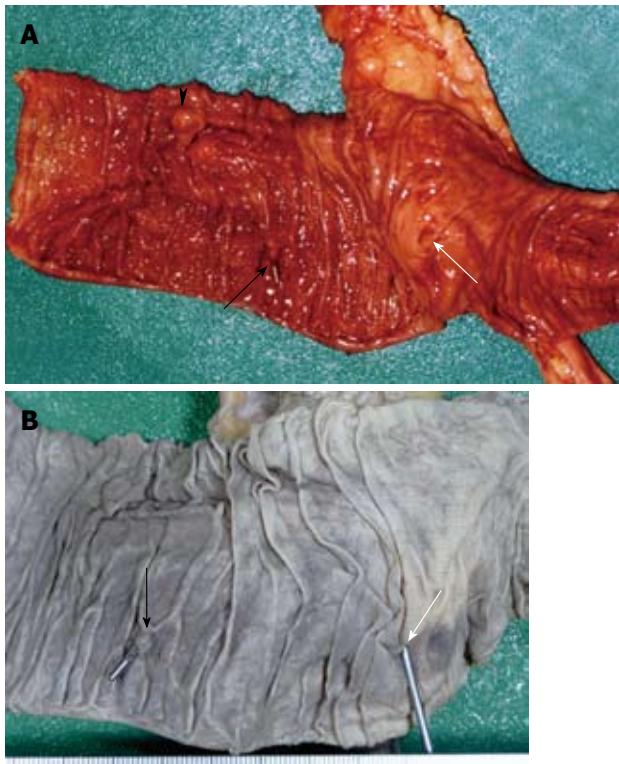


**Figure 4** A rupture and the size of a pinhole with no degeneration or inflammation of the third part of the duodenal mucosa as indicated by the white arrow.

(Figure 5). No other origin of bleeding was found in the gastrointestinal tract.

#### Microscopic investigations

Histological examination revealed a fibrous adhesion between the aorta and duodenal serosa. The aneurysm contained atheromatous degeneration covered by a mural thrombus with a layer of fibrin. The internal and external elastic membranes were destroyed and the media of the artery was thinned under the most advanced plaque. Fibroblast proliferation and granulation tissue were revealed in the adventitia of the aorta and perivascular tissue. Inflammation of the duodenum was more serious at the serosa, but there were no signs of inflammation of the duodenal mucosa (Figure 6). Because repair reaction of the aorta was broader than that of



**Figure 5** A scar on the clipped ulcer indicated by the black arrow, papilla Vater indicated by the black triangle, and a rupture on the third part of duodenal mucosa located in front of the firm adhesion indicated by the white arrow in the duodenal mucosa specimen (A), and a postclipping scar indicated by the black arrow and a rupture indicated by the white arrow in the formalin-fixed specimen of the duodenal mucosa (B). The rupture was located approximately 6 cm from the anal side of the postclipping scar.

the duodenum, inflammation may originate out of the aorta.

## DISCUSSION

A PAEF, defined as a communication between the native aorta and gastrointestinal tract, is a rare cause of gastrointestinal bleeding<sup>[2,3,8,9]</sup>. A large autopsy series reported an incidence of PAEF of 0.04%-0.07%<sup>[1,2,7]</sup>. A fistula most commonly originates from an AAA, of which 85% are atherosclerotic. AAA is a common disease among middle-aged and older subjects, and its clinical consequences depend on its location and size. The major complications of AAA include rupture into the peritoneal cavity, occlusion of a branch vessel and embolism from atheroma or mural thrombus. PAEF is the rarest complication of AAA. The most frequent site of a fistula is the third part of the duodenum, as in the current case<sup>[1,4,10]</sup>. The literature reports that PAEFs are often fatal, with a total mortality rate of 80%-100% and a perioperative mortality rate of 18%-63%<sup>[1-3,7,9-11]</sup>. However, the actual incidence and mortality rates are not known because many patients die of PAEFs before they have been correctly diagnosed<sup>[1,8]</sup>.

The classical triad of symptoms, i.e. gastrointestinal bleeding, abdominal pain, and a pulsating abdominal mass is overemphasized<sup>[1,3,4]</sup>, as it occurs in less than 25% of PAEF cases<sup>[6]</sup>. The diagnosis of a PAEF is difficult because of its nonspecific and subtle clinical presenta-



**Figure 6** Formalin-fixed duodenum and aorta specimen demonstrating a sonde inserted from the rupture on the duodenal mucosa to the inside of the aortic lumen (A), horizontal cross sections showing a firm adhesion between the aorta and its wall as well as a rupture (black arrow) on the third part of the duodenum (B); and a microphotograph of a horizontal cross section showing an adhesion between the duodenum and the aorta with atherosclerosis-contained clotted blood (C). The histology of the duodenal appears normal (HE,  $\times 5$ ).

tion<sup>[2,4]</sup>. However, PAEFs usually present with a herald bleeding prior to exsanguination<sup>[1-4,9]</sup>. Herald bleeding is usually minor and self-limiting, and it is probably due to a spasm of the intestinal wall musculature in response to sudden distention<sup>[1,3]</sup>. Bleeding can be further limited by hypotension and thrombus formation<sup>[1-3]</sup>. Consequently, excessive volume therapy and endoscopy may promote fatal exsanguination<sup>[1,12]</sup>. The time interval between a herald bleeding and exsanguination is known to range from hours to months<sup>[1-4,9]</sup>. The interval was about 2 wk in the current patient. When the patient dies of fatal exsanguination after going to the hospital due to a herald bleeding, his family may suspect an error in medical treatment.

Frequently, the choice of a diagnostic procedure is based on the clinical condition<sup>[1,3,5]</sup>. Diagnostic imaging techniques, such as contrast-enhanced computed tomography (CT) and angiography, are useful investigation modalities for identifying PAEFs<sup>[1-4]</sup>. Even if there is limited bleeding at the examination, CT might reveal the size, location, and degree of calcification of an AAA. Fibrous adhesion between the aorta and duodenum might facilitate PAEF identification. In a hemodynamically stable patient with gastrointestinal bleeding, endoscopy is the preferred primary procedure which provides valuable information<sup>[1-3,7]</sup>. However, endoscopy rarely reveals confirmatory evidence of a PAEF because stable patients do not often have an active bleeding<sup>[1,2,11,13]</sup>. Additionally, endoscopic visualization of a fistula that is present

lower than the third part of the duodenum, which is the most common site of PAEF occurrence, is extremely difficult<sup>[2,4,14]</sup>. In the clinical setting, the absence of identifiable bleeding lesions with initial gastrointestinal endoscopy is regarded by some as a strong indicator for laparotomy<sup>[3,13]</sup>. Recently, since the introduction of capsule endoscopy for clinical use, small bowel bleeding from the ampulla of Vater into the terminal ileum can be easily visualized and defined<sup>[15]</sup>. Capsule endoscopy, which non-invasively captures images of the gastrointestinal mucosa without any pressure load, might be the best strategy for identifying a PAEF. However, if upper endoscopy or colonoscopy detects other coexisting bleeding sites in PAEF patients, the findings may be misleading<sup>[3,4,6,7,13]</sup>. Furthermore, concomitant gastrointestinal lesions are occasionally found in PAEF patients, and this incidence is 25% as indicated in previous series<sup>[5,7,13,16]</sup>. In the current patient, a bleeding ulcer was identified as the source of the gastrointestinal bleeding. Hence, the possibility of a PAEF might have been overlooked. Ultimately, the key to early diagnosis of PAEF is an endoscopist's heightened index of suspicion<sup>[4,5,13]</sup>. Endoscopists need to recognize PAEFs as a potential cause of gastrointestinal bleeding.

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CASE REPORT

# Carcinoma *in situ* arising in a tubulovillous adenoma of the distal common bile duct: A case report

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## Abstract

Tubulovillous adenomas are common in the colon and rectum, but are rare in the common bile duct. Biliary adenomas may produce obstructive jaundice, which can be easily confused with a malignant neoplasm or stone. We report a case of a carcinoma *in situ* arising in a tubulovillous adenoma of the distal common bile duct causing obstructive jaundice. A 55-year-old male presented with a 10-d history of pruritus and progressive jaundice. Abdominal sonography and computed tomography showed a mass in the distal common bile duct. Endoscopic retrograde cholangiopancreatography showed luminal narrowing of the bile duct due to a polypoid mass. Positron emission tomography demonstrated no abnormal uptake. It was thought that this mass was a malignant tumor, thus a pylorus-preserving pancreaticoduodenectomy was performed. The final pathology showed a tubulovillous adenoma with carcinoma *in situ* of the distal common bile duct. At follow-up 8 mo later, endoscopy showed multiple polyps in the rectum, colon and stomach. The polyps were removed by endoscopic mucosal resection and shown to be tubular adenomas with high grade dysplasia. Biliary adenomas require careful follow-up for early detection of recurrence and malignant transformation.

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**Key words:** Common bile duct; Adenoma; Carcinoma *in situ*

## INTRODUCTION

Biliary adenomas are very rare tumors, which may pose diagnostic dilemmas preoperatively. Due to recent advances in diagnostic techniques and early diagnosis, biliary adenomas are often detected as high grade dysplasias or carcinomas *in situ*. The optimal therapeutic strategy for adenomas of the distal common bile duct (CBD) or ampillary region has not been established. We report herein a case of a bile duct tubulovillous adenoma with carcinoma *in situ* presenting painless jaundice in a man who was treated with a pylorus-preserving pancreaticoduodenectomy (PPPD) and presented 8 mo later with a tubular adenoma in the gastrointestinal tract with high grade dysplasia. Our patient did not exhibit any clinical signs of familial adenomatous polyposis<sup>[1]</sup> or Gardner's syndrome<sup>[2]</sup>. We also reviewed the literature about the common bile duct adenoma found in the English literature<sup>[3-22]</sup> (Table 1).

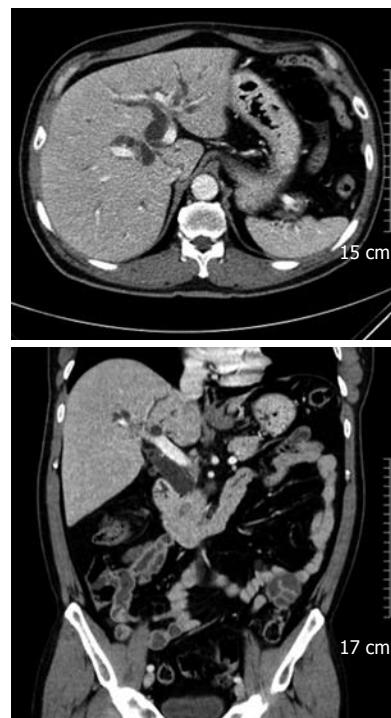
## CASE REPORT

A 55-year-old man was admitted to our hospital with a 10-d history of painless obstructive jaundice and pruritus. He had diabetes mellitus which had been controlled by an oral hypoglycemic agent. He had no family history of colorectal cancer or polyposis. On physical examination, he had icteric sclerae. Abdominal examination revealed no palpable mass or tenderness. Laboratory tests showed elevations of total bilirubin (8.2 mg/dL), direct bilirubin (6.3 mg/dL), alkaline phosphatase (302 IU/L), alanine transaminase (27 IU/L) and gamma glutamyl transpeptidase (110 IU/L). Endoscopic retrograde cholangiopancreatography (ERCP) showed a 10-mm polypoid mass in the distal CBD. Endoscopic mucosal resection (EMR) was performed and the specimen was sent for histopathological examination. The final pathology showed a tubulovillous adenoma with carcinoma *in situ* of the distal common bile duct. At follow-up 8 months later, endoscopy showed multiple polyps in the rectum, colon and stomach. The polyps were removed by endoscopic mucosal resection and shown to be tubular adenomas with high grade dysplasia.

**Table 1** Reported cases of common bile duct adenomas

Author	Sex	Age(yr)	Presentation	Treatment	Histology
Hulten, 1970 <sup>[3]</sup>	M (n = 2)	61, 80	Biliary colic (n = 1) and jaundice (n = 2)	Local excision (n = 2)	Papilloma (n = 2) with moderate atypia
Styne, 1986 <sup>[4]</sup>	F (n = 1)	59	Recurrent cholangitis	Local excision	Papilloma
Saxe, 1988 <sup>[5]</sup>	M (n = 1)	64	Painful jaundice and pruritus	Whipple	Villous adenoma
Harshfield, 1990 <sup>[6]</sup>	M (n = 1)	78	Chronic right upper quadrant pain	Local excision	Villous adenoma
Sturgis, 1992 <sup>[7]</sup>	F (n = 1)	81	Right upper quadrant pain	Endoscopic excision	Tubulovillous adenoma
Hanafy, 1993 <sup>[8]</sup>	M (n = 1)	76	Mild jaundice and abdominal mass	Local excision	Villous adenoma
Buckley, 1993 <sup>[9]</sup>	M (n = 1)	34	Chronic jaundice and abdominal pain	Whipple	Villous adenoma with malignant foci
Blot, 1996 <sup>[10]</sup>	M (n = 1)	84	Febrile jaundice	Local excision	Villous adenoma
Kawakatsu, 1997 <sup>[11]</sup>	F (n = 3)	60.6	Febrile jaundice	Whipple (n = 2), local excision (n = 3)	Villous adenoma with mild dysplasia (n = 1), malignant foci (n = 4)
	M (n = 2)	(mean)			
Chae, 1999 <sup>[12]</sup>	M (n = 1)	77	Painless jaundice and pruritus	Local excision	Villous adenoma with malignant foci
Inagaki, 1999 <sup>[13]</sup>	M (n = 1)	73	Epigastric pain and jaundice	Whipple	Papilloma
Chang, 2001 <sup>[14]</sup>	M (n = 1)	51	Febrile jaundice, abdominal mass	Operation refused	Papilloma with focal dysplasia
Oshikiri, 2002 <sup>[15]</sup>	F (n = 1)	69	Jaundice	Whipple	Papilloma
Ariche, 2002 <sup>[16]</sup>	F (n = 1)	77	Abdominal pain	Local excision	Villous adenoma with adenocarcinoma
Aggarwal, 2003 <sup>[17]</sup>	M (n = 1)	55	Abdominal pain	Whipple	Adenoma with moderate dysplasia
Jao, 2003 <sup>[18]</sup>	M (n = 1)	60	Abdominal screening ultrasound	Endoscopic excision	Tubulovillous adenoma
Lou, 2003 <sup>[19]</sup>	M (n = 1)	47	Fever, abdominal pain	Local excision	Tubular adenoma with moderate dysplasia
Fletcher, 2004 <sup>[20]</sup>	M (n = 1)	74	Painless jaundice and pruritus	Whipple	Papilloma
Katsinelos, 2006 <sup>[21]</sup>	M (n = 1)	58	Painful jaundice	Whipple	Villous adenoma with atypia
Xu, 2008 <sup>[22]</sup>	F (n = 1)	27	Painless jaundice and pruritus	Whipple	Villous adenoma with mild dysplasia
Present case	M (n = 1)	55	Painless jaundice and pruritus	PPPD	Tubulovillous adenoma with carcinoma <i>in situ</i>

PPPD: Pylorus-preserving pancreaticoduodenectomy.

**Figure 1** Ultrasound examination reveals a 2 cm sized non-shadowing mass and a dilated common bile duct (arrow).**Figure 2** Computed tomography shows a diffuse dilatation of the common bile duct with an intraluminal mass in the distal common bile duct.

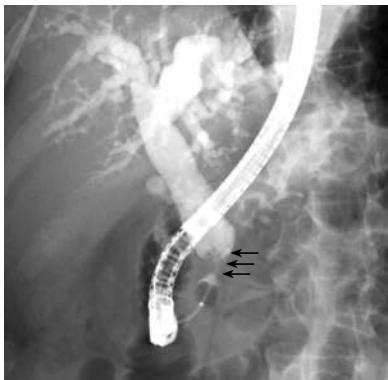
tidase (321 IU/L). The serum amylase was within the normal range. The carbohydrate antigen 19-9 level was 131.6 U/mL (normal range,  $\leq 27$  U/mL) and the carcinoembryonic antigen level was 1.5 ng/mL (normal range, 5 ng/mL). The hepatitis serologic markers were all negative. On abdominal ultrasonography, the CBD was dilated with a distal non-shadowing polypoid mass, however, there was no pancreatic duct dilatation (Figure 1). The CT findings were similar to the ultrasonographic findings and therefore a distal CBD tumor was suspected (Figure 2). Endoscopic retrograde cholangiopancreatography showed a 2cm polypoid mass and stricture in the distal CBD (Figure 3). Bile cytology revealed no malignancy. Positron emission tomography (PET) showed no hypermetabolic lesions. Based on the preoperative diagnosis of a distal tumor, a PPPD was performed.

The resected specimen revealed a 2 cm  $\times$  1.5 cm polypoid mass in the distal CBD (Figure 4). The final pathology showed a tubulovillous adenoma with carcinoma *in situ* in the distal CBD (Figure 5). There was no lymph node metastasis. The patient recovered

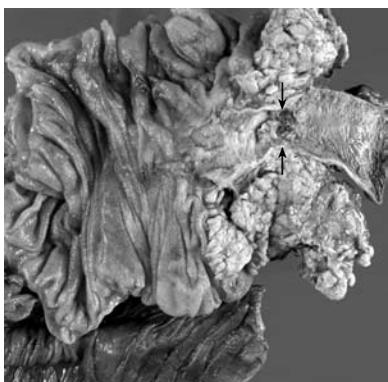
uneventfully. Eight months later, he developed multiple polyps (two in rectum, three in the colon and two in the stomach). An endoscopic mucosal resection was performed, which revealed a tubular adenoma with high grade dysplasia.

## DISCUSSION

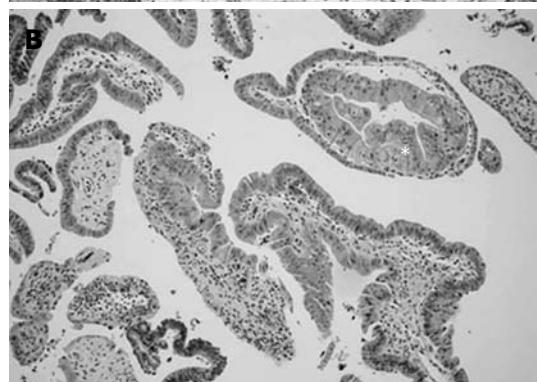
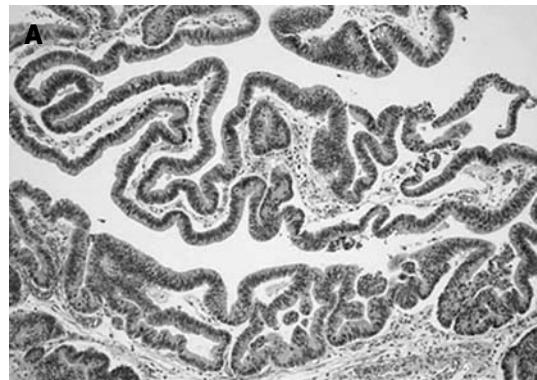
Tubulovillous adenomas are usually encountered in the gastrointestinal tract, but as a primary site, the CBD is



**Figure 3** Endoscopic retrograde cholangiopancreatography shows a 2 cm round lobulated filling defect in the distal common bile duct (arrows).



**Figure 4** A photograph of the pylorus-preserving pancreaticoduodenectomy specimen shows a polypoid mass in the distal common bile duct, 2 cm x 1.5 cm x 1.5 cm size (arrows).



**Figure 5** Microscopic features of the tubulovillous adenoma. Hematoxylin-eosin stain. **A:** The elongated and pseudostratification of nuclei are stained ( $\times 40$ ); **B:** Higher magnification on this slide demonstrates carcinoma *in situ* (asterisk;  $\times 100$ ).

rare. Adenomas arising from the CBD are summarized in Table 1. Saxe *et al*<sup>[5]</sup> was first to report a case of a villous adenoma in the CBD. The clinical manifestations of a biliary adenoma include jaundice, right upper quadrant abdominal pain, dyspepsia, nausea and vomiting in a fashion similar to ampullary tumors. Villous adenomas are benign tumors, but are considered to be premalignant. It is possible that the adenoma-to-carcinoma sequence occurs in biliary tumors<sup>[23-26]</sup>. Considering the similarity of the histologic and biologic characteristics of adenomas in the other segments of the GI tract, such an adenoma-to-carcinoma carcinogenic process involving the rectum, ampulla, gallbladder, and biliary duct occurs within the biliary tract<sup>[27,28]</sup>. Therefore, complete resection of the lesion makes it possible to avoid development of carcinoma<sup>[29]</sup>. It is difficult to differentiate biliary adenomas from other malignant lesions with radiologic imaging<sup>[23]</sup>. Predicting the presence of malignant foci preoperatively is difficult. However, suspicion of malignancy could be made by an experienced biliary endoscopist.

Appropriate management of these lesions in the distal CBD has not been clearly defined. In 1992, Sturgis *et al*<sup>[7]</sup> first reported that high-risk patients with tubulovillous adenomas of the CBD were best treated by endoscopic resection but the risk of recurrence is high. Other treatment options, such as local resection, are performed in high-risk patients thought preoperatively to have benign tumors<sup>[10]</sup>. Ariche *et al*<sup>[10]</sup> proposed that resection with free margins of the CBD with lymph node dissection of the hepatoduodenal ligament for tumors in the mid part of the CBD is an appropriate treatment option. If the remaining duct length is inadequate, local resection is impossible and

PD should be considered mandatory in cases involving cancer of the distal CBD. If malignancy is suspected or the size is larger than approximately 2 cm, radical resection is needed. We considered the other treatment option, duodenum preserving pancreatic head resection(DPPHR) which was first introduced by Beger *et al*<sup>[27]</sup> for chronic pancreatitis and has been increasingly used in neoplastic lesions, cystadenoma, borderline lesions, and carcinoma *in situ*<sup>[28-30]</sup>. However, we suspected that this tumor would be malignant and performed PPPD considering the size, site and clinical findings. Compared to Whipple-type resection, duodenum preserving pancreatic head resection has benefits in regard to postoperative morbidity and mortality, maintenance of glucose metabolism, absence of delay of gastric emptying, shorter hospital stay offers better quality of life of patients. However, duodenum preserving pancreatic resection has two major problems, incomplete lymph node dissection and ischemia of duodenum and has not been used yet as a surgical option of adenoma of common bile duct. Maeda *et al*<sup>[31]</sup> reported that duodenum preserving pancreatic resection in the treatment of pancreatic metastasis from renal cell carcinoma should be considered as radical lymph node dissection is not necessary. And then, if the nature and extent of common bile duct adenoma is suggestive of benign tumor and lymph node enlargement is absent, preoperatively, DPPHR should be considered in the treatment of common bile duct adenoma.

In this case, colonoscopy has not been performed and gastroscopy revealed no tumors in the stomach and

duodenum preoperatively. At the time of follow-up 8 mo postoperatively, colonoscopy and gastroscopy revealed multiple polyps in the rectum, sigmoid colon, and stomach. They were removed by endoscopic mucosal resection and confirmed as tubular adenomas with high grade dysplasia. In view of the risk of recurrence of adenomas, careful follow-up is in order.

Järvinen *et al*<sup>[32]</sup> have reported biliary involvement in familial adenomatous coli patients. The present report is the first case of a tubulovillous adenoma with carcinoma *in situ* of the distal CBD and several tubular adenomas with high grade dysplasia of the GI tract confirmed 8 mo apart. We think that although adenoma of the biliary tract and GI tract did not exist concurrently, tubulovillous adenoma of the distal CBD may have developed by a similar mechanism to that of the GI tract. In conclusion, this case suggests that adenomas arising from the distal CBD can transform into carcinoma and support the existence of an adenoma-to-carcinoma sequence given that carcinoma *in situ* with an adenomatous lesion of the distal CBD and tubular adenoma of the GI tract adenoma ultimately developed.

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CASE REPORT

# Coexistence of small cell neuroendocrine carcinoma and villous adenoma in the ampulla of Vater

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## Abstract

Small cell neuroendocrine carcinoma of the ampulla of Vater is extremely rare and different from the common ampullary adenocarcinoma. The ampillary adenoma is also a rare neoplasm and has the potential to develop an adenocarcinoma. Their coexistence has been rarely reported in the literature. We herein describe an unusual case of a small cell neuroendocrine carcinoma associated with a villous adenoma in the ampulla of Vater with emphasis on computed tomography (CT) and histopathological findings. We also discuss their clinical, histopathological and radiological features as well as possible histogenesis.

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**Key words:** Small cell neuroendocrine carcinoma; Adenoma; Ampulla of Vater; Computed tomography; Histopathology

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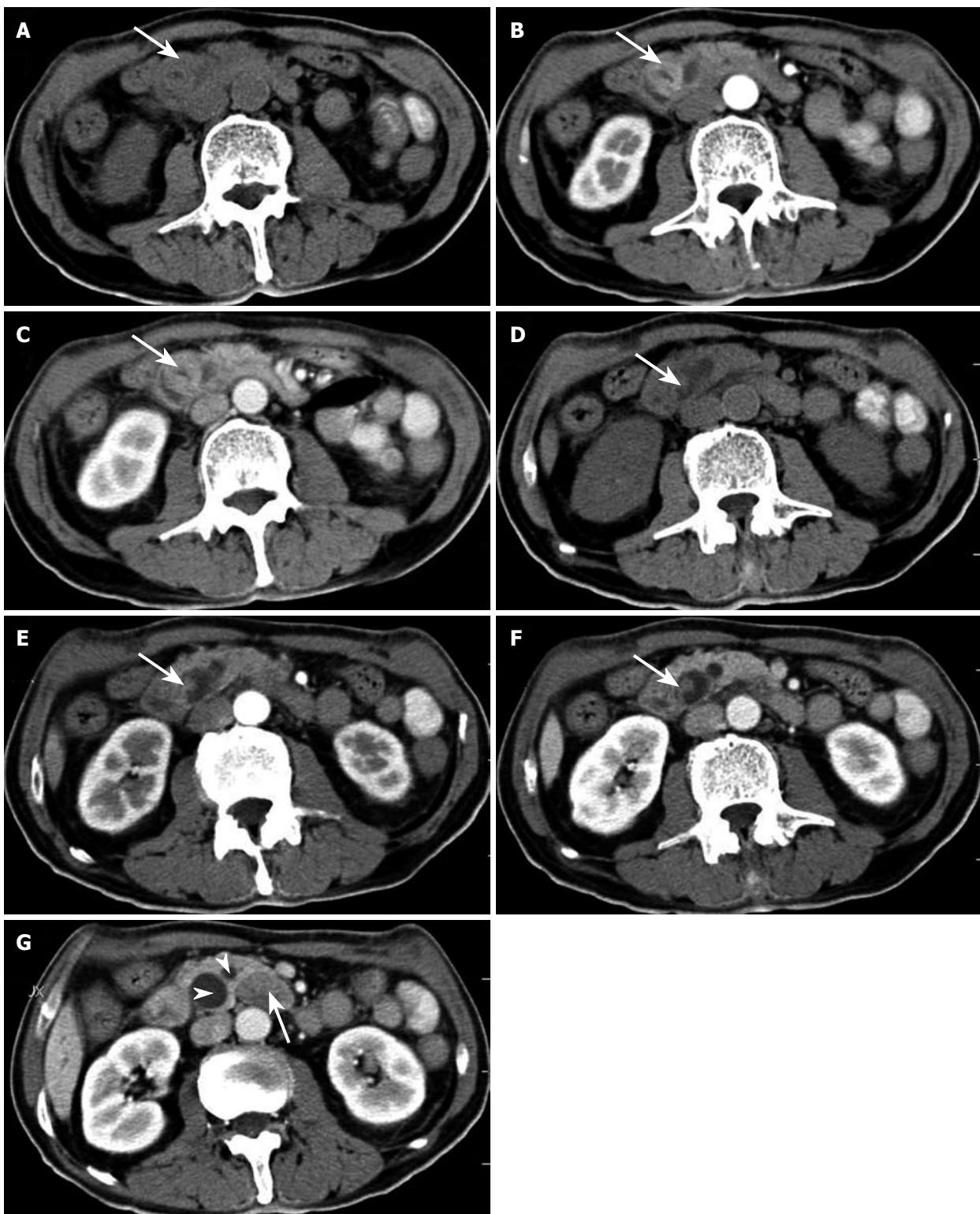
## INTRODUCTION

Primary extrapulmonary small cell neuroendocrine carcinoma is rare, occurring in only 4% of all patients with small cell carcinomas<sup>[1]</sup>. Small cell neuroendocrine carcinoma presents 0.1%-1% of all gastrointestinal malignant tumors with varying incidence in different organs<sup>[2]</sup>. Small cell neuroendocrine carcinoma of the ampulla of Vater is extremely rare and different from the common ampullary adenocarcinoma<sup>[3]</sup>. The ampillary adenoma is also a rare neoplasm and has the potential to develop an adenocarcinoma<sup>[4]</sup>. Only two cases of a small cell neuroendocrine carcinoma associated with an adenoma have been described by Nassar *et al*<sup>[5]</sup>. To our knowledge, there has been no report describing the imaging features of these coexisting tumors of the ampillary region. Here we report the computed tomography (CT) and magnetic resonance (MR) findings of an unusual case of a small cell neuroendocrine carcinoma associated with a villous adenoma in the ampulla of Vater, and review their clinical and histopathologic features.

## CASE REPORT

A 74-year-old man presented with a 3-wk history of abdominal discomfort and jaundice. Physical examination showed icterus and a palpable gall bladder. Laboratory findings were: total bilirubin, 11.19 mg/dL (normal 0.3-1.5); direct bilirubin, 6.91 mg/dL (normal 0.01-0.35); serum albumin, 3.14 g/dL (normal 3.5-5.0); alanine aminotransferase, 209 U/L (normal 0-50); aspartate aminotransferase, 197 U/L (normal 0-40); γ-glutamyl transpeptidase, 1610 U/L (normal 0-40); alkaline phosphatase, 1419 IU/L (normal 30-140); and carbohydrate antigen 19-9, 657.4 U/mL (normal < 37).

Gastroduodenal endoscopy showed a swollen duodenal papilla with the intact mucosal surface. Abdominal ultrasonography revealed a heterogeneous hypoechoic mass in the ampulla of Vater and markedly dilated bile ducts over the neoplasm. Abdominal CT showed two well-defined masses, dilated bile ducts and peripancreatic lymphadenopathy (Figure 1). On precontrast CT scanning, the two masses were isoattenuated to the surrounding pancreatic parenchyma (Figure 1A and D). On arterial and portal phase images after contrast enhancement, the larger oval mass was slightly hyperattenuated to the surrounding pancreas and showed the stenosis of the ampulla of



**Figure 1** Abdominal CT reveals two well-defined masses in the ampulla of Vater. On precontrast CT scanning, the two masses (arrow) show isoattenuation to the surrounding pancreatic parenchyma (A and D). On arterial and portal phase images after contrast enhancement, the larger oval mass (arrow) shows slightly higher attenuation than the surrounding pancreas (B and C). The smaller polypous mass (arrow) with a long pedicle and stalk shows slightly lower attenuation than the surrounding pancreas (E and F). Contrast-enhanced CT scan (G) shows peripancreatic lymphadenopathy (arrow) and dilatation of the common bile duct and the pancreatic duct (arrowheads).

Vater in its central portion (Figure 1B and C). The smaller polypous mass with a pedicle was slightly hypoattenuated to the surrounding pancreas (Figure 1E and F). MR cholangiopancreatography (MRCP) showed the dilated biliary tree up to the level of the ampulla, where the

common bile duct ended abruptly (Figure 2). The low-signal intensity mass was demonstrated immediately inferiorly to the level of the obstruction. The pancreatic duct was moderately dilated.

The patient underwent Whipple's operation with



**Figure 2** MRCP demonstrates dilatation of intrahepatic and extrahepatic bile ducts and the pancreatic duct as well as the low signal intensity mass (arrow) in the ampulla of Vater.

local lymph node dissection. At operation there was a 1.5 cm × 2 cm yellowish gray tumor located mainly in the submucous layer of the ampulla. A polyp with a 6 mm × 6 mm spherical head and a relatively long pedicle and stalk was found in the vicinity of the tumor and extended to the distal common bile duct. Histologically, the tumor proliferated in a nesting pattern. Patchy necrosis was present. The tumor was composed predominantly of small to medium-sized, round or oval cells with a high nucleus-to-cytoplasm ratio, finely dispersed chromatin, inconspicuous nucleoli, and high mitotic activity (more than 10 per 10 high power microscopic fields) (Figure 3A). Lymphatic permeation was seen, but no obvious venous involvement was identified. Immunohistochemically, neoplastic cells were positive for synaptophysin and low-molecular-weight cytokeratin (Figure 3B and C). Ki-67 immunostaining revealed a proliferation index of more than 80%. The associated adenomatous polyp with villous structures as well as foci of areas of moderate and severe dysplasia was seen in the ampillary mucosa (Figure 3D). The dysplastic epithelium of the adenoma was in continuity with the invasive tumor (Figure 3E). Unfortunately, the patient died of pneumonia 3 mo after the operation.

## DISCUSSION

Ampullary small cell neuroendocrine carcinoma is extremely rare and has only been documented in few case reports and retrospective studies<sup>[2,5]</sup>. The patients with ampullary small cell neuroendocrine carcinoma usually presented after the age of 60 years (mean age, 63.5 years), and a male predilection was observed<sup>[6]</sup>. The tumor is a poorly differentiated, high-grade neuroendocrine carcinoma and resembles its counterpart originating from the tracheobronchial tree in its aggressive behavior and high propensity for early lymph node metastases and distant metastases<sup>[2,3,5]</sup>.

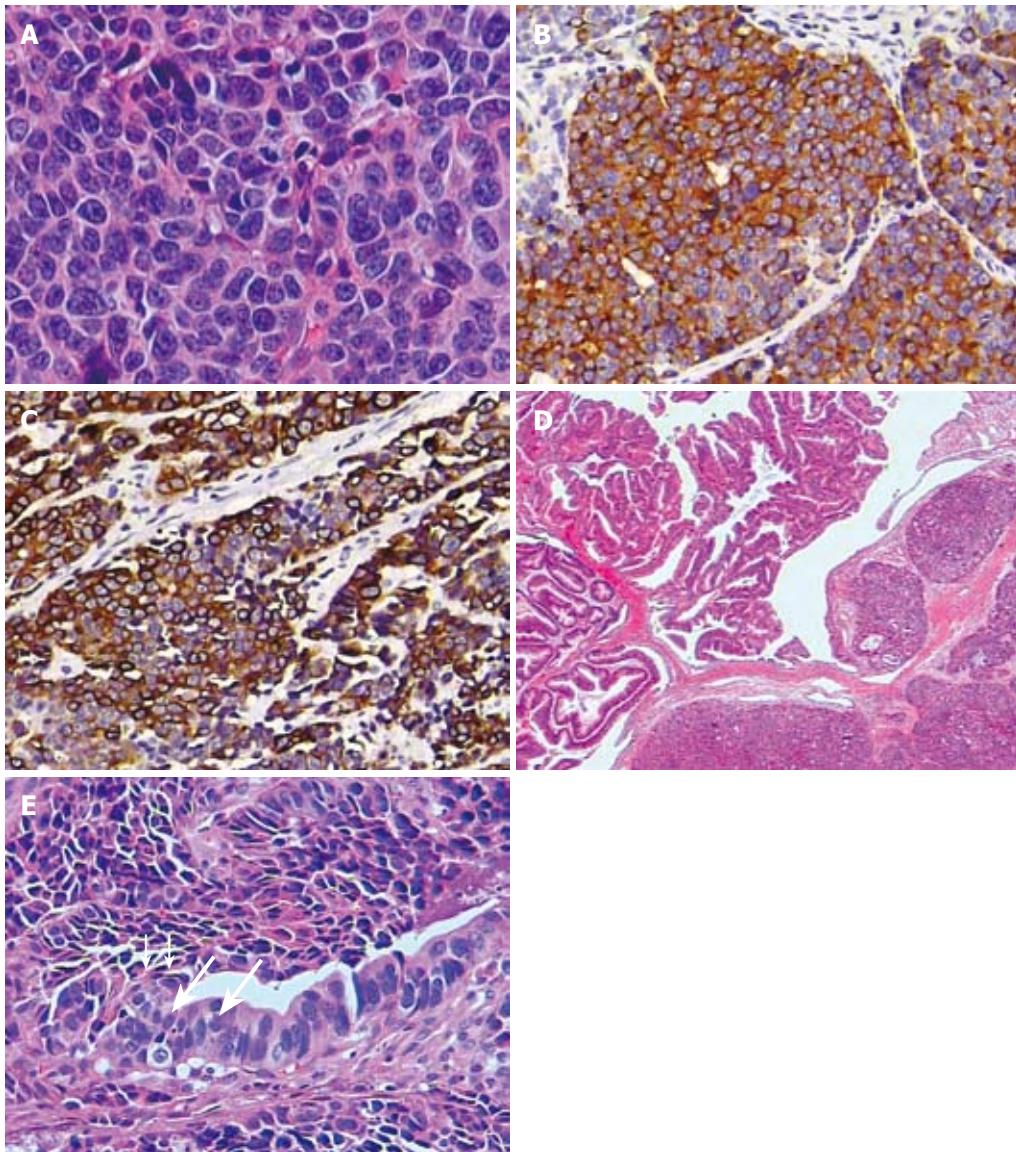
Pancreatoduodenectomy with local lymph node dissection is currently the standard surgical treatment for the ampullary cancer<sup>[7]</sup>. No effective postoperative chemotherapies for ampullary small cell neuroendocrine carcinoma have been established. Chemotherapy or

radiation has been reported to be effective for some cases of the tumor of the gastrointestinal tract and the biliary system<sup>[2,8]</sup>. The prognosis of these patients with small cell neuroendocrine carcinoma was poor, the survival period even after operation usually being less than a year<sup>[2,6,9]</sup>.

Ampullary adenoma and adenocarcinoma are tumors that originate in the glandular epithelium of the ampulla of Vater. The progression from adenoma to carcinoma in the ampulla of Vater is similar to that in colon<sup>[10]</sup>. Some cases of ampullary small cell neuroendocrine carcinoma associated with or mixed with adenoma, adenocarcinoma and squamous cell carcinoma have been reported<sup>[5,11]</sup>. These associations suggest a common origin of small cell neuroendocrine carcinoma and adenoma at this site. It is likely that the two neoplasms originate from non-neoplastic multipotent stem cells that terminally differentiate into all kinds of epithelial cells including ciliated cells, mucous cells and endocrine cells. However, the molecular data suggest that there are significant differences in the pathogenesis of the various types of tumors of the ampulla.

Ampullary small cell neuroendocrine carcinomas have histologic appearance similar to their pulmonary counterparts<sup>[6]</sup>. They are usually comprised of solid sheets of pleomorphic cells with small round-to-oval hyperchromatic nuclei, inconspicuous nucleoli, a high nuclear-to-cytoplasmic ratio. On immunohistochemical examination, the diagnosis of neuroendocrine tumors must be confirmed by positive immunohistochemical staining for at least 1 neuroendocrine marker, such as chromogranin or synaptophysin<sup>[12]</sup>. In our case, the diagnosis of small cell neuroendocrine carcinoma was based on typical morphological and immunohistochemical findings. In addition, neoplastic cells exhibited cytoplasmic immunostaining for cytokeratin and nuclear Ki-67 positivity (a proliferation index of more than 80%). We postulate that this neoplastic histogenesis was from epithelial stem cells with both epithelial and neuroendocrine characteristics.

The preoperative diagnosis of these rare tumors in the ampulla is potentially problematic. In most cases with ampullary tumors, endoscopic retrograde cholangiopancreatography (ERCP) with biopsy is valuable for making a definitive preoperative diagnosis in patients with ampullary tumors. However, it is very difficult to diagnose intramural ampullary neoplasm at endoscopy because the papilla is covered with normal duodenal mucosa. Although MRCP and CT can show obstruction of the ampulla of Vater, CT is known to be more accurate for demonstrating the location and extent of the involved biliary duct than MRCP and ERCP<sup>[13]</sup>. In our case, CT revealed two well-defined masses in the ampulla of Vater. The radiological finding of a polypous mass with a long pedicle was helpful to diagnose an adenoma. The ampullary small cell neuroendocrine carcinoma presented as a well-enhancing mass with local lymphadenopathy and differentiating it from ampullary adenocarcinoma may not be possible. Thus, small cell neuroendocrine carcinoma should be included in the differential diagnosis of ampullary masses especially when the patient is an elderly man.



**Figure 3** Histopathological findings. **A:** The tumor cells are composed predominantly of small to medium-sized, round or oval cells with hyperchromatic nuclei, inconspicuous nucleoli and scanty cytoplasm (HE,  $\times 400$ ); Immunohistochemically, the tumor cells are positive for synaptophysin (**B**) and low-molecular-weight cytokeratin (**C**) ( $\times 200$ ). **D:** Light microscopy shows the coexistence of villous adenoma and small cell neuroendocrine carcinoma (HE,  $\times 20$ ); **E:** The epithelium of the adenoma (long arrows) is in continuity with the small cell neuroendocrine carcinoma (short arrows) (HE,  $\times 200$ ).

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CASE REPORT

# Abrupt onset of type 1 diabetes mellitus during recombinant interferon-alpha 2b therapy in a patient with chronic hepatitis B

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## INTRODUCTION

Interferon-alpha (IFN- $\alpha$ ) is now widely used in the treatment of chronic hepatitis B and C. Systemic side effects of IFN- $\alpha$  therapy can affect numerous organ systems. These adverse reactions include flu-like syndrome, hematological abnormalities, cardiovascular and central nervous symptoms, gastrointestinal symptoms, pulmonary dysfunction, depression and retinopathy. Besides, IFN- $\alpha$  has been shown to be related to the development of a variety of autoimmune diseases, including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and autoimmune thyroid diseases (AITDs). However, the development of type 1 diabetes mellitus (DM) is relatively rare and few patients with positive islet cell autoantibody (ICAb) or glutamic acid decarboxylase autoantibody (GADAb) have been reported<sup>[1-3]</sup>. Here, we report the first case of a patient suffering from chronic hepatitis B who had an unexpected onset of type 1 DM during IFN- $\alpha$  2b therapy in China.

## CASE REPORT

A 33-year-old woman with a body mass index (BMI) of 22.49 kg/m<sup>2</sup> was treated with recombinant human IFN- $\alpha$  2b at a dosage of 3 million units (MU) once every other day from April 2006 because of hepatitis B. Ten months after IFN- $\alpha$  2b treatment, both hepatitis B e antigen (HBeAg) and HBV DNA became negative, and serum aminotransferases returned to normal. However, 13 mo after initiation of IFN- $\alpha$  2b treatment (reaching 585 MU of total dose in May 2007), the patient complained of weakness, polydipsia, polyuria and a rapid weight loss (5 kg within 10 d). She was then admitted to our hospital. Her clinical data on admission are shown in Table 1. Urinalysis showed glucosuria and ketonuria.

## Abstract

We describe a case of a 33-year-old female patient with chronic hepatitis B who developed type 1 diabetes mellitus (DM) after a 13-mo period of treatment with recombinant human interferon-alpha (IFN- $\alpha$ ) 2b. The patient presented with polydipsia, polyuria, hyperglycemia, diabetic ketoacidosis, combined with C-peptide secretion deficiency and positive islet cell autoantibody (ICAb). IFN- $\alpha$  2b treatment was terminated and instead insulin treatment was initiated. Five months after cessation of the recombinant human IFN- $\alpha$  2b therapy, the patient remained insulin-dependent. Her serum HBV DNA became negative and serum transaminase returned to the normal level after a 10-mo period of IFN therapy. Type 1 DM induced by IFN- $\alpha$  is relatively rare in patients with chronic hepatitis B. We should pay more attention to patients on IFN- $\alpha$  therapy to avoid destruction of pancreatic beta cells. This is the first case report from China.

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**Key words:** Interferon-alpha; Islet cell autoantibody; Type 1 diabetes mellitus; Autoimmune disease; Chronic hepatitis B

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**Table 1** Laboratory findings in the patient on admission

	Laboratory findings
Urinalysis	
Glucose	(+++)
Ketobody	(++++)
Protein	(-)
CBC	
RBC	$3.94 \times 10^{12}/\text{L}$
Hb	124 g/L
WBC	$5.1 \times 10^9/\text{L}$
Plt	$62 \times 10^9/\text{L}$
Blood chemistry	
Fasting plasma glucose	31.7 mmol/L (570.6 mg/dL)
Arterial blood gas analysis	
pH	7.314
PCO <sub>2</sub>	35.1 mmHg
PO <sub>2</sub>	70.6 mmHg
HCO <sub>3</sub> <sup>-</sup>	11.5 mmol/L
BE	-3.6 mmol/L
TP	87.1 g/L
ALB	48 g/L
AST	36 IU/L
ALT	40 IU/L
ALP	98 IU/L
GGT	21 IU/L
Serology	
HbA1c	10.0%
HBs Ag	(+)
HBs Ab	(-)
HBe Ag	(-)
HBe Ab	(-)
HBcAb	(+)
HBV DNA (PCR)	< 1000 copies/mL
IgG	18.37 g/L
IgA	2.79 g/L
IgM	1.87 g/L
TSH	0.636 mIU/mL
FT3	4.03 pmol/L
FT4	16.3 pmol/L
TPOAb	(-)
TgAb	(-)
ANA	(-)
Anti-DNA Ab	(-)
RF	(-)

Fasting plasma glucose and glycosylated hemoglobin (HbA1c) were 31.7 mmol/L (570.6 mg/dL) and 10.0%, respectively. Arterial blood gas analysis showed metabolic acidosis. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were within normal range. Hepatitis B surface antigen (HBsAg) and anti-HB core antibody (HBcAb) in serum were positive. The fasting plasma C-peptide level was low. The curve of C-peptide response to 75 g glucose load was flat (Table 2). The serum ICAb was positive (Figure 1). The patient had no family history of type 1 DM or other autoimmune disorders. She had no symptoms of other autoimmune diseases. Other autoantibodies including thyroid peroxidase antibody (TPOAb), thyroglobulin antibody (TgAb), antinuclear antibody (ANA), anti-dsDNA antibody, anti-RNP, anti-SSA, anti-SSB, and rheumatoid factor (RF) were all negative. Taken together, clinical and laboratory data confirmed the diagnosis of type 1 DM with ketoacidosis. Administration of IFN was immediately terminated. Her clinical condition improved with diet

**Table 2** Results of oral glucose tolerance test (75 g)

Time (min)	Blood glucose (mmol/L)	C-peptide (pmol/mL)
0	7.5	0.29
30	10.8	0.40
60	15.0	0.61
120	18.3	0.77
180	14.1	0.78

**Figure 1** Positive islet cell autoantibody in pancreas from a mouse at 1:40 serum dilution tested by immunofluorescence assay ( $\times 200$ ).

therapy, intravenous fluids and insulin therapy (isophane protamine biosynthetic human insulin, Novolin® R, Novo Nordisk, Denmark). Ketonuria disappeared after 12 h insulin therapy for. Following normalization of the acute metabolic disturbances, intensive insulin therapy was recommended with four daily doses of subcutaneous insulin: each before every meal and last at bedtime. Four days later, fasting plasma glucose decreased to 5.6 mmol/L (100 mg/dL). Five months after cessation of IFN- $\alpha$  2b therapy, the patient remained insulin dependent with a daily requirement of 20 units Novolin® 30R. The plasma C-peptide level was still low. Serum HbA1c level was 6.5%. Serum aminotransferase was normal, and serum HBV DNA remained undetectable.

## DISCUSSION

IFN- $\alpha$  has different biologic effects (antiviral, antiproliferative, immunomodulatory), and has been used in treatment of chronic viral hepatitis for nearly 20 years<sup>[4]</sup>. IFN- $\alpha$  acts on many target cells and organs. The thyroid represents the main target for autoimmunity associated with IFN- $\alpha$  therapy. Although several reports indicate a beneficial effect on glucose metabolism<sup>[5-7]</sup>, IFN- $\alpha$  has been considered to have a variety of effects on pancreatic beta cells<sup>[1-3,8,9]</sup>. In 1992, Fabris *et al*<sup>[10]</sup> reported the first case of type 1 DM in a chronic hepatitis C patient treated with IFN- $\alpha$  and concluded that IFN could trigger autoimmune destruction of pancreatic beta cells.

IFN- $\alpha$  therapy-induced autoimmunity of pancreatic beta cells has been evaluated in some studies<sup>[11-14]</sup>. The prevalence of ICAb, GADAb, insulin autoantibody (IAA), and/or tyrosine-like phosphatase autoantibody

(IA2Ab) is not generally increased in patients with chronic viral infection prior to IFN- $\alpha$  therapy compared with normal control subjects. In 50% of the previously reported patients, markers of pancreatic autoimmunity predated treatment, the majority of cases having a genetic predisposition (HLA-DR3/-DR4 was positive)<sup>[15]</sup>. However, after IFN- $\alpha$  treatment, the prevalence of pancreatic autoantibodies may increase from 3% to 7% with type 1 DM developed in a few cases. Therefore, IFN- $\alpha$  therapy may induce type 1 DM in genetically and immunologically predisposed individuals.

The exact mechanism underlying the development of type 1 DM in chronic viral hepatitis patients treated with IFN- $\alpha$  is unclear. A variety of mechanisms may account for the effect of IFN- $\alpha$  on pancreatic beta cell dysfunction<sup>[14]</sup>. First, IFN- $\alpha$  activates the oligoadenylyate synthase-RnaseL pathway and the protein kinase R pathway, thus inducing apoptosis of pancreatic beta cells. Second, IFN- $\alpha$  may stimulate a counter regulatory hormone secretion (growth hormone, glucagon, etc.), thus resulting in impaired glucose tolerance. Third, regarding type 1 DM, IFN- $\alpha$  may favor the development of Th1 immune reaction and thereby contribute to the development of autoimmune disease by activating CD4 lymphocytes secreting IL-2, IFN- $\gamma$ , and tumor necrosis factor. IFN- $\alpha$  is also associated with over-expression of MHC class I antigens in human islets of pancreas. In addition to IFN- $\alpha$ , HCV infection can increase the frequency of pancreatic autoimmunity. That is why IFN- $\alpha$ -induced type 1 DM is relatively rare in chronic hepatitis B patients.

Transient insulin dependency was observed in some cases and permanent insulin administration was required in the other reported cases. These data demonstrate that in some cases the autoimmune attack is at least partially reversible with interruption of interferon therapy<sup>[15]</sup>.

In conclusion, development of type 1 DM should be considered as one of the risk consequences after IFN- $\alpha$  therapy. In order to avoid it, administration of IFN- $\alpha$  in special patients should be evaluated, weighing the risk of diabetes and the benefit of the treatment. We conclude that patients having positive islet autoantibodies, HLA-DR3/-DR4, impaired glucose regulation, or positive family history of diabetes mellitus, should be considered to have a higher risk of developing type 1 DM following IFN- $\alpha$  treatment<sup>[13]</sup>. Physicians should be cautious of the use of IFN therapy.

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## Meetings

### Events Calendar 2008-2009

#### FALK SYMPOSIA 2008

January 24-25, Frankfurt, Germany  
Falk Workshop: Perspectives in Liver Transplantation

#### International Gastroenterological Congresses 2008

February 14-16, Paris, France  
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies  
[www.easl.ch/hepatitis-conference](http://www.easl.ch/hepatitis-conference)

February 14-17, Berlin, Germany  
8<sup>th</sup> International Conference on New Trends in Immunosuppression and Immunotherapy  
[www.kenes.com/immuno](http://www.kenes.com/immuno)

February 28, Lyon, France  
3<sup>rd</sup> Congress of ECCO - the European Crohn's and Colitis Organisation Inflammatory Bowel Diseases 2008  
[www.ecco-ibd.eu](http://www.ecco-ibd.eu)

February 29, Québec, Canada  
Canadian Association of Gastroenterology  
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK  
British Society of Gastroenterology Annual Meeting  
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China  
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea  
Asian Pacific Association for the Study of the Liver  
18<sup>th</sup> Conference of APASL: New Horizons in Hepatology  
[www.apaslseoul2008.org](http://www.apaslseoul2008.org)

March 29-April 1, Shanghai, China  
Shanghai-Hong Kong International Liver Congress  
[www.livercongress.org](http://www.livercongress.org)

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco  
OESO 9<sup>th</sup> World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas  
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA  
SAGES 2008 Annual Meeting - part of Surgical Spring Week  
[www.sages.org/08program/html/](http://www.sages.org/08program/html/)

April 18-22, Buenos Aires, Argentina  
9<sup>th</sup> World Congress of the International Hepato-Pancreato Biliary Association  
Association for the Study of the Liver  
[www.ca-ihpba.com.ar](http://www.ca-ihpba.com.ar)

April 23-27, Milan, Italy  
43<sup>rd</sup> Annual Meeting of the European Association for the Study of the Liver  
[www.easl.ch](http://www.easl.ch)

May 2-3, Budapest, Hungary

Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA  
Digestive Disease Week 2008

May 21-22, California, USA  
ASGE Annual Postgraduate Course  
Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion  
E-mail: education@sgo.org

June 4-7, Helsinki, Finland  
The 39<sup>th</sup> Nordic Meeting of Gastroenterology  
[www.congrex.com/ngc2008](http://www.congrex.com/ngc2008)

June 5-8, Sitges (Barcelona), Spain  
Semana de las Enfermedades Digestivas  
E-mail: sepd@sepde.es

June 6-8, Prague, Czech Republic  
3<sup>rd</sup> Annual European Meeting: Perspectives in Inflammatory Bowel Diseases  
E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey  
ESGAR 2008 19<sup>th</sup> Annual Meeting and Postgraduate Course  
E-mail: fca@netvisao.pt

June 11-13, Stockholm, Sweden  
16<sup>th</sup> International Congress of the European Association for Endoscopic Surgery  
E-mail: info@aes-eur.org

June 13-14, Amsterdam, Netherlands  
Falk Symposium 165: XX International Bile Acid Meeting. Bile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic  
Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management  
E-mail: idca2008@guarant.cz

June 25-28, Barcelona, Spain  
10<sup>th</sup> World Congress on Gastrointestinal Cancer  
Imedex and ESMO  
E-mail: meetings@imedex.com

June 25-28, Lodz, Poland  
Joint Meeting of the European Pancreatic Club (EPC) and the International Association of Pancreatology (IAP)  
E-mail: office@epc-iap2008.org  
[www.e-p-c.org](http://www.e-p-c.org)  
[www.pancreatology.org](http://www.pancreatology.org)

June 26-28, Bratislava, Slovakia  
5<sup>th</sup> Central European Gastroenterology Meeting  
[www.ceurgem2008.cz](http://www.ceurgem2008.cz)

July 9-12, Paris, France  
ILTS 14<sup>th</sup> Annual International Congress  
[www.ilts.org](http://www.ilts.org)

September 10-13, Budapest, Hungary  
11<sup>th</sup> World Congress of the International Society for Diseases of the Esophagus  
E-mail: isde@isde.net

September 13-16, New Delhi, India  
Asia Pacific Digestive Week  
E-mail: apdw@apdw2008.net

III FALK GASTRO-CONFERENCE  
September 17, Mainz, Germany

Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany  
Falk Symposium 166: GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic  
Prague Hepatology Meeting 2008  
[www.czech-hepatology.cz/phm2008](http://www.czech-hepatology.cz/phm2008)

September 20-21, Mainz, Germany  
Falk Symposium 167: Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France  
Third Annual Meeting  
European Society of Coloproctology  
[www.escp.eu.com](http://www.escp.eu.com)



October 8-11, Istanbul, Turkey  
18<sup>th</sup> World Congress of the International Association of Surgeons,  
Gastroenterologists and Oncologists  
E-mail: orkun.sahin@serenash.com.tr

October 18-22, Vienna, Austria  
16<sup>th</sup> United European Gastroenterology Week  
[www.negf.org](http://www.negf.org)  
[www.acv.at](http://www.acv.at)

October 22-25, Minnesota, USA  
Anstralian Gastroenterology Week 2008  
E-mail: gesa@gesa.org.au

October 22-25, Brisbane, Australia  
71<sup>st</sup> Annual Colon and Rectal Surgery Conference  
E-mail: info@colonrectalcourse.org

October 31-November 4, Moscone West Convention Center, San Francisco, CA  
59<sup>th</sup> AASLD Annual Meeting and Postgraduate Course  
The Liver Meeting  
Information: [www.aasld.org](http://www.aasld.org)

November 6-9, Lucerne, Switzerland  
Neurogastroenterology & Motility Joint International Meeting 2008  
E-mail: ngm2008@mci-group.com  
[www.ngm2008.com](http://www.ngm2008.com)

November 12, Santiago de Chile, Chile  
Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

November 28-29, Cairo, Egypt  
1<sup>st</sup> Hepatology and Gastroenterology Post Graduate Course  
[www.egyptgastrohep.com](http://www.egyptgastrohep.com)

December 7-9, Seoul, Korea  
6<sup>th</sup> International Meeting Hepatocellular Carcinoma: Eastern and Western Experiences  
E-mail: sglee@amc.seoul.kr

INFORMATION FOR ALL  
FALK FOUNDATION e.V.  
E-mail: [symposia@falkfoundation.de](mailto:symposia@falkfoundation.de)  
[www.falkfoundation.de](http://www.falkfoundation.de)

Advanced Courses - European

Institute of Telesurgery EITS - 2008  
Strasbourg, France  
January 18-19, March 28-29, June 6-7, October 3-4

N.O.T.E.S  
April 3-5, November 27-29  
Laparoscopic Digestive Surgery

June 27-28, November 7-8  
Laparoscopic Colorectal Surgery  
July 3-5  
Interventional GI Endoscopy Techniques  
Contact address for all courses:  
E-mail: info@eits.fr

International Gastroenterological Congresses 2009  
March 23-26, Glasgow, Scotland  
Meeting of the British Society of Gastroenterology (BSG)  
E-mail: bsg@mailbox.ulcc.ac.uk

May 17-20, Denver, Colorado, USA  
Digestive Disease Week 2009

November 21-25, London, UK  
Gastro 2009 UEGW/World Congress of Gastroenterology  
[www.gastro2009.org](http://www.gastro2009.org)



#### Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London, The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.



## Instructions to authors

### GENERAL INFORMATION

*World Journal of Gastroenterology* (*World J Gastroenterol* ISSN 1007-9327 CN 14-1219/R) is a weekly open access peer-reviewed journal supported by an editorial board consisting of 1208 experts in gastroenterology and hepatology from 60 countries. The aim of the journal is to deliver the most clinically relevant original and commentary articles to readers, and to make the full text publicly available to all clinicians, scientists, patients and biomedical students on an unrestricted platform, so that they can access and learn about the most recent key advances in the field.

In addition to the open access nature, another key characteristic of *WJG* is its reading guidance for each article which includes background, research frontier, related reports, breakthroughs, applications, terminology, and comments of peer reviewers for the general readers.

*WJG* publishes articles on esophageal, gastrointestinal, hepatobiliary and pancreatic tumors, and other esophageal, gastrointestinal, hepatic-biliary and pancreatic diseases in relation to epidemiology, immunology, microbiology, motility & nerve-gut interaction, endocrinology, nutrition & obesity, endoscopy, imaging and advanced hi-technology.

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#### Indexed and abstracted in

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#### Published by

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Manuscripts should be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Number all pages consecutively, and start each of the following sections on a new page: Title Page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables, Figures, and Figure Legends. Neither the editors nor the publisher are responsible for the opinions expressed by contributors. Manuscripts formally accepted for publication become the permanent property of The WJG Press, and may not be reproduced by any means, in whole or in part, without the written permission of both the authors and the publisher. We reserve the right to copy-edit and put onto our website accepted manuscripts. Authors should follow the relevant guidelines for the care and use of laboratory animals of their institution or national animal welfare committee. For the sake of transparency in regard to the performance and reporting of clinical trials, we endorse the policy of the International Committee of Medical Journal Editors to refuse to publish papers on clinical trial results if the trial was not recorded in a publicly-accessible registry at its outset. The only register now available, to our knowledge, is <http://www.clinicaltrials.gov> sponsored by the United States National Library of Medicine and we encourage all potential contributors to register with it. However, in the case that other registers become available you will be duly notified. A letter of recommendation from each author's organization should be provided with the contributed article to ensure the privacy and secrecy of research is protected.

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An informative, structured abstract of no more than 350 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections: AIM: Only the purpose should be included. METHODS: The materials, techniques, instruments and equipment, and the experimental procedures should be included. RESULTS: The observed and experimental results, including data, effects, outcome, etc. should be included. Authors should present *P* value where necessary, and also include any significant data. CONCLUSION: Accurate view and the value of the results should be included.

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#### Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

#### Text

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Figures should be numbered as 1, 2, 3, etc., and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: <http://www.wjgnet.com/1007-9327/13/4520.pdf>; <http://www.wjgnet.com/1007-9327/13/4554.pdf>; <http://www.wjgnet.com/1007-9327/13/4891.pdf>; <http://www.wjgnet.com/1007-9327/13/4986.pdf>; <http://www.wjgnet.com/1007-9327/13/4498.pdf>. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: ...etc. It is our principle to publish high resolution-figures for the printed and E-versions.

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### Notes in tables and illustrations

Data that are not statistically significant should not be noted. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 should be noted (*P* > 0.05 should not be noted). If there are other series of *P* values, <sup>c</sup>*P* < 0.05 and <sup>d</sup>*P* < 0.01 are used. A third series of *P* values can be expressed as <sup>e</sup>*P* < 0.05 and <sup>f</sup>*P* < 0.01. Other notes in tables or under illustrations should be expressed as <sup>1</sup>F, <sup>2</sup>F, <sup>3</sup>F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, □, ▲, △, etc., in a certain sequence.

### Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscripts and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

## REFERENCES

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The author should number the references in Arabic numerals according to the citation order in the text. Put reference numbers in square brackets in superscript at the end of citation content or after the cited author's name. For citation content which is part of the narration, the coding number and square brackets should be typeset normally. For example, "Crohn's disease (CD) is associated with increased intestinal permeability<sup>[1,2]</sup>". If references are cited directly in the text, they should be put together within the text, for example, "From references<sup>[19,22-24]</sup>, we know that..."

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### Style for journal references

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### Style for book references

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### Format

#### Journals

English journal article (list all authors and include the PMID where applicable)

- 1 Jung EM, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

- 2 Lin GZ, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixudiarrhoea. *Shijie Huaren Xiaohua Zaishi* 1999; **7**: 285-287

In press

- 3 Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

- 5 Vallancien G, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

Volume with supplement

- 7 Geraud G, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment

of migraine and in comparison with sumatriptan. *Headache* 2002; 42 Suppl 2: S93-99 [PMID: 12028325]

*Issue with no volume*

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (401): 230-238 [PMID: 12151900]

*No volume or issue*

- 9 Outreach: Bringing HIV-positive individuals into care. *HRS&A Careaction* 2002; 1-6 [PMID: 12154804]

## Books

*Personal author(s)*

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

*Chapter in a book (list all authors)*

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

*Author(s) and editor(s)*

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

*Conference proceedings*

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Write as mean  $\pm$  SD or mean  $\pm$  SE.

## Statistical expression

Express  $t$  test as  $t$  (in italics),  $F$  test as  $F$  (in italics), chi square test as  $\chi^2$  (in Greek), related coefficient as  $r$  (in italics), degree of freedom as  $v$  (in Greek), sample number as  $n$  (in italics), and probability as  $P$  (in italics).

## Units

Use SI units. For example: body mass,  $m$  (B) = 78 kg; blood pressure,  $p$  (B) = 16.2/12.3 kPa; incubation time,  $t$  (incubation) = 96 h, blood glucose concentration,  $c$  (glucose)  $6.4 \pm 2.1$  mmol/L; blood CEA mass concentration,  $p$  (CEA) = 8.6 24.5  $\mu$ g/L;  $CO_2$  volume fraction, 50 mL/L  $CO_2$ , not 5%  $CO_2$ ; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

## Italics

Quantities:  $t$  time or temperature,  $c$  concentration,  $A$  area,  $l$  length,  $m$  mass,  $V$  volume.

Genotypes:  $gyrA$ ,  $arg\ 1$ ,  $c\ myc$ ,  $c\ fos$ , etc.

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## Chemokine receptor CXCR4-prognostic factor for gastrointestinal tumors

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### INTRODUCTION

Chemokines and their receptors, such as CXCR4, induce chemotaxis of diverse immune cells [CD4<sup>+</sup> cells, CD8<sup>+</sup> cells, macrophages, and dendritic cells (DC)] into areas of inflammation<sup>[1]</sup>. CXCR4 and CCR5 were initially reported to represent co-receptors for human immunodeficiency virus (HIV) infection in man. Gp120 binds CXCR4 and CCR5, mediating fusion of HIV with the host cell membrane. Therefore, CXCR4 was previously termed *Fusin*. However, after viral binding to the chemokine receptor CXCR4, HIV induces an immunologic T helper (Th) cell response, called a Th1 to Th2 switch, which clearly supports the immune evasion of HIV-infected lymphocytes<sup>[2,3]</sup>. In addition, *in vitro* studies on HIV revealed that the chemokine receptor CXCR4 ligand SDF-1 $\alpha$  induced apoptosis of CD8<sup>+</sup> cells, again inhibiting strong immune defence mechanisms<sup>[4]</sup>. In HIV therapy, much attention has been paid to CXCR4, to which the fusion-mediating and migration-inducing chemokine SDF-1 $\alpha$  binds selectively. However, the sole blockade of this receptor, which is expressed on CD4<sup>+</sup> cells, CD8<sup>+</sup> cells, and APC, does not lead to a sufficient inhibition of the fusion and, therefore, to a persisting HIV infection of lymphocytes.

During the last 5 years, diverse human cancers were reported to express high levels of CXCR4 multiplying the metastasis process *via* their aggressive-invasive and migratory phenotypes<sup>[5,6]</sup>. We and others recently reported that gastrointestinal cancers co-express CXCR4 and its ligand SDF-1 $\alpha$  in an autocrine and paracrine fashion, thereby increasing tumor cell dissemination<sup>[7-9]</sup>.

### IMMUNOLOGICAL RELEVANCE OF THE CXCR4/SDF-1 AXIS

The CXCR4/SDF-1 pathway has been reported to impact the progression of various malignomas. However, the influence on the immune system was only analyzed at a basic level, so far. Moreover, only brief investigations have focused on the influence of SDF-1 $\alpha$  in comparison

### Abstract

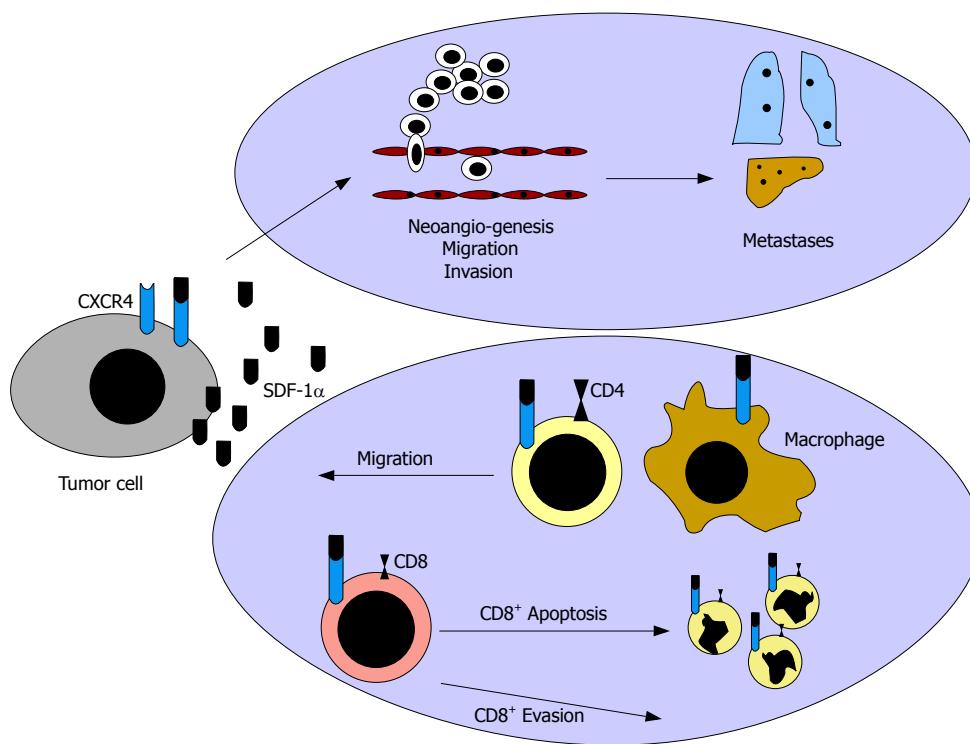
To review the implication of CXCR4 for gastrointestinal cancer, a "Pubmed" analysis was performed in order to evaluate the relevance of CXCR4 and its ligands for gastrointestinal cancers. Search terms applied were "cancer, malignoma, esophageal, gastric, colon, colorectal, hepatic, pancreatic, CXCR4, SDF-1 $\alpha$ , and SDF-1 $\beta$ ". CXCR4 expression correlated with dissemination of diverse gastrointestinal malignomas. The CXCR4 ligand SDF-1 $\alpha$  might act as "chemorepellent" while SDF-1 $\beta$  might act as "chemorepellent" for CTLs, inducing tumor rejection. The paracrine expression of SDF-1 $\alpha$  was furthermore closely associated with neoangiogenesis. CXCR4 and its ligands influence the dissemination, immune rejection, and neoangiogenesis of human gastrointestinal cancers. Inhibition of CXCR4 might be an interesting therapeutic option.

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**Key words:** CXC chemokine receptor-4; CXCL12; Stromal-derived-factor-1; Cancer; Malignoma

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**Figure 1** CXCR4 mediates tumor dissemination via induction of tumor cell migration and invasion and via stimulation of neoangiogenesis. The CXCR4 ligand SDF-1 $\alpha$  might act as "chemorepellent" preventing tumor rejection.

to the splicing variant SDF-1 $\beta$  on "immune escape" mechanisms (Figure 1). SDF-1 is the only known ligand of CXCR4 and exists in several splicing variants<sup>[10]</sup>. Its expression is restricted to some cell types, as endothelial cells, epithelial thymus cells, bone marrow cells, mucosal epithelial cells, and tumor cells<sup>[11-13]</sup>. Increased expression occurs, especially in areas of inflammation and neoangiogenesis, and thus leads to a fulminate chemotaxis of CXCR4 expressing immune cells, such as CD4 $^{+}$  cells and DC along the SDF-1 gradient<sup>[14]</sup>.

Interestingly, SDF-1 $\alpha$  exposition of anti-CD3-activated CD4 $^{+}$  cells induced Th1-(IFN- $\gamma$ ) as well as Th2 (IL-4 and IL-10)-specific cytokine expression<sup>[15]</sup>. A SDF-1 $\alpha$  exposition of activated T cells inhibited CD4 $^{+}$  cell apoptosis by reduction of BCC-2 and activation of PI3-K and MAPK<sup>[16]</sup>. However, this effect was linked to the SDF-1 $\alpha$  concentration and the activation state of the CD4 $^{+}$  cells, as a higher concentration of SDF-1 $\alpha$  induced apoptosis of CD4 $^{+}$  Jurkat cells by up-regulation of CD95 and CD95 ligands<sup>[17]</sup>.

Moreover, SDF-1 $\alpha$  induced CD8 $^{+}$  apoptosis after up-regulation of membrane-bound TNF- $\alpha$  on APC and TNF- $\alpha$  receptor II on CD8 $^{+}$  cells and a consecutive interaction of the ligand with the receptor<sup>[4]</sup>.

Further results implied that SDF-1 $\alpha$  expressing tumor cells may protect themselves from a cytotoxic immune reaction, as SDF-1 $\alpha$  promotes apoptosis in cytotoxic T cells and might induce a local Th1 → Th2 shift in the tumor bed. This theory is supported by data reporting that SDF-1 $\alpha$  induces a chemorepulsion of CTL supporting an immune evasion in melanoma<sup>[18]</sup>. In contrast, SDF-1 $\beta$  acts as chemoattractant for CTLs, inducing tumor rejection<sup>[19]</sup>. Interestingly, CXCR4 blockade not only inhibited dissemination but also sensitized tumor cells for immune therapy<sup>[20]</sup>. However,

the influence on the immune system and differences between the splicing variants SDF-1 $\alpha$  and SDF-1 $\beta$  have been analysed only marginally.

## ONCOLOGICAL ASPECTS OF THE CXCR4/SDF-1 $\alpha$ / $\beta$ AXIS

The presence of different chemokine/cytokine receptors and their ligands, such as IL-8, IP10, Rantes, or SDF-1 $\alpha$  could be confirmed in the tumor bed of diverse malignancies<sup>[7]</sup>. Current investigations reveal an expression by tumor cells, but also by endothelial cells<sup>[7,14]</sup>. Thus, these findings suggest that both tumor-expressed CXCR4 and SDF-1 $\alpha$  play important roles in local progression, dissemination, and immune evasion of tumor cells.

SDF-1 $\alpha$  binding to the second extracellular loop of CXCR4 induces activation of classical G-protein-coupled PI3-K/AKT/mTOR and RAF/MEK/ERK signalling pathways in the respective cells<sup>[7,21]</sup>. Proliferation, adhesion, migration, and invasion are the most relevant consequences. In addition, current publications report that CXCR4 activation induces EGFR1, HER2neu, and VEGFR transactivation. Most interestingly, CXCR4 upregulation was observed after EGFR/Her2neu activation, implying a compensatory pathway possibly inducing resistance to tyrosine-kinase inhibition<sup>[11]</sup>.

Autocrine SDF-1 $\alpha$  expression has initially been described in breast cancer and glioblastoma cell lines, where it has been correlated with an increased migratory and invasive potential of the tumor cells<sup>[7-9]</sup>. These effects are closely associated with the receptor affinity of SDF-1 $\alpha$ , since a SDF-1 $\alpha$  gene polymorphism, which is associated with an increased SDF-1 $\alpha$  receptor affinity

occurring in 5% of the general population<sup>[22]</sup>, can be frequently observed in patients with breast cancer and HIV-associated non-Hodgkin lymphoma<sup>[23,24]</sup>.

The paracrine expression of SDF-1 $\alpha$  is also closely associated with neoangiogenesis. On the one side, SDF-1 $\alpha$  and VEGFB attract endothelial precursor cells towards the tumor. On the other side, hemangiocytes (VEGFR1 $^+$ , CXCR4 $^+$ ) are released in the bone marrow by VEGFA and directed towards the tumor by a SDF-1 $\alpha$  gradient, where they might differentiate to pericytes stabilizing the neo-vasculature<sup>[25,26]</sup>.

In contrast to benign tissues, tumors often reveal a significant up-regulation of CXCR4. A key investigation concerning the relevance of chemokine receptors links CXCR4 expression with the localization of metastases in breast cancer<sup>[27]</sup>. In a variety of human tumor entities, an increased CXCR4 expression correlated with hematogenous and lymphogenic metastasis and reduced survival times<sup>[5,6,28]</sup>. *In vitro*, the activation of this receptor was associated with increased proliferation, migration, and invasion. These properties could be efficiently inhibited by CXCR4 blockade in tumor models<sup>[29,30]</sup>. The important role of CXCR4 for dissemination of tumor cells was confirmed in animal models. AMD3100, a recently developed CXCR4-inhibiting “small molecule”, blocked CXCR4 in a glioblastoma model with high receptor affinity and induced a massive tumor regression *in vivo*<sup>[31]</sup>. T22, another CXCR4 antagonist, strongly inhibited pulmonary metastasis in a murine B16 melanoma model<sup>[32]</sup>.

## INFLUENCE OF SDF-1 $\alpha$ /CXCR4 ON THE CLINICAL OUTCOME OF GASTROINTESTINAL TUMORS

The relevance of CXCR4 expression for tumor progression has been described in various gastrointestinal malignancies.

Over-expression of CXCR4 in colorectal cancer was significantly associated with advanced UICC tumor stages III/IV and with lymphatic or haemotogenous metastasis, respectively<sup>[28]</sup>. Moreover, the CXCR4 ligand SDF-1 $\alpha$  stimulated migration and invasion in the CXCR4-expressing colon carcinoma cells SW480 and SW620 *in vitro*<sup>[28]</sup>.

In esophageal cancer, the median overall survival of patients with CXCR4 expressing tumors was 20 months compared to 76 months for CXCR4 negative cases. CXCR4 expression was furthermore significantly associated with increased lymph node and bone marrow involvement. In multivariable analysis, CXCR4 expression was an independent variable and strongly associated with reduced disease-specific survival and overall survival. In another publication, which discriminated squamous cell from adenocarcinoma, a strong CXCR4 expression revealed a poorer long-term prognosis following curative esophagectomy for both histological subtypes, though with lack of statistical significance<sup>[33]</sup>.

In gastric cancer, CXCR4 expressing primary gastric

carcinomas significantly correlated with the development of peritoneal carcinomatosis and malignant ascites which contained high concentrations of CXCL12<sup>[34]</sup>.

In pancreatic cancer, an over-expression of CXCR4 correlated with advanced UICC stages III/IV and revealed a trend for hematogenous metastasis and progressed local tumor stages without affecting survival<sup>[35]</sup>.

In hepatocellular cancer, a high expression of CXCR4 or CCR7 was associated with locally advanced primary tumors and lymphogenic metastasis<sup>[36]</sup>. Moreover, high expression of CXCR4 led to a significant increase in haematogenic metastasis and to a decreased 3-year survival<sup>[36]</sup>. In Huh7 hepatoma cells, SDF-1 $\alpha$  exposition induced receptor internalization and perinuclear localization as well as an increase of proliferation and invasion. As a result of a CXCR4 receptor defect, these phenomena did not occur in HepG2 cells. While Hep3B and Huh7 showed high levels of autocrine and paracrine SDF-1 $\alpha$  expression, this effect could barely be observed in HepG2 cells.

In summary, CXCR4 increases the metastatic phenotype for diverse gastrointestinal malignancies. Current analyses are investigating whether CXCR4 expression might be valuable predictor for tumor recurrence.

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## Hepatitis G virus

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## HISTORY OF THE DISCOVERY OF HEPATITIS G VIRUS

GBV-C, or hepatitis G virus (HGV), was discovered by two independent groups of investigators in the study of cases of hepatitis non-A, non-B, non-E<sup>[1,2]</sup>. The discovery of a new viral agent associated with liver diseases has attracted considerable attention due to the fact that there are hepatides of unknown etiology. This determined the urgency of investigations aimed at comprehensively studying the properties of the virus, its association with liver disease and infection rates in different countries of the world.

In 1966, the 34-year-old surgeon G. Barker (GB) fell ill with acute hepatitis of moderate enzymatic activity and three-week icteric period. Patient blood taken on icteric day 3 was used for intravenous inoculation of nonhuman primates (bare-faced marmosets, the Callithricidae family). Hepatitis was recorded in all animals when four monkey-to-monkey passages were performed. The findings suggested that the cause of this hepatitis was a yet unidentified viral agent that was named GBV.

Investigations of the GB agent recommenced 25 years later when new methods for qualitative viral analysis and recognition evolved. Serum taken in the acute stage of hepatitis from infected marmosets was found to contain two viral genomes: GBV-A and GBV-B belonging to closely-related viruses of the Flaviviridae family. Both viruses were able to replicate in the marmosets, but only GBV-B caused hepatitis. Attempts to detect GBV-A or GBV-B in human beings failed. A third virus GBV-C was soon isolated from patient material by means of specially designed primers to the conserved part of the NS3 region of the viruses GBV-A, GBV-B and HCV. GBV-C was assigned to the GBV group as it was slightly similar to GBV-B protein in immunoassays and largely identical to GBV-A in nucleotide sequence. GBV-C proved to be genetically related to another independent isolate that had been originally called HGV. They are virtually indistinguishable in the routine diagnosis by polymerase chain reaction (PCR). Since the signs of GBV-C/HGV became more commonly detected in patients with hepatitis and persons at risk for parenteral hepatitis, hepatitis G was considered

## Abstract

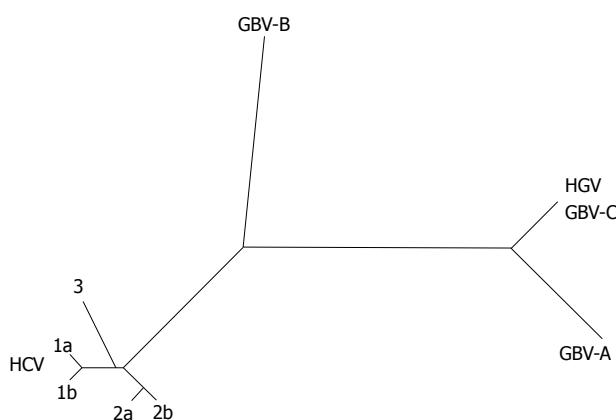
A number of new hepatitis viruses (G, TT, SEN) were discovered late in the past century. We review the data available in the literature and our own findings suggesting that the new hepatitis G virus (HGV), disclosed in the late 1990s, has been rather well studied. Analysis of many studies dealing with HGV mainly suggests the lymphotropicity of this virus. HGV or GBV-C has been ascertained to influence course and prognosis in the HIV-infected patient. Until now, the frequent presence of GBV-C in coinfections, hematological diseases, and biliary pathology gives no grounds to determine it as an "accidental tourist" that is of no significance. The similarity in properties of GBV-C and hepatitis C virus (HCV) offers the possibility of using HGV, and its induced experimental infection, as a model to study hepatitis C and to develop a hepatitis C vaccine.

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**Key words:** Hepatitis G virus; Markers of GBV-C; Epidemiology; Clinical manifestations

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**Figure 1** Affinity of HCV, GBV-C, GBV-A, and GBV-B (From Robertson BH, 2001)<sup>[5]</sup>.

to be an independent hepatotropic entity.

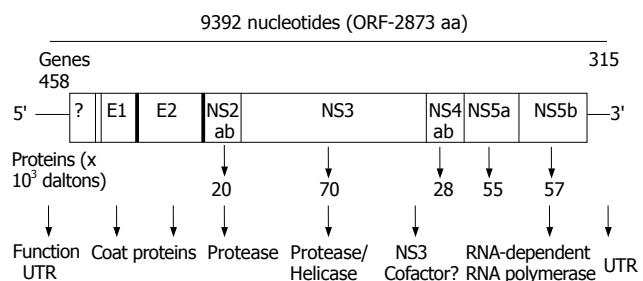
Experiments infecting chimpanzees with the GBV-C RNA-containing plasma taken from patients with chronic hepatitis G (CHG) yielded rather unexpected results. All the infected animals developed persistent and continuing (as long as 20 mo) viremia. However, no case showed a rise in the levels of indicator enzymes or detectable abnormal liver tissue changes in liver biopsy specimens taken weekly throughout the follow-up. Javan macaques were also observed to have viremia without signs of liver damage. By contrast, signs of hepatitis in the form of hyperenzymaemia and necrotic and inflammatory changes in the liver appeared by day 30 after inoculation of the marmosets that had received the same GBV-C-containing materials<sup>[3]</sup>.

Further serological screening-based investigations have indicated that the GBV-C isolate is of widespread occurrence; however, there is no evidence for an association of viremia with the development of some known diseases, such as hepatitis<sup>[4]</sup>.

## TAXONOMY AND GENOTYPIC VARIETY OF GBV-C

GBV-C virus, like GBV-A, GBV-B, and HCV, belongs to the Flaviviridae family. Comparison of the genomes of GBV-C, GBV-A, GBV-B, and HCV has demonstrated that their RNA does not bear a more than 32% similarity, thereby supporting the hypothesis that these viruses are independent (Figure 1)<sup>[5]</sup>.

Five HGV genomes (the divergence between them was 12%) have been described<sup>[6,7]</sup>. Investigations dealing with the classification of GBV-C were conducted by measuring restriction fragment length polymorphisms. The isolates from West Africa are referred to as genotype 1 wherein 2 subtypes: 1a and 1b are identified. Genotypes 2a and 2b are more frequently detected in North America and Europe; genotypes 3, 4 and 5 are more common in Asia, South-Eastern Asia, and South Africa, respectively. Phylogenetic analysis of genomic nucleotide sequences of the 5' and NS5 regions made by Novikov in 2000<sup>[8]</sup> has established that the GBV-C



**Figure 2** Schematic representation of GBV-C RNA structure, coded proteins, and their functions (from Kim JP, Frey KE)<sup>[14]</sup>.

isolate belonging to viral genotype 2 circulates in Russia, Kazakhstan, Kyrgyzstan, and Turkmenistan. Analysis of GBV-C 5'-untranslated region sequences revealed a new sixth genotype of virus in Indonesia<sup>[9]</sup>. In addition to genomic variability in different GBV-C isolates, some authors propose GBV-C genomic variability within one isolate, i.e. they suggest that there are quasispecies, thereby emphasizing their similarity with HCV<sup>[10]</sup>. But, the opponents of this theory argue that based on the absence of a hypervariable region in the E2 gene, the presence of quasispecies is impossible<sup>[11,12]</sup>.

## GBV-C STRUCTURE

The genome of the virus is represented by single-chain RNA with positive polarity<sup>[13]</sup>. The GBV-C genome is similar to hepatitis C virus (HCV) RNA in its organization, i.e. the structural genes are located at the genomic 5' region and non-structural genes are at the 3' end (Figure 2)<sup>[14]</sup>. The untranslated region at the 5' end may serve as an internal ribosomal embarkation site, which ensures translation of a RNA coding region<sup>[15]</sup>. The extent of the genome in different viral isolates ranges from 9103 to 9392 nucleotides<sup>[16,17]</sup>. An open reading frame carries information on the virus-specific polypeptide consisting of 2873-2910 amino acid residues. GBV-C RNA codes for two structural proteins (E1 and E2) which are envelope proteins. Unlike HCV, the proportion of glycosylated E2 is much lower in GBV-C. It has a total of three potential N-glycosylation sites as compared with HCV E2, which has eleven sites. The complete structure of viral nucleocapsid is still to be determined as the genomic region coding for core proteins has not been identified yet<sup>[18]</sup>.

Five non-structural proteins: NS2, NS3, NS4b, NS5a, and NS5b with molecular weights of 20, 70, 28, 55, and 57 kDa, respectively, have been found<sup>[19,20]</sup>. These proteins perform the function of protease, helicase, and RNA-dependent RNA-polymerase. The sequencing of the E1 and E2 regions has shown that they are not hypervariable unlike the respective regions of HCV<sup>[12]</sup>. Of interest are the data obtained while studying the buoyant density of GBV-C particles in a sucrose gradient before and after treatment with the nonionic detergent Tween-80. These data suggest that there is a lipid envelope in the virus whose association with lipids reduces antibody formation.

**Table 1** Detection rate of GBV-C RNA in blood donors

Authors, year, country	GBV-C RNA (%)
Jarvis et al, 1996, United Kingdom <sup>[33]</sup>	3.2
Alter et al, 1997, USA <sup>[34]</sup>	1.7
Mikhailov, 1997, Russia <sup>[11]</sup>	3.3
de Lamballerie et al, 1997, Belgium <sup>[35]</sup>	1.1
Lefrere et al, 1997, France <sup>[36]</sup>	4.2
Guilera et al, 1998, Spain <sup>[37]</sup>	3.0
Masuko et al, 1996, Japan <sup>[38]</sup>	0.9
Novikov, 2000, Russia <sup>[8]</sup>	3.2
Kalkan et al, 2005, Turkey <sup>[39]</sup>	4.1
Mastouri et al, 2005, Tunis <sup>[40]</sup>	5.3
Grabarczyk et al, 2006, Poland <sup>[41]</sup>	3.2

## MARKERS OF GBV-C

The basic marker used to diagnose GBV-C is RNA that is detectable by the amplification technique with a preliminary stage of reverse transcription in which cDNA is synthesized [reverse-transcriptase polymerase chain reaction (RT-PCR)]. Data on the sequence of the RNA region coding for helicase (NS3) and the NS5A region are used to synthesize oligonucleotide primers. This choice is made due to the high (83%-99%) stability of this region in various viral isolates (the sensitivity was as high as 200 copies/mL).

Further investigations indicated that there might be false negative results in the testing of some samples despite the fact that the latter contained the virus. By taking this into account, primers with the information coded in the 5'-untranslated region (the sensitivity was as high as 100 copies/mL) came into additional use for the designing of diagnostic kits<sup>[10]</sup>. The above primer kits had a high sensitivity, but also a rather high level of errors due to the incomplete conservatism of respective viral RNA regions.

An alternate primer kit for the region coding for E2 has been developed. These primers had 100% specificity for this RNA region; however, their sensitivity was not greater than 76.6%. Recent investigations propose the use of the two different primer kits (for viral RNA NS3, NS5A, 5'UTR, or E2 regions) for the accurate diagnosis of GBV-C RN<sup>[21]</sup>.

GBV-C RNA has been detected in hepatocytes<sup>[19,20,22]</sup>, peripheral blood lymphocytes and monocytes<sup>[23,24]</sup>, vascular endothelial cells<sup>[25]</sup>, and other tissues<sup>[7]</sup>. GBV-C viremia may persist for a few years. The infection is accompanied by the formation of specific antibodies against the envelope protein E2 (anti-E2). These antibodies have a long survival and may prevent the body from reinfection.

An enzyme immunoassay has been developed to detect serum GBV-C antibodies. The envelope E2 antigen (glycoprotein) was used as a viral antigen. Analysis of the sera from healthy individuals and patients with hepatitis demonstrated that most anti-E2-positive sera were GBV-C RNA negative, which enabled anti-E2 to be regarded as a marker of previous infection<sup>[8,26-28]</sup>. As a rule, GBV-C antibodies and RNA are not simultaneously encountered in a patient despite the fact that HCV, the nearest relation of GBV-C, is

**Table 2** Detection rate of anti-E2 in blood donors

Authors, year, country	Anti-E2 (%)
Jarvis et al, 1996, United Kingdom <sup>[33]</sup>	3.0
Masuko et al, 1996, Japan <sup>[38]</sup>	4.9
Bouchardieu et al, 2000, France <sup>[45]</sup>	42.1
Novikov, 2000, Russia <sup>[8]</sup>	13.7
Mastouri et al, 2005, Tunis <sup>[40]</sup>	4.9
Grabarczyk et al, 2006, Poland <sup>[41]</sup>	23.6

typified by this an inverse correlation between anti-E2 and viremia. The presence of serum viral RNA is also indicative of continuing infection so is that of E2 protein antibodies for clearance of viral particles from the patient's body. It has been shown that the production of GBV-C antibodies and the cessation of viremia in most (60%-75%) immunocompetent patients occur spontaneously and they are followed by the generation of antibodies to the envelope protein E2<sup>[29,30]</sup>. Two markers (RNA and anti-E2) of GBV-C have been concurrently detected in single studies (in 5% of cases)<sup>[8]</sup>. The highest detection rates of GBV-C antibodies are observed in individuals aged above 50 years<sup>[31,32]</sup>.

## EPIDEMIOLOGY OF GBV-C

Infection with HGV is common in the world. The detection rate of GBV-C in the population averages 1.7%. GBV-C, like other parenteral hepatitis viruses, occurs universally, but nonuniformly (Table 1)<sup>[8,11,33-41]</sup>. GBV-C is detectable in all ethnic groups. Analysis of the results of examining 13 610 blood donors described in 30 reports revealed viral RNA in 649 (4.8%) of cases. These included Caucasians (4.5%), Asians (3.4%), and Africans (17.2%)<sup>[42]</sup>. The authors propose to test blood samples due to the high risk of infection with GBV-C<sup>[42,43]</sup>.

An investigation of the prevalence of HGV among north-eastern Thai blood donors carrying HBsAg and anti-HCV revealed the high frequency of GBV-C RNA (10% and 11%, respectively) in the co-infected as compared with the controls (0%)<sup>[44]</sup>.

The development of an anti-E2 detection method has promoted a complete definition of the prevalence of GBV-C. E2 antibodies are several times more frequently detectable than RNA in blood donors (Table 2)<sup>[8,33,38,40,41,45]</sup>.

GBV-C is a parenterally transmitted infection<sup>[28-30]</sup>. The first verification of this fact were the experiments dealing with inoculation of primates with the blood of the surgeon who fell ill in 1966<sup>[2]</sup>. Cases of acute posttransfusion hepatitis along with the enhanced activity of serum aminotransferases and the detection of blood GBV-C RNA in the absence of other markers of viral hepatitis has been documented<sup>[3,31,32]</sup>. Indirect evidence that HGB is parenterally transmitted lies in its more frequent detection in the groups at higher risk for infection with hepatitis viruses by similar routes of transmission (Table 3)<sup>[8,11,34,36,38,41,46-51]</sup>, as well as the increased risk for infection in patients treated with multiple hemodialysis procedures and higher units of transfused blood products<sup>[33-35]</sup>.

**Table 3** Detection rate of HGV RNA in high infection-risk groups

Authors, year, country	Risk group	GBV-C RNA (%)
Mazuko <i>et al</i> , 1996, Indonesia <sup>[38]</sup>	Patients on hemodialysis	55
Alter <i>et al</i> , 1997, USA <sup>[34]</sup>	Patients on hemodialysis	20
Mikhailov, 1997, Russia <sup>[11]</sup>	Drug abusers	35
Karayiannis <i>et al</i> , 1997, United Kingdom <sup>[46]</sup>	Patients with hemophilia	14
	Recipients of immunoglobulins	5.4
	Drug abusers	13.5
Martin <i>et al</i> , 1999, USA <sup>[47]</sup>	Patients on hemodialysis	17.1
Rubio <i>et al</i> , 1997, Germany <sup>[48]</sup>	Patients on hemodialysis	5
	Renal or hepatic posttransplantation patients	14-20
Lefrere <i>et al</i> , 1997, France <sup>[36]</sup>	Patients on hemodialysis	5
Miyakawa <i>et al</i> , 1997, Japan <sup>[49]</sup>	Patients on hemodialysis	57.5
	Patients on hemodialysis	3.1
Novikov, 2000, Russia <sup>[8]</sup>	Patients on intravenous drug injection	16
Kumar <i>et al</i> , 2005, India <sup>[50]</sup>	Patients with hemophilia	28
Kachko <i>et al</i> , 2005, Russia <sup>[51]</sup>	Patients on hemodialysis	6
Grabarczyk <i>et al</i> , 2006, Poland <sup>[41]</sup>	Drug abusers	25
	Patients on hemodialysis	23.7

The use of infected blood and its products promotes the prevalence of HGV. In the USA, 18%-20% of all blood preparations are infected with GBV-C, of them plasma being in 33%-84%<sup>[33]</sup>. In the United Kingdom, 94%-100% of coagulation factor VIII-IX preparations are infected with this virus<sup>[34]</sup>. Despite the fact that this persistent infection is present in a considerable number of healthy blood donors and in more than 35% of the human immunodeficiency virus (HIV)-infected, the world food and drug administration considers it unnecessary to recommend donor blood to be tested for serum GBV-C RNA.

There may be a sexual transmission in hepatitis G, as in hepatitis B and C. This is evidenced by the high detection rate of GBV-C RNA in homosexuals and prostitutes: 13.4%-63.0%<sup>[48,52]</sup> and 13.9%-24.8%<sup>[48,53]</sup>, respectively. Yeo *et al*<sup>[54]</sup> studied sexual transmission risk in 161 hemophilic patients. 21% of the females in sexual contact with them were found to be GBV-C RNA seropositive. The more frequent detection of markers of GBV-C in persons at increased risk for sexually transmitted diseases is also indirect evidence for its sexual transmission. Wachtler *et al*<sup>[31]</sup> revealed HGV RNA in 27% and anti-E2 in 35% of the HIV-infected, while in the control group these were 2% and 6%, respectively.

The vertical transmission of GBV-C from infected mother to infant may now be considered proven<sup>[36,55-57]</sup>. There may be intranatal infection of a baby at delivery by the maternal passage, as confirmed by the data on a significant reduction in the infection rates of neonates after cesarean section of their mothers<sup>[55]</sup>. There is also postnatal GBV-C infection. On examining 288 mothers, Lefrere *et al* revealed that 89% of the GBV-C-positive babies were infected at 3 mo after birth<sup>[36]</sup>.

The level of viremia is a factor that is of importance in the transmission of the virus. By following up 24 babies born to mothers with a GBV-C RNA level of more than  $10^6$  copies/mL, Ohto *et al* revealed GBV-C in 23 (96%) of them. The viremia index in the mothers whose babies proved to be infected was significantly higher than that in those whose babies were seronegative ( $P < 0.001$ ). Most

babies had no clinical or biochemical signs of liver disease despite one-year HGV persistence<sup>[58]</sup>. In the opinion of Wejstal *et al*, the vertical transmission of GBV-C amounts to 75%-80% of cases and that of HCV is 2.8%-4.2% ( $P < 0.001$ )<sup>[56]</sup>. The frequent maternal-infant transmission of GBV-C may account for the high prevalence of the virus among the adult population at low risk of parenteral and sexual transmissions. The detection of GBV-C increases with age. HGV was detectable in 9% and 28.6% of the children under 15 years and above 16 years of age, respectively<sup>[59]</sup>.

## GBV-C TROPISM

GBV-C predominantly replicates in peripheral blood mononuclear cells, mainly in B and T (CD4+ and CD8+) lymphocytes and bone marrow<sup>[23-25,60]</sup>. The mechanism responsible for the development of GBV-C-induced hepatitis is not clear so far. Despite the described cases of acute and chronic hepatitis G, its hepatotropism remains controversial. Table 4<sup>[11,27,28,61-74]</sup> shows data that both confirm and rule out viral tropism to liver tissue.

Viral hepatotropism is supported by the detection of GBV-C RNA in hepatocytes and by the development of acute and fulminant hepatitis following the transfusion of infected blood and its products. Lang *et al* reported interesting data on the immunohistochemical detection of GBV-C NS5 Ag in the liver biopsy specimens taken from patients with various liver diseases<sup>[68]</sup>. Like RNA-containing HCV, GBV-C does not integrate into the genome of an infected cell, but it is located in its cytoplasm and the "positive" cells are diffusely arranged. The indirect evidence for the liver tissue GBV-C replication is a considerable reduction in the serum content of viral RNA after liver transplantation ( $12.4 \pm 3.9 \times 10^7$  copies/mL vs  $2.8 \pm 0.7 \times 10^7$  copies/mL)<sup>[69]</sup>.

Primary replication of HGV in the hepatocytes has been questioned. Thus, the level of GBV-C RNA in the serum was higher than that in the liver tissue (there is an inverse correlation for HCV). In a third of serum-positive patients, RNA was undetectable in

**Table 4 Data on the hepatotropicity of GBV-C**

Hepatotropicity	No hepatotropicity
Detection of GBV-C RNA in the sera of patients with acute hepatitis non-A-non-E <sup>[61,66]</sup>	The equal detection rate of GBV-C RNA in donors with normal and increased alanine aminotransferase activities (1.7 and 1.5%, respectively) <sup>[70]</sup>
Histological pattern of hepatitis in GBV-C infection <sup>[27,28,66,67]</sup>	Normal aminotransferase activity values in the presence of GBV-C RNA <sup>[11,71]</sup>
Detection of GBV-C RNA and NS5 Ag in the liver tissue <sup>[68]</sup>	No correlation between the level of GBV-C RNA and the activity of alanine aminotransferase <sup>[72]</sup>
A significant reduction in the serum level of GBV-C RNA after liver transplantation <sup>[69]</sup>	The level of GBV-C RNA in the liver tissue is lower than that in the serum <sup>[73,74]</sup>

the hepatocytes despite the fact that tissue had been repeatedly taken from different lobes of the liver<sup>[73]</sup>. A study of liver biopsy specimens from 12 GBV-C-positive patients revealed no RNA “minus” strand responsible for replication and a RNA “plus” strand only in half the patients with low titers, which may be indicative of GBV-C contamination from blood. Laskus *et al* reported similar results investigating liver tissue and sera from 10 patients co-infected with HCV and GBV-C<sup>[74]</sup>.

After establishing that the hepatotropicity of GBV-C was low, the next stage of elucidating the pathogenicity of the virus was to study its tropism to other tissues. Handa *et al* determined the presence of a RNA “minus” strand in the vascular endotheliocytes<sup>[25]</sup>. In the authors’ opinion, isolation of GBV-C RNA from a liver biopsy specimen may reflect viral replication in the endothelium of the vessels located in the liver<sup>[25]</sup>. Tucker *et al* reported the detection of RNA “plus” strands in all 23 study organs taken for analysis from GBV-C-infected patients who had suddenly died<sup>[7]</sup>. However, both RNA strands were found only in the spleen and bone marrow.

The comparison of nucleotide sequences in the E2-region and the lack of occurrence of mutant viral forms during antiviral therapy with interferons suggested that the mechanisms that are responsible for persistent infection are different from those for HCV. Thus, during 2-year follow-up, the average amino acid sequence replacement in the E2-region was 100 times lower in GBV-C than in HCV<sup>[75]</sup>. Investigations indicated that viremia in GBV-C-infected patients was low and equal to  $10^3\text{-}10^4$  copies/mL<sup>[76]</sup>. It has been suggested that the viral particles that are present in the blood use low-density lipoprotein receptors for penetration into the target cell and generate lipid complexes similar to those seen for HCV particles. An experiment was made on cultured peripheral blood mononuclear cells (PBMC)<sup>[60,77]</sup>.

GBV-C may replicate in PBMC and interferon-resistant Daudi cells<sup>[60]</sup>. Experiments were carried out to inoculate human PBMC lines and hepatocytes with GBV-C RNA *in vitro*. The same lines were infected with HCV as a control. These experiments demonstrated that GBV-C replicates only in CD4+ cells<sup>[60,78]</sup>. Studies of cells from different organs of GBV-C-infected patients were conducted in parallel. They also detected traces of RNA “minus” strand virus. Thus, the *in vitro* and *in vivo* studies provide evidence that PBMC are the primary site of GBV-C replication.

The contribution of not only the immune system,

but also genetic predisposition to prolonged viral circulation is suggested. HLA typing in GBV-C-infected patients with hemophilia showed that 22% of the RNA-positive patients and 72% of the anti-E2-positive patients had HLA DQ7, HLA DR15 and HLA DR8. There is also evidence for low content of CD4+ and the high level of CD8+ lymphocytes in anti-E2-positive patients, which makes it possible to predict GBV-C clearance<sup>[79]</sup>.

HGV replication in peripheral blood monocytes and lymphocytes, and the spleen and bone marrow, combined with long viral persistence suggest that GBV-C replicates predominantly in the hematopoietic system. On examining 44 patients with non-Hodgkin’s lymphoma, African *et al* revealed markers of HCV infection in 5% of cases<sup>[80]</sup>. None of them was found to have HGV RNA. However, meta-analysis of 178 cases of non-Hodgkin’s lymphoma and 355 healthy volunteers indicated GBV-C RNA in 8.4% (15/176) and 0.8% (3/355) of the examinees, respectively, which points to the high risk of HGV in patients with lymphoma<sup>[81]</sup>. There is evidence for the frequent detection of GBV-C RNA in patients with leukemia as compared to those with myeloproliferative diseases<sup>[82]</sup>. Crespo *et al* reported the development of aplastic anemia in a 24-year-old male patient with acute hepatitis G<sup>[83]</sup>. Frequent transfusions in these patients may be one of the causes of HGV infection.

There are higher detection rates of GBV-C RNA (11%) and anti-E2 (17%) in autoimmune hepatitis than in the control group (2%)<sup>[84]</sup>. Heringlake *et al* revealed serum GBV-C RNA in 6.7%, 10.0% and 12.5% of the patients with types I, II and III autoimmune hepatitis, respectively<sup>[85]</sup>. GBV-C is typified by a long-term (as long as 16 years) persistence in human blood<sup>[86]</sup>.

## CLINICAL MANIFESTATIONS OF GBV-C

The clinical picture of GBV-C infection is commonly similar to that of the subclinical and anicteric types of hepatitis with normal or low aminotransferase activities<sup>[87]</sup>. GBV-C-associated hepatitis runs with normal biochemical parameters in 75% of patients<sup>[80]</sup>. There are reports on the occurrence of acute (Table 5)<sup>[62-65,88,89]</sup>, fulminant<sup>[61,90,91]</sup> and chronic (mild and moderate)<sup>[32,76,92,93]</sup> hepatitis and hepatic fibrosis<sup>[27,86]</sup>. Some author’s note the younger age of the GBV-C-infected<sup>[28,37,93]</sup>. The incubation period of acute viral

**Table 5 Detection rate of GBV-C RNA in acute nonA-nonE hepatitis**

Authors, year, country	Number of patients	GBV-C RNA (%)
Alter <i>et al</i> , 1997, United Kingdom <sup>[62]</sup>	45	9.0
Romano <i>et al</i> , 2000, Italy <sup>[63]</sup>	98	3.1
Chu <i>et al</i> , 1999, Taiwan, China <sup>[64]</sup>	53	3.0
Parana <i>et al</i> , 1999, Brazilia <sup>[65]</sup>	25	16
Yashina <i>et al</i> , 1997, USA <sup>[88]</sup>	28	3.6
Uchaikin <i>et al</i> , 2000, Russia <sup>[89]</sup>	35	5.7

hepatitis G averages 14-20 d. The outcome of acute hepatitis may be: (1) recovery with the disappearance of serum GBV-C RNA and the emergence of anti-E2; (2) development of chronic hepatitis (CH) with serum GBV-C RNA being persistently detectable; (3) presence of GBV-C RNA without biochemical or histological signs of liver disease.

The alanine aminotransferase (ALT) activity in GBV-C unlike HCV, does not correspond to the degree of viremia and the severity of hepatic histological changes. By examining 1075 patients with isolated hypertransaminasemia for 6 mo, Berasain *et al* revealed GBV-C RNA in 74 (6.9%) patients<sup>[94]</sup>. Only one (0.09%) patient was monoinfected. There is also evidence for two-fold increases in the activity of alkaline phosphatase (AP) and  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GTP) in GBV-C positive patients<sup>[95]</sup>.

## HISTOLOGICAL CHANGES

Fibrosis of the portal tract without lymphoid-cell infiltration<sup>[96]</sup>, steatosis and insignificant inflammatory infiltration of the portal tract<sup>[67,97,98]</sup> were detectable in isolated persistent GBV-C infection. The histological activity index in patients infected with GBV-C alone was observed to be much lower than that in patients with HCV+GBV-C or HCV<sup>[37,99,100]</sup>. In GBV-C monoinfected patients, moderate or mild focal portal hepatitis was prevalent with slight periportal infiltration and lobular components being found in single cases. The bile tract displayed epithelial fragmentary swelling and flattening and no nuclei in some epitheliocytes. Some bile ducts demonstrated partially desquamated epithelium in the case of higher activities<sup>[99,101]</sup>.

Intraoperative biopsies from GBV-C positive patients with cholelithiasis who were monoinfected with GBV-C, indicated that they had mild chronic hepatitis and, in some cases, viral RNA in the liver tissue and gallbladder mucosa. It is suggested that GBV-C may play a role in the production of lithogenic bile and in the development of cholelithiasis<sup>[102]</sup>.

Coinfection of HGV with hepatitis B, C, and D viruses is significantly more frequently detected than mono-infection<sup>[103]</sup>. In patients with acute viral hepatitis A (HAV), -B (HBV), -C (HCV), the detection rate of GBV-C RNA was 2.9%-25%, 19%-32%, and 20%-48.3%, respectively<sup>[88,89]</sup>. GBV-C RNA was detectable in 8%-16% of patients with chronic hepatitis (CH) B<sup>[30,77,100]</sup>, 5.6%-21% of CH C<sup>[30,77,100]</sup>, and 58% of

CH B+D<sup>[98]</sup>. No differences were found in the clinical manifestations (including those in the chronic pattern and outcome) of the disease, biochemical parameters, or the severity of hepatic histological changes in patients with HBV and/or HCV as compared in those with HBV+GBV-C and/or HCV+GBV-C<sup>[104-106]</sup>. Patients with CHC alone and in combination with HGV have been meticulously examined. By examining 420 patients, Tanaka *et al* revealed a higher ALT activity in the group of patients coinfected with HCV and GBV-C than in those infected with HCV<sup>[107]</sup>.

By comparing histological changes in the liver tissue of patients with HCV and HCV + GBV-C, Moriyama *et al* detected more significant bile duct damages, perivenular and pericellular fibrosis in the latter group<sup>[108]</sup>. These data were supported by the examination of 312 patients with CH<sup>[99]</sup>. Of them 28 (9%) patients were found to have RNA for HCV and GBV-C. Complaints and clinical symptoms did not differ in the groups of patients with HCV and HCV+GBV-C. There was no evidence for the impact of HGV on the clinical manifestations and the course of concomitant HCV infection. However, analysis of liver tissue morphological changes in patients coinfected with HCV and GBV-C revealed slightly more frequent epithelial damage in the bile duct (89%) than in those infected with HCV (67%), which manifested itself as lysis of the epitheliocytic nuclei, as well as flattening, destruction, and swelling of the epithelium and its lymphocytic infiltration.

Whether GBV-C influences the course of CHC and whether therapy with interferon is effective are currently being discussed. Most studies demonstrate no differences in the clinical course of the disease, biochemical parameters, or the magnitude of hepatic histological changes in both HCV alone and in combination with GBV-C<sup>[109-111]</sup>. A study for the therapy of HGV is based on the evaluation of interferon treatment in patients coinfected with HCV+GBV-C. HGV was ascertained to be sensitive to interferon. Administration of  $\alpha$ -interferon ( $\alpha$ -IFN) to patients at a dose of 3 000 000 IU thrice weekly for 6 mo resulted in ALT activity normalization and serum GBV-C RNA clearance in 18%-40% of the patients treated with  $\alpha$ -IFN<sup>[112,113]</sup>. Six months after termination of a course of therapy, there were persistent biochemical and virological responses in 55%-57% of patients<sup>[114]</sup>. The therapeutic efficiency was observed to depend on baseline GBV-C RNA levels. The patients who had a low RNA titer (mean,  $3.3 \times 10^5$  copies/mL) more frequently responded to the therapy than those who had a higher one (mean,  $3.5 \times 10^8$  copies/mL)<sup>[104,109]</sup>. There is now a prevailing opinion that GBV-C has no impact on the efficiency of  $\alpha$ -interferon treatment for chronic hepatitis C<sup>[114,115]</sup>. At the same time some investigations suggest that the therapy causes more frequent adverse reactions in patients with HCV+GGV-C and that after its termination, this group of patients has a higher histological activity index<sup>[116,117]</sup>.

The implications of HGV for the development of chronic liver diseases has not been appraised to date.

As for GBV-C infection, investigators could not trace the clinical stages characteristic of HBV and HCV: acute hepatitis-chronic hepatitis-liver cirrhosis (LC)-hepatocellular carcinoma (HCC). A long-term (less than 16 years) follow-up of patients permitted discussion only of the likelihood of development of chronic hepatitis. GBV-C RNA was detectable in 8%-25.4% of patients with chronic hepatitis non-A-non-E<sup>[118]</sup>, 6%-15% of patients with cryptogenic liver cirrhosis<sup>[119,120]</sup>, and 3.1%-8.3% with HCC<sup>[121,122]</sup>.

The similarity of the properties of GBV-C and HCV offers a possibility of using HGV and its induced experimental infection as a model to study hepatitis C. Unlike hepatitis C, hepatitis G infection may be modeled in nonhuman primates, which considerably reduces the cost these studies that are in great demand for the designing of hepatitis C vaccine.

Unexpected results were obtained while studying the impact of GBV-C on the course of HIV infection<sup>[123,124]</sup>. Co-infection with GBV-C in the HIV-infected was established to cause a reduction in mortality rates and better clinical parameters of infection. Furthermore, the efficiency of high-activity antiretroviral therapy significantly increased. The positive effect of GBV-C is accounted for by the fact that the envelope proteins of this virus bind CD81+ on T cells and induce dose-dependent secretion of RANTES (regulated on activation, normal T-cell expressed and secreted), the natural ligand that binds CCR5 on the target cell, thereby blocking the penetration of HIV<sup>[21,125]</sup>. *In vitro* studies showed an increase in the expression of the chemokines-RANTES, macrophage inflammatory proteins (MIP-1 $\alpha$ , MIP-1 $\beta$ ), and stromal-cell derived factor (SDF-1) in the blood of patients. There was also a reduction in the expression of CCR5 onto the surface of GBV-C-infected cells. All these factors may provide indirect evidence for the diminished sensitivity of GBV-C-infected cells to HIV<sup>[125-127]</sup>.

A review of available data in the literature and the authors' own data suggest that the new HGV discovered in the late 1990s has been rather well studied. The structure of the virus is almost completely known; its genotypes have been ascertained; its prevalence (epidemiology) shown and the clinical picture of the disease, routes of viral transmission, and the types of coinfection described. The predominant site of replication of the virus in the blood mononuclear cells, spleen, and bone marrow has been indicated. The lack of hepatotropism of virus G (which is rarely detected in the liver), its frequent detection in the body and tissues of a patient without any clinical signs of hepatitis, and clinical improvement in the HIV-infected patients coinfected with GBV-C cast doubt on the appropriateness of the concept "viral hepatitis G". The interest shown in HGV is likely to be associated with the similarity of its properties to those of HCV.

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REVIEW

# Therapy for acute pancreatitis with platelet-activating factor receptor antagonists

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## Abstract

Acute pancreatitis (AP) causes release of platelet-activating factor (PAF), which induces systemic effects that contribute to circulatory disturbances and multiple organ failure. PAF is a cell surface secretion of bioactive lipid, which could produce physiological and pathological effects by binding to its cell surface receptor called platelet-activating factor receptor (PAF-R). Studies showed that PAF participates in the occurrence and development of AP and administration of platelet-activating factor receptor antagonists (PAF-RAs) could significantly reduce local and systemic events after AP. PAF has also been implicated as a key mediator in the progression of severe AP, which can lead to complications and unacceptably high mortality rates. Several classes of PAF-RAs show PAF-RAs significant local and systemic effects on reducing inflammatory changes. As a preventive treatment, PAF-RAs could block a series of PAF-mediated inflammatory injury and thus improve the prognosis of AP. This review introduces the important role of PAF-RAs in the treatment of AP.

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**Key words:** Acute pancreatitis; Platelet-activating

factor; Platelet-activating factor receptor antagonist; BN52021; Lexipafant

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## INTRODUCTION

Acute pancreatitis (AP) is a kind of inflammatory disease that can develop into severe acute pancreatitis (SAP). SAP refers to AP associated with organ failure and/or local complications such as necrosis, pseudocyst or abscess<sup>[1]</sup>. The overall mortality of SAP has decreased in recent years to around 15%-20%<sup>[2]</sup>. A variety of inflammatory mediators play a crucial role in AP. Synthesis of platelet-activating factor (PAF) is sensitive to biologically active mediators seen in many inflammatory processes, which could produce physiological and pathological effects by binding to its cell surface receptor called platelet-activating factor receptor (PAF-RA). PAF significantly potentiates pancreatic tissue damage and increases serum amylase and lipase levels, causes scattered haemorrhages and may serve as a primary mediator of inflammation. Many identified PAF-RAs exhibit varied structures, and interact with the PAF-RA in different ways. It has been postulated that these differences could be due to conformation changes in the receptor protein itself, by G-protein coupling or changes on the cell membrane. Several classes of PAF-RAs show significant local and systemic effects on reducing inflammatory changes. PAF-RAs used as a preventive treatment can block a series of inflammatory injury caused by PAF, thereby improving AP prognosis<sup>[3]</sup>. Research on such as a potential therapy has helped to elucidate the role of PAF in AP<sup>[4]</sup>. Progress in treatment of AP with PAF-RAs is summarized below.

## BIOLOGICAL ACTIVITY OF PAF

PAF released by various cells including endothelial,

polymorphonuclear, gastrointestinal epithelial cells and macrophages, is a potent proinflammatory phospholipid mediator that belongs to a family of biologically active, structurally related alkyl phosphoglycerides with diverse pathological and physiological effects. These bioactive phospholipids mediate such diverse processes as wound healing, physiological inflammation, angiogenesis, apoptosis, reproduction, long-term potentiation and immunoregulatory role<sup>[5]</sup>. The data suggest that PAF enhances PAF-R expression<sup>[6,7]</sup>. PAF activates multiple intracellular signaling pathways by binding to PAF-R. In the inflammatory process, the primary function of PAF is to activate the interleukin system in endothelial cells to surface rolling, adhesion and through endothelial cells to stroma cells. PAF has neurological toxicity<sup>[8]</sup>. It is also involved in etiopathogenesis of type-1 diabetes<sup>[9]</sup> and liver tissue repair post-acetaminophen treatment<sup>[10]</sup>.

## RELATIONS BETWEEN PAF AND AP

In the development of AP, the pancreas and its surrounding tissues secrete digestive enzymes to digest their own components, causing acute inflammation. AP is divided into acute edema and acute hemorrhagic pancreatitis according to its histology and clinical manifestations. Acute hemorrhagic pancreatitis is also called as severe acute pancreatitis (SAP). Studies found that SAP is closely related to neutrophil excessive activation and cytokines-mediated cascade of systemic inflammatory response syndrome (SIRS)<sup>[11]</sup>. The considerable release of PAF and direct or indirect systemic response facilitates formation of SIRS and causes circulation dysfunction, resulting in multiple organ dysfunction syndrome (MODS)<sup>[4]</sup>. *In vitro* experiments showed that PAF can stimulate a large number of trypsin release<sup>[12]</sup>, while *in vivo* experiments showed that PAF could induce and aggravate AP through injection into pancreatic arteries<sup>[13]</sup>. PAF is increased in pancreas, ascites, immune complexes, cerulein and AP induced by cows iodine acid salt.

## PAF-RA

PAF-RA can successfully repress the effect mediated by PAF in AP. PAF-RAs are divided into five categories according to their chemical structure and properties: nitrogen heterocyclic compounds (such as WEB2086, WEB2170, etc), PAF analogues (such as CV3988, SDZ63072, etc), dihydropyridines (such as PCV4233, PVA4248, etc), natural medicines (such as BN52021, Kadsurenone, etc), and others (such as 52770 RP, TCV309, etc).

PAF-RAs exert their effects by inhibiting the activity of neutrophils and depressing pulp peroxidase, competing targets with PAF and inhibiting the activity of PAF<sup>[14]</sup>, inhibiting increasing PAF in AP, reducing vascular permeability and improving microcirculation of blood flow velocity, reducing plasma cytokines and inflammatory mediators, enzyme activity and the role of self-digestion of pancreatic tissue. At present, there are

several compounds with obvious antagonism of PAF, their chemical codes are BN52021, WEB2170, TCV309, WEB2086 and BB882 (*lexipafant*), which can reduce the inflammatory response and significantly affect local and systemic status in AP.

### WEB2170

WEB2170 is a nitrogen heterocyclic compound. Nitrogen binds to the receptor through the hydrogen bond, facilitating the activity of PAF-RAs, thus reducing the NO production by liver cells in pancreatitis. PAF is likely to be the main contributing factor for endotoxin effect. WEB2170 can significantly reduce intestinal necrosis mediated by lipopolysaccharide (LPS)<sup>[15]</sup> and WEB2170 does not affect the TNF levels<sup>[16]</sup>. WEB2170 can reduce vascular permeability and infiltration of neutrophils and macrophages.

WEB2086 (Apafant) can inhibit angiogenesis in atherosclerotic plaque<sup>[17]</sup>. Oral WEB2086 can elevate low blood pressure induced by PAF.

### TCV309

TCV309 is an important PAF antagonist, which can inhibit the biological activity of PAF both *in vivo* and *in vitro*. Oral TCV309 can significantly reduce the concentration of neutrophil chemokine, but does not lead to significant changes in amylase<sup>[18]</sup>. TCV309 has more advantages than PAF analogues in avoiding hemolysis and vascular injury when the inhibitory activity of PAF binding to its receptor is higher. In addition to injection, TCV309 can be taken orally.

### Lexipafant

Lexipafant has entered clinical trials. Lexipafant is a molecule specifically designed to bind to PAF-R<sup>[4]</sup> and an imidazole derivative of heterocyclic sp<sup>2</sup> nitrogen compounds and has been shown to be considerably more potent than other PAF receptor antagonists with a much greater affinity than PAF itself for its binding to human platelet PAF-R.

Lexipafant can prevent liver ischemia/reperfusion injury<sup>[19]</sup> and reduce the severity of inflammatory response to liver injury induced by bile duct ligation in rats<sup>[20]</sup>, leading to impairment of AP endothelial barrier function, leukocyte accumulation and IL-1 level in the pancreas<sup>[21]</sup>. Treatment of pancreatitis with lexipafant reduces the severity of pancreatitis-associated intestinal dysfunction, systemic IL-1 concentration and local leukocyte recruitment<sup>[22]</sup>.

Animal experiments showed that lexipafant improves inflammation of AP<sup>[23]</sup>. Lexipafant treatment can improve acute necrotizing pancreatitis caused by bacteria shift<sup>[24]</sup>. In clinical trials, it was shown that lexipafant could reduce the morbidity and mortality of acute pancreatitis, but may not reduce the morbidity and mortality of severe acute pancreatitis. Further investigation is expected about the effect of lexipafant<sup>[25]</sup>.

Vincent *et al*<sup>[26]</sup> also claimed that there is no difference in survival, hemodynamics, respiratory function and MOF score between treatment with lexipafant and

placebo. Johnson *et al*<sup>[27]</sup> used lexipafant intervention therapy to study 290 cases of AP patients and found that the organ failure scores of AP are significantly reduced, suggesting that lexipafant cannot change the SAP organ failure process in patients. Further study is needed to observe the effect of lexipafant.

### BN52021

BN52021 is a terpenoid extracted from *Ginkgo biloba* leaves. In 1988, Jancar found that treatment of AP in immune-complex-induced mouse model with BN52021 could significantly relieve edema of pancreas<sup>[28]</sup>. Since BN52021 competitively inhibits the binding to PAF and its platelet receptor, PAF cannot activate phospholipase C through G-protein transduction, adenosine cyclase and tyrosine protein kinase, thereby blocking the PAF receptor signal transduction and the biological effects of PAF. BN52021 exerts its biological effects by competitively inhibiting the expression of PAF and PAF-R rather than by decreasing the expression of PAF receptor in pancreatic tissues<sup>[29]</sup>. BN52021 is a non-competitive GABA receptor antagonist<sup>[30]</sup> and inhibits gene transcription of adrenal peripheral benzodiazepine receptor and synthesis of steroids<sup>[31]</sup>.

BN52021, a most promising PAF-R antagonist, can effectively inhibit PAF-induced neutrophil chemotaxis, adhesion, aggregation and other inflammatory factors, thus reducing inflammation injury. BN52021 significantly reduces the mortality of AP, prolongs the average survival time, maintains a lower serum amylase activity, reduces pancreatic injury as well as Ca<sup>2+</sup> content and malondialdehyde (MDA) level and superoxide dismutase (SOD) activity in pancreatic tissue. Bedirli *et al*<sup>[32]</sup> reported that BN52021 can inhibit intestinal bacterial shift to the pancreas. BN52021 protects the stability of macrophage membrane against lysis<sup>[33]</sup>. PAF may play an important role in liver injury and regeneration. Ginkgolide B attenuates liver damage, thus improving liver function following acetaminophen intoxication<sup>[34]</sup>.

To sum up, BN52021 has a wide range of pharmacological effects. Currently, pharmacology of BN52021 mainly focuses on the PAF-R antagonists. Whether other mechanisms of BN52021 exert effects through different pathways is worthy of further study.

### CONCLUSION

Progress has been made in research about the therapy for AP, especially for SAP, with PAF-RA. However, there is still a long way to go. Further study is needed to confirm the encouraging results obtained from animal experiments and find other key inflammatory mediators in the pathogenesis of AP.

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# Nuclear factor kappa B: A marker of chemotherapy for human stage IV gastric carcinoma

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## Abstract

**AIM:** To detect the nuclear factor kappa B (NF- $\kappa$ B) condition in human stage IV gastric carcinoma patients and to explore the correlation between NF- $\kappa$ B activation and survival of these patients after chemotherapy.

**METHODS:** Expression of NF- $\kappa$ B-p65 was determined by immunohistochemical analysis. Activity of NF- $\kappa$ B DNA-binding in carcinoma tissue was detected by electrophoretic mobility shift assay. Kaplan-Meier survival analysis was performed to show the relation between NF- $\kappa$ B and progression-free survival (PFS) or overall survival (OS) of the patients.

**RESULTS:** The positive expression rate of NF- $\kappa$ B-p65 in 60 gastric cancer tissue samples was 76.7% (46/60). The expression of NF- $\kappa$ B-p65 was reduced in adjacent carcinoma and normal tissue samples. Electrophoretic mobility shift assay (EMSA) analysis showed a strong activation of NF- $\kappa$ B in cancer tissue samples. A survival difference was found in NF- $\kappa$ B-p65 positive and negative patients. NF- $\kappa$ B-p65 expression was negative in cancer tissue samples ( $n = 14$ ). PFS was  $191.40 \pm 59.88$  d and  $152.93 \pm 16.99$  d, respectively, in patients with positive NF- $\kappa$ B-p65 expression ( $n = 46$ ) ( $P = 0.4028$ ). The

survival time of patients with negative and positive NF- $\kappa$ B-p65 expression was  $425.16 \pm 61.61$  d and  $418.85 \pm 42.98$  d, respectively ( $P = 0.7303$ ). Kaplan-Meier analysis showed no significant difference in PFS or OS. The 46 patient tissue which positive NF- $\kappa$ B-p65 expression was found in the tissue samples from the 46 patients whose PFS and OS were  $564.89 \pm 75.94$  d and  $352.37 \pm 41.32$  d, respectively ( $P = 0.0165$ ).

**CONCLUSION:** NF- $\kappa$ B is activated in gastric carcinoma tissue, which is related to the OS after chemotherapy.

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**Key words:** Gastric carcinoma; Nuclear factor kappa B; Activation; Survival analysis; Therapy

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## INTRODUCTION

Nuclear factor kappa B (NF- $\kappa$ B) contributes to cell differentiation, proliferation, and death, as well as plays a prominent role in the immune response of mammals<sup>[1]</sup>. It consists of homodimers or heterodimers of the Rel family members: NF- $\kappa$ B1 (p50), NF- $\kappa$ B2 (p52), c-Rel, RelA (p65), and RelB<sup>[2]</sup>. The ability of NF- $\kappa$ B to suppress apoptosis and regulate cell-cycle transition indicates that NF- $\kappa$ B may participate in oncogenesis<sup>[3]</sup>.

There is evidence that NF- $\kappa$ B level is higher in gastric carcinoma cells than in normal adjacent epithelial cells, and activation of NF- $\kappa$ B in gastric carcinoma is related to lymphatic invasion<sup>[4]</sup>. It was reported that patients with high NF- $\kappa$ B activation in carcinoma tissue would not survive as long as those with low NF- $\kappa$ B activation<sup>[5]</sup>, and p65, but not NF- $\kappa$ B, is a prognostic indicator of gastric carcinoma<sup>[5]</sup>. It was also reported that NF- $\kappa$ B is frequently activated in the early-stage gastric carcinoma, and negatively associated with lymphatic invasion, but

significantly associated with a better prognosis<sup>[6]</sup>.

Although NF-κB is activated in human gastric carcinoma tissue, there is no evidence that NF-κB activation is associated with the clinicopathological features of cancer<sup>[6]</sup>. Our present study was to detect whether NF-κB is activated in human stage IV gastric carcinoma and explore the correlation between its activation with the survival of gastric cancer patients after chemotherapy.

## MATERIALS AND METHODS

### Materials

The criteria for inclusion of patients in this study were (1) those with histologically or cytologically proved gastric cancer, (2) those with stage IV gastric cancer diagnosed according to the 2002 American Joint Committee on Cancer (AJCC) modified staging criteria, (3) those treated in our hospital with follow-up data, (4) those not receive chemotherapy prior to admission to our hospital, (5) those who received treatment with chemotherapeutics FOLFOX or paclitaxel/LV5Fu2 (PLF), (6) those with their therapeutic effect divided into complete remission (CR), partial remission (PR) and progression of disease (PD). The exclusion criteria for patients from this study were those with less than two cycles of treatment with FOLFOX or PLF, those with their therapeutic effect not estimate after two cycles of treatment with FOLFOX or PLF, those with stable effect of chemotherapeutics after several cycles of treatment, those having received two cycles of chemotherapy with FOLFOX and PLF. The response evaluation criteria used were the WHO and UICC criteria. We collected 60 gastric carcinoma samples from the First Affiliated Hospital of Sun Yat-Sen University in November 1999-June 2005. The data obtained from patients are shown in Table 1.

### Immunohistochemistry for NF-κB-p65 detection

All samples were fixed with 100 mL/L formalin and embedded in paraffin. Each paraffin block was cut into 5-μm thick sections. Streptavidin-Biotin Complex (SABC) Immunohistochemistry was performed according to the manufacturers' instructions to detect the expression of NF-κB-p65. Briefly, the tissue sections were deparaffinized in xylene at 37°C for 20 min. Endogenous peroxide was blocked by incubating the sections with 30 mL/L H<sub>2</sub>O<sub>2</sub> for 10 min at 37°C. The sections were incubated with primary antibodies to NF-κB-p65 at 4°C overnight. Staining was visualized with diaminobenzidine (DAB) for 10 min at room temperature. Finally, the sections were counterstained for nuclei with hematoxylin solution. Each section was observed microscopically in eight visual fields ( $\times 400$  magnification) and at least 100 cells were counted in each field. Positive staining for NF-κB-p65 appeared as buffy grains in the nuclei and cytoplasm. A semi-quantitative method was adopted to judge the results as previously described<sup>[7]</sup>. Immunohistochemistry score was calculated by combining an estimate of the percentage of immunoreactive cells (quantity score) with

**Table 1 Data obtained from patients in the two treatment groups**

	FOLFOX group (n = 44)	PLF group (n = 16)	P
Sex (male/female)	29/15	11/5	0.547
Age (yr)	51.51 ± 13.10	49.06 ± 12.36	0.521
General state of health			
ECOG patients			
0 score	1	1	0.721
1 score	32	12	
2 scores	11	4	
Primary location			
Cardiac orifice	11	2	0.431
Stomach	19	12	
Gastric remnant	4	2	
Accumulative organs			
1	3	1	0.1642
2	23	3	
3	16	8	
> 3	2	4	
Pathologic classification			
Moderately-differentiated	9	4	0.691
Poorly-differentiated	33	12	0.336
Undifferentiated	2	0	0.132

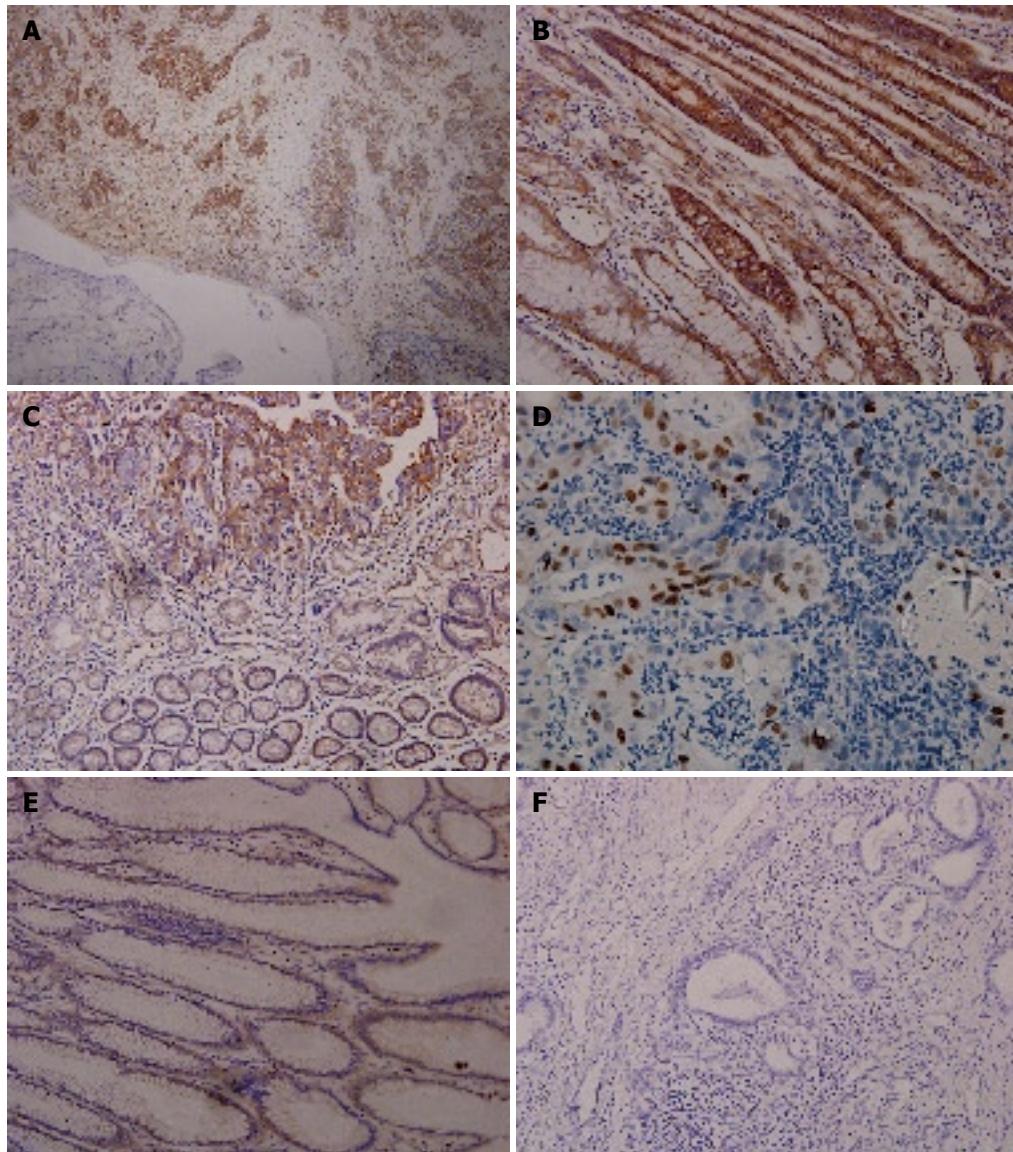
an estimate of the staining intensity (staining intensity score) as follows: no staining as 0, 1%-10% of cells stained as 1, 11%-50% cells stained as 2, 51%-80% cells stained as 3, and 81%-100% cells stained as 4. Staining intensity was rated on a scale of 0-3, with 0 = negative, 1 = weak, 2 = moderate, and 3 = strong. The staining was evaluated by color intensity and density of grade products as follows: 0-3 = negative (-), > 3 = positive (+). All specimens were evaluated by two pathologists without any knowledge about the clinical data obtained from the patients. Negative control was designed using PBS instead of primary antibody. Adjacent glandular epithelium served as an internal positive control of NF-κB-p65 protein expression.

### Electrophoretic mobility shift assay (EMSA) of NF-κB

Activity of NF-κB DNA-binding was detected by EMSA as previously described<sup>[8]</sup> with certain modifications. Double-stranded oligonucleotide sequence of 5'-AG TTGAGGGGACTTCCCCAGGC-3', 5'-GCCTGGG AAAGTCCCCTCACT-3', which corresponds to the NF-κB binding site, was end-labeled with [ $\gamma$ -32P] ATP by T4 polynucleotide kinase (Promega). Labeled probes were purified and detected with Whatman DE81 (> 5000 r/min). Nuclear extract (15 μg) was mixed with 5 × 2 μL binding buffer at room temperature for 10 min, then labeled probe (0.0175 pmol) was incubated at room temperature for 20 min. The mixture was then subjected to electrophoresis on 4% polyacrylamide gel at 150 V in 0.5 × TBE buffer for 40 min at 4°C. After drying, the gel was exposed to an imaging system of FX P screen (Bio-Rad, America) for 24 h. The result was analyzed with software Bandscan 4.0 (denary logarithm of total gray value).

### Follow-up methods

All patients were followed up in out-patient clinic, or by



**Figure 1** Immunohistochemical staining for NF- $\kappa$ B-p65 showing p65 expression in cancer tissue samples but not in adjacent non-neoplastic tissue samples (SABC,  $\times$  100) (A), in gastric adenocarcinoma epithelial tissue samples (SABC,  $\times$  200) (B), in intestinal metaplasia samples (SABC,  $\times$  200) (C), in nuclei of cancer cells (SABC,  $\times$  200) (D), and very weak expression of NF- $\kappa$ B-p65 in gastric mucosa samples (SABC,  $\times$  200) (E), and in negative control (SABC,  $\times$  200) (F).

telephone or letters, two times each year, until June 4, 2006. We calculated progression-free survival (PFS) and overall survival (OS) rates from the day of treatment. Life-table methods were used to estimate PFS and OS<sup>[9]</sup>. For OS, deaths, irrespective of cause, were coded as events. For PFS, an event was defined as relapse, progression, or death during the period of active follow-up. Kaplan-Meier curves for PFS and OS were calculated for the subgroups with positive or negative NF- $\kappa$ B-p65 staining. In addition, other sets of Kaplan-Meier curves were derived to determine the PFS and OS of patients after FOLFOX and PLF therapy when NF- $\kappa$ B-p65 staining was positive. To assess variables influencing PFS and OS, univariate analysis using log-rank tests and proportional hazards models was performed. This study was approved by the local human investigation committee.

#### Statistical analysis

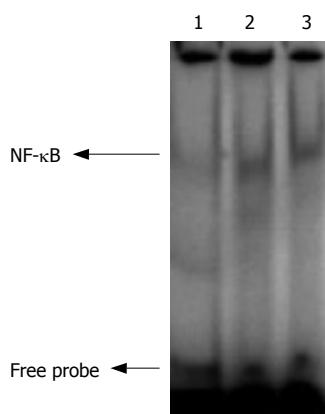
Statistical analysis was performed using SPSS 10.0

software. Significant differences were compared using one-way ANOVA test. Chi square test was performed for numerical data. Survival analysis was carried out using the Kaplan-Meier product-limit method, and survival curves were plotted. Differences were evaluated by the log rank test.  $P < 0.05$  was considered statistically significant.

## RESULTS

### NF- $\kappa$ B-p65 expression by SABC

The positive expression rate of NF- $\kappa$ B-p65 in 60 gastric cancer tissue samples was 76.7% (46/60). Most positive expression of NF- $\kappa$ B-p65 showed brown-stained signals in cell cytoplasm or membrane (Figure 1A-C). Only a small amount of expression was found in the nuclei (Figure 1D). The immunohistochemistry score was  $7.18 \pm 2.72$ . The expression of NF- $\kappa$ B-p65 was reduced in the carcinoma tissue samples and the expression rate



**Figure 2** Images of EMSA. Lanes 1-3: NF- $\kappa$ B activity in normal, moderately- and poorly-differentiated gastric carcinoma tissue samples.

was 26.7% (16/60, Figure 1C). Weak expression of NF- $\kappa$ B-p65 was observed in normal tissue samples (Figure 1E). The immunohistochemistry score was  $1.85 \pm 1.29$  and  $0.17 \pm 0.38$ , for carcinoma and normal tissue samples, respectively ( $P < 0.00001$ ). No stained signal was detectable in negative control (Figure 1F).

#### EMSA for NF- $\kappa$ B

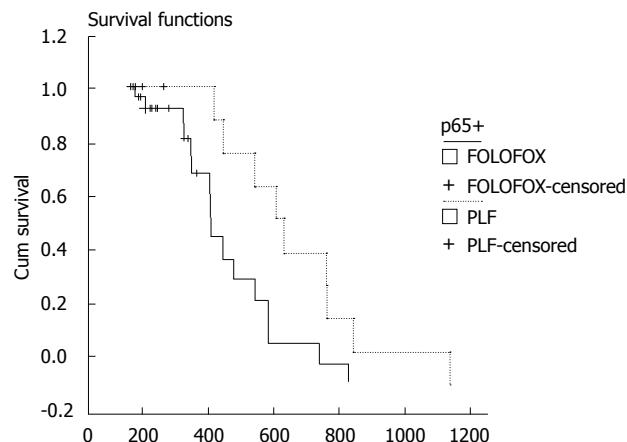
Nuclear extracts from normal, moderately- and poorly-differentiated gastric carcinoma tissue samples were analyzed by EMSA, which showed a strong activation of NF- $\kappa$ B in cancer tissue samples (Figure 2).

#### Different survival time of positive and negative NF- $\kappa$ B-p65 patients

We analyzed PFS and OS using the Kaplan-Meier method. The results showed that the expression of NF- $\kappa$ B-p65 was negative in cancer tissue samples (immunohistochemistry score ranged from 1 to 3 scores,  $n = 14$ ) and PFS was  $191.40 \pm 59.88$  d (95% CI = 74.04 - 308.76), while it was positive in adjacent and normal tissue samples (immunohistochemistry score  $> 3$ ,  $n = 46$ ) and PFS was  $152.93 \pm 16.99$  d (95% CI = 119.62 - 186.24). The survival time of patients with negative and positive expression of NF- $\kappa$ B-p65 was  $425.16 \pm 61.61$  d (95% CI = 304.41 - 545.92) and  $418.85 \pm 42.98$  d (95% CI = 334.62 - 503.08), respectively.

#### Different survival time of patients after treatment with FOLFOX and PLF

Kaplan-Meier analysis showed that the PFS time of patients after treatment with PLF and FOLFOX was  $135.79 \pm 15.09$  d (95% CI = 106.22 - 165.35) and  $183.08 \pm 30.97$  d (95% CI = 122.37 - 243.78), respectively. The OS time of patients after treatment with PLF and FOLFOX was  $539.51 \pm 69.82$  d (95% CI = 402.66 - 676.35) and  $363.12 \pm 35.70$  d (95% CI = 293.15 - 433.09), respectively. There was no significant difference in PFS or OS time of patients after treatment with FOLFOX and PLF ( $P = 0.2931$  and  $P = 0.0548$ , respectively). The NF- $\kappa$ B-p65 expression was positive in the tissue samples from 46 patients. PFS time of patients with positive p65 expression was  $132.60 \pm 16.62$  d (95% CI = 100.03 - 165.17) and  $163.64 \pm 23.99$  d (95% CI = 116.62 - 210.65), respectively, after treatment with



**Figure 3** Survival cures for OS time of patients after treatment with PLF and FOLFOX.

PLF and FOLFOX ( $P = 0.2407$ ). However, the OS time of patients with negative p65 expression was  $564.89 \pm 75.94$  d (95% CI = 416.04 - 713.74) and  $352.37 \pm 41.32$  d (95% CI = 271.38 - 433.36), respectively, after treatment with PLF and FOLFOX ( $P = 0.0165$ ). Their survival cure is shown in Figure 3.

## DISCUSSION

NF- $\kappa$ B plays a role in oncogenic transformation. v-Rel, a highly oncogenic retroviral homologue of c-Rel, causes carcinogenesis in avian lymphoid cells<sup>[3,10]</sup>. Inhibition of NF- $\kappa$ B, by over expression of a degradation-resistant I $\kappa$ B, delays the development of T-cell lymphomas and prolongs the survival of v-Rel transgenic mice<sup>[3,10]</sup>. Chromosomal alterations in NF- $\kappa$ B family genes provide additional evidence for the role of NF- $\kappa$ B in oncogenesis. It has been demonstrated that genes encoding c-Rel, NF- $\kappa$ B2 (p100/p52), p65/RelA, and Bcl-3 proteins are all located within breakpoint regions of the genome involving oncogenic rearrangements or amplifications<sup>[3,11]</sup>. Indeed, increased NF- $\kappa$ B activity is evident in a number of human cancers, including breast cancer, non-small cell lung carcinoma, thyroid cancer, T- or B- lymphocyte leukemia, melanoma, colon cancer, bladder cancer, and several virus-induced tumors<sup>[12]</sup>.

It was reported that NF- $\kappa$ B is constitutively activated in gastric carcinoma tissue<sup>[4-6,13,14]</sup>. The expression rate of NF- $\kappa$ B-p65 in gastric carcinoma is 18%-78.3%, which is higher than in normal adjacent epithelial cells. Sasaki *et al*<sup>[4]</sup> hold that increased NF- $\kappa$ B expression is correlated to the clinicopathological features of tumor aggression (especially its invasive ability) of gastric carcinoma. In the present study, NF- $\kappa$ B-p65 expression was mainly observed in gastric carcinoma cytoplasm with an expression rate of 77.6% (46/60), which is consistent with the previously reported data<sup>[4,14]</sup>. Some representative tumor specimens showed that p65 staining was increased in nuclei (Figure 1D), suggesting that subunit of NF- $\kappa$ B is translocated from cytoplasm to nuclei. We also determined p65 expression in samples from normal gastric glands and

intestinal metaplasia except in cancer tissue samples. Weaker staining was found in such tissue samples, suggesting that NF-κB activation is associated with cell proliferation. EMSA showed that the increased nuclear translocation of NF-κB-p65 showed a higher NF-κB DNA-binding activity in differently differentiated tumor tissue samples than in adjacent normal tissue samples (Figure 2). The NF-κB-p65 expression rate is not consistent with the previously reported rate<sup>[6]</sup>. The discrepancy might be due to the following reasons. (1) The classification reference for positively-stained cells might have decreased the expression rate of NF-κB-p65. Lee *et al*<sup>[6]</sup> reported that cells showing p65 nuclear staining irrespective of cytoplasmic staining display constitutive NF-κB activation. (2) Carcinoma stage was different. In our study, the 60 patients had stage IV gastric cancer compared with 15.5% reported in the study of Lee *et al*<sup>[6]</sup>, who found that NF-κB activation is more prominent in early-stage pTNM tumors than in late-stage tumors.

Sasaki *et al*<sup>[4]</sup> analyzed the relation between NF-κB activation and traditional clinicopathological parameters, showing that NF-κB activation is correlated with tumor size and lymphatic invasion. However, Lee *et al*<sup>[6]</sup> showed that NF-κB activation, as a prognostic factor, is not correlated with the prognosis of patients after curative resection of their tumors, but significantly associated with a better prognosis of early-stage gastric carcinoma patients, suggesting that NF-κB activity is less reliable than TNM criteria as a prognostic biomarker of gastric cancer<sup>[6]</sup>. In the present study, we selected specimens from stage IV gastric cancer patients to discriminate p65 positive and negative expression. Sixty samples were divided into positive p65 expression group ( $n = 46$ ) and negative p65 expression group ( $n = 14$ ). When no significant difference in age, sex, accumulative organs and pathologic classification was found between the two groups, we analyzed the PFS and OS time of the patients in the two groups. The PFS time and OS time were longer in the group with positive p65 expression than in the group with negative p65 expression ( $P > 0.05$ ), which is not consistent with previous reports<sup>[5,6]</sup>. We speculate that this discrepancy is caused by (1) the different carcinoma stages, (2) different treatment modalities, and (3) the number of tumor cases not taken into account.

In the present study, the 60 patients were treated with PLF ( $n = 16$ ) and FOLFOX ( $n = 44$ ), respectively (Table 1). Their PFS time and OS time had no statistical significance ( $P > 0.05$ ). However, when we divided the 46 patients into a PLF treatment group ( $n = 12$ ) and a FOLFOX treatment group ( $n = 12$ ) with positive p65 expression ( $n = 34$ ), the OS time of the patients in the two groups was  $564.89 \pm 75.94$  d (95% CI = 416.04 - 713.74) and  $352.37 \pm 41.32$  d (95% CI = 271.38 - 433.36), respectively ( $P = 0.0165$ ), suggesting that NF-κB activation is associated with the prognosis of gastric cancer patients.

In conclusion, NF-κB-p65 is constitutively activated in late-stage gastric carcinoma patients and NF-κB

activation is correlated to the survival time of gastric cancer patients after chemotherapy.

## COMMENTS

### Background

Nuclear factor kappa B (NF-κB) contributes to cell differentiation, proliferation, and death. There is experimental evidence that NF-κB plays a major role in the development and progression of various human cancers including gastric carcinoma. The effect of anti-cancer drugs is related to NF-κB in cancer cells.

### Research frontiers

As it is known, there is evidence that NF-κB plays a major role in the development and progression of various human cancers including gastric carcinoma. On the other hand, NF-κB can suppress tumor growth by promoting apoptotic signals in response to certain cancer therapeutic agents. However, the molecular mechanism of NF-κB underlying cancer development remains to be elucidated.

### Innovations and breakthroughs

NF-κB was found to be a biomarker of gastric cancer. Further study is needed to prove it.

### Applications

NF-κB activation is correlated to the survival of gastric cancer patients after chemotherapy and may affect treatment of choice for late human gastric carcinoma.

### Peer review

In the present work, the authors revealed the differential expression of NF-κB p65 in normal and gastric carcinoma tissue samples, and the potential importance of p65 up-regulations was underscored in chemotherapy for human gastric carcinoma. Their findings support the theory that p65 functions as a tumor promoter in human gastric carcinoma. Therefore, identification of the functional and phenotypic characteristics of p65 is essential for designing a rational anti-cancer therapy, as suggested by the authors. The study is well designed, and the data are reliable.

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S- Editor Zhong XY L- Editor Wang XL E- Editor Zhang WB



CLINICAL RESEARCH

## Revaluation of clinical and histological criteria for diagnosis of dysmetabolic iron overload syndrome

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### Abstract

**AIM:** To re-evaluate the diagnostic criteria of insulin resistance hepatic iron overload based on clinical, biochemical and histopathological findings.

**METHODS:** We studied 81 patients with hepatic iron overload not explained by known genetic and acquired causes. The metabolic syndrome (MS) was defined according to ATPIII criteria. Iron overload was assessed by liver biopsy. Liver histology was evaluated by Ishak's score and iron accumulation by Deugnier's score; steatosis was diagnosed when present in  $\geq 5\%$  of hepatocytes.

**RESULTS:** According to transferrin saturation levels, we observed significant differences in the amount of hepatic iron overload and iron distribution, as well as the number of metabolic abnormalities. Using Receiving Operating Curve analysis, we found that the presence of two components of the MS differentiated

two groups with a statistically significant different hepatic iron overload ( $P < 0.0001$ ). Patients with  $\geq 2$  metabolic alterations and steatosis had lower amount of hepatic iron, lower transferrin saturation and higher sinusoidal iron than patients with  $< 2$  MS components and absence of steatosis.

**CONCLUSION:** In our patients, the presence of  $\geq 2$  alterations of the MS and hepatic steatosis was associated with a moderate form of iron overload with a prevalent sinusoidal distribution and a normal transferrin saturation, suggesting the existence of a peculiar pathogenetic mechanism of iron accumulation. These patients may have the typical dysmetabolic iron overload syndrome. By contrast, patients with transferrin saturation  $\geq 60\%$  had more severe iron overload, few or no metabolic abnormalities and a hemochromatosis-like pattern of iron overload.

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**Key words:** Iron overload; Hepatic iron distribution; Transferrin saturation; Metabolic syndrome; Hepatic steatosis

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Riva A, Trombini P, Mariani R, Salvioni A, Coletti S, Bonfadini S, Paolini V, Pozzi M, Facchetti R, Bovo G, Piperno A. Revaluation of clinical and histological criteria for diagnosis of dysmetabolic iron overload syndrome. *World J Gastroenterol* 2008; 14(30): 4745-4752 Available from: URL: <http://www.wjgnet.com/1007-9327/14/4745.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4745>

### INTRODUCTION

Iron overload includes a wide spectrum of conditions from mild to marked tissue iron accumulation and iron-related organ dysfunctions<sup>[1-3]</sup>. Apart from the known hereditary and acquired conditions of iron overload<sup>[4]</sup>, there are other conditions in which iron overload cannot be explained and the pathophysiology of iron accumulation is still obscure. Increased hepatic iron

deposits have been frequently described in association with overweight and alterations of lipid or glucose metabolism. These abnormalities are part of the metabolic syndrome (MS) that is closely linked to insulin resistance, affecting a large number of adults in Western countries, and is associated with non-alcoholic fatty liver disease (NAFLD), now considered the hepatic manifestation of MS<sup>[5]</sup>. This form of iron overload has been variably named dysmetabolic or insulin resistance hepatic iron overload syndrome (IR-HIO)<sup>[6]</sup>. However, the etiopathogenetic mechanisms that link metabolic abnormalities, insulin resistance and/or NAFLD to iron accumulation are still undefined. The fact that only a proportion of subjects with metabolic abnormalities are at risk for iron overload<sup>[7]</sup> suggests that other factors may be involved. In addition, the original definition of IR-HIO<sup>[6]</sup> includes hyperferritinemia with normal or high transferrin saturation, presence or absence of NAFLD, and even a single metabolic alteration among the following: body mass index (BMI) > 25 kg/m<sup>2</sup>, dyslipidemia and abnormal glucose metabolism. These criteria are generous compared with those more recently established to define the presence of MS<sup>[8,9]</sup>.

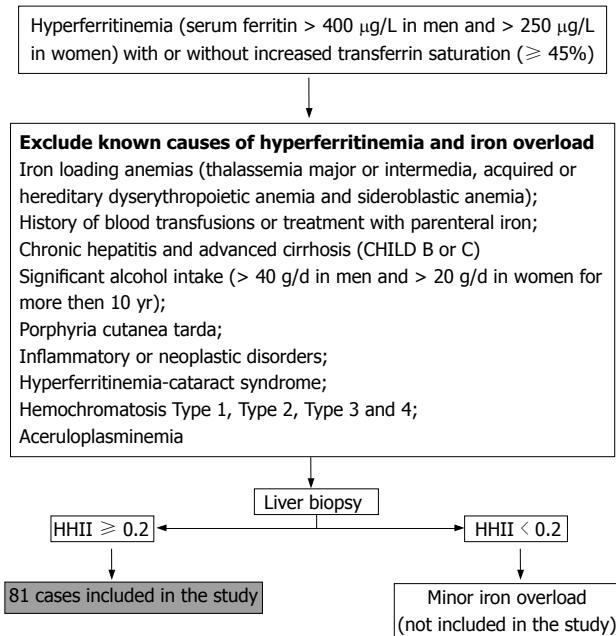
In the present study, we evaluated patients with alterations of serum iron indices and hepatic iron overload not explained by mutations in iron related genes or by acquired factors that can lead to iron overload through known pathogenetic mechanisms<sup>[3]</sup>. We defined this condition as hepatic iron overload (HIO) irrespective of the presence of metabolic alterations. Our aims were to obtain information in order to differentiate the variegated phenotypes in patients with HIO and to better characterize the dysmetabolic iron overload syndrome.

To this end, we carried out the following: (1) Analyzed iron status either at biochemical and histological levels. We hypothesized that the level of transferrin saturation and the cellular distribution of iron deposits in the liver might give insight into the mechanism leading to iron accumulation<sup>[3,10]</sup>; (2) Determined the frequency of abnormalities that are part of the MS (according to the more recent criteria), the frequency and severity of hepatic steatosis and their relation with iron indices. We hypothesized that the presence and the number of metabolic alterations might be associated with a peculiar iron phenotype; (3) Studied a control group of patients with HFE-related hemochromatosis (HFE-HH), taken as the model of primary iron overload, in order to compare hepatic iron distribution, the frequency of metabolic alterations and hepatic steatosis with those found in the group of patients with HIO.

## MATERIALS AND METHODS

### Materials

We studied 81 Italian unrelated adult patients (67 males and 14 females) with HIO. They were consecutively selected, since 1998, from outpatients who were referred to us for abnormalities of serum iron indices. Selection



**Figure 1** Selection criteria of the 81 patients included in the study. HHII: Histological Hepatic Iron Index [Total iron score/Age (years)].

criteria are reported in Figure 1.

To compare patients' data we also selected from our own database 57 patients affected by HFE-HH (51 were C282Y homozygotes, two were compound heterozygous for C282Y/E168X and four for C282Y/H63D). Criteria for selection of HFE controls were: availability of liver biopsy, semiquantitative iron grading and metabolic indices. All patients gave their written consent to liver biopsy, which was done for diagnostic and/or prognostic evaluation according to international guidelines<sup>[11]</sup>.

### Methods

All data were recorded at the time of diagnosis. All patients with HIO and HFE-HH underwent liver biopsy. Liver sections were stained with standard methods for histological evaluation and with Perl's stain for iron grading and assessed by an independent observer (G.B.).

### Biochemical and metabolic data

The following data were collected: glucose and insulin, total cholesterol, HDL, triglycerides, serum alanine and aspartate-aminotransferases, and  $\gamma$ -glutamyl transferase, which were measured using commercial kits, BMI, waist circumference and blood pressure. Overweight was defined by BMI > 25 kg/m<sup>2</sup> and < 30 kg/m<sup>2</sup> and obesity by BMI  $\geq$  30 kg/m<sup>2</sup><sup>[12]</sup>. Diagnoses of arterial hypertension, diabetes mellitus and glucose intolerance were based on the ESH/ESC and American guidelines, respectively<sup>[13,14]</sup>. The presence of dyslipidemia and MS was based on NCEP-ATP III criteria<sup>[8]</sup>. HOMA index was available in 68 patients (84%)<sup>[15]</sup>. A HOMA index of 2.77 was chosen to distinguish insulin resistant and insulin sensitive patients according to Bonora *et al*<sup>[16]</sup>.

### Iron status

Serum iron indices were determined using standard

**Table 1** Main clinical, biochemical and histological data of the HIO patients (as a whole and according to gender) and HFE controls

	HIO (n = 81)	P	HFE-HH (n = 57)	Men (n = 67)	P	Women (n = 14)
Age (yr)	52 (28-76)	> 0.05	50 (26-71)	54 (28-76)	> 0.05	51 (29-70)
Transferrin saturation (%)	39 (21-118)	< 0.0001	76 (29-100)	38 (21-118)	0.02	56 (28-99)
Serum ferritin ( $\mu\text{g/L}$ )	982 (413-5050)	> 0.05	1100 (274-10990)	964 (413-5050)	> 0.05	1286 (520-3987)
AST (U/L)	27 (12-174)	> 0.05	27 (17-87)	27 (12-174)	> 0.05	28 (13-65)
ALT (U/L)	38 (11-271)	> 0.05	43 (11-114)	38 (14-271)	> 0.05	36 (11-118)
Total Iron Score	19 (12-46)	0.0071	24 (8-49)	18 (12-40)	0.0006	32 (12-46)
Hepatocytic iron score	15 (6-27)	0.001	18 (3-27)	12 (6-27)	0.0022	21 (9-27)
Sinusoidal iron score	5 (3-8)	< 0.0001	3 (0-10)	5 (3-7)	> 0.05	6 (3-8)
Portal iron score	0 (0-12)	0.0002	2 (0-12)	0 (0-8)	< 0.0001	3.5 (0-12)
SIS/TIS	0.25 (0.09-0.5)	< 0.0001	0.13 (0-0.63)	0.25 (0.10-0.5)	0.001	0.16 (0.09-0.29)
HH II	0.38 (0.20-1.21)	0.0012	0.53 (0.15-0.97)	0.36 (0.20-1.08)	0.004	0.64 (0.23-1.21)
Hepatic fibrosis (Ishak's score)	34 (42)	> 0.05	33 (57.8)	26 (38.8)	> 0.05	8 (57.1)
Hepatic cirrhosis (Ishak's score)	7 (8.6)	0.027	13 (22.8)	3 (4.5)	0.015	4 (28.6)
Patients with hepatic steatosis	47 (58)	0.015	20 (36.3)	41 (61.2)	> 0.05	6 (42.9)
Patients with moderate-severe hepatic steatosis	35 (43.2)	< 0.0001	6 (10.5)	31 (46.3)	> 0.05	4 (28.6)
Metabolic syndrome	20 (24.7)	> 0.05	9 <sup>1</sup> (16.1)	19 (28.4)	> 0.05	1 (7.1)

Data are reported as median (range) or number (%). Hepatic steatosis: > 5% of hepatocytes involved by steatosis; Moderate-severe steatosis: ≥ 33% of hepatocytes involved by steatosis. <sup>1</sup>Not available in one HFE-control. SIS/TIS: Sinusoidal/total iron score; HHII: Histological hepatic iron index (ratio of TIS to age).

methods. Transferrin saturation was calculated as follows: serum iron/(serum transferrin  $\times$  1.4)  $\times$  100<sup>[17]</sup>. A semi-quantitative evaluation of hepatic iron was done according to Deugnier *et al*<sup>[18]</sup>. Iron deposits were assessed according to size, cellular and lobular locations in Rappaport's acinus, leading to three different scores: hepatocytic (HIS; range, 0-36), sinusoidal (SIS; range, 0 to 12) and portal iron scores (PIS; range, 0-12). The sum of these scores defined the total iron score (TIS; range, 0-60). Previous studies have shown that age-related histological hepatic iron index (the ratio of TIS to age, HHII) is an accurate means of predicting the genetic status of HH patients (heterozygotes *vs* homozygotes)<sup>[19]</sup>. Accordingly, all the patients with HIO and HFE-HH had a histological hepatic iron index in the range of homozygous HH.

### Hepatic fibrosis and steatosis

Hepatic fibrosis was graded according to Ishak *et al*<sup>[20]</sup>. Hepatic steatosis was diagnosed when at least 5% of hepatocytes were involved and steatosis was graded as follows: absence ( $\leq 5\%$  of hepatocytes), mild ( $> 5\% \leq 33\%$ ), moderate ( $> 33\% \text{ and } \leq 66\%$ ), severe ( $> 66\%$ )<sup>[21]</sup>.

### Statistical analysis

Data were expressed as median and range due to the non-Gaussian distribution of iron indices. The Student's *t* test was used for quantitative variables with a normal distribution. All the comparisons involving hepatic iron distribution according to Deugnier *et al*<sup>[18]</sup> and variables with non-skewed distributions were performed on medians by non-parametric methods (Mann-Whitney or Kruskall-Wallis when more than two groups were involved). The Fisher's exact test or the Chi-square test was used for qualitative data. Correlations between variables were evaluated by the Spearman's test. The

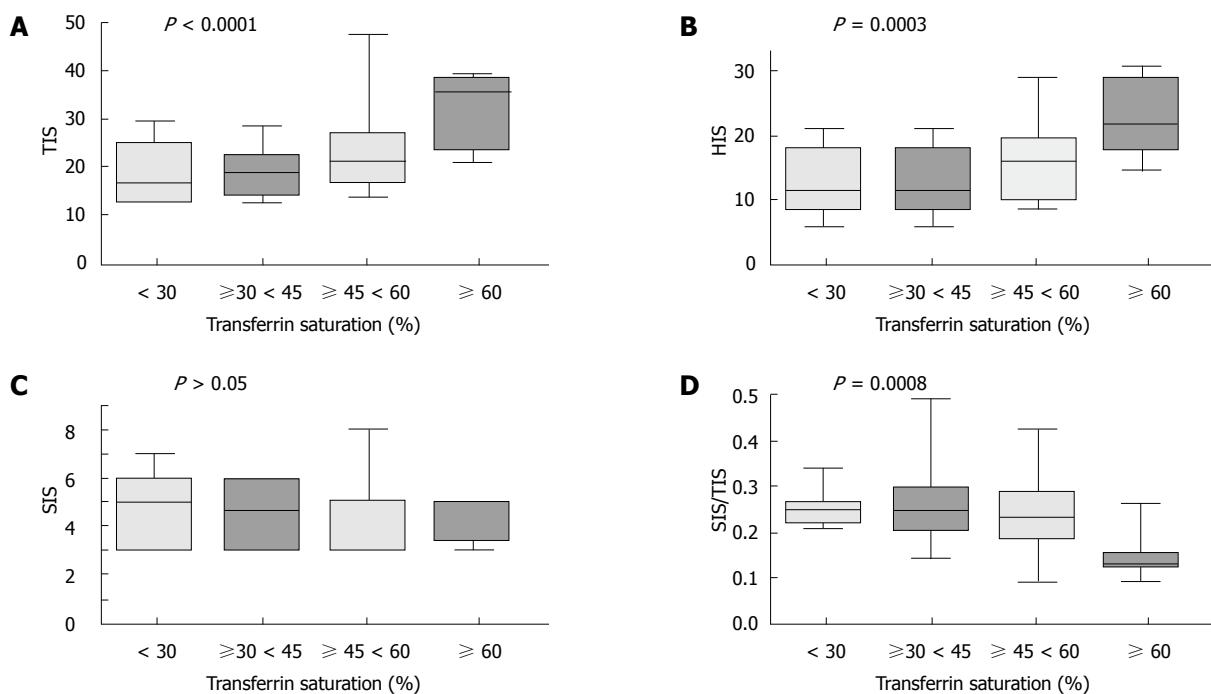
receiving operating curve (ROC) was used to evaluate if a threshold number of MS components was associated with different hepatic iron overload as defined by the HHII. We compared the Area under the curve (AUC) of the ROCs relative to 1, 2, 3, 4 and 5 components of the MS according to HHII. The influence of age, gender, alcohol intake, BMI, serum iron indices and hepatic iron distribution (HIS, SIS and PIS) on hepatic fibrosis, was evaluated by a logistic regression model. All tests were two-sided and with a significance level of  $P \leq 0.05$ .

## RESULTS

### Iron status

Seven patients (8.6%) were heterozygous the C282Y and 27 (33.3%) the H63D mutation. Table 1 shows the main clinical, biochemical and histological data of patients with HIO (as a whole and according to gender) and with HFE-HH.

Thirty patients (37%) had transferrin saturation repeatedly  $\geq 45\%$  and 39 patients (48%) had serum ferritin  $> 1000 \mu\text{g/L}$ . All patients showed homogeneous hemosiderin deposits in the hepatic lobules and a decreasing hepatocytic iron gradient from Rappaport I to III. In this series, females had more iron overload and a higher frequency of cirrhosis, lower BMI ( $P = 0.0007$ ) and a number of MS components more than males. TIS correlated with age in males ( $r = 0.4$ ,  $P = 0.0007$ ), but not in females. TIS significantly correlated with several indices, as reported in Table 2, showing an inverse correlation with metabolic indices. At linear regression analysis, gender ( $P = 0.0005$ ), grade of fibrosis ( $P = 0.0005$ ), transferrin saturation ( $P < 0.0001$ ) and severity of steatosis ( $P < 0.0001$ ) retained statistical significance with TIS.



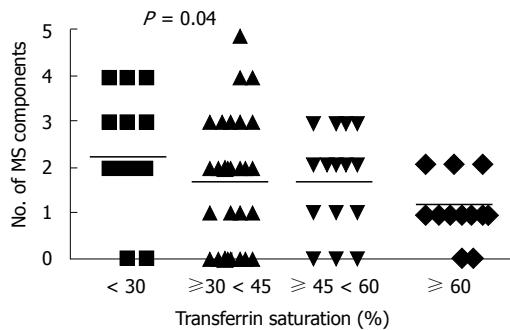
**Figure 2** Total iron score (TIS,  $P < 0.0001$ ), hepatocytic iron score (HIS,  $P = 0.0003$ ), sinusoidal iron score (SIS,  $P > 0.05$ ) and sinusoidal to total iron score ratio (SIS/TIS,  $P = 0.0008$ ) according to transferrin saturation. The box includes all the observations between the first and the third quartile, the horizontal bar represents the median. Whiskers extend from the edges of the box to the extreme values.

**Table 2 Correlation between total iron score (TIS) and several parameters in HIO patients**

	<i>r</i>	<i>P</i>
Hepatocytic iron score	0.94	< 0.0001
Sinusoidal iron score	0.54	< 0.0001
Portal iron score	0.58	< 0.0001
Serum ferritin ( $\mu\text{g/L}$ )	0.53	< 0.0001
Transferrin saturation (%)	0.43	< 0.0001
Age (yr)	0.23	0.04
Hepatic fibrosis	0.28	0.012
Body mass index ( $\text{kg}/\text{m}^2$ )	-0.48	< 0.0001
Degree of hepatic steatosis	-0.53	< 0.0001
No. of components of MS	-0.33	0.0026
HOMA index	-0.3	0.0125

Degree of hepatic steatosis refers to the percentage of hepatocytes involved by steatosis. Hepatic fibrosis refers to Ishak's score.

Dividing HIO patients according to transferrin saturation, we observed that TIS, HIS and PIS significantly increased with the increasing transferrin saturation. By contrast, SIS did not change, whereas the SIS to TIS ratio significantly decreased (Figure 2). We observed that the higher the transferrin saturation level, the lower was the number of MS components (Figure 3). Patients with transferrin saturation  $< 45\%$  ( $n = 51$ ) had lower amount of iron overload in hepatocytic ( $P = 0.0004$ ) and portal ( $P = 0.0007$ ) compartments than patients with increased transferrin saturation but with a proportionally greater amount of iron in sinusoidal compartment (SIS/TIS:  $0.25 \pm 0.07$  vs  $0.2 \pm 0.08$ ,  $P = 0.003$ ). These patients also had a lower prevalence of fibrosis (Ishak's score  $\geq 1$ :  $16/51$  vs  $18/30$ ,  $P = 0.019$ ). Patients with the highest levels of transferrin saturation



**Figure 3** Number of MS components by transferrin saturation ( $P = 0.04$ ).

( $\geq 60\%$ ) had a SIS to TIS ratio (median, 0.15; range, 0.1-0.25) not different than that observed in HFE-HH (median, 0.13; range, 0-0.63). They also had a prevalence of hepatic steatosis and hepatic fibrosis comparable to that observed in patients with HFE-HH (data not shown) but without MS.

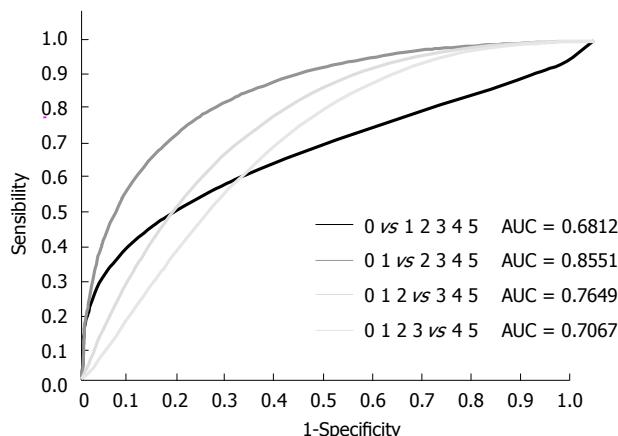
#### Metabolic alterations

One patient (1.2%) had all five components of MS, five (6.2%) had four components, 14 (17.2%) had three, 27 (33.3%) had two, 17 (20.9%) had one and 17 (20.9%) had none. The frequency and severity of hepatic steatosis and MS prevalence in HIO are reported in Table 1 and are compared with those observed in HFE-HH patients. Thirty-one patients with HIO (45.6%) were insulin resistant. Steatosis was more common in Rappaport zones 2 and 3 and more prevalent in HIO patients with MS than in those without (90% vs 47.5%,  $P = 0.0007$ ). The number of MS components correlated positively with age, severity of steatosis and

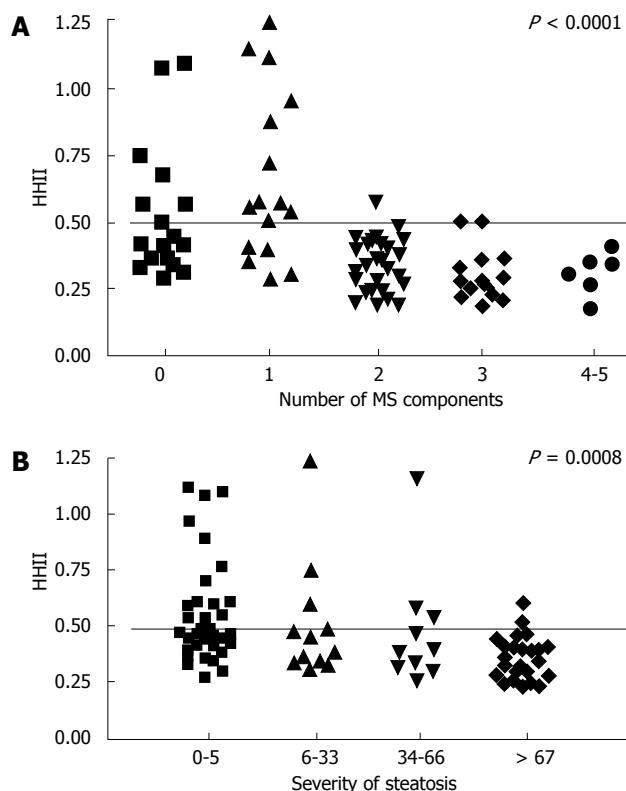
**Table 3** Correlation between number of MS components and several parameters in HIO patients

	<i>r</i>	<i>P</i>
Age (yr)	0.3	0.0055
Degree of hepatic steatosis (%)	0.57	< 0.0001
HOMA index	0.59	< 0.0001
Transferrin saturation (%)	-0.25	0.0293
Total iron score	-0.33	0.0053
Histological hepatic iron index	-0.53	< 0.0001
Sinusoidal iron score/Total iron score	0.13	> 0.05

Degree of hepatic steatosis refers to the percentage of hepatocytes involved by steatosis.



**Figure 4** Receiving operating curve relative to 1, 2, 3, 4 and 5 components of the MS.



**Figure 5** Distribution of HHII (histological hepatic iron index) according to number of MS components (A) and the degree of hepatic steatosis (B).

**Table 4** Iron indices in patients according to the presence of  $\geq 2$  components of MS or steatosis

	Number of MS components		Hepatic steatosis			
	0-1 ( <i>n</i> = 34)	<i>P</i>	$\geq 2$ ( <i>n</i> = 47)	Absent ( <i>n</i> = 34)	<i>P</i>	Present ( <i>n</i> = 47)
Transferrin saturation (%)	44 (28-118)	0.0058	36 (21-99)	39 (23-118)	> 0.05	39 (21-76)
Total Iron Score	24 (12-46)	0.0008	17 (12-39)	24.5 (12-46)	< 0.0001	16 (12-39)
HHII	0.51 (0.3-1.21)	< 0.0001	0.33 (0.2-0.57)	0.43 (0.23-1.1)	0.0004	0.34 (0.19-1.2)
SIS/TIS	0.21 (0.1-0.5)	0.0185	0.25 (0.11-0.4)	0.22 (0.1-0.5)	0.032	0.25 (0.09-0.4)
MS components $\geq 2$	-	-	-	11 (32)	< 0.0001	36 (77)
Degree of hepatic steatosis (%)	0 (0-80)	< 0.0001	40 (0-98)	-	-	-

Data are reported as median (range) or number (%). HHII: Histological hepatic iron index (ratio of TIS to age); SIS/TIS: Sinusoidal/total iron score. Hepatic steatosis, absent when  $\leq 5\%$  of hepatocytes are involved; present when at least 5% of hepatocytes are involved.

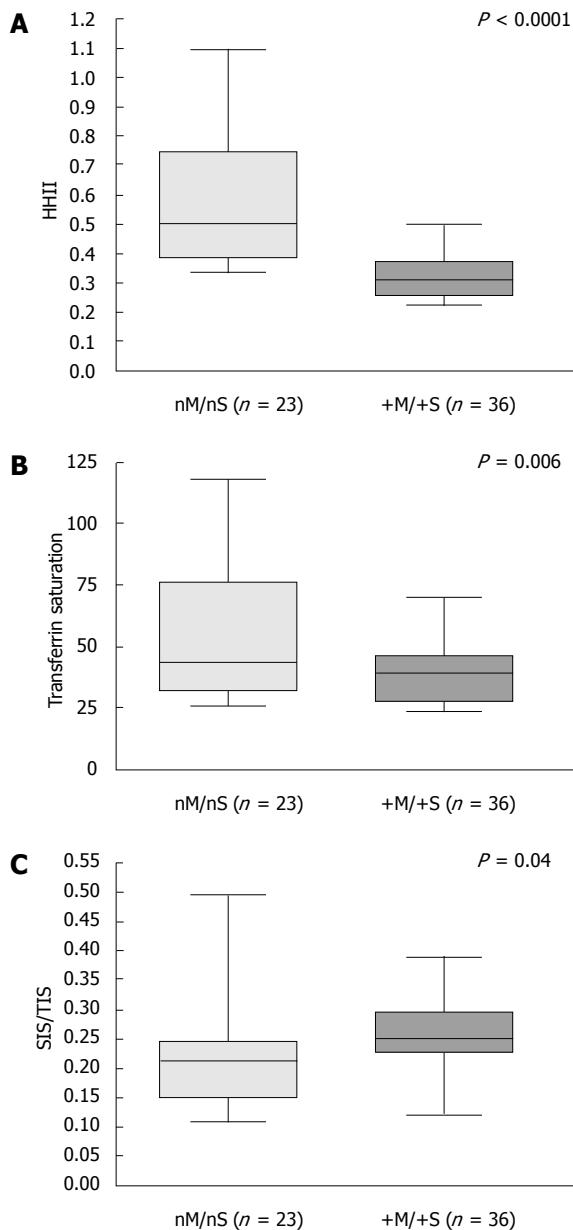
HOMA index, and inversely with transferrin saturation and hepatic iron indices, but not with serum ferritin (Table 3).

By the ROC analysis (Figure 4), we found that the presence of two MS alterations best differentiates HIO patients according to HHII ( $P < 0.0001$ ). Patients with 0-1 MS components showed a wide range of HHII from low to very high, whereas in patients with two or more MS features the HHII was  $\leq 0.50$  in all but one (Figure 5A). Similar findings were observed when patients were divided according to the presence of steatosis (Figure 5B). In addition, the percentage of hepatocytes involved by steatosis inversely correlated with HIS ( $r = -0.52$ ,  $P < 0.0001$ ) and with the amount of iron removed by phlebotomies ( $r = -0.45$ ,  $P = 0.0019$ ).

Table 4 compares iron indices, in patients according to the presence of  $\geq 2$  components of MS or steatosis. Figure 6 compares the same data in two subgroups: patients with a “metabolic phenotype” (+M/+S, patients with  $\geq 2$  MS alterations and hepatic steatosis) and patients without (nM/nS, patients with 0-1 MS components and absence of hepatic steatosis).

#### Hepatic damage in HIO patients

Data on hepatic damage in HIO patients are summarized in Table 1. TIS correlated with fibrosis ( $r = 0.28$ ,  $P = 0.01$ ), but PIS showed the highest correlation ( $r = 0.51$ ,  $P < 0.0001$ ) and SIS none. Logistic regression modelling indicated that PIS, age, HOMA index and presence of steatosis independently correlated with hepatic fibrosis



**Figure 6** Distribution of HHII (histological hepatic iron index), transferrin saturation and Sinusoidal/Total Iron Score (SIS/TIS) in patients with 0-1 MS components and absence of steatosis (nM/nS) and patients with 2 MS alterations and steatosis (+M/+S).

( $P < 0.0001$ ,  $P = 0.004$ ,  $P < 0.0001$  and  $P = 0.04$ , respectively).

## DISCUSSION

In the present study, we evaluated 81 patients with hepatic iron overload not explained by known genetic or acquired disorders. HIO as expected, patients showed a wide range of variability in the biochemical iron status, in hepatic iron deposits and lobular iron distribution, and had a variable number of metabolic alterations that are part of the MS, with or without NAFLD. There was an inverse relationship between transferrin saturation and sinusoidal iron. Patients with transferrin saturation  $< 45\%$  had a lower amount of iron overload in the hepatocytic and portal compartments than

patients with increased transferrin saturation, but have a proportionally greater amount of iron in sinusoidal compartment as shown by the higher SIS to TIS ratio. Accordingly, when compared to HFE-controls, patients with transferrin saturation  $< 45\%$  had higher SIS and SIS to TIS ratios (data not shown). These findings indicate that, in HIO patients with normal transferrin saturation, the mechanism of hepatic iron accumulation is different than that of the classical HH<sup>[1,2]</sup> and suggest the existence of macrophage iron retention and an iron recycling defect. By contrast, in patients with increased transferrin saturation, hepatic iron distribution was comparable to that observed in HFE-HH, thus supporting the idea that the pathogenetic mechanism of iron overload was due to increased iron absorption<sup>[22]</sup>. The strongest identity with HFE-HH was observed in patients with the highest transferrin saturation ( $\geq 60\%$ ), suggesting that between 45% and 60% there is a grey zone that includes mixed conditions. In addition, patients with transferrin saturation  $\geq 60\%$  had the same prevalence of hepatic steatosis and of organ damage as HFE-HH, however, none had MS. Thus, transferrin saturation can distinguish, among HIO patients, two main subgroups with likely different pathogenetic mechanisms of iron overload.

Another part of the study concerns the metabolic aspects and their relation with serum and hepatic iron indices. First, in agreement with the theory that NAFLD is the hepatic manifestation of the MS, we found that the number of MS components correlated with the severity of hepatic steatosis and hyperinsulinemia and that 90% of the patients with the MS had NAFLD<sup>[5]</sup>. Second, we found that the presence of metabolic abnormalities and/or NAFLD was associated with a peculiar iron phenotype characterised by a lower hepatic iron overload, a more prevalent iron in the sinusoidal compartment and a lower transferrin saturation that corresponds to the more typical features of IR-HIO<sup>[6]</sup>. We demonstrated that this phenotype was common in patients presenting at least two of the MS features and in the subgroup with  $\geq 2$  MS abnormalities and NAFLD.

An inverse relation between steatosis and hepatic iron has been previously observed and variably explained<sup>[23-25]</sup>. The significant inverse correlation that we found between the degree of steatosis and the amount of iron removed by phlebotomies provides the strongest evidence that the higher the percentage of hepatocytes involved by steatosis, the lower the amount of iron accumulation. Recent studies in severely obese patients<sup>[26]</sup> and in an animal model of insulin resistance<sup>[27]</sup> suggest that obesity or hyperinsulinemia favour the development of iron deficiency. Increased adipocyte hepcidin production has been reported in morbid obesity that could result in reduced intestinal iron absorption, macrophage iron release and eventually in low iron stores<sup>[26]</sup>. Although these findings need to be further elucidated and confirmed, they support the hypothesis that metabolic abnormalities associated with insulin resistance and MS might protect from iron overload. Accordingly, in C282Y homozygous patients, obesity (in

females)<sup>[28]</sup> and hepatic steatosis<sup>[29]</sup> were associated with a decreased transferrin saturation<sup>[28]</sup> and hepatic iron concentration<sup>[28,29]</sup>. It was suggested that the overweight-related chronic inflammatory state may increase hepcidin production (likely at extrahepatic sites) and partially compensates for the defect of hepcidin synthesis due to the HFE mutation<sup>[7,28]</sup>. We recently found that urinary hepcidin levels, although inappropriate for the iron overload, were indeed significantly higher in patients with dysmetabolic iron overload than in controls<sup>[30]</sup>. Overall, these findings seem to contradict the hypothesis that iron overload and metabolic abnormalities are linked by a common pathogenetic mechanism in IR-HIO, unless a hepcidin-resistance state is hypothesized. Another possible explanation is that IR-HIO is a multifactorial disease that results from the association of a mild-moderate, maybe polygenic, defect of hepcidin production and insulin resistance or MS. This may explain some of the features of the disease: late-onset and mild-moderate iron overload tending to a plateau<sup>[31]</sup>. As observed in subjects carrying the low expressing C282Y/H63D HFE genotype<sup>[32]</sup>, these patients may retain some ability to increase hepcidin production in response to iron load that possibly explains their slight phenotype. In patients with dysmetabolic iron overload, NAFLD and MS might foster hepcidin production leading to the typical IR-HIO phenotype. Further studies are needed to clarify this issue and the role of dysmetabolisms in dysregulating iron regulatory pathways. This is not unimportant considering that the dysmetabolic iron overload syndrome is currently the most prevalent iron overload disorder in the adult population.

Based on our findings and in the absence of a clear pathogenetic explanation of IR-HIO, we suggest a more strict clinical definition of this syndrome characterised by the presence of two or more components of the MS, hepatic steatosis and normal transferrin saturation. Liver biopsy should confirm the presence of steatosis and iron overload with typical involvement of sinusoidal compartment. In fact, in agreement with other studies<sup>[23,24,33]</sup>, we showed that serum ferritin is not a reliable index of iron overload in patients with metabolic abnormalities and might overestimate the true amount of hepatic iron overload due to NAFLD-related hepatocellular damage, local cytokine activation or higher mesenchymal iron retention<sup>[7]</sup>. Noninvasive methodologies, such as magnetic resonance (MR) for iron quantification, can be an alternative option when liver biopsy is not feasible, provided the MR apparatus is calibrated and validated<sup>[34]</sup>.

Finally, we showed that a number of HIO patients presented a classical hemochromatosis phenotype (high transferrin saturation and prevalent hepatocytic iron overload). Some of them, including a few young women, had a severe iron overload that lead to clinical complications. We had no etiopathogenetic explanation for this form of iron overload, which may represent the severe boundary of a complex trait due to gene-gene and gene-environmental interactions, or the result

of a still undefined major locus defect. Studies aimed to clarify the genetic background and the complex pathways causing these forms of unexplained iron overload are needed to improve the diagnostic setting of iron overload disorders.

## COMMENTS

### Background

Hepatic iron overload includes a wide spectrum of conditions from mild to marked tissue iron accumulation. In some cases the pathophysiology of iron accumulation is still obscure. The association of hepatic iron overload and metabolic alterations and/or non-alcoholic fatty liver is common in Western population. A new syndrome, named Dysmetabolic Hepatic Iron Overload or Insulin Resistance Hepatic Iron Overload was described in 1999, based on the presence of hepatic iron overload in association with even a single metabolic abnormality. These criteria were generous compared to those more recently established for the metabolic syndrome. In addition, the pathogenetic mechanisms that link insulin resistance and/or steatosis to iron overload are still undefined.

### Research frontiers

To characterize clinical, biochemical and histological features of hepatic iron overload not explained by known genetic or acquired disorders, including the form associated to metabolic alterations. To understand the pathogenetic mechanisms of hepatic iron accumulation. To characterize the phenotype of the dysmetabolic iron overload syndrome, according to the more recent criteria defining the metabolic syndrome, and the presence of fatty liver disease.

### Innovations and breakthroughs

We demonstrated that patients with hepatic iron overload, not explained by known genetic or acquired disorders, need a systematic diagnostic approach, including the evaluation of hepatic iron overload and cellular iron distribution. We suggested the existence of two main different pathogenetic mechanisms leading to forms of unexplained iron overload: an iron recycling defect and an increased intestinal iron absorption. We suggested a new, updated definition of the dysmetabolic iron overload syndrome. We speculated on the pathogenesis of the iron overload syndrome based on our results and current knowledge of iron metabolism.

### Applications

This study may improve the diagnostic approach in patients with unexplained forms of iron overload including the dysmetabolic iron overload syndrome. This is not trivial considering that the latter disorder is currently the most prevalent iron overload disease in adult population. In addition, our findings indicate simple and valid criteria to better classify these forms of iron overload. This will be useful: (a) to better understand the etiopathogenesis of these disorders and the dysregulation of iron homeostasis in future studies; (b) to better address therapeutic strategies, e.g. iron depletion vs treatment of metabolic abnormalities and hepatic steatosis.

### Peer review

In this manuscript authors give a different angle to a problem that has interested the scientific world recently. The study is well conducted.

## ACKNOWLEDGMENTS

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# Prevention of hepatotoxicity due to anti tuberculosis treatment: A novel integrative approach

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## Abstract

**AIM:** To evaluate the ability of *Curcuma longa* (CL) and *Tinospora cordifolia* (TC) formulation to prevent anti-tuberculosis (TB) treatment (ATT) induced hepatotoxicity.

**METHODS:** Patients with active TB diagnosis were randomized to a drug control group and a trial group on drugs plus an herbal formulation. Isoniazid, rifampicin, pyrazinamide and ethambutol for first 2 mo followed by continuation phase therapy excluding Pyrazinamide for 4 mo comprised the anti-tuberculous treatment. Curcumin enriched (25%) CL and a hydro-ethanolic extract enriched (50%) TC 1 g each divided in two doses comprised the herbal adjuvant. Hemogram, bilirubin and liver enzymes were tested initially and monthly till the end of study to evaluate the result.

**RESULTS:** Incidence and severity of hepatotoxicity was significantly lower in trial group (incidence: 27/192 vs 2/316,  $P < 0.0001$ ). Mean aspartate transaminase (AST) ( $195.93 \pm 108.74$  vs  $85 \pm 4.24$ ,  $P < 0.0001$ ), alanine transaminase (ALT) ( $75.74 \pm 26.54$  vs  $41 \pm 1.41$ ,  $P < 0.0001$ ) and serum bilirubin ( $5.4 \pm 3.38$  vs  $1.5 \pm 0.42$ ,  $P < 0.0001$ ). A lesser sputum positivity ratio at the end of 4 wk (10/67 vs 4/137,  $P = 0.0068$ ) and decreased incidence of poorly resolved parenchymal lesion at the end of the treatment (9/152 vs 2/278,  $P$

= 0.0037) was observed. Improved patient compliance was indicated by nil drop-out in trial vs 10/192 in control group ( $P < 0.0001$ ).

**CONCLUSION:** The herbal formulation prevented hepatotoxicity significantly and improved the disease outcome as well as patient compliance without any toxicity or side effects.

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**Key words:** Hepatoprotection; Anti-tuberculous treatment; Curcumin longa; Tinospora cordifolia

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## INTRODUCTION

During the first few years of introduction of Isoniazid (INH), it was considered so safe that in 1963, The American Thoracic Society recommended for all tuberculin-positive persons to receive a year of INH chemoprophylaxis regardless of age or duration of the tuberculin positivity<sup>[1]</sup>. A retrospective study reported a 1% incidence of clinical hepatitis and a 0.1 % death in patients taking INH chemoprophylaxis<sup>[2]</sup>. A large multicenter prospective surveillance study by United States Public Health Service revealed a 1% incidence of hepatitis and 0.06% deaths from hepatitis due to INH<sup>[3]</sup>. After introduction of Rifampicin (RMP), several reports suggested that hepatitis was more frequent and severe in patients receiving both INH and RMP than in those receiving INH alone<sup>[4]</sup>. For preventing acquired

resistance and a successful treatment; it is recommended to start with a combination chemotherapy containing INH, RMP, and Pyrazinamide (PZA)-one more hepatotoxic agent- with or without ethambutol for the initial 2 mo followed by a continuation phase of 4-6 mo of INH + RMP<sup>[5]</sup>. It is well known that drug induced hepatotoxicity is a potentially serious adverse effect of anti-tuberculosis treatment (ATT) containing INH, RMP and PZA<sup>[6]</sup>. Preventive therapy of latent TB with 2-mo course of RMP and PZA has been associated with fatal and severe hepatotoxicity more often than does 6 mo of INH therapy or curative treatment of clinical TB<sup>[7-9]</sup>. Similar high risk was observed with PZA and ethambutol or a fluoroquinolone when given to contacts of multi-drug resistant TB patients<sup>[10]</sup>. Now integrating these observations with the fact that about one third of the world's population has latent TB and roughly 9 million cases of active TB emerge annually resulting in 2-3 million deaths<sup>[11]</sup>, exemplifies the magnitude and importance of the problem, especially when most new cases occur in the most populated nations like India and China<sup>[12]</sup>. Also, a higher risk of hepatotoxicity has been reported in Indian patients (up to 11.5%) than in their western counterpart (up to 4.3%)<sup>[4]</sup>. In a study of European patients, the incidence of ATT induced hepatotoxicity was found to be 18.2% in group having risk factors like, old age, extensive TB, malnutrition, alcoholism, HIV and chronic viral hepatitis B and C infections, as against 5.8% in group without risk factors indicating the significance of risk factors<sup>[13]</sup>.

The only measure available for managing ATT induced hepatotoxicity in clinical cases is stopping the offending agents, once there is an evidence of liver damage and reintroducing the same after normalization of liver enzymes<sup>[14,15]</sup>. To reduce the incidence of hepatotoxicity in latent TB patients, recommendations for drugs and patient selection criteria have been revised several times by organizations like Center for Disease Control, American Thoracic Society, Joint Tuberculosis Committee of British Thoracic Society, and Hong Kong Tuberculosis Service *etc.*, but until today no drug has been developed for prevention of hepatotoxicity.

The pathogenesis of hepatotoxicity is not entirely clear, but INH and RMP induced damage may involve oxidative stress<sup>[16]</sup>, lipid peroxidation<sup>[17]</sup>, choline deficiency leading to lowering of phospholipids protein synthesis with alteration in cell wall configuration<sup>[18]</sup>, reduced glutathione level<sup>[19]</sup> and activation of CYP2E1<sup>[20]</sup>. It is well known that some non-toxic herbs are having opposite activities in the form of membrane stabilizing, anti-oxidative and CYP2E1 inhibitory effects<sup>[21]</sup>. A review of available literature suggests that reduction in lipid peroxide content in tissue and increase in superoxide dismutase, catalase, glutathione, glutathione-s-transferase and glutathione peroxidase activities should help to maintain liver cell integrity and control the increase in level of liver enzymes.

In a previous preclinical study we found *Curcuma longa* (CL) and *Tinospora cordifolia* (TC) to offer protection in guinea pig model of ATT induced

hepatotoxicity<sup>[22]</sup>. Both these herbs have an excellent safe toxicological profile<sup>[23,24]</sup>. Phase-I clinical trials on curcumin showed that it is safe to humans up to 12000 mg/d when taken orally<sup>[25,26]</sup>. Several animal experiments for various activities of TC did not reveal any toxicity at dosage as high as 400 mg/kg while no adverse reactions have been noted in international adverse reaction database in spite of several clinical trials and wide spread usage in Ayurvedic system of medicine<sup>[27]</sup>. So it was logical and ethical to conduct a randomized controlled clinical trial to evaluate the efficacy of CL and TC to control the hepatotoxic episodes in patients diagnosed to have TB and undergoing ATT. The primary aim of the present study was to estimate to what extent CL and TC addition in the standard regime affects hepatotoxicity profile and the secondary being whether the well known immunomodulatory and tonic activity of these herbs have any impact on the outcome of TB itself. To the best of our knowledge, such a clinical trial using hepatoprotective herbs as an adjuvant medicine to prevent ATT induced hepatotoxicity has not been performed anywhere.

## MATERIALS AND METHODS

### **Study population**

All the cases between the ages of 15 to 85 years having evidence of pulmonary or extra-pulmonary TB requiring a full curative course of ATT and coming to the clinic run by Shree Gurudev Sarvajanik Charitable Trust (SGSCT) from April 2005 to March 2007 were screened for enrollment in the trial with reservations as shown in Table 1. As the six centers of the mobile clinic were attending patients on a weekly basis for any illness, catering more than 200 villages of the District Surat and adjoining state Maharashtra, the local primary care agencies were advised and allowed to give routine treatment for inter-current illnesses and patients were advised to inform about the episodes and treatment given.

As shown in Figure 1, out of 578 patients screened 528 were found eligible. Periodic review was done by an independent review committee at 48, 100, 148 and 200 patients' recruitment in each group. The interim analysis of first 400 patients performed in October, 2006 revealed zero incidence of hepatotoxicity in the trial as against 22 patients with any grade of hepatotoxicity and 16 patients with no improvement in functional status in control group. The trial was granted with an intention to treat and as the criteria for outcome assessment were objective and as the sample size exceeded the WHO criteria for adequacy of sample size with power > 80%, the control arm was truncated on ethical ground and later all the patients were recruited in trial arm till March, 2007; only to find out whether any case of hepatotoxicity was encountered.

During their baseline visit, patients underwent a review of symptoms that included history of nausea, vomiting, jaundice, abdominal pain, weight loss, arthralgia, headache and neuropathy.

**Table 1** Protocol for selection of patients for recruitment

Protocol for selection of patients for recruitment	
Criteria for selection	
Evidence of active pulmonary or extra-pulmonary tuberculosis	
Age between 15 and 85 years	
Readiness to comply with randomization, treatment and follow-up protocols	
Patients giving written informed consent	
Absence of diseases warranting treatment with systemic steroids, antimetabolites or warfarin	
Concomitant conditions allowed	
Patients with relapse, multiple relapse, treatment failure of DOTS regimen	
Patients with hypertension, diabetes mellitus, COPD (chronic obstructive pulmonary disease) with continuation of required medications	
Patients with asthma on inhalational steroid	
Skin conditions requiring topical small area steroid application	
Patients with HIV positivity but without symptoms suggestive of AIDS	
Hepatitis B/C virus carrier	
Criteria for rejection	
Presumptive diagnosis or lack of evidence of active tuberculosis	
Age < 15 or > 85	
Patients taking other alternative therapies for tuberculosis	
Patients declining to give consent or comply with protocol	
Pregnant females	
Heavy alcoholism history, > 80 g of alcohol/d for males and > 20 g of alcohol/d for females <sup>[50]</sup>	
Concomitant conditions not allowed	
Preexisting liver disease with AST, ALT raised > twice upper normal, evidence of portal hypertension.	
Preexisting renal disease with S. Creatinine raised > twice upper normal	
Sickle cell disease with history of crisis, anemia and jaundice	
History of gout	
Recent drop-outs from other TB centers due to complications and side effects	
Patients on steroid and/or antimetabolite for other collagen, autoimmune or neoplastic diseases	

**Table 2** Chemical constituents of formulation

Chemical constituents of formulation with active principles	
Chemical constituents of <i>curcuma longa</i> with active principles	
Curcuminoids	Desmethoxycurcumin Bisdesmethoxycurcumin Curcumin
Turmerones	Ar-turmerone $\alpha$ -turmerone $\beta$ -turmerone
Curcumenes	$\gamma$ -Curcumene, ar-Curcumene, Dehydrocurcumene zingiberene $\beta$ -bisabolene $\beta$ -sesquiphellandrene
Miscellaneous	Terpinolene, P-Cymene, 1-8-Cineole, Currone
Chemical constituents of <i>Tinospora cordifolia</i> with active principles	
Alkaloids	Berberine, Palmatine,Tembatarine, Magnoflorine, Choline,Tinosporin, Isocolumbin, Tetrahydropalmatine
Glycosides	18-norclerodane glycoside, Furanoid diterpene glucoside, Tinocordiside,Tinocordifolioside, Syringin, Syringin-apiosylglycoside, Palmatosides C, PalmatosidesP Cordifolioside A, Cordifolioside B Cordifolioside- A, -B, -C, -D and -E Furanolactone, Clerodane derivatives,
Diterpenoid	
Lactones	Tinosporon, Tinosporides, Jateorine, Columbin
Steroids	$\beta$ -sitosterol, $\delta$ -sitosterol, 20 $\beta$ -hydroxyecdysone Ecdysterone, Makisterone A, Giloinsterol.
Sesquiterpenoid	Tinocordifolin
Aliphatic compounds	Octacosanol, Heptacosanol, Nonacosan-15-one
Miscellaneous compounds	3, ( $\alpha$ , 4-dihydroxy-3-methoxy-benzyl)-4-(4-hydroxy-3-methoxy-benzyl)- tetrahydrofuran, Jatrorrhizine, Tinosporidine, cordifol, cordifelone N-trans-feruloyl tyramine as diacetate, Giloin, Giloinin

### Herbal formulation

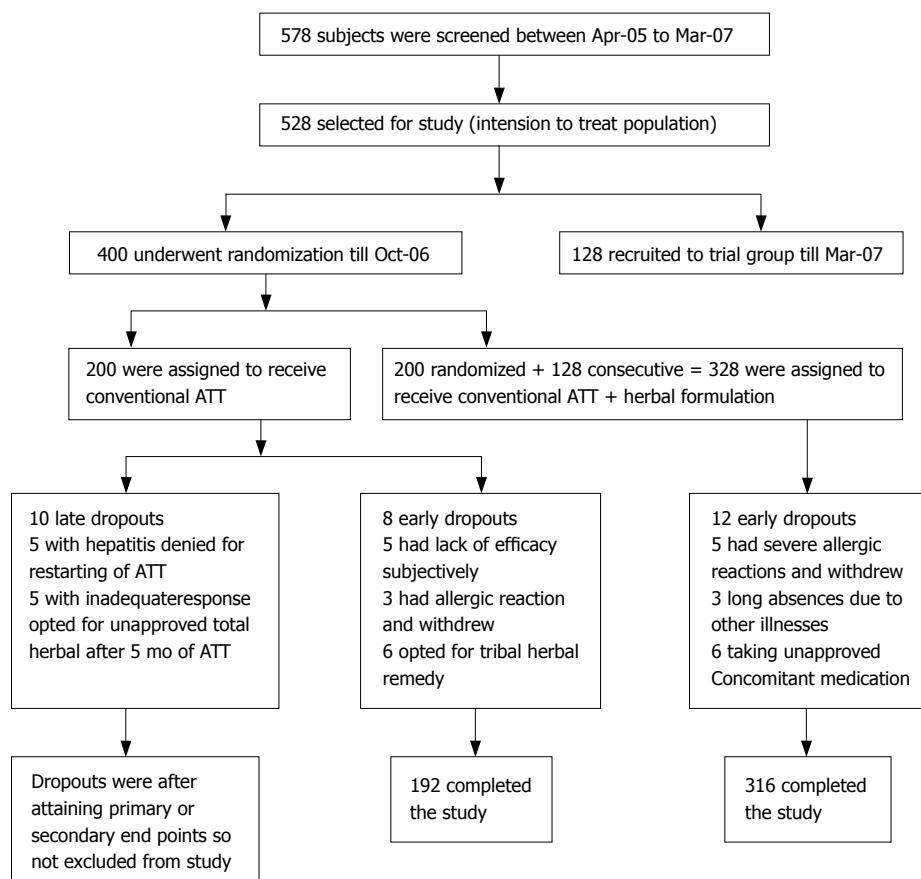
By high performance thin layer chromatography (HPTLC) analysis of different samples, we found that Salem types of turmeric powder contained highest 4% of curcuminoids that are the main active molecules. In Ayurvedic literature, the dose for turmeric has been described to be 6-12 g/d<sup>[28]</sup>. Our patient population being malnourished with an average wt of 38 kg, we preferred equivalent of 6 g/d by enriching the turmeric powder curcumin content to 25% so that 1 g dose would suffice. The dose of *Tinospora cordifolia* is also described to be 6-12 g/d<sup>[29]</sup> and again we preferred to make 1 g/d equivalent of 6 g/d by enriching the crude *Tinospora cordifolia* with 10:1 strength hydro-ethanolic extract to 50%. The phytochemical groups with active principles of both the herbs are shown in Table 2<sup>[30-32]</sup> and HPTLC fingerprinting of the formulations used were done for standardization purpose and have been shown in Figure 2.

### Study design

Ninety percent of all the patients were tribal, either working as laborers or owner of small piece of

agricultural land, while 10% were belonging to suburban area and belonging to other casts and occupations. Hence patients were explained in their local language about the protocol and those agreeing to comply were assigned the group by permuted block randomization for a balanced distribution. Because the evaluation criteria were fully objective and as the biochemical assessments were done in laboratories at a distance by unknown technicians, patients were not blinded conventionally about the therapy. Being a joint project it was approved by both the institutional ethical committee and informed consents were obtained from all the patients.

All of the eligible patients in the control group were started with an intensive phase ATT comprising of INH 300 mg/d, RMP 450 mg/d and PZA 20 mg/kg body wt to the closest 250 mg in two divided doses and Ethambutol 800 mg HS for initial 2 mo and than three drugs excluding PZA for 4 mo. Similar ATT medications supplemented with curcumin enriched (25%) CL and a hydro-ethanolic extract enriched (50%) TC 1 g/d each in two divided doses started from day one in trial group. Other supplemental medications like vitamins, iron, H2 blockers, proton pump inhibitors, anti-emetics,



**Figure 1** Study design and enrollment and follow-up of patients.

gut motility enhancers and calcium were also being given on an individual basis to patients in any group as deemed necessary by the physician. Two weeks quota of medications was given for self administration and patients were advised to return to the clinic every two weeks or earlier if any troublesome symptoms mentioned earlier were experienced. Blood sample for liver enzymes and bilirubin were taken every alternate visit and a pre-filled requisition form was given with the instruction to get it done at nearby center having pathology laboratory facility if patient could not come at time of appearance of symptoms. At each visit the patients were evaluated for signs and symptoms of adverse events by trained staff and adherence to treatment schedule was confirmed for both the groups. Sputum examination was repeated at fourth week in all sputum AFB positive cases.

Hepatotoxicity gradation was based on the WHO classification and defined by liver enzyme level, however, aspartate transaminase (AST) level was preferred instead of alanine transaminase (ALT) level as it is well known that AST/ALT ratio > 2 is one important criteria for differential diagnosis between drug or chemical and viral hepatitis<sup>[33]</sup>. Grade I for any AST level of 51-125 U/L; grade II for any AST level of 126-250 U/L; Grade III for AST level of 251-500 U/L and grade IV for any level greater than 500 U/L or more than 250 U/L with symptoms of fulminant hepatitis. In case of grade I or II liver injury ATT was continued for 2 wk and enzymes were repeated. In grade III and IV

hepatitis ATT was stopped till enzyme level came to normal. AST was checked weekly, once its rise beyond 2 fold was confirmed after initial 2 wk follow-up till it came to normal level. Patients were called for monthly follow-up visits after completion of scheduled treatment for 3 mo in both the groups.

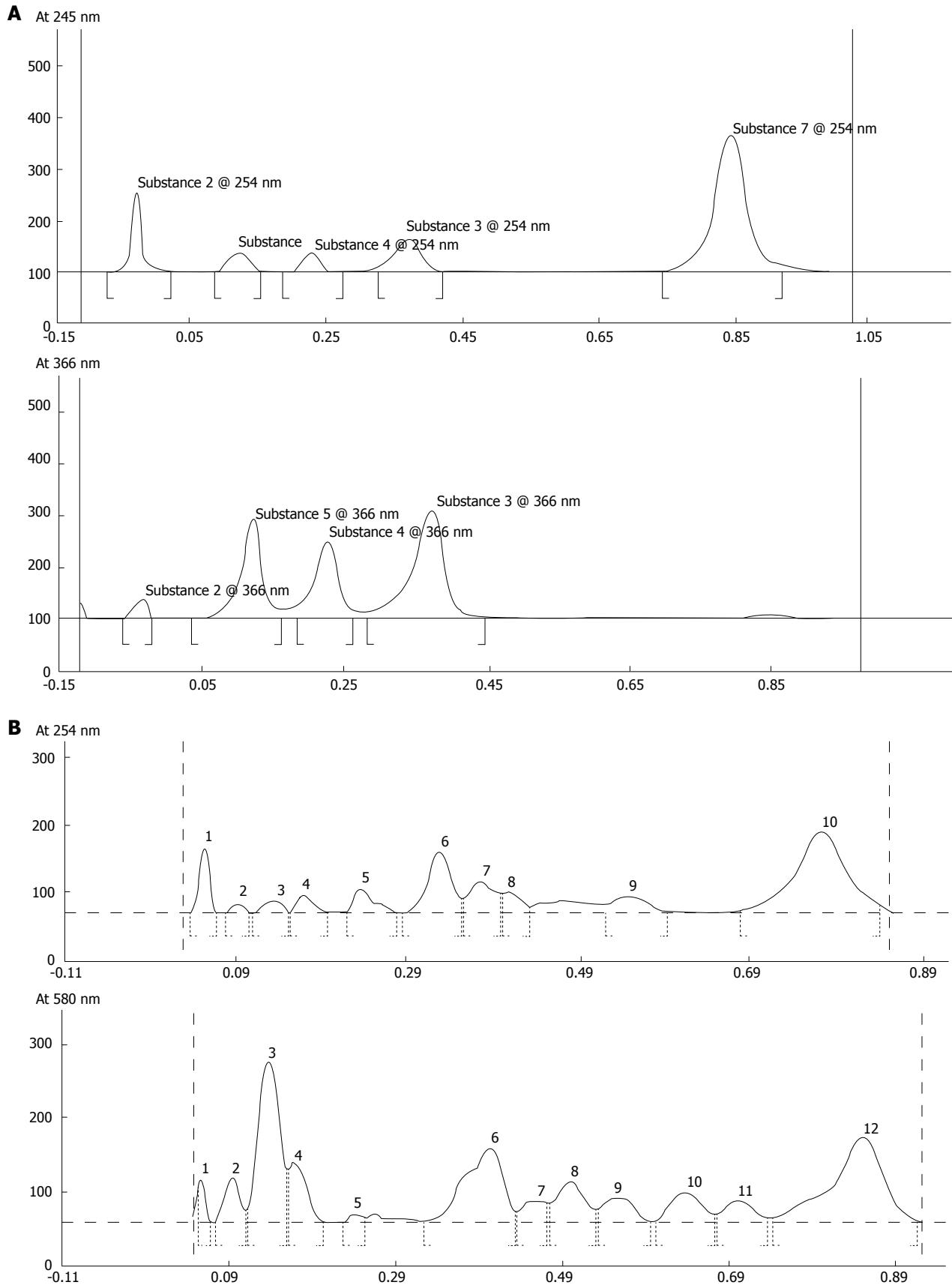
### Outcomes

Primary endpoints were to observe the development of hepatotoxicity in both the groups with assessment of severity by biochemical parameters and liver function tests if the hepatotoxicity exceeded Grade III parameters. Secondary outcome was to assess the impact of adjuvant medicine on the outcome of TB itself as defined by follow-up investigations, clinical cure and functional improvement<sup>[34]</sup> by the end of scheduled ATT and follow-up period. Completion of the regimen was defined as taking > 80% of scheduled medication for both the groups. Occasional breaks of one or two weeks due to some tribal festivals or an episodes of minor illness were not considered to be a break in the treatment.

### Statistical analysis

A sample of approximately 500 patients would provide 99% power in two sided tests with an  $\alpha$  value of 0.05 to detect a decrease in expected incidence of hepatotoxicity of any grade from 15% in control to < 3% in trial group.

Nominal and ordinal data in patient characteristics were described with percentage, median and inter



**Figure 2** A: HPTLC finger print of formulation of *Curcuma longa*. Sample was extracted in MeOH and Solvent system for Mobile phase used was Chloroform: MeOH (99.5: 0.5). Note: Before loading the material on TLC plate, it was impregnated with di NaHPO<sub>4</sub> (anhydrous)-1.25 g dissolved in 25 mL double distilled water (DDW). B: HPTLC finger print of formulation of *Tinospora cordifolia*. Sample was extracted in-MeOH and NH<sub>4</sub>OH: MeOH (9:1) and solvent system for mobile phase used was -Chloroform: MeOH (8:2).

quartile range (IQR) between 25% and 75%, while measurement data by mean  $\pm$  SD. Significance for each

characteristic was tested to ensure basic homogeneity of both the groups. Normality test was performed as the patients were representative of a tribal ethnicity and belonged to a disease that may have specific propensity for a subgroup, so the distribution may not be Gaussian despite a large sample. Those parameters failing the normality test were further analyzed by Wilcoxon's rank sum test and those passing it were tested by unpaired *T* test with Welch's modification where variance was unequal. Student's *T* test and  $\chi^2$  test with Yate's correction were done as needed.  $P \leq 0.05$  was considered significant. Number needed to treat (NNT) was decided for the adjuvant as test therapy. Finally more common outcomes including completion of treatment and response to treatment were analyzed by Fisher Exact test. Analyses were done by using KyPlot Version 2 beta 15, GraphPad InStat Version 3.06 and GraphPad StatMate 2 version 2.00.

## RESULTS

### Clinical and demographic characteristics at baseline

As shown in Figure 1, 528 patients were enrolled at six centers of SGSCT mobile clinic. A high proportion of patients were of tribal ethnicity from Chaudhari, Gamit, Vasava and Bhil community known to carry sickle cell trait in up to 30% of population<sup>[35]</sup> and disease in 1.5%<sup>[36]</sup>, as reported by different governmental agencies. Eight patients from control and 12 patients from trial group were excluded from study due to violation of the protocol, hence control had 192 and trial had 316 patients for final analysis. As seen in Table 3 the median age and IQR for both the groups were quite similar. Also the two groups were similar in sex, body weight, habits, disease profile, baseline hematologic means and liver function except that trial group contained significantly higher number of patients with Cavitary Koch's than the control group.

### Hepatotoxicity

Tables 4 and 5 depict the effects of herbal adjuvant on primary outcome in the form of significantly decreased incidence of hepatotoxicity (2/316 vs 27/192) in the trial group as compared to control group. Mean onset of asymptomatic hepatitis was delayed in trial group and none in the trial group suffered from symptomatic hepatitis as against 63% of control group hepatitis patients. Grades II and III hepatotoxicity were observed in control group only. Serum AST, ALT and bilirubin level were raised in control group much significantly when compared with trial group hepatotoxicity cases.

### Disease outcome

As shown in Table 6, the number of sputum-positive cases after 1 mo of treatment in the trial group (2.9%) was significantly lower than control group (14.93%). Incidence of poorly resolved parenchymal lesion by roentgenogram and failure to improve functional status by ECOG score<sup>[34]</sup> being significantly lower and treatment dropouts nil in the trial group proved the efficacy of herbal adjuvant formulation.

**Table 3 Baseline demographic and disease characteristics in the intention-to-treat population**

Characteristics of patients	Control	Trial	P
Sex			
Male (%)	104 (54)	169 (53)	0.934
Female (%)	88 (46)	147 (47)	0.927
Age-yr median(IQR 25th & 5th)	35 (23.75-45)	35 (27-45)	0.229
Weight (kg, mean $\pm$ SD)	37.61 $\pm$ 5.94	38.32 $\pm$ 6.08	0.199
Ethnicity (%) <sup>1</sup>			
Tribals	171(89)	287(91)	0.883
Others	21(11)	29(9)	0.559
Habits (%)			
Smoking	58 (30.2)	103 (32.6)	0.685
Alcohol	23 (12)	33 (10.4)	0.633
Both	26 (13.5)	53 (16.7)	0.403
Socio-Economic status (%)			
Upper-Middle	20 (10.4)	40 (12.6)	0.499
Middle	30 (15.6)	52 (16.4)	0.833
Lower	142 (74)	224 (71)	0.763
Pulmonary (%) <sup>2</sup>			
Open cases (sputum +ve)	67 (35)	137 (43.4)	0.214
Parenchymal	152 (79.2)	258 (81.6)	0.822
Cavitory	18 (9.4)	66 (21)	0.003
Effusion	9 (4.6)	20 (6.3)	0.464
Fibrosis	27 (14)	68 (21.5)	0.081
Extra pulmonary (%) <sup>2</sup>			
Cervical lymph node	18 (9.4)	31 (9.8)	0.883
Abdominal	4 (2.1)	6 (2)	0.886
Laryngeal	7 (3.6)	11 (3.5)	0.925
Central nervous system	6 (3.1)	6 (2)	0.389
Bones/Joints	10 (5.2)	19 (6)	0.72
Skin	0	3 (1)	0.598
Genito-Urinary	2 (1.04)	1 (0.3)	0.304
Type of case (%)			
New	140 (73)	195 (61.7)	0.245
Relapse	43 (22.4)	97 (30.7)	0.122
Chronic	9 (4.7)	24 (7.6)	0.225
Hematology			
Hb (%)	8.84 $\pm$ 1.93	8.95 $\pm$ 1.95	0.808
Total leucocyte count/mm <sup>3</sup>	10 179 $\pm$ 4 680	9575 $\pm$ 4075	0.478
ESR	66.79 $\pm$ 30.64	70.49 $\pm$ 27.91	0.517
Liver Function (mean, 95%CI)			
Serum bilirubin	1.07 (0.7-1.3)	1.09 (0.8-1.4)	0.952
AST	34.35 (17-63)	32.4 (11-61)	0.658
ALT	27.45 (9-57)	31.15 (6-52)	0.655
ALP	100 (50-260)	107 (50-260)	0.092

Unless stated otherwise, P values were calculated with the use of a two sided *t*-test with Welch's modification (when unequal variance) for continuous data and a  $\chi^2$  test for categorical data. <sup>1</sup>Ethnic group was assessed by investigation on screening. Tribal consists of Chaudhari, Gamit, Vasava, Bhil, Valvi, Nayka and Halpati. <sup>2</sup>Patients may belong to more than one category. ALP: Alkaline phosphatase.

### Weight and hematologic outcome

As seen in Table 3 and Table 6, the mean weight in control increased to  $39.17 \pm 5.5$  kg from  $37.61 \pm 5.94$  kg before ATT ( $P = 0.0063$ ) and in trial group mean weight was  $40.65 \pm 6.22$  kg from  $38.32 \pm 6.08$  kg before ATT and herb treatment ( $P < 0.0001$ ). While comparing the weight gain, trial group showed significantly higher weight gain than control group. Mean Hb of control and trial groups increased significantly after therapy; however, the inter group difference was nonsignificant. Erythrocyte sedimentation rate (ESR) in both groups decreased significantly compared from start to end of the treatment period; however, inter group comparison

**Table 4** Effects of herbal formulation on incidence, onset, duration and severity of hepatitis

ATT induced hepatitis outcome (Table 2)	Control (n = 192)	Trial (n = 316)	P
Patients with raised AST (%)	27 (14)	2 (0.63)	< 0.0001
Patients with raised S bilirubin (%)	17/27 (63)	0	< 0.0001
Mean onset of hepatitis symptomatic	49.12 ± 17.22	-	< 0.0001
Mean onset of hepatitis asymptomatic	29.9 ± 17.63	39 ± 11.31	0.443
Mean onset of any hepatitis	42 ± 19.48	39 ± 11.31	0.776
Mean duration of raised AST	27.5 ± 7.25	21	< 0.0001
Hepatitis gradation			
Grade- I	10	2	< 0.0001
Grade- II	9	0	< 0.0001
Grade- III	8	0	< 0.0001
Grade- IV	0	0	
Reinstitution of ATT in patients (%)	22/27 (81.5)	NA	

P calculated by  $\chi^2$  (categorical data) and two-sided Student's t-test with Welch's procedure (continuous data with unequal variance). NA: Not applicable.

**Table 5** Comparison of liver enzymes and bilirubin in patients developing hepatitis

	Control (n = 27)	Trial (n = 2)	P
AST	195.93 ± 108.74	85 ± 4.24	< 0.0001
ALT	75.74 ± 26.54	41 ± 1.41	< 0.0001
AST/ALT	2.35 ± 0.74	2.08 ± 0.18	0.207
ALP	241.74 ± 140.96	147.5 ± 45.96	0.118
Serum bilirubin	5.4 ± 3.4	1.5 ± 0.42	< 0.0001

Two-sided Student's t-test with Welch's procedure for continuous data with unequal variance. ALP: Alkaline phosphatase.

**Table 6** Effects of herbal formulation addition on disease outcome

Treatment outcome for ATT	Control	Trial	P
Number of sputum +ve case at start (%)	67/192 (35)	137/316 (43)	0.03
Number of sputum +ve case after 1-month of treatment (%)	10/67 (14.93)	4/137 (2.9)	0.0068
Poorly resolved parenchymal lesion (%)	9/152 (5.92)	2/258 (0.78)	0.0037
Failure to improve functional status (%)	19/192 (10)	6/316 (2)	0.00013
Treatment dropouts (%)	10/192 (5.2)	0	0.0001
Weight (kg)	39.17 ± 5.5	40.65 ± 6.22	0.0016
Hb (%)	10.92 ± 2.01	11.17 ± 1.97	0.176
ESR mm in 1st hour	45.96 ± 18.52	38.84 ± 22.37	0.0001

P calculated by  $\chi^2$  (categorical data) and two-sided Student's t-test with Welch's procedure (continuous data with unequal variance).

that was nonsignificant at the start showed a significantly more decrease in trial group at completion.

#### Non-hepatotoxic adverse events (data not shown)

Non-hepatotoxic adverse events occurred occasionally in both the groups and included nausea, vomiting, skin rash, epigastric pain and discomfort, malaise, dizziness,

arthralgia, peripheral neuropathy, anorexia and insomnia and sickle crisis. They were treated symptomatically with appropriate medications by the physician. Early dropouts occurred in both groups due to allergic reactions to ATT and they were excluded from the analysis as shown in Figure 1.

## DISCUSSION

The present clinical trial was based on unpublished results of previous preclinical studies at our center. As the conventional ATT has been well established and the Ayurvedic herbs have excellent safety profile as described previously, a phase I trial omission was granted. As the efficacy of the herbs was shown in concurrent administration design in an animal model of ATT induced hepatotoxicity<sup>[22]</sup>, it was considered scientific, as well as ethical, to try a similar model for a human clinical trial, hence herb treatment was started along with ATT at the same time and continued until completion of ATT.

Curcumin has anti-inflammatory, free radical scavenging and hepatoprotective activities tested *in vitro*. It suppresses production of superoxide by macrophages, exerts potent anti-inflammatory action that inhibits production of tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin (IL) 1- $\beta$  and the activation of NF- $\kappa$ B in human monocytic derived cells<sup>[37,38]</sup>. Also, it has a strong antioxidant property and it inhibits lipid peroxidation in rat liver microsomes, erythrocyte membranes and brain homogenates, by maintaining the activity of SOD, catalase and glutathione peroxidase at a higher level<sup>[39]</sup>. These properties clearly explain the hepatoprotective activity of CL in preclinical studies and present trial as well. Curcumin has antiviral, antiprotozoal, antibacterial, anti inflammatory, and antioxidant actions, in addition to its hepatoprotective activity, making it a suitable adjuvant agent that actually enhanced efficacy of ATT in present study.

In preclinical studies, TC has been shown to induce enzymes of drug metabolism and antioxidant system and to inhibit lipid peroxidation in mice. Cytochrome P450, NADP-Cytochrome (P sub 450) reductase, glutathione-s-transferase, DT diaphorase, SOD and catalase are enhanced. These effects improve liver function, protect against toxic assaults and increase protein synthesis by liver<sup>[40]</sup>. These activities can account for its hepatoprotective potential. It is a known immunostimulant enhancing cell mediated as well as humoral immune response in mice. Treatment of mice with dried stem crude extract prevented cyclophosphamide induced myelosuppression as well as immunosuppression<sup>[41]</sup>. Improved humoral immunity, enhanced macrophage and Kupffer cell function, enhanced neutrophil function, antioxidant activity and excellent hepatoprotective potential might explain the result of present clinical trial. Since many years the concept of classical phytotherapy using herbal drug combinations with superior efficacy and lesser side effects in comparison with single isolated constituents

of plant extracts has been repeatedly assessed clinically as well as pharmacologically. The exact mechanisms of action underlying these synergy effects are unknown. It could be explained by a multitarget action of compounds on a molecular level or partly by an improved resorption rate and a change of pharmacokinetics<sup>[42]</sup>. Hence, a formulation of CL and TC was used with an idea to enhance the potency due to synergistic action that was backed by promising results of a small preclinical pilot study (unpublished). As such, many standard Ayurvedic preparations also contain both of these herbs, albeit in a lower dose along with other multiple herbs.

Regarding the design of the trial, the strength lied in the base line characteristics which were remarkable similar and all the parameters for hepatotoxicity were objective and being tested by laboratory technicians at different places and totally unaware about the trial or outcome, so there could not be any chance for a bias in the detection and gradation of hepatotoxicity. In many complex clinical situations adaptive random allocation methods are implemented to maintain balance between stratification variables<sup>[43]</sup>; however, periodic evaluation revealed acceptably similar distribution among groups even with permuted block randomization. When one treatment or an intervention is expected to be superior; a Bayesian adaptive randomization (BAR) method is considered desirable to conventional randomization because it utilizes the accruing data in a "learn-as-you-go" fashion that arguably makes more sense than ignoring the trial's data until it is completed<sup>[44]</sup>. BAR would allocate more numbers of patients to the potentially advantageous group without compromising the randomization, but making the trial design very complicated. Instead the control arm was truncated for ethical reasons with caution at periodic evaluations so that no dissimilarity in favor of the trial group should occur.

Though sickle cell disease patients were excluded from the study, persons having sickle cell traits are prone to have anemia, repeated chest infection and theoretically sickle cell crisis in extremes of situations with dehydration and acidosis. In the present trial two events of sickle cell crisis occurred in the control group. They might have phenotypic sickle cell disease<sup>[45]</sup>.

The average weight of the study population was around 38 kg in both the groups confirming malnutrition in addition to effect of TB alone. The significance of this characteristic could not be overemphasized because a low weight would culminate in to a higher per kg dosage of the hepatotoxic ATT. Considering the average weight to be 38 kg, the per kg dose of INH, RFM, PZA and EMB would come to 7.9, 11.84, 26.31 and 21 mg, respectively. Comparing this dose with a previous study<sup>[46]</sup> in which the per kg doses for INH, RFM and PZA were 5, 10 and 25-30 mg, respectively, and the period for observation was 59 d with reported hepatotoxicity to be 11%; it is apparent that the chances of developing hepatotoxicity would be definitely more for the patients in present study. In addition to malnutrition as a known risk factor, a higher per kg dose could also play some role.

With all these background, the results of present trial carried utmost importance that hepatotoxicity did not occur in the trial group despite a slightly unfavorable profile in the form of significantly higher number of patients with Cavitary pulmonary TB. Only two instances of raised AST were detected during monthly surveillance of liver enzymes and ATT was not stopped as it was asymptomatic grade I hepatotoxicity. Extensive disease, relapse cases, multiple relapse cases, miliary TB cases and cases with malnutrition, HIV carrier, and hepatitis B and C carrier, sickle cell trait, other back ground illnesses like COPD, hyper-eosinophilic syndrome, MB leprosy, diabetes mellitus, asthma, rheumatoid arthritis were all recruited in the control as well as the trial group and yet no incidence of clinically significant hepatotoxicity occurred in trial group, suggesting that regular administration of adjuvant herbs had taken care of all the known, hypothesized and unknown risk factors predisposing to ATT induced hepatotoxicity in clinical cases of TB.

Though with a power of 99% and highly significant out-come, this was a pilot study and much has remained to be done to exploit the full potential of the hepatoprotective ability of these herbs in cost-effective manner with defined recommendation for different subclasses of patients including latent TB cases and different high risk groups of clinical cases. A separate study with a low risk control, a high risk control and a high risk trial would serve both the purposes of testing the efficacy of herbs in high risk group as well as the validity of the hypothesis that low risk group has lower incidence of hepatotoxicity in the same population having similar ethnicity. The answer to the question whether low risk patients should be subjected to adjuvant medication or not, could also be answered from the results of study with such a design.

As latent TB cases on different preventive regimes have been shown to be at a greater risk for developing hepatotoxicity<sup>[7-9]</sup>, a separate well-designed trial for this class of patients could not be over-emphasized. The efficacy of these herbs may also be tested in patients showing liver enzyme increase detected by surveillance to decide whether ATT could be continued without risk of it to progress to higher grade hepatotoxicity as it is observed that sooner the detection, lesser the risk and later the appearance of hepatitis, greater the chances of fulminant failure<sup>[47]</sup>.

The NNT analysis of the study with a single outcome of hepatoprotection would come to eight. This is because the herbs were given to all as an adjuvant medicine where the possibility of developing hepatitis itself was expected to be between 11% and 15%. Looking to the result on disease profile it is clear that the sputum conversion to negativity was significantly superior in herb treated group despite an unfavorable sample, resolution of parenchymal lesion was significantly higher, failure to improve functional status was significantly lower and dropout rate came to nil. If these treatment factors are added then NNT would surely come lower.

Weight and hemoglobin both increased and ESR reduced significantly on intra group pre- and post-treatment comparison in both the groups proving the efficacy of conventional ATT alone too. The inter group comparison post treatment showed a significant advantage in weight gain and reduction in ESR, but not in hemoglobin level in trial group suggesting the superiority of ATT plus adjuvant herbs over ATT alone. It is apparent that iron and vitamin supplements were being given to anemic patients as per diagnosis and role of ATT must be supportive only in rising the Hb. Tumor necrosis factor alpha (TNF) has been shown to cause hypoferremia and reduced intestinal iron absorption in mice and Curcumin inhibits TNF thereby having the potential to enhance iron absorption and help alleviate anemia<sup>[48,49]</sup>. Rise in Hb was not a direct function of both modalities and effects of curcumin on iron absorption in humans has not yet been tested; so the only valid comment would be that the theoretical advantage of CL was not observed practically in present study.

In conclusion CL and TC given as an adjuvant to standard ATT to any kind of TB patients prevented hepatotoxicity very significantly in terms of incidence, duration and severity and also helped improve outcome in terms of quicker and more efficient achievement of sputum negativity in open, potentially infectious cases; better response in parenchymal lesion resolution and helped improve patient compliance. Treated cases had better weight gain and significantly more reduction in ESR. A prospective trial for latent TB and MDR TB contacts can further exemplify its usefulness. Post-treatment design on detection of hepatotoxicity and concurrent administration design for different subgroups may clarify its efficacy in different clinical situations and provide a basis for evidence based recommendation for different sub groups of TB patients.

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## COMMENTS

### Background

Isoniazid, rifampicin and pyrazinamide, three major drugs of intensive phase of conventional anti-tuberculosis (TB) treatment (ATT), are hepatotoxic and the incidence varies between 4%-11%. Healthy contacts of TB patients (latent TB cases) are more prone to develop hepatotoxicity by presently recommended regimens. There is no established drug therapy to cure or prevent hepatotoxicity except stopping the treatment for a while and restoring it gradually at a later date when liver enzymes come to a normal level. Stopping the therapy may increase morbidity or prolong disability, while shifting to suboptimal non-hepatotoxic regime for time being poses the risk of emergence of resistant strains.

### Research frontiers

Search for non-toxic and highly effective new compounds for treating

tuberculosis or an effective vaccine conferring sustained protective immunity have largely failed. In literature there are many published preclinical studies showing herbs to prevent CCl<sub>4</sub>, and paracetamol induced liver damage in rodents. Recently, four Ayurvedic herbs were shown to prevent ATT induced hepato-toxicity and immunosuppression in Guinea pigs. (<http://www.wjgnet.com/1007-9327/13/3199.asp>). But no clinical trial of hepatoprotective compound has ever been carried out in patients taking conventional ATT.

### Innovations and breakthroughs

The present study is the first of its kind and the results are encouraging for the prevention of hepato-toxicity and improving the disease outcome by greater sputum clearance in Cavitary lesions and better resolution of parenchymal lesions. Thus, addition of two simple herbs makes the conventional ATT much safer and efficacious.

### Applications

Incorporation of such a formulation in current conventional ATT at a mass level may contribute a lot to minimize the risk of liver injury, improve patient compliance and disease outcome that might ultimately help to control emergence of MDR strains of AFB. If similar protection is observed in latent tuberculosis by a prospective trial, it would be a boon for the latent TB cases subjected to a serious risk by preventive ATT.

### Peer review

The manuscript is well written. This is an interesting trial. The study is nicely designed and the analytical data appear to be technically and scientifically sound. The outcome is remarkable and clearly shows beneficial effect of the herbal formulation in hepatic injury by ATT.

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# Ultrastructural view of colon anastomosis under propolis effect by transmission electron microscopy

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that administration of propolis accelerated the healing of colon anastomosis following surgical excision.

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## Abstract

**AIM:** To evaluate the effect of propolis administration on the healing of colon anastomosis with light and transmission electron microscopes.

**METHODS:** Forty-eight Wistar-Albino female rats were divided into two groups and had colon resection and anastomosis. In group I, rats were fed with standard rat chow pre- and postoperatively. The rats in group II were fed with standard rat chow and began receiving oral supplementation of propolis 100 mg/kg per day beginning 7 d before the operation and continued until they were sacrificed. Rats were sacrificed 1, 3, 7 and 14 d after operation, and anastomotic bursting pressures measured. After the resection of anastomotic segments, histopathological examination was performed with light and transmission electron microscopes by two blinded histologists and photographed.

**RESULTS:** The colonic bursting pressures of the propolis group were statistically significantly better than the control group. Ultrastructural histopathological analysis of the colon anastomosis revealed that propolis accelerated the phases of the healing process and stimulated mature granulation tissue formation and collagen synthesis of fibroblasts.

**CONCLUSION:** Bursting pressure measurements and ultra structural histopathological evaluation showed

## INTRODUCTION

Despite the use of optimal surgical techniques and medical treatments, the integrity of intestinal anastomosis may be compromised, resulting in wound dehiscence<sup>[1]</sup>. The frequency of this complication, which carries a high morbidity and mortality rate, increases if surgery has to be performed in the presence of high-risk situations, such as surgeries performed in emergency settings, on infected and necrotic stumps, in unsafe anatomical areas (rectum, esophagus), or in patients with metabolic derangements<sup>[2,3]</sup>. If we consider these high-risk situations annihilated, there are several factors in the etiology of colon anastomotic leakage. These factors can be separated into two groups, as systemic (advanced age, malignancy, malnutrition, anemia, hypovolemia, hypoproteinemia) and local (colonic perfusion disorders, septic colonic contents, perfusion impairment at anastomotic line, anastomotic tension, haematoma, inadequate surgical technique, type of preparation prior to surgery and primary colonic pathology)<sup>[4,5]</sup>. Many studies focused on the detrimental effects of these local and systemic factors. However, in the last century researchers have drawn attention to the cellular mechanisms of the healing and suggested many agents to decrease anastomotic insufficiency and leakage. This

experimental study was performed to investigate the cellular ultrastructure of the anastomotic healing process besides to examine the effects of propolis.

Propolis is a resinous material collected by bees from various plants. Bees use material actively secreted by plants or exuded from wounds. Once collected, this material is enriched with salivary and enzymatic secretions of bees. Propolis is used by bees to cover hive walls, fill cracks or gaps, and embalm killed invader insects<sup>[6]</sup>. It also found its way into a wide spectrum of applications from scolicidal efficacy to cosmetic products such as face creams, ointments, lotions, and solutions<sup>[7]</sup>. Propolis contains a variety of flavonoids, phenols, alcohols, terpenes, sterols, vitamins, and amino acids<sup>[8]</sup>. Chemical composition of propolis samples was detected by gas chromatography mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC)<sup>[9]</sup>. Antimicrobial properties of propolis seem attributable mainly to the flavonoids, pinocembrin, galangin, and pinobanksin. Pinocembrin also exhibits antifungal properties. Other active compounds are esters of coumaric and caffeic acids. Prenylated p-coumaric and diterpenic acids possess antibacterial and cytotoxic activities. Caffeoylquinic acid derivates show immunomodulatory and hepatoprotective actions and furofuran lignans inhibit the growth of some bacteria. It also has antioxidative, immunostimulative and regenerative properties<sup>[6]</sup>.

According to these properties, we planned to use propolis for determining the effects on the healing of colonic anastomoses in the rats after colonic resection and anastomosis.

## MATERIALS AND METHODS

### **Animals**

Forty-eight Wistar-Albino female rats, weighing  $225 \pm 25$  g were included in this study. Twelve hours before anesthesia, animals were deprived of food, and had free access to water until two hours before anesthesia. No enteral or parenteral antibiotics were administered at all. Rats were housed under constant room temperature ( $21 \pm 2^\circ\text{C}$ ) individually in wire cages with a 12-h light-dark cycle. The rats, which died during the experimental period, were excluded from the study and were not replaced with new rats. Additionally, 6 sham-operated rats (no surgery performed on colons) were used to measure basal bursting pressure. The procedures in this experimental study were performed in accordance with the National Guidelines for the Use and Care of Laboratory Animals and approved by the Animal Ethics Committee of Ankara Research and Training Hospital.

### **Study groups**

Rats were randomly divided into two groups each including 24 animals. Colon resection and anastomosis were performed on all animals. In group I, rats were fed with standard rat chow pre- and postoperatively. The rats in group II were fed with standard rat chow and began receiving oral supplementation of propolis

100 mg/kg per day beginning 7 d before the operation and continued until they were sacrificed. Propolis was given by nasogastric tube everyday at 8 am.

### **Surgical procedure**

Animals were anesthetized by intramuscular injection of 30 mg/kg ketamine hydrochloride (Ketalar®; Parke-Davis, Istanbul, Turkey) and 5 mg/kg xylazine (Rompun®; Bayer, Istanbul, Turkey). Under sterile conditions, midline laparotomy was performed. The left colon was transected 3 cm proximal to the peritoneal reflection and a 1 cm-long colon segment was resected. End-to-end anastomosis was performed by one-layer, inverted, interrupted sutures by using 6-0 polydioxanone (PDS, Ethicon, UK). The same surgeon, who was unaware of the grouping of each rat, performed all anastomoses. The fascia and skin layers of the abdomen were closed separately with continuous 3-0 silk sutures. Animals had free access to food after the operation. The rats were sacrificed on postoperative day 1, 3, 7, and 14 by high-dose diethyl ether inhalation. The abdominal cavity was inspected through a U-shaped incision.

### **Measurement of bursting pressure**

The anastomotic segment was separated from the surrounding organs. The tissues that adhered to the anastomosis too tightly were not forced away. A colon segment including the anastomosis with 2.5 cm proximal and distal parts was removed. One end of the segment was tied by a 3-0 silk suture. The cannula was inserted into the proximal colonic segment. A catheter was inserted into the other end and tied by 3-0 silk suture to avoid air leaks; it was connected to an infusion pump and to the computer for pressure readings (Logger computer program). Air was pumped into the colon segment at a constant rate of 8 mL/min. The maximum pressure reading before the pressure declined suddenly was recorded as the bursting pressure.

### **Histological examination**

The histopathological analysis of this study was carried out in the Histology and Embryology Department of Ankara University Faculty of Medicine. For light microscopic analyses, a colonic anastomosis-site segment was removed from each rat and the specimens were fixed in 10% neutral buffered formalin solution for 2 d. Tissues were washed in flowing water and dehydrated with increasing concentrations of ethanol (50%, 75%, 96%, and 100%). After dehydration, specimens were put into xylene to obtain transparency and then infiltrated with and embedded in paraffin. Embedded tissues were cut into sections of 5-μm thickness using a Leica RM 2125 RT. Systematically randomly selected sections were stained with hematoxylin and eosin and mallory-azan dyes. Histopathological examinations were performed by two histologists blinded to the study design and photographed with a Nikon eclipse E 600.

For the transmission electron microscope (TEM) analyses, samples were fixed with phosphate buffered (pH 7.3) 2.5% glutaraldehyde and a 2% PFA mixture

**Table 1** The colonic bursting pressures

Time	Sham group	Control group	Propolis group	Sham vs Control	Sham vs Propolis	Control vs Propolis
1st day	214.7 ± 5.50	33.8 ± 2.99	43.3 ± 1.51	P = 0.002	P = 0.002	P = 0.002
3rd day		43.7 ± 1.86	52.7 ± 3.33	P = 0.002	P = 0.002	P = 0.002
7th day		146.5 ± 6.53	179.8 ± 6.71	P < 0.001	P < 0.001	P < 0.001
14th day		176.7 ± 4.41	200.8 ± 7.19	P < 0.001	P = 0.002	P < 0.001

solution for 2 h at room temperature. They were washed with phosphate-buffered saline solution (PBS, pH 7.3) and fixed with 1% osmium tetroxide for 2 hours as the secondary fixative. After washing, they were embedded in Araldite 6005 and cut with a Leica EM FCS (Vienna-Austria) ultramicrotome. One micrometer semi-thin sections were stained by toluidine blue-Azur II to select the region of interest for the following procedures. Sixty-seventy nm thin sections were stained with uranyl acetate and lead citrate. The sections were examined and photographed using a LEO 906 E TEM (80 kV, Oberkochen, Germany) microscope.

### Statistical analysis

Data analysis was performed using the SPSS (Statistical Package for Social Science, version 11.5) software package. Whether the bursting pressure measurements were normally distributed or not was determined by using the Shapiro Wilk test. Descriptive statistics were shown as mean ± standard deviation (SD). The differences among groups and days were evaluated by one-way ANOVA post-hoc Tukey test or Kruskal Wallis test, where appropriate. When the P-value from the Kruskal-Wallis test statistics was statistically significant, Bonferroni adjusted Mann Whitney U test was used to know which groups or days differ from which others. Bonferroni correction was applied for all possible within group or day comparisons. A P value less than 0.05 was considered statistically significant.

## RESULTS

### Surgical results

The rats were sacrificed on postoperative day 1, 3, 7, and 14. One rat from each group died in the early postoperative period, probably due to anesthesia. On gross evaluation, no signs of anastomotic leakage, intraabdominal abscess, peritonitis, or ileus were detected.

Anastomotic wound healing was evaluated by means of bursting pressure and histopathological assessment. The biggest P value among the P values as P < 0.001 was found as 5.6E-6. To obtain a standardized projection the notation was preferred as P < 0.001 for most of the P values. The colonic bursting pressures of the propolis group were significantly better than the control group (Table 1).

### Histopathological results

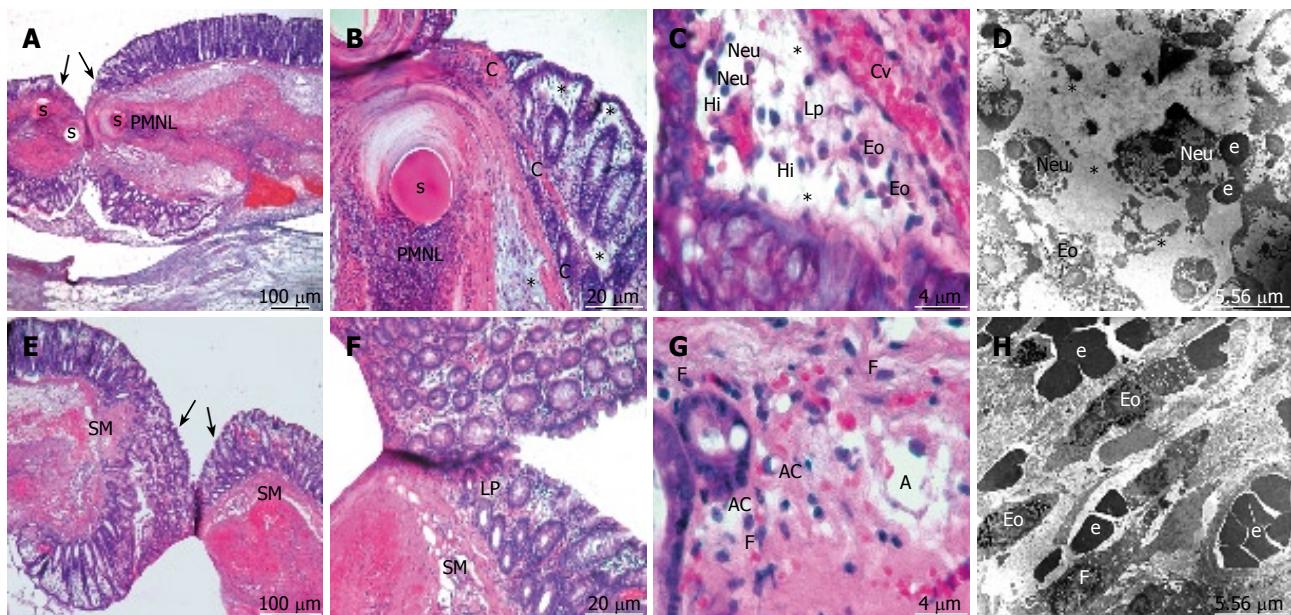
On the first postoperative day of the control group, healing began with a strong influx of polymorphonuclear

leucocytes (PMNL), most prominent in the anastomotic line. There was a massive infiltration of neutrophils, eosinophils, and histiocytes in the lamina propria, submucosa, and the area surrounding the suture material. The subepithelial region of the mucosal layer and the submucosa were highly congested and edematous. The subepithelial edema was expanded to the wide adjacent area of the anastomotic line (Figure 1A-D). In the propolis group, the subepithelial edema was not striking and not expanded very far from the anastomotic line. Congestion and infiltration of PMNL was also present; additionally the fibroblasts' migration became apparent in the wound edges and around the suture material. The thing to note was that angiogenesis became visible in focal areas of the submucosa (Figure 1E-H).

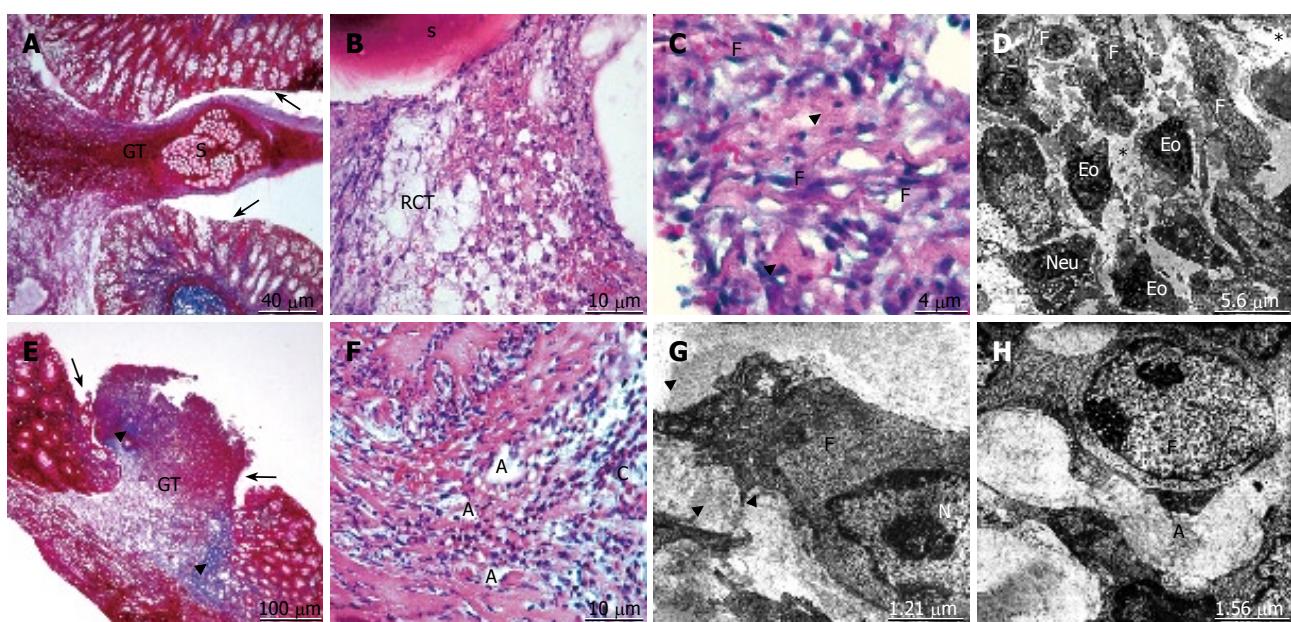
On the third postoperative day, edema of the control group was persistent. The existence of PMNL and a slight increase in the lymphocyte infiltration was seen. The hemorrhage, inflammation and the intensity of eosinophils in the lamina propria were recognizable. The granulation tissue formation and the resorption of the suture material were observed. The reticular connective tissue formation by fibroblasts and the wrapping of this tissue around the suture line were evident (Figure 2A-D). In the propolis group, the edema and the swelling of the tissue ends regressed. The fibroblastic activity and the synthesis of new collagen were observed. Angiogenesis, a component of granulation tissue formation, was prominent. The spatial arrangement of collagen fibers in the submucosal layer and inclining towards the anastomotic line to form bridging of the gap was recognizable (Figure 2E-H).

On the seventh postoperative day, edema of the control group had almost disappeared. The granulation tissue was well organized and wrapped around the suture material particles. The collagen formation with increased fibroblastic activity was present but irregular. The macrophage infiltration removing the debris was observed (Figure 3A-D). In the propolis group, a very small tissue defect was still visible in the mucosal layer. Many bands of collagen fibers and well-developed capillary vessels were distinguished. The collagen bundles were well arranged to form the bridging between mucosal and submucosal layers. The macrophage cells clearing off the debris and the mature cells of the connective tissue as fibrocytes were easily viewed (Figure 3E-H).

On the fourteenth postoperative day for the control group, the mucosal integrity was still not gained. There was a very small (< 1 mm) tissue defect between the anastomotic ends. The regeneration of the epithelium



**Figure 1** An overview of healing at day 1 after operation. The micrographs "A-C, E-G" represent hematoxylin-eosin stained sections and "D, H" T.E.M. images of the control (upper panels) and propolis (bottom panels) group. Arrow: the anastomosis site; Asterisk: Edema; PMNL: Polymorphonuclear leucocytes; s: Suture material; C: Congestion; Cv: Capillary vessel; Lp: Lamina propria; Eo: Eosinophil; Neu: Neutrophil; Hi: Histiocyte; e: Erythrocyte; SM: Submucosa; AC: Apoptotic cell; F: Fibroblast; A: Angiogenesis.



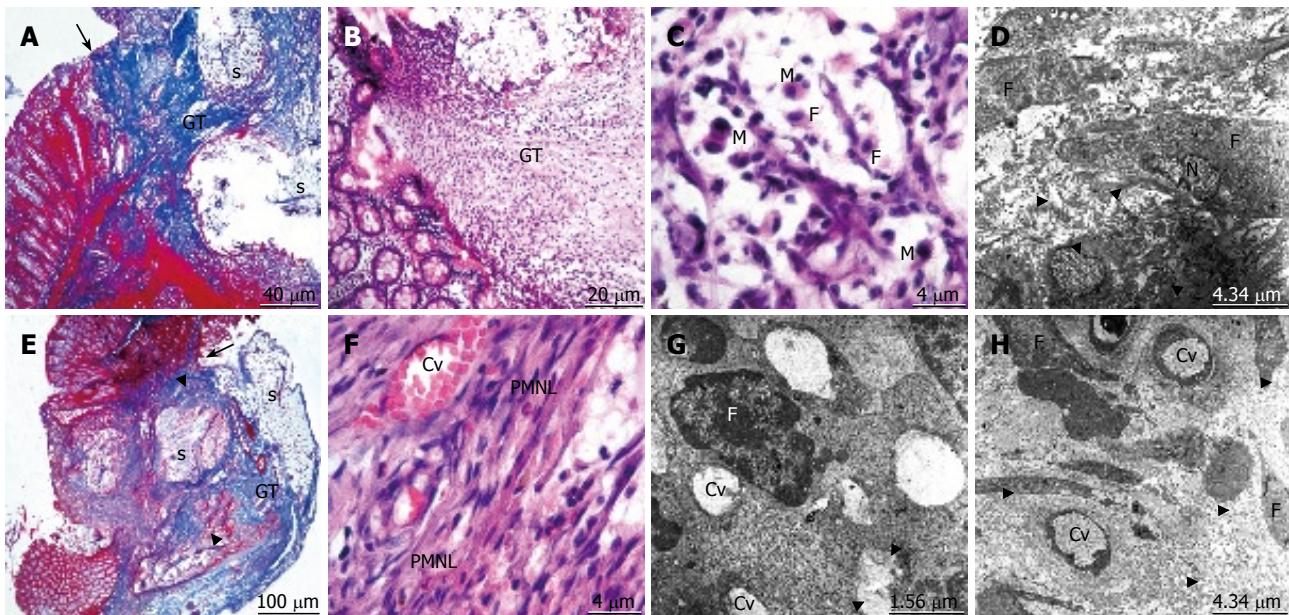
**Figure 2** An overview of healing at day 3 after operation. The micrographs "A, E" represent mallory-azan stained sections, "B, C, F" hematoxylin-eosin stained sections and "D, G, H" T.E.M. images of the control (upper panels) and propolis (bottom panels) group. Arrow: The anastomosis site; Arrowhead: Collagen; Asterisk: Edema; GT: Granulation tissue; s: Suture material; RCT: Reticular connective tissue; F: Fibroblast; Eo: Eosinophil; Neu: Neutrophil; C: Congestion; A: Angiogenesis; N: Nucleus.

and the mucosa only partially covered by simple squamous and cuboidal epithelium was observed. The apoptotic cells and the macrophages clearing the debris were still remarkable. The granulation tissue and the epithelium of the anastomotic ends were very close (Figure 4A-D). In the propolis group, the mucosa had completely healed. The regeneration of epithelium was with glandular columnar epithelium. The lamina propria of the anastomotic line under the restored epithelium was cleaned out from the debris. There was

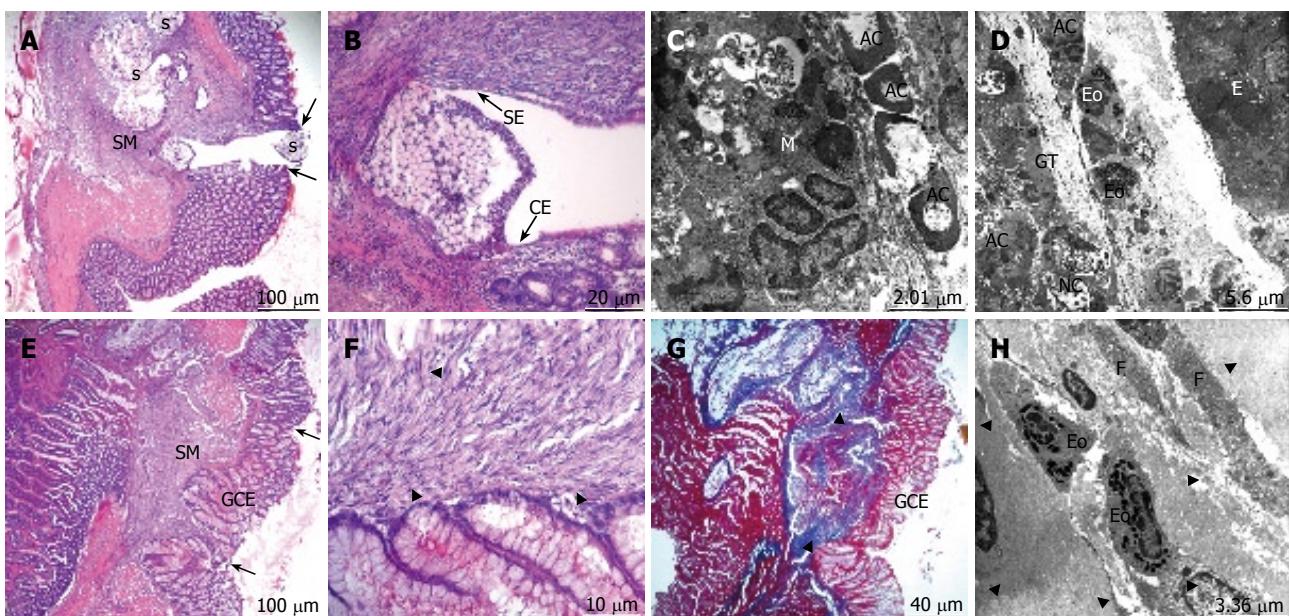
a dense collagen deposition in the submucosal layer. The collagen bundles were regular and harmoniously organized (Figure 4E-H).

## DISCUSSION

Leakage of anastomoses is a very serious and severe complication in gastrointestinal surgery. Morbidity and mortality rates are still high despite advances in operative techniques and suture materials. Therefore, basic



**Figure 3** An overview of healing at day 7 after operation. The micrographs "A, E" represent mallory-azan stained sections, "B, C, F" hematoxylin-eosin stained sections and "D, G, H" T.E.M. images of control (upper panels) and propolis (bottom panels) group. Arrow: The anastomosis site; Arrowhead: Collagen; GT: Granulation tissue; s: Suture material; M: Macrophage; F: Fibroblast; N: Nucleus; Cv: Capillary vessel.



**Figure 4** An overview of healing at day 14 after operation. The micrographs "A-F" represent hematoxylin-eosin stained sections, "G" mallory-azan stained section, and "C, D, H" T.E.M. images of the control (upper panels) and propolis (bottom panels) group. Arrow: The anastomosis site; Arrowhead: Collagen; s: Suture material; SM: Submucosa; SE with arrow: Squamous epithelium; CE with arrow: Cuboidal epithelium; M: Macrophage; AC: Apoptotic cell; NC: Necrotic cell; GT: Granulation tissue; Eo: Eosinophil; E: Epithelium; GCE: Glandular columnar epithelium; F: Fibroblast.

research on the mechanisms of intestinal healing and the factors affecting it is very crucial<sup>[10]</sup>. Even though wound healing is a complex and dynamic process, it can be divided into three distinct, but at the same time overlapping phases of (1) hemostasis and inflammation, (2) proliferation, and (3) maturation and remodeling<sup>[2]</sup>. Intestinal anastomotic healing differs slightly from the general healing process, as it is a controlled full-thickness injury, followed by reconstitution of luminal integrity with artificial sutures. During this period, an organized

and complex cascade of cellular and biochemical events take place, and many disturbances in any phase of this healing process may result in complications<sup>[11]</sup>.

The first phase of the healing, described as the inflammatory phase is seen on the first 3 d of the healing process. As Steed and Portera *et al* reported, this is the essential phase in which the polymorphonuclear leucocytes (PMNL) migrate from the circulation to the wound<sup>[12,13]</sup>. It is really an essential phase because of the large microorganism content of the colonic

lumen. During the first three-day period, we found the regressive effect of propolis on edema might be by decreasing the level of IL-5 as Leticia *et al* reported<sup>[14]</sup>. The cellular content on the first day were supporting the regression of edema with the rare number of eosinophils. Marcucci and later Krol *et al* studied the antiinflammatory activity of propolis and 19 phenolic compounds<sup>[15,16]</sup>. Also supporting these observations, we found in our study that, while in the control group the PMNLs were leading on the first and third postoperative days, in the propolis group proliferation of fibroblasts began immediately following the decrease in the amount of PMNLs.

Extensive bacterial invasion was well known to impair healing at anastomotic sites. This is most likely secondary to increased PMNLs invasion and activation by microorganisms. Granulocyte collagenase activity of PMNLs' has been implicated at increasing collagen breakdown and delaying the anastomotic healing process. The potential protective effect of propolis may be suggested by our findings of early decreased number of PMNLs and less inflammatory reaction at the anastomotic line and further away during first and third day<sup>[17,18]</sup>.

Propolis has a constructive antibacterial effect besides antiinflammatory properties on bacterial invasion, which plays a crucial role in the healing process. There are a number of studies documenting the antimicrobial functions of propolis, its extracts, and constituents. This is a broad-spectrum activity against Gram-positive and Gram-negative rods and cocci, and viruses<sup>[19]</sup>. Park *et al* tested antimicrobial activity on oral microorganisms<sup>[20]</sup>; and Sosa *et al* observed that propolis samples inhibited the growth of some gram-positive bacteria and *Candida albicans*<sup>[21]</sup>. Kujumgiev *et al* further examined the antibacterial, antifungal, and antiviral activity of propolis from different geographical origins. All the propolis samples were active against the fungal and gram-positive bacterial test strains, and most showed antiviral activity. The antibacterial activities of these samples were very similar, despite differences in their chemical composition<sup>[22]</sup>. We had also shown its protective effect on ileal mucosa and reduced bacterial translocation in an experimental obstructive jaundice model in our previous researches<sup>[23]</sup>. Although the chemical composition of propolis varies depending on the site of its collection, antimicrobial properties seem attributable mainly to the flavonoids pinocembrin, galangin, and pinobanksin<sup>[24]</sup>. Other active compounds are esters of coumaric acid and caffeic acids. Phenylated p-coumaric and diterpenic acids possess antibacterial and cytotoxic activities. Caffeoylquinic acid derivatives show immunomodulatory and hepatoprotective actions and furofuran lignans inhibit the growth of some bacteria<sup>[25]</sup>.

Propolis has been used in the treatment of cutaneous lesions such as burns, wounds, and ulcers. Morales *et al*<sup>[26]</sup> used a hypoallergic formula of propolis and obtained a very satisfactory evolution and cicatrization in cases of wounds with and without infection. A fast cure, shorter treatment period, and less septic complications were

also obtained. The cicatrization was evident between the 4th and 5th day by the early formation of granulation tissue. The antimicrobial capacity was evident with a fast regression of the septic component of the suppurated wound. Claus *et al* reported that, propolis also exhibits immunostimulatory and immunomodulatory effects *in vitro* on macrophages<sup>[27]</sup>, while Kimoto *et al* reported it increases the ratio of CD4/CD8 T cells *in vivo* in mice<sup>[28]</sup>. An intact cellular immune response, particularly of T lymphocytes, is not necessary for the initial phase of wound healing but seems to be essential for a normal outcome of tissue repair. In our study, the propolis group indicated that lymphocytes appear earlier in contrast with the control group. Propolis exerts a wound healing effect by minimizing acute inflammatory exudate and stimulating macrophage and CD4 T lymphocyte activity.

In this study, angiogenesis was remarkable in the propolis group beginning on the third postoperative day; unfortunately, the capillaries were poor in the control group. We know that oxygen is critical for anastomotic wound healing<sup>[2]</sup>. The exact cellular and molecular mechanisms caused by hypoxia, which results in impaired anastomotic healing, are not well understood. While hypoxia may act as an early stimulus for neovascularization, it is unlikely to sustain it. The process of angiogenesis, whereby new capillaries are formed from pre-existing blood vessels, is essential for wound healing<sup>[29]</sup>. At the molecular level of angiogenesis, vascular endothelial growth factor (VEFG) and nitric oxide (NO) are the two key factors that are relevant<sup>[30]</sup>. Tan-no *et al* reported the anti-inflammatory effect of propolis through inhibition of nitric oxide production<sup>[31]</sup> and also Attard *et al* reported the expression of both VEGF and inducible nitric oxide synthase (iNOS) significantly increased at the anastomotic site exposed to a hypoxic environment but the healing was not enhanced<sup>[32]</sup>. These two hypotheses above warrant further evaluation. The molecular mechanisms of angiogenesis and the reflection of it to the healing process are not clearly understood yet; however, we definitely observed new capillary formation in the early period of the propolis group.

Propolis mostly contains flavonoids and phenolic compounds, which have been reported to have antioxidative properties. Due to its antioxidative effects, propolis may protect humans from deleterious oxidative processes. Several groups of authors thus studied the antioxidative properties of propolis and their active constituents<sup>[33-35]</sup>. Propolis may also act as a scavenger against oxygen radicals. Recent studies indicate that propolis is able to inhibit the formation of superoxide anion, which is produced during autoxidation of  $\beta$ -mercaptoethanol<sup>[25]</sup>.

In the healing process, the first phase of inflammation is followed by the second phase, fibroblastic proliferation. Fibroblasts are normally found in the later phases of normal wound healing. They are responsible for the production of collagen and for establishing the structural extracellular matrix. Although

the healing process can be divided into phases as we mentioned before, they are overlapping. The maturation and remodeling phase following the fibroblastic proliferation are very close to each other. Because remodeling of tissue requires the deposition of adequate amounts of extracellular matrix components, particularly collagen fibers, in the wound area. As comprehended, fibroblasts have a key position. Graham *et al* reported, in contrast to dermal tissue, both smooth muscle cells and fibroblasts manufacture collagen in the gastrointestinal tract<sup>[36]</sup>. We know that the bulk of collagen within the intestinal tract is contained within the submucosa since intestinal smooth muscle cells in the lamina propria produce and maintain intestinal collagen. As a result, most of the strength of the intestine is found in the submucosal layer. Furthermore, it is responsible for anchoring the sutures that hold anastomosed bowel ends together<sup>[11]</sup>.

Simpson and Ross reported that, as early as 3 d after wounding, immature fibroblasts could be identified within the wound exudate<sup>[37]</sup>. We observed the fibroblasts on the third postoperative day of the control group, but interestingly, they were present on the first postoperative day of the propolis group. On the first day, possibly they were immature fibroblasts, but on the third day as these cells matured, they acquired the characteristic ultrastructural features of actively synthetic fibroblasts. By day 5, small collagen fibers were observable by electron microscopy within the extracellular spaces; the collagen fibers were distinguishable on the third postoperative day of the propolis group. The collagen fibers were in a spatial arrangement and inclining towards the anastomotic line.

Although healing in the gastrointestinal tract essentially follows the same phases as skin wound healing, our understanding of these events is much more limited. Just as with dermal reepithelialization, if the mucosa is the only injured layer, it can be reformed by migration and proliferation. Full-thickness injury provokes a fibroblastic response resulting in scar formation<sup>[38]</sup>. During the first few postoperative days, anastomotic strength is low, as collagen is degraded secondary to collagenase activity at the site of the injury. Early anastomotic strength is therefore dependent on the suture-holding capacity of existing collagen until large amounts of new collagen can be synthesized by both fibroblasts and smooth muscle cells. The final phase of healing involves maturation of newly formed anastomosis by the transformation of collagen into thick bundles and contractile units<sup>[39]</sup>. In the propolis group, the proliferation, activation, and synthesis capacity of fibroblasts were much better than the control group. As expected, the bridging of the gap between the anastomotic edges as the means of remodeling was completed.

All of the bee products, especially honey, have had a valued place in traditional medicine for centuries. In the last century, researchers rediscovered this old remedy and honey has been of proven value in many pathologies and also in the surgical wound. Honey is

a supersaturated sugar solution which the half of its content is fructose and glucose with other chemical compounds in small quantities like flavonoids and phenolics. Consequently as a bee product, propolis (100 mg/kg per day) is recommended in this study because it is more effective than the same doses of honey (10 g/kg per day)<sup>[7,40]</sup>.

In conclusion, the results of this study clearly demonstrate that, according to the histopathological parameters, administration of propolis, as an additional strategy to surgical excision, accelerated the healing of colon anastomosis. Propolis, a beehive product, possesses a broad spectrum of biological activities including hepatoprotective, antitumour, antioxidative, antimicrobial, and antiinflammatory effects. Current opinion is that the use of standardized preparations of propolis is safe and less toxic than many synthetic products. Our results suggest that propolis enhances not only the early phase of colon anastomotic healing by inhibiting the inflammatory response, but also stimulates the collagen synthesis of fibroblasts. The exact mechanisms of improved anastomotic wound healing by propolis administration are not known, although increase of neoangiogenesis, mononuclear cell infiltration, and the stimulation of collagen synthesis are all involved. These findings may provide insight into potential therapeutic approaches to improve and promote healing of colonic anastomosis and to minimize the morbidity and mortality associated with anastomotic leaks. We conclude that propolis should be further investigated for its capacity to enhance anastomotic healing and to reduce the incidence of the anastomotic dehiscence.

## COMMENTS

### Background

Inadequate healing of colon anastomosis may still be a cause of postoperative morbidity and mortality despite the use of optimal surgical techniques.

### Research frontiers

This study was to evaluate the effect of propolis administration on the healing of colon anastomosis with light and transmission electron microscopes.

### Innovations and breakthroughs

The achievement of this article is to show the stages of anastomotic healing with clear light and electron micrographs without any doubt and draw attention to the cellular mechanisms of the healing. The breakthrough of our experience from the present study is that: propolis which is a natural, cheap and easily obtained material can be used safely in colonic anastomosis.

### Applications

Administration of propolis, as an additional strategy to surgical excision, might be used to accelerate the healing of colon anastomosis and to prevent the anastomotic leakage, in clinical settings.

### Peer review

Our results suggest that propolis enhances not only the early inflammatory phase of colon anastomotic healing but also stimulates the collagen synthesis of fibroblasts. These findings may provide a new insight into potential therapeutic approaches to improve and promote healing of colonic anastomosis.

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RAPID COMMUNICATION

# Prevalence of metabolic syndrome, obesity and diabetes type 2 in cryptogenic cirrhosis

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## Abstract

**AIM:** To evaluate the prevalence of metabolic syndrome (MS), obesity and type 2 diabetes mellitus (T2DM) in a group of Mexican Mestizo patients with cryptogenic cirrhosis (CC) and to compare this group with patients with cirrhosis secondary to other causes (disease controls).

**METHODS:** Patients with CC, diagnosed between January, 1990 and April, 2005, were included in a retrospective study. Patients with cirrhosis caused by chronic hepatitis C, alcohol abuse or autoimmune hepatitis (AIH) served as disease controls.

**RESULTS:** A total of 134 patients with CC were analyzed. Disease controls consisted of 81 patients with chronic hepatitis C, 33 with alcohol abuse and 20 with AIH. The median age of patients with CC was 57 years (range, 16-87); 83 (61.9%) patients were female; 53 (39.6%) were Child A, 65 (48.5%) Child B, and 16 (11.9%) were Child C cirrhosis. The prevalence of MS (29.1% vs 6%;  $P < 0.001$ ), obesity (16.4% vs 8.2%;  $P = 0.04$ ) and T2DM (40% vs 22.4%;  $P = 0.013$ ) was higher in CC patients than in disease controls. There were no differences in sex, age or liver function tests between the two groups.

**CONCLUSION:** The prevalence of MS, obesity

and T2DM were higher in patients with CC than in patients with cirrhosis secondary to others causes. Our findings support the hypothesis that non-alcoholic steatohepatitis (NASH) plays an under-recognized role in CC.

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**Key words:** Cryptogenic chronic hepatitis; Metabolic syndrome; Obesity; Diabetes mellitus

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Tellez-Avila FI, Sanchez-Avila F, García-Saenz-de-Sicilia M, Chavez-Tapia NC, Franco-Guzman AM, Lopez-Arce G, Cerda-Contreras E, Uribe M. Prevalence of metabolic syndrome, obesity and diabetes type 2 in cryptogenic cirrhosis. *World J Gastroenterol* 2008; 14(30): 4771-4775 Available from: URL: <http://www.wjgnet.com/1007-9327/14/4771.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4771>

## INTRODUCTION

The diagnosis of "cryptogenic" cirrhosis is made after an extensive evaluation has excluded recognizable etiologies<sup>[1]</sup>. The prevalence of cryptogenic cirrhosis (CC) ranges from 5% to 30% in cirrhotic patients<sup>[1]</sup>. In Mexico, the etiology of cirrhosis remains unclear in 10% patients despite an extensive evaluation<sup>[2]</sup>. Several etiological possibilities are offered in such patients. These include occult alcohol abuse, silent autoimmune hepatitis (AIH), occult viral (non-B, non-C) hepatitis, and progression of nonalcoholic steatohepatitis (NASH)<sup>[3]</sup>.

The prevalence of clinically silent autoimmune hepatitis in patients with CC is unknown; however, several studies have suggested that a significant number of patients with CC may have burnt-out AIH<sup>[4-6]</sup>. Occult virus disease (Non-B, non-C hepatitis) is considered to account for about 15% of post-transfusion hepatitis<sup>[7]</sup> and may exist in a silent form for several years<sup>[8]</sup>. Obesity and non-insulin dependent diabetes mellitus are the

two most common conditions associated with NASH<sup>[9]</sup>, which is frequently asymptomatic<sup>[10]</sup> and can progress silently to cirrhosis with definitive histological features<sup>[10]</sup>.

The aim of the present study was to characterize the metabolic disturbances [prevalence of metabolic syndrome (MS), obesity and type 2 diabetes mellitus (T2DM)] in a group of Mexican Mestizo patients with CC. In particular, we compared the prevalence of metabolic disturbances in the cryptogenic group with patients with cirrhosis due to other causes: hepatitis C without prior alcoholism, alcohol abuse and AIH.

## MATERIALS AND METHODS

In a retrospective manner, we examined the medical records (paper and electronic-based records) of all patients with CC diagnosed from January, 1990 to April, 2005. We also included in a random fashion, disease controls consisting of patients with cirrhosis caused by chronic hepatitis C, alcohol abuse and AIH.

Diagnosis of CC was made after an exhaustive evaluation failed to provide a specific etiology. The data collected included the hepatologic diagnosis, comorbid conditions, complications of portal hypertension if present, and major forms of treatment. Additional information was obtained from clinical charts, hospital records, the clinic and hospital laboratory databases, and by personal or telephone interview. Patients were included in the study if sufficient data was available and if the diagnosis was confirmed on review of all the available information.

The diagnosis of cirrhosis was made on the basis of clinical, laboratory and imaging data. In addition, histological findings were available in 56 (42%) CC patients. Biopsy was not performed in 78 patients, either because of refusal by the patient or their in-charge physician. Data collected included gender, age at diagnosis of cirrhosis, presenting symptoms, potential occupational exposure to hepatotoxins, family history of liver disease, and family or personal history of autoimmune diseases. Risk assessment for viral hepatitis included history of exposure to intravenous drugs, blood transfusions, tattoos, other known percutaneous needle exposures, and high-risk sexual behavior. All patients underwent extensive serological testing including hepatitis B and C screening [hepatitis B surface antibody, surface antigen, and anticore antibody, and hepatitis C enzyme-linked immunosorbent assay (Abbott Laboratories, Abbott Park, IL)], iron studies (ferritin, iron, iron binding capacity, and tissue assessment if the diagnosis was questionable), ceruloplasmin, antinuclear antibody (ANA), antimitochondrial antibody, and  $\alpha$ 1-antitrypsin. Quantitative immunoglobulin levels (IgG, IgM, IgA) were obtained in all patients. Assessment of  $\alpha$ 1-antitrypsin level was performed using isoelectric focusing (pH range, 4.0-5.0).

Patients with CC who had a positive antinuclear antibody (positive > 1:80) test, an index of autoimmune hepatitis, were evaluated by the International Autoimmune Hepatitis (IAH) score, based on clinical

and laboratory parameters as previously described<sup>[11]</sup>. None of the patients received steroid therapy, and thus the IAH score was calculated using the Minimal Required Parameters, wherein a score of 10 to 15 is suggestive of autoimmune hepatitis, and a score of greater than 15 is considered definitive. The term overweight was defined as body mass index (BMI) greater than 25, while obesity was defined as a BMI greater than 30. BMI was calculated by dividing the patients' body weight by the square of their height expressed as kg/m<sup>2</sup>. BMI was calculated using the average adult weight reported by the patient and the patient's height. In all cases, type 2 DM was diagnosed by the presence of recurrent fasting hyperglycemia ( $\geq 126$  mg/dL), requiring treatment with dietary management, oral hypoglycemic agents, or insulin therapy. Dyslipidemia was considered in the presence of high serum triglycerides ( $> 150$  mg/dL) and/or low high-density lipoproteins ( $< 50$  mg/dL in women and  $< 40$  mg/dL in men). The diagnosis of MS was made according to the NCEP (ATP) III consensus<sup>[12,13]</sup>.

The absolute and relative frequencies were used for summary. The data is presented as mean  $\pm$  SD. The one-way ANOVA test or Kruskal-Wallis was used to compare parametric or nonparametric variables, respectively. The  $\chi^2$  test was used for categorical variables. A *P* value ( $\alpha$ ) of  $< 0.05$  was considered significant. Bonferroni correction for *P*-value was applied for multiple comparisons, calculated as  $\alpha/n$ . For multiple comparisons a *P* value of  $< 0.016$  was considered significant. All statistical analyses were conducted using the statistics program SPSS/PC version 12.0 (Chicago, IL, USA).

## RESULTS

After careful review of the medical records, 50 patients who were originally classified as CC in the hospital registry were found to have other causes of liver disease. The main reason for this discrepancy was incomplete investigation or erroneous interpretation of the test results when the patients were referred to our center. These patients were initially listed as CC, but the diagnosis was not corrected in the registry when the new information became available. Other less common reasons for patient exclusion were incomplete medical information and indeterminate test results. For the final analysis, a total of 134 patients with CC were included in the study. In addition, EIGHTY ONE patients with chronic hepatitis C, thirty-three with alcohol abuse and twenty with AIH were evaluated as disease controls. The demographic, clinical, and laboratory characteristics of the study subjects are summarized in Table 1. In patients with CC, the median age was 57 years (range 16-87); 83 (61.9%) were female; and 53 (39.6%) had Child A cirrhosis, 65 (48.5%) were Child B and 16 (11.9%) were Child C.

Five patients were determined to have moderate alcohol consumption (< 2 drinks/d), but this was not considered to be the cause of their liver disease, either by the hepatologist or their primary care physician. None of the patients had a history of intravenous drug

**Table 1 Demographic, clinical and laboratory parameters of patients with cryptogenic cirrhosis and non-cryptogenic cirrhosis**

Variable	Cryptogenic (n = 134, %)	Non-cryptogenic (n = 134, %)	P
Sex (female)	83 (62)	75 (56)	0.32
DM	53 (40)	30 (22.4)	0.013
HBP	24 (18)	14 (10.4)	0.08
Hyperuricemia	13 (10)	2 (1.5)	0.003
Dyslipidemia	72 (54)	8 (6)	< 0.001
Overweight (BMI > 25)	103 (77)	106 (79)	0.65
Obesity (BMI > 30)	22 (16.4)	11 (8.2)	0.04
MS	39 (29.1)	8 (6)	< 0.001
Age (yr, mean ± SD)	54.6 ± 14.3	56.8 ± 11.4	0.15
BMI (mean ± SD)	27 ± 4.6	26 ± 4	0.22
ALT (U/L, mean ± SD)	52.5 ± 59	57.5 ± 33	0.72
AST (U/L, mean ± SD)	67.1 ± 60	77.8 ± 46	0.19

DM: Diabetes mellitus; HBP: High blood pressure; BMI: Body mass index (calculated as patient's body weight divided by the square of the height expressed in kg/m<sup>2</sup>); MS: Metabolic syndrome.

use. Seven patients had a history of blood transfusions, but none of them had hepatitis C or hepatitis B virus infections. Seven patients had a positive family history of liver disease. A positive antinuclear antibody test was present in 13 patients (10%), but a definite score for autoimmune hepatitis was not present in any patient. Serum α1-antitrypsin deficiency was assessed in 6 patients. However, none of the patients had biochemical or histological evidence of α1-antitrypsin deficiency. Serum ferritin and iron saturation tests were measured in all patients and were within normal/non-diagnostic limits. Genetic testing for hemochromatosis was not performed, and thus carriage of abnormal alleles cannot be excluded. There was no difference in the liver function tests or the Child Pugh score between patients with CC who had a liver biopsy (n = 56, 42%) and those did not (n = 78, 58%). However, patients without liver tissue examination had higher prevalence of metabolic disturbances (Table 2).

The prevalence of MS, obesity and T2DM were greater in CC patients compared to patients without CC (Table 1). When patients without CC were classified by etiology (hepatitis C, alcohol, and AIH), significant differences in MS prevalence were observed: 6.2% in hepatitis C, 6% in patients with alcohol abuse, and 5% in AIH vs 29.1% in CC patients (P < 0.001). The differences in the prevalence of T2DM persisted, but when Bonferroni correction for multiple-comparison was used, only obesity showed a statistical trend (Table 3). The prevalence of the different components of MS were analyzed separately; Dyslipidemia (P < 0.001) and abnormal glucose (P = 0.01) were more common in CC patients than in disease controls, while high blood pressure (HBP) showed a trend towards significance (P = 0.08). Hyperuricemia was more frequent in CC patients (10% vs 1.5%, P = 0.003).

## DISCUSSION

The present study shows a high prevalence of MS,

**Table 2 Comparison of patients with cryptogenic cirrhosis with and without liver tissue examination**

Variable	Liver biopsy (n = 56, %)	No liver biopsy (n = 78, %)	P
Sex (female)	37 (66)	46 (59)	0.47
DM	13 (23)	40 (51)	0.001
HBP	7 (13)	17 (22)	0.18
Hyperuricemia	3 (5)	10 (13)	0.03
Dyslipidemia	21 (38)	51 (65)	0.002
Overweight (BMI > 25)	46 (82)	57 (73)	0.29
Obesity (BMI > 30)	4 (7)	18 (23)	0.017
MS	10 (18)	29 (37)	0.02
Child-Pugh A	29 (52)	24 (31)	0.02
Age (yr, mean ± SD)	55.8 ± 14.5	53.7 ± 14	0.42
BMI (mean ± SD)	26.2 ± 4.6	27.3 ± 4.6	0.28
ALT (U/L, mean ± SD)	51.8 ± 38	52.9 ± 70	0.91
AST (U/L, mean ± SD)	66 ± 48	68 ± 69	0.86
Albumin (g/dL, mean ± SD)	3.3 ± 0.7	2.9 ± 0.6	0.01
Alkaline Phosphatase (U/L, mean ± SD)	150 ± 72	154 ± 74	0.7
Child-Pugh score (mean ± SD)	6.9 ± 2.7	7.7 ± 1.8	0.06

DM: Diabetes mellitus; HBP: High blood pressure; BMI: Body mass index (calculated as patient's body weight divided by the square of the height expressed in kg/m<sup>2</sup>); MS: Metabolic syndrome.

obesity, and T2DM in Mexican Mestizo population with CC. The relationship between T2DM, obesity, and cirrhosis has been much debated<sup>[14-17]</sup>. To our knowledge, this is the first study that shows an association between MS and CC. There is less controversy regarding an association between MS, obesity, T2DM, and NASH<sup>[18]</sup>, and several previous studies have shown a relationship between components of MS and NASH as well as the severity of liver fibrosis<sup>[19-21]</sup>. MS is a worldwide problem with a high prevalence rate<sup>[22]</sup>, and in agreement with our data this abnormality, along with some of its components, is more frequent in CC than in patients with cirrhosis caused by other etiologies. This finding is very important because it provides further evidence to support the theory that NAFLD/NASH can progress to cirrhosis in some patients.

The prevalence of MS was 500% higher in patients with CC compared to patients without CC. When the prevalence of each of the MS components in patients with and without CC was analyzed, only abnormal glucose values and Dyslipidemia showed statistically significant differences between the two groups (Table 1). There was no difference between the two groups with respect to the prevalence of HBP and being overweight. This may be related to the hemodynamic changes and malnutrition, seen commonly in cirrhotic patients. The mean ± SD of HDL and triglyceride levels in CC patients were similar in women (43.4 ± 10.9 mg/dL and 92.4 ± 49 mg/dL) and men (39.5 ± 8.5 mg/dL and 111.3 ± 59 mg/dL). Both of these test values were abnormal when the NCEP guidelines were taken into consideration (abnormal HDL serum levels < 50 mg/dL for women and < 40 mg/dL for men); prevalence of low HDL levels was seen in 76.7% women and 41.5% men. An observation not previously reported is the finding of higher prevalence (statistically significant) of hyperuricemia in CC compared to disease controls. Hyperuricemia is not accepted as

**Table 3 Comparison of patients with cryptogenic cirrhosis and disease controls separated by the etiology of cirrhosis**

<b>Variable</b>	<b>Cryptogenic (n = 134, %)</b>	<b>CHC (n = 81, %)</b>	<b>Alcohol (n = 33, %)</b>	<b>AIH (n = 20, %)</b>	<b>P</b>
Sex (female)	83 (62)	54 (66.7)	7 (21.2)	14 (70)	< 0.001
DM	53 (40)	17 (21)	10 (30.3)	3 (15)	0.013
HBP	24 (18)	8 (10)	4 (12.1)	2 (10)	0.36
Hyperuricemia	13 (10)	1 (1.2)	0 (0)	1 (5)	0.027
Dyslipidemia	72 (54)	5 (6)	1 (3)	2 (10)	< 0.001
Overweight (BMI > 25)	103 (77)	63 (78)	27 (81.8)	16 (80)	0.93
Obesity (BMI > 30)	22 (16.4)	5 (6.2)	5 (15.2)	1 (5)	0.10
MS	39 (29.1)	5 (6.2)	2 (6)	1 (5)	< 0.001
Age (yr, mean ± SD)	54.6 ± 14.3	56.8 ± 11.4	58 ± 12.6	55.6 ± 14.1	0.48
BMI (mean ± SD)	27 ± 4.6	26 ± 4	26.4 ± 3.5	26.1 ± 5.1	0.65
ALT (U/L, mean ± SD)	52.5 ± 59	57.5 ± 33	52.6 ± 47.9	46.2 ± 27.7	0.79
AST (U/L, mean ± SD)	67.1 ± 60	77.8 ± 46	73.1 ± 52	73.5 ± 69	0.59

CHC: Cirrhosis by hepatitis C virus; AIH: Autoimmune hepatitis; DM: Diabetes mellitus; HBP: High blood pressure; BMI: Body mass index (calculated as patients' body weight divided by the square of the height expressed in kg/m<sup>2</sup>); MS: Metabolic syndrome.

a criterion of MS; however, it is a common metabolic disturbance in this group of patients. We believe that the higher prevalence of hyperuricemia in CC may be another piece in the puzzle in the relationship between MS, NASH and cirrhosis.

In 1999, Caldwell *et al*<sup>[17]</sup> described the prevalence of obesity and T2DM in 70 patients with CC, and compared the findings with three patient groups: NASH, cirrhosis with hepatitis C, and primary biliary cirrhosis (PBC). The prevalence of these risk factors (obesity and T2DM) were similar between patients with NASH and patients with CC, both of which had a higher prevalence compared to patients with hepatitis C and PBC. In another study by Poonawala *et al*<sup>[16]</sup>, the prevalence of obesity and T2DM in patients with CC was compared with the prevalence in control patients. The various causes of cirrhosis in the control group were alcohol, chronic viral hepatitis, AIH, PBC and primary sclerosing cholangitis. Similar to the findings by Caldwell *et al*<sup>[17]</sup>, the prevalence of obesity (55% vs 24%) and T2DM (47% vs 22%) were significantly higher in patients with CC compared with disease controls. Both authors concluded that their data supported the hypothesis that NASH may be an etiological factor in some of the patients with CC<sup>[16,17]</sup>. We obtained similar results, but in a different population (Mexican Mestizo) and with a bigger sample size. When we classified the patients as CC vs no CC, important differences in the prevalence of obesity and T2DM were observed (16.4% vs 8.2% and 40% vs 22.4%, respectively). However, when patients without CC were classified by etiology, only the prevalence of T2DM was statistically significant (Table 3). With respect to obesity, the prevalence between CC and patients with cirrhosis secondary to alcohol abuse was similar, and both showed a higher frequency than patients with cirrhosis due to hepatitis C and AIH.

An interesting finding in the present study was that patients with CC without a liver biopsy had greater prevalence of MS, obesity and T2DM compared with patients with CC who had a liver biopsy, despite similar liver function tests. This finding may be related to the

presence of metabolic disturbances, suggesting to the physician the diagnosis of CC secondary to NASH; thus creating a different situation from patients with CC without metabolic disturbances.

The present study suffered from some limitations. First, the study design. Second, we did not record the waist circumference for the diagnosis of MS, but used BMI as a substitute for waist circumference. The use of BMI may have had a small impact on the number of cases diagnosed with MS, since there is a strong correlation between these parameters ( $r = 0.8$ )<sup>[23,24]</sup>. We recognize that this may have resulted in underestimating the number of cases that fulfilled the NCEP definition.

In conclusion, the prevalence of MS, obesity and T2DM in patients with CC is higher than that seen in patients with cirrhosis secondary to others causes. Moreover, the prevalence of hyperuricemia was higher in patients with CC compared to patients with cirrhosis secondary to others causes, a finding not reported previously. Our results support the hypothesis that NASH plays an under-recognized role in some patients with CC.

## COMMENTS

### Background

Nonalcoholic steatohepatitis (NASH) is the main etiology suspected in patients with Cryptogenic Cirrhosis (CC). The association of NASH with Metabolic Syndrome (MS) is well-known; however, the association of CC with MS has not been well examined.

### Research frontiers

The possible association of MS and CC remains unknown.

### Innovations and breakthroughs

This study shows an association between MS with CC, and raises the possibility of an under-recognized role of NASH in CC.

### Applications

Further prospective studies may clarify the association between MS and CC.

### Peer review

The findings in the present study imply that non-alcoholic steatohepatitis is frequently associated with cryptogenic cirrhosis. This paper is well written and the results suggest an under-recognized role of NASH in patients with cryptogenic cirrhosis.

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RAPID COMMUNICATION

## Modification of end-loop ileostomy for the treatment of ischemic or radiation enteritis

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**Author contributions:** Tepetes K conceived the technique and the study, is the main surgeon of the reported cases; Liakou P performed data acquisition and follow up of the patients; Balogiannis I wrote the manuscript; Kouvaraki M had a supportive contribution and revised the manuscript; Hatzitheofilou K supervised data acquisition and follow up of the patients.

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### INTRODUCTION

Fecal diversion by colostomy or ileostomy represents an important treatment in colorectal surgery. Fecal diversion may be either temporary for decompression, or permanent for palliation. The indications for fecal diversion include inflammatory bowel diseases, familial adenomatous polyposis, colorectal carcinoma, nongastrointestinal obstructing tumors, pelvic sepsis, trauma, diverticulitis, fistulas, ischemic bowel disease, radiation enteritis, pseudomembranous enterocolitis, fecal incontinence and paraplegia<sup>[1]</sup>.

An ileostomy may be performed as an end stoma, or as a loop stoma constructed either for decompression of the gastrointestinal tract or for the protection of a distal colonic anastomosis. The majority of loop ileostomies are performed as a temporary treatment, except for those performed for palliation (i.e. non-respectable tumors in patients with low expected survival)<sup>[2-5]</sup>. Occasionally, a small bowel stoma is required in patients with high risk of small intestinal anastomosis.

In this report, we present a new technique of ileal anastomotic stoma, following small bowel resection, in patients in whom the anastomosis is anticipated to have borderline margins with dubious viability. These include patients with mesenteric ischemia, radiation induced stenosis or fistulas, bowel necrosis due to hernias or adhesions, intestinal trauma and necrotizing enterocolitis in children<sup>[6]</sup>.

### MATERIALS AND METHODS

Five patients underwent enterectomy and partially anastomosed end-loop ileostomy at the University

### Abstract

**AIM:** To evaluate a new technique of temporary ileal anastomotic stoma, following small bowel resection, in patients where the anastomosis is anticipated to have borderline margins with dubious viability.

**METHODS:** Five patients underwent enterectomy and partially anastomosed end-loop ileostomy at the University Hospital of Larissa between 2000 and 2006. Enterectomy was performed because of conditions such as mesenteric vascular occlusive disease, radiation enteritis and small bowel injury.

**RESULTS:** Postoperatively, none of the patients developed any stoma-related or anastomotic complications. There were no major complications. All patients were discharged between the 8th and 15th day after the procedure, and the stoma was closed 3 wk to 4 wk later.

**CONCLUSION:** We believe that our proposed modification of end-loop ileostomy is a simple, quick and safe technique with minimal stoma-related morbidity, and with simple and safe reversion. This technique can be considered as a useful option in the treatment of ischemic or radiation-induced enteritis, and in the management of severe intestinal trauma.

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**Key words:** Anastomotic stoma; Loop ileostomy; Ischemic enteritis; Radiation enteritis

**Table 1** Clinical characteristics of the study patients

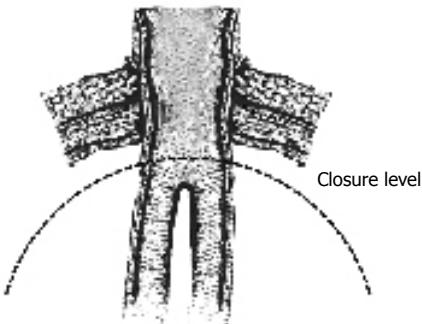
Patient	Age/Sex	Diagnosis	Procedure	Time of stoma closure (wk)
1	65/F	Radiation enteritis ileal obstruction	50 cm small bowel resection	4
2	71/F	Radiation enteritis ileal obstruction	60 cm small bowel resection	4
3	74/F	Intestinal ischemia	60 cm small bowel resection	3
4	72/M	Intestinal ischemia	80 cm small bowel resection	4
5	52/M	Blunt abdominal injury, ileal rupture	50 cm small bowel resection	3

**Figure 1** Afferent and efferent limbs brought out together.**Figure 2** Side-to-side anastomosis performed, with the blind end forming a stoma.

Hospital of Larissa between 2000 and 2006. The patient's mean age at stoma formation was 66 years (range, 52-74 years). There were three female and two male patients, and all were Caucasian. Enterectomy was performed secondary to mesenteric vascular occlusive disease, radiation enteritis and small bowel injury. The clinical characteristics of patients are shown in Table 1.

Following resection of the diseased intestinal segment, the afferent and efferent limbs were anastomosed side-to-side using a GIA 100-38 linear stapler (Autosuture, Norwalk, Connecticut, USA; Figure 1). The common blind end remained open and was brought out as a stoma (Figure 2). Using this technique the integrity and the viability of both limbs could be inspected and both the proximal and distal bowel loops were vented.

Closure of the enterostomy is a simple procedure that can be performed under local anesthesia and sedation. The common blind end-stoma was mobilized from the skin, subcutaneous tissue and fascia and was closed just below the fascia level using a TA-55 stapler (Autosuture, Norwalk, Connecticut, USA).

**Figure 3** Closure of the blind end stoma results in a patent side-to-side anastomosis.

The short amputation of the common limb (4-5 cm) enabled side-to-side anastomosis to restore anatomic continuity without sacrificing any length of the intestines (Figure 3).

Closure of the stoma was accomplished in 3 wk to 4 wk. Direct inspection of both limbs of the already fashioned side-to-side anastomosis was possible during that period.

## RESULTS

Postoperatively, none of the patients developed any stoma-related or anastomotic complication. There were no major complications. Two of the five patients developed a mild wound infection which was managed conservatively with local measures. All patients were discharged between the 8th and 15th day after the procedure and the stoma was closed 3 wk to 4 wk later.

## DISCUSSION

Small bowel enterostomies are occasionally required following emergent small bowel resection in conditions such as bowel ischemic, and inflammatory or traumatic disorders. The clinical setting of these patients requires a quick and safe procedure preserving as much of the intestinal length as possible. A small intestinal diverting stoma, however, carries significant morbidity, mostly due to fluid, electrolyte and nutrient imbalance. Furthermore, the restoration of intestinal continuity usually requires meticulous dissection of the afferent and efferent segments and the formation of a new anastomosis, usually under general anesthesia.

Even a loop or "end loop" stoma requires an anastomosis for its closure, as well as peritoneal breaching for the intraabdominal position of the anastomotic segment.

Because of the aforementioned reasons, closure itself is associated with complications such as small bowel obstruction (0%-15%), anastomotic leak (0%-8%), fistula formation (0%-7%), wound infection (up to 18%) and development of hernia at the stoma site (1%-12%)<sup>[7,8]</sup>.

Finally, morbidity related to general anesthesia should also be taken into consideration, because the majority of these patients have poor general health and nutritional status secondary to the underlying medical disorders<sup>[9]</sup>.

A special variant of ileal stoma was first described by Bishop, in 1957, for the treatment of meconium ileus, and shortly afterwards by Santuli, in 1961, for the treatment of congenital atresia of the intestines in pediatric patients. After resection of the diseased bowel, either the distal (Bishop) or the proximal (Santuli) limb is brought out as a stoma, while the remaining limb is anastomosed end-to-side to the former, a few centimeters below the abdominal wall, in order to avoid creating a blind loop. These stomas provide satisfactory decompression of the proximal bowel, along with the passage of some amounts of intestinal content into the distal limb. Although the construction of such stomas is rather demanding, they have the advantage of easy closure, even under local anesthesia.

However, the presence of an intrabdominal anastomosis carries several risks such as obstruction, breakdown, leakage and sepsis. Nevertheless, the Santuli enterostomy, the mirror image of the Bishop procedure, has been reported to be efficacious in adult patients as well, in conditions such as mesenteric ischemia, bowel necrosis due to an incarcerated hernia and trauma<sup>[10-12]</sup>.

In the present study, we modified the anastomotic stoma, a "partially anastomosed end-loop ileostomy" for the treatment of adult patients, who presented with conditions such as small intestinal necrosis due to extended mesenteric vascular occlusive disease, radiation-induced stenosis, and intestinal trauma with macroscopically borderline margins with respect to the viability of the intestinal wall after extended bowel resection. In order to preserve as much length of the small intestine as possible, and also to protect the terminal ileum, we constructed a side-to-side anastomosis between the remaining bowel limbs and fastened the common blind end as a stoma. Thus, spontaneous decompression was achieved along with the ability to easily inspect the bowel integrity through the stoma. Furthermore, both limbs were vented, and the distal limb received bowel contents within 48 h, minimizing massive intestinal fluid loss. Thus, we created a low output ileo-cutaneous fistula. The formation of the anastomosis in a side-to-side fashion provides richer blood supply, augmenting the viability of its intrabdominal portion. Moreover, the procedure requires less time than an end-to-side anastomosis. The formation of such a stoma was simple and quick, with no risk of stenosis or kinking. We believe that the short duration of our technique is an important advantage since operative time is a critical factor in the survival of these patients (urgent cases, unstable patients, "damage control" in trauma)<sup>[6]</sup>.

In addition, closure of the stoma can be performed easily and quickly under local anesthesia, by mobilizing the common limb from the abdominal wall and stapling it just below the fascia without entering the abdominal cavity. Moreover, it can be performed within 4 wk, since the integrity of the intestine can be assured by direct inspection of both limbs.

We believe that our proposed modification of end-loop ileostomy is a simple, fast and safe technique

with minimal stoma-related morbidity, and with simple and safe reversion. It should be considered as a useful treatment option in patients with ischemic and radiation-induced enteritis, and in the management of severe intestinal trauma.

## COMMENTS

### Background

Enterostomies are usually high output stomas, that often result in fluid and electrolyte depletion.

### Research frontiers

A variety of techniques have been proposed for the formation of a low morbidity ileostomy.

### Innovations and breakthroughs

Our technique is a combination of a low output stoma and an anastomosis with minimal risk of rupture.

### Applications

This technique should be taken into consideration in patients who are at a high risk of small bowel resection such as those with ischemic and radiation enteritis. We believe that our technique provides a fast, simple and safe surgical treatment of patients with small bowel necrosis.

### Peer review

Our proposed modification of end-loop ileostomy appears to be simple, fast and safe technique with minimal stoma-related morbidity, and with simple and safe reversion. It should be considered as a useful option in the treatment of ischemic and radiation-induced enteritis, and in the management of severe intestinal trauma.

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RAPID COMMUNICATION

## Thalidomide effect in endothelial cell of acute radiation proctitis

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### Abstract

**AIM:** To determine whether thalidomide prevents microvascular injury in acute radiation proctitis in white rats.

**METHODS:** Fourteen female Wistar rats were used: six in the radiation group, six in the thalidomide group, and two in normal controls. The radiation and thalidomide groups were irradiated at the pelvic area using a single 30 Gy exposure. The thalidomide (150 mg/kg) was injected into the peritoneum for 7 d from the day of irradiation. All animals were sacrificed and the rectums were removed on day 8 after irradiation. The microvessels of resected specimens were immunohistochemically stained with thrombomodulin (TM), von Willebrand Factor (vWF), and vascular endothelial growth factor (VEGF).

**RESULTS:** The microscopic scores did not differ significantly between the radiation and thalidomide groups, but both were higher than in the control group. Expression of TM was significantly lower in

the endothelial cells (EC) of the radiation group than in the control and thalidomide groups ( $P < 0.001$ ). The number of capillaries expressing vWF in the EC was higher in the radiation group ( $15.3 \pm 6.8$ ) than in the control group ( $3.7 \pm 1.7$ ), and the number of capillaries expressing vWF was attenuated by thalidomide ( $10.8 \pm 3.5$ ,  $P < 0.001$ ). The intensity of VEGF expression in capillaries was greater in the radiation group than in the control group and was also attenuated by thalidomide ( $P = 0.003$ ).

**CONCLUSION:** The mechanisms of acute radiation-induced proctitis in the rats are related to endothelial cell injury of microvessel, which may be attenuated with thalidomide.

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**Key words:** Radiation proctitis; Von Willebrand factor; Thrombomodulin; Vascular endothelial growth factor; Thalidomide

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Kim KT, Chae HS, Kim JS, Kim HK, Cho YS, Choi W, Choi KY, Rho SY, Kang SJ. Thalidomide effect in endothelial cell of acute radiation proctitis. *World J Gastroenterol* 2008; 14(30): 4779-4783 Available from: URL: <http://www.wjgnet.com/1007-9327/14/4779.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4779>

### INTRODUCTION

Radiation therapy is performed widely in the suppression of the growth of malignant tumors, but it is occasionally accompanied by acute or late complications. Acute radiation injury to the intestine induces mucosal inflammation and damage to the endothelial cells (EC) of microvessels<sup>[1,2]</sup>. In radiation enteropathy, there may be a causal relationship between EC apoptosis and crypt cell apoptosis<sup>[3]</sup>. In addition to EC damage, increased chemotaxis and thrombogenesis of damaged vessels are the main mechanisms causing radiation enteropathy<sup>[1,3-6]</sup>. These events are related to complex molecular mechanisms involving many kinds of cytokines in the coagulation system, including thrombomodulin

(TM)<sup>[1,3,4]</sup>. In inflammatory bowel disease (IBD), microvascular inflammation also plays a crucial role in the pathogenesis<sup>[7]</sup>.

In studies of IBD, thalidomide has been used to treat both animal models of colitis and human refractory Crohn's disease. Thalidomide suppresses the production of many kinds of cytokines including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and it also suppresses angiogenesis<sup>[8-12]</sup>. Angiogenesis, or new vessel formation, is considered a novel target of therapy for IBD<sup>[7,13-15]</sup>. The factors promoting angiogenesis in IBD are tissue hypoxia and many kinds of cytokines secreted by infiltrating inflammatory cells<sup>[13]</sup>. We investigated the expression of von Willebrand factor (vWF), TM and vascular endothelial growth factor (VEGF) in acute radiation-induced proctitis and the response to thalidomide treatment.

## MATERIALS AND METHODS

### **Experimental animals**

Fourteen 10-wk-old female Wistar rats (body weight, 220-300 g) were used as the experiment subjects. White rats were purchased from Charles River Japan (Kanagawa, Japan), maintained in a standard steel net cage for 7 d (12-h light and dark cycle) at 24°C, and fed with standard animal feed and water. Three groups were assigned randomly: six animals were assigned to the radiation group, six animals to the thalidomide group, and two animals to the control group. Both the radiation group and thalidomide group were irradiated in the pelvic area with 30 Gy as a single dose. For the thalidomide group, thalidomide was dissolved in dimethyl sulfoxide at 10 mmol/L, and 150 mg/kg thalidomide was injected intraperitoneally for 7 d from the day of irradiation. On day 8 after irradiation, all animals were killed under anesthesia by injecting pentobarbital (40 mg/kg) and the rectum was removed from each animal.

### **Microscopic examination**

The rectum was resected and examined macroscopically and then fixed in 10% formalin. The sample was dehydrated with ethanol, 7  $\mu$ m-thick paraffin sections were cut, paraffin removed with xylene, and the sections stained with hematoxylin-eosin and examined. For microscopic examination, the hematoxylin-eosin-stained samples were evaluated by a pathologist who was unaware of the groups or treatment. Each sample was scored into one of five stages<sup>[16]</sup> as 0 = normal; 1 = slight radiation damage, mild inflammation or slight crypt change; 2 = mild damage, more significant inflammation, or crypt change; 3 = moderate damage, loss of epithelium, degree of inflammation variable; and 4 = severe damage (ulcers, necrosis).

### **Immunohistochemical staining**

Tissue samples were immunohistochemically stained according to the manufacturer's protocols for TM using a polyclonal antibody (1:100; Santa Cruz, CA, USA), for vWF using a polyclonal antibody (1:600; Abcam PLC, Cambridge, UK), and for VEGF using a rabbit

polyclonal IgG (1:300; Santa Cruz, CA). The intensity of VEGF and TM expression in endothelial cells of microvessels was measured separately at the mucosa and submucosa, and it was scored into one of four grades (none, weak, moderate, strong). Expression of vWF was measured quantitatively by observing five random areas of stained microvessels under the light microscope in both the mucosa and the submucosa, and is presented as the mean  $\pm$  SD<sup>[17,18]</sup>.

### **Statistical analysis**

Parametric data were analyzed by Student's unpaired *t* test, and nonparametric data were analyzed by *F* test. *P* value less than 0.05 was considered significant.

## RESULTS

### **Histological findings**

All rats in the radiation and thalidomide groups showed grade 2-3 pathology, and this differed noticeably from the control group. The pathology (i.e. the change of crypt, the level of mucosal inflammation, and the infiltration of inflammatory cells) was more severe in the radiation and thalidomide groups than in the control group. The scores of the microscopic findings did not differ significantly between the radiation group ( $2.3 \pm 0.5$ ) and the thalidomide group ( $2.5 \pm 0.4$ ).

### **TM expression**

The EC of normal controls showed only strong (3/4) and moderate (1/4) expression of TM. In the radiation group, there was weak (58%, 7/12) or no (42%, 5/12) TM expression with no samples of strong or moderate TM expression. The thalidomide-treated group showed moderate TM expression in 50% (6/12) of samples and strong expression in 50% (6/12) of samples. TM expression was significantly greater in the control and thalidomide groups than in the radiation group (*P* < 0.001, Table 1, Figure 1).

### **Intensity of vWF expression**

The mean number of capillaries expressing vWF in both the mucosa and submucosa was  $3.7 \pm 1.7$  in the control group,  $15.3 \pm 6.8$  in the radiation group, and  $10.8 \pm 3.5$  in the thalidomide group (*P* < 0.001). The radiation-induced capillary damage was significantly greater in the radiation group than in the control group, and the damage was reduced by thalidomide treatment (Figure 2).

### **Intensity of VEGF expression**

VEGF expression was weak in all capillaries (4/4, 100%) of the control group. Most capillaries of the radiation group (11/12, 92%) had moderate expression except one severe case. Capillaries of the thalidomide group had weak (7/12, 58%) or moderate expression (5/12, 42%, *P* = 0.003; Table 1, Figure 3).

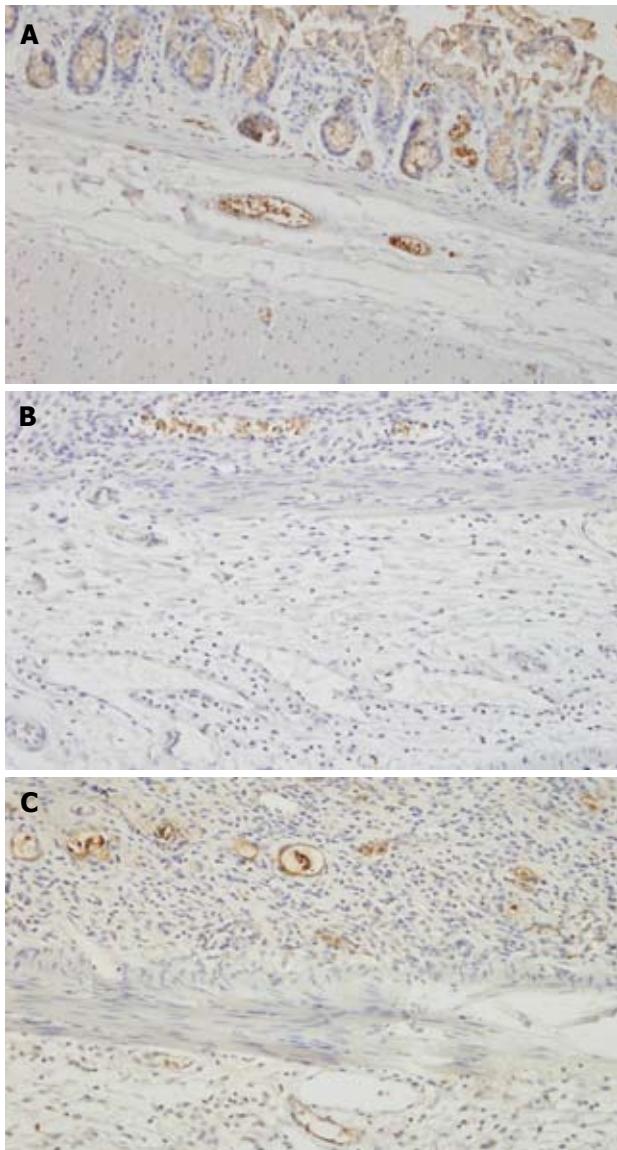
## DISCUSSION

Acute radiation injury induced histological changes

**Table 1** The intensity of TM and VEGF expression in EC in the mucosa and submucosa

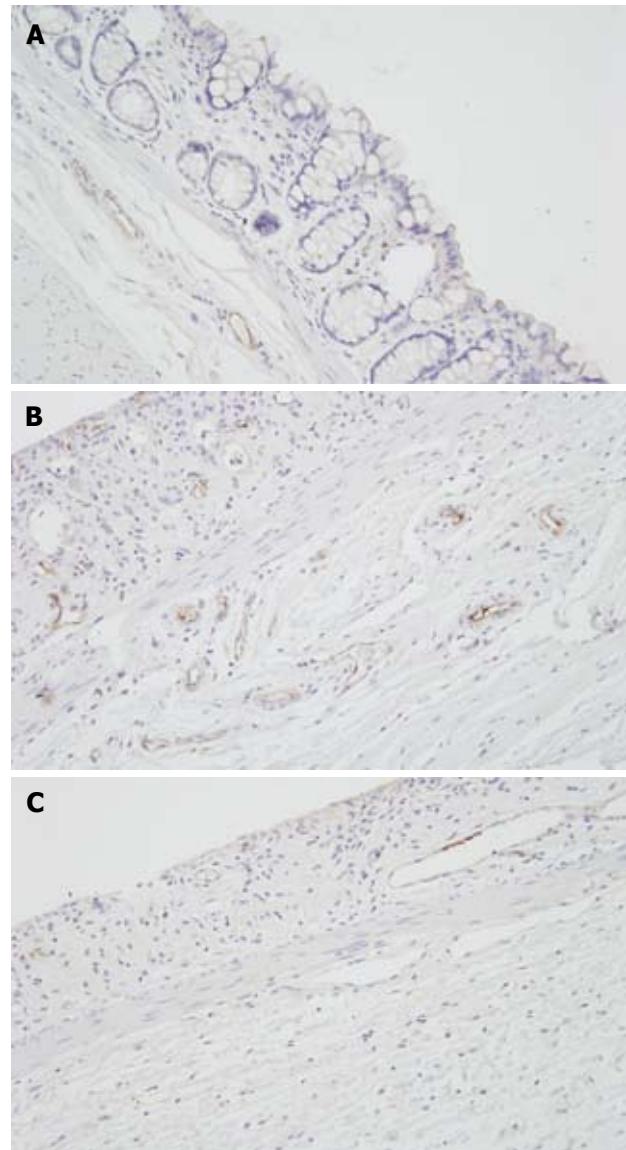
	Control group				Radiation group				Thalidomide group				<i>P</i>
	0	+	++	+++	0	+	++	+++	0	+	++	+++	
TM	0	0	1	3	5	7	0	0	0	0	7	5	< 0.001
VEGF	0	4	0	0	0	0	11	1	0	7	5	0	0.003

0: None; +: Weak; ++: Moderate; +++: Strong expression.



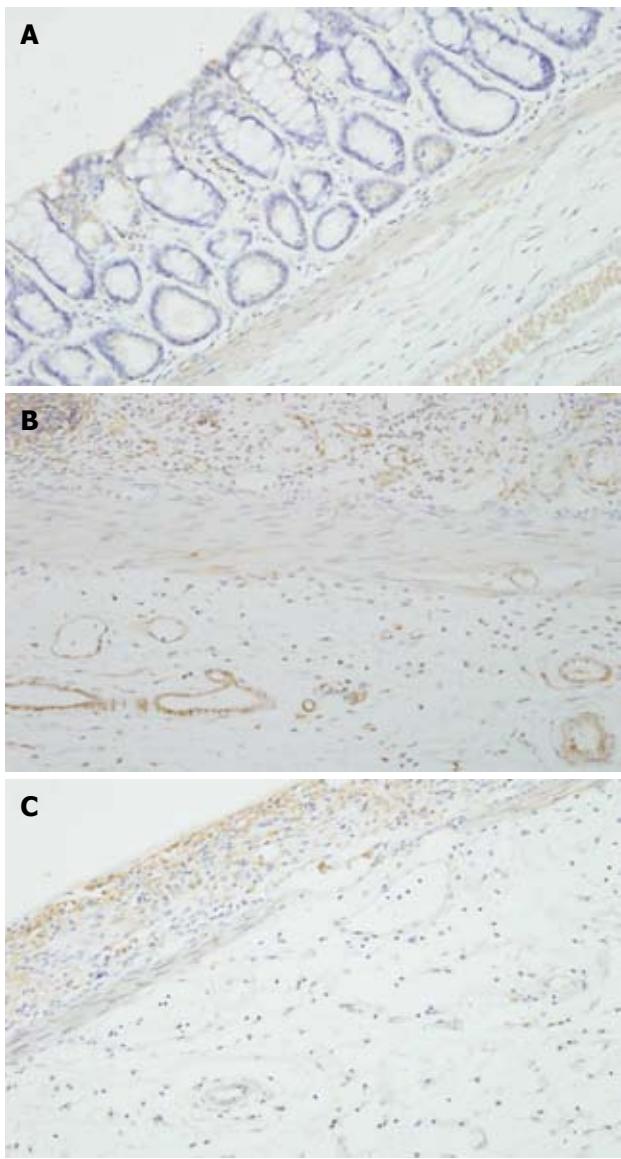
**Figure 1** Immunohistochemical staining of TM (thrombomodulin) expression in the endothelial cells of the mucosa and submucosa. The intensity of TM expression in capillaries is significantly higher in the control group (A) and thalidomide group (C) than in the radiation group (B) ( $\times 200$ ).

including edema, hyperemia, crypt abscess, ulcers, and necrosis of the intestine. It is thought that both acute and chronic radiation injury results from tissue ischemia secondary to thrombogenesis induced by thrombin formation in microvessels<sup>[1,6]</sup>. This loss of endothelial thromboresistance in radiation enteropathy may be related to the actions of many kinds of molecular markers such as tissue factor, vWF, platelet activating factor, TM, and prostacyclin 1<sup>[1,3-6]</sup>.



**Figure 2** Immunohistochemical staining of vWF expression in the endothelial cells of the mucosa and submucosa. Both the total number of microvessels and the number of vWF expressing microvessels are markedly higher in the radiation group (B) than in the control group (A), which are attenuated in the thalidomide group (C) ( $\times 200$ ).

In this study, the expression of TM in the EC was significantly lower in the radiation group than in the control group. These findings are consistent with previous studies suggesting that the mechanism of radiation-induced injury is related to endothelial thrombogenesis<sup>[1,6,19,20]</sup>. This reduced expression of TM in radiation group was restored by treatment with thalidomide. It is known that thalidomide treatment



**Figure 3** Immunohistochemical staining of VEGF expression in the endothelial cells of the mucosa and submucosa. The number of VEGF-positive microvessels and the intensity of VEGF expression were significantly higher in the radiation group (**B**) than in the control (**A**) and thalidomide (**C**) groups ( $\times 200$ ).

suppresses the increase in myeloperoxidase activity and production of TNF- $\alpha$ , interleukin-1 $\beta$ , and intercellular adhesion molecule-1 in an animal model of dinitrobenzene sulphonic acid-induced colitis and *in vitro* model via nuclear factor- $\kappa$ B signaling<sup>[10,12,21,22]</sup>. We found here that thalidomide treatment increased the TM expression in the EC of acute radiation proctitis. This finding might be due to the suppression of inflammatory cytokines including TNF- $\alpha$  by thalidomide. However, thalidomide may rarely cause thrombogenesis as a side effect when used in the treatment of multiple myeloma, although the underlying mechanism is not clear<sup>[23-26]</sup>. In our study, thalidomide seemed to contribute to thromboresistance rather than to thrombogenesis. If thalidomide is used to prevent radiation injury in the future, the clinician should consider that it can unintentionally cause aggravated enteropathy.

Because increased vWF expression in the EC is

regarded as a marker of endothelial damage<sup>[27,28]</sup>, we counted the number of vessels expressing vWF in the mucosa and submucosa of rats with radiation-induced proctitis. The number of vWF-expressing vessels was much higher in the radiation group than in the control group, but was reduced by thalidomide treatment. In the normal EC, the expression of vWF is low because high TM levels on EC surface degrade coagulation factor V and vWF<sup>[5]</sup>. Also in our study, some decrease of vWF expression in the EC of the thalidomide group might be explained by degradation of vWF by elevated TM.

We also found greater VEGF expression in the EC from the radiation group compared with the control group. It is thought that two factors induced by radiation injury contribute to elevated VEGF expression in EC. First, radiation-induced thrombus formation and ischemia of microvessels contribute to the increased production of angiogenic factors such as VEGF<sup>[29-32]</sup>. Secondly, inflammatory cytokines induce angiogenesis, and this may contribute to increased VEGF expression, as in other forms of IBD. This suggests that VEGF expression might be a candidate marker of radiation injury in radiation enteropathy, as in other IBD<sup>[13,15,33]</sup>. The effect of thalidomide on VEGF expression in the EC may be related to either direct inhibition of the EC, as shown in a study of human EC lines, or to inhibition by inflammatory cytokines of colitis, as in another colitis model<sup>[12,13]</sup>. In addition, it is thought that thalidomide might contribute to decreased VEGF expression by inhibiting thrombus formation by elevating TM production, as shown in our data. In other words, thalidomide may enhance the endothelial thromboresistance in radiation enteropathy, suggesting that thalidomide may help prevent radiation injury. We found no histological differences between the radiation and thalidomide groups, probably because of the near-complete denudation of all crypts in both the radiation and thalidomide groups. It is likely that the radiation dosage (30 Gy) was too high for thalidomide to salvage the radiation toxicity.

## COMMENTS

### Background

Tissue hypoxia or ischemia secondary to the microvessel damage and coagulopathy has been considered as a main mechanism of radiation injury in various organs including intestine. Thalidomide as anti-angiogenic or anti-TNF- $\alpha$  agent, has been used for the treatment of malignant disease and inflammatory bowel disease (IBD). Several studies about thalidomide therapy support that it might be useful to prevent radiation-induced enteropathy.

### Research frontiers

Recently, a few studies have been reported about both tissue hypoxia and microvessel injury in radiation injury of the intestine with related molecular markers such as vascular endothelial growth factor (VEGF).

### Innovations and breakthroughs

To evaluate the effect of thalidomide for prevention of radiation-induced proctitis, the markers related to radiation-induced vascular damage were investigated. Our results show that thalidomide has beneficial effects in these markers in spite of no salvation of microscopic damage.

### Applications

By applying the markers of microvascular injury (von Willebrand Factor (vWF),

VEGF and thrombomodulin (TM)] in radiation-induced proctitis, we provide scientific basis for the thalidomide therapy in the prevention of acute radiation-induced proctitis. In the future, it could be useful as a preventive agent for the prevention of radiation-induced proctitis although thalidomide has some vascular side effects.

#### Peer review

The manuscript describes that irradiation to the rectum induces vWF and VEGF in vascular endothelial cells and inhibits TM expression as a marker of thromboresistance. Thalidomide treatment attenuates the effects of the radiation injury in endothelial cells.

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RAPID COMMUNICATION

# Irsogladine maleate suppresses indomethacin-induced elevation of proinflammatory cytokines and gastric injury in rats

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**CONCLUSION:** The mucosal protective effects afforded by irsogladine maleate on gastric injury induced by indomethacin are mediated by inhibition of mucosal proinflammatory cytokine production and neutrophil infiltration, leading to suppression of mucosal inflammation and subsequent tissue destruction.

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**Key words:** Irsogladine; Gastric injury; Indomethacin; Cytokine; Myeloperoxidase

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## Abstract

**AIM:** To investigate the mucosal protective effect and the mechanisms of action of the anti-ulcer drug irsogladine maleate in gastric injury induced by indomethacin in rats.

**METHODS:** Gastric mucosal injury was induced in male Hos:Donryu rats by oral administration of indomethacin at a dose of 48 mg/kg. One hour before indomethacin treatment, animals were orally pretreated with irsogladine maleate at doses of 1 mg/kg, 3 mg/kg or 10 mg/kg. Four hours after indomethacin administration, the animals were sacrificed and their stomachs were rapidly removed and processed for the evaluation of gastric mucosal damage and the determination of the concentrations of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-8 and myeloperoxidase (MPO) in mucosal tissues.

**RESULTS:** Linear hemorrhagic mucosal lesions were observed primarily in the glandular stomach 4 h after oral administration of indomethacin. Pretreatment with irsogladine maleate markedly reduced the number and severity of these lesions in a dose-dependent manner. The mucosal concentrations of proinflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , and IL-8) and MPO, which indicates the degree of mucosal infiltration by neutrophils, increased concomitantly with the occurrence of gastric injury in the indomethacin-treated rats. Pretreatment with irsogladine maleate significantly decreased the levels of these inflammatory factors in gastric tissue elicited by indomethacin.

## INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs), such as indomethacin and aspirin, are widely prescribed in clinical practice because they exhibit excellent efficacy in the management of pain, fever and inflammation through their suppression of the synthesis of prostaglandins (PGs) from arachidonic acids resulting from their inhibition of cyclooxygenase (COX)<sup>[1]</sup>. However, the use of NSAIDs is associated with significant risks of adverse gastrointestinal events, such as gastric mucosal erosion, ulceration, bleeding, and perforation<sup>[2,3]</sup>. Such side effects considerably limit the use of these drugs. In addition to the decrease in

the intrinsic production of PGs in the gastric mucosa, other processes, such as increased mucosal production of proinflammatory cytokines, increased production of reactive oxygen species, and increased lipid peroxidation as well as increased mucosal infiltration of neutrophils, are closely associated with NSAID-induced gastric mucosal injury<sup>[4-6]</sup>.

Although the anti-ulcer drug irsogladine maleate has been shown in animals to exhibit potent mucosal protective effects on various experimental gastrointestinal injuries induced by a variety of stimuli, including indomethacin<sup>[7-10]</sup>, the exact mechanisms underlying the gastroprotective effect of irsogladine maleate remains to be fully elucidated. We have recently found that irsogladine maleate increases the intracellular levels of the second messenger cyclic adenosine monophosphate (cAMP) in rat glandular stomach and in human neutrophils through its previously unreported ability to inhibit cyclic nucleotide phosphodiesterase 4 (PDE4), and this inhibitory activity is probably involved in the antiulcer effects of the drug<sup>[11-13]</sup>. PDE4 inhibitors have been demonstrated to have multiple anti-inflammatory effects mediated by the suppression of proinflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ) and IL-8, as well as by inhibiting neutrophil infiltration by suppressing the expression of adhesion molecules<sup>[14-16]</sup>.

Paying particular attention to the novel inhibitory action of irsogladine maleate on PDE4, in the present study we investigated the mucosal protective effect of irsogladine maleate and its underlying mechanisms of action by using a rat model of gastric injury elicited by indomethacin, focusing on the effect of the drug on cytokine production and neutrophil infiltration in the gastric mucosa. We found that irsogladine maleate markedly reduced gastric injury in indomethacin-treated rats with a concomitant decrease in levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-8 and myeloperoxidase (MPO) in gastric mucosal tissue. The possible contribution of PDE4 inhibitory activity to the mucosal protective effect of irsogladine maleate was also discussed.

## MATERIALS AND METHODS

### Animals

Male Hos: Donryu rats (Japan SLC, Hamamatsu, Japan) at 6 weeks of age were used following quarantine and acclimation for a week after delivery to the laboratory. The rats were housed in rooms at 20-26°C with a relative humidity of 35%-75% in a 12 h light-dark cycle (lights on, 8:00-20:00) and a ventilation frequency of at least 15 times per hour. The rats were fed standard laboratory chow (F-2; Funabashi Farm, Funabashi, Chiba, Japan) with free access to tap water. All the animals were fasted for 18 h before the experiments. However, free access to tap water was allowed 1 h before the experiments. All experimental procedures were approved by the Committee for the Institutional Care and Use of Animals of Nippon Shinyaku Co.

### Preparation of drugs

Irsogladine maleate, synthesized at Nippon Shinyaku Co., was suspended in 5 g/L methylcellulose solution. Indomethacin, purchased from Sigma Chemicals (St. Louis, MO, USA), was dissolved in 50 g/L NaHCO<sub>3</sub> solution.

### Production of indomethacin-induced gastric mucosal injury in rats

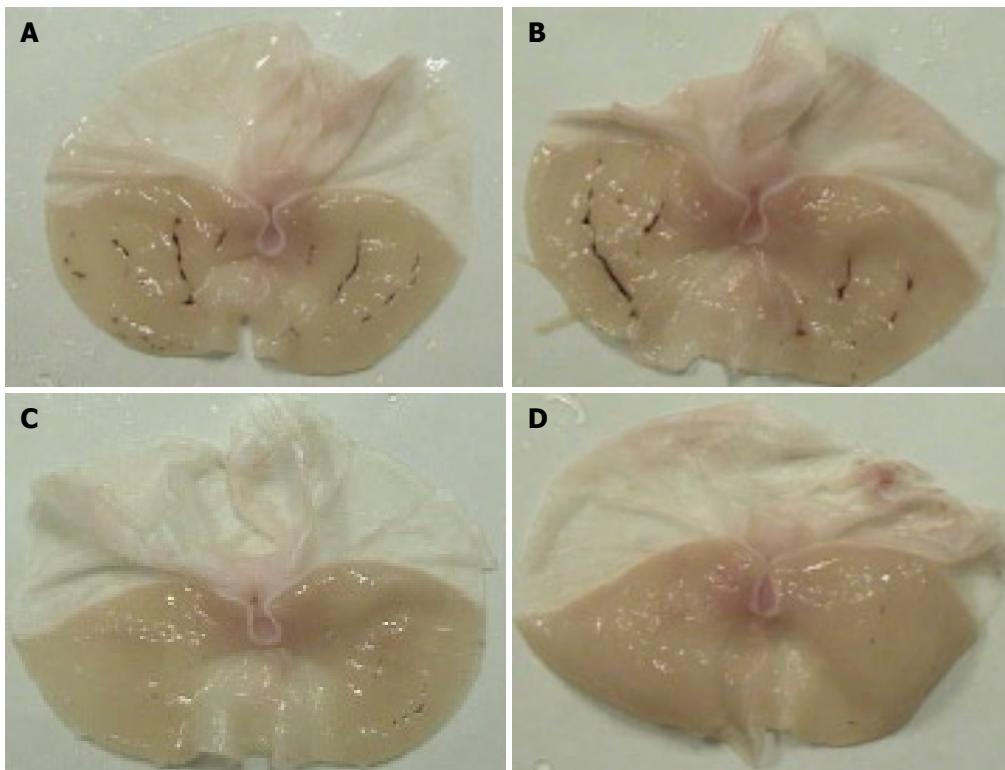
Gastric mucosal injury was produced by intragastric gavage of indomethacin at a dose of 48 mg/kg. Irsogladine maleate was given orally 1 h before indomethacin treatment. Four hours after indomethacin treatment, the animals were sacrificed with an overdose of diethyl ether and their stomachs were rapidly removed and processed for the evaluation of gastric mucosal damage and determination of TNF- $\alpha$ , IL-1 $\beta$ , IL-8 and MPO in the mucosal tissues.

### Evaluation of gastric mucosal injury

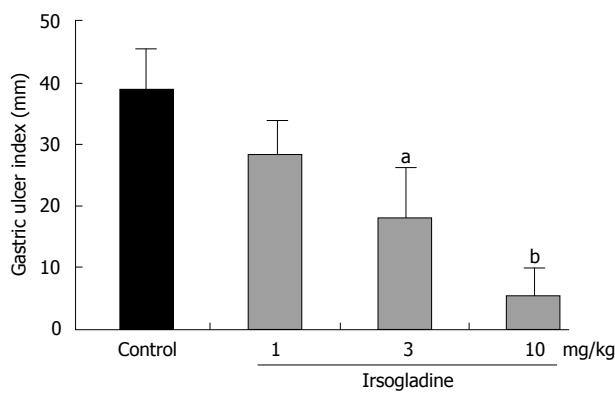
The stomach was distended with 10 mL of 8 g/L formaldehyde and immersed in the formaldehyde solution for 10 min. The lightly fixed stomach was then opened along the line of the greater curvature and spread out on a board. The hemorrhagic lesions in the mucosal layer were examined under a dissecting microscope (Olympus SZ40, Olympus Optical Co., Tokyo, Japan) at  $\times 10$  magnification by an observer who was unaware of the treatments. The gastric ulcer index was calculated for each rat as the sum of the lengths in millimeters of the hemorrhagic lesions (seen as red streaks).

### Determination of the concentrations of TNF- $\alpha$ , IL-1 $\beta$ and IL-8 in gastric mucosa

To evaluate the effect of irsogladine maleate on the mucosal production of cytokines elicited by treatment with indomethacin, the concentrations of TNF- $\alpha$ , IL-1 $\beta$ , and IL-8 in gastric mucosa were determined. Briefly, the gastric mucosa was rapidly scraped from the gastric wall of each rat and immediately frozen on dry ice and kept at -70°C until analysis. Mucosal samples were homogenized with a Phycotron NS-310E mechanical microhomogenizer (Niti-on, Tokyo, Japan) in 1 mL of PBS containing complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA). The homogenate was centrifuged for 30 min at 15 000 r/min (4°C) in a Hitachi Himac CF 15D2 centrifuge (Hitachi High-Technologies, Tokyo, Japan) and the supernatant was retained for determination of proinflammatory cytokines. TNF- $\alpha$  and IL-1 $\beta$  were determined by enzyme-linked immunosorbent assay (ELISA) with commercial kits from R&D Systems (Minneapolis, MN, USA). IL-8 was determined with a commercial ELISA kit from Panapharm Laboratories (Kumamoto, Japan). The total protein in the supernatant was determined with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), and the concentrations of TNF- $\alpha$ , IL-1 $\beta$  and IL-8 were expressed as ng per gram of total protein.



**Figure 1** Protective effect of irsogladine maleate on gastric mucosal lesions induced by indomethacin in rats. Stomachs from rats not pretreated with irsogladine maleate (A) and pretreated with irsogladine maleate at the doses of 1 mg/kg (B), 3 mg/kg (C) or 10 mg/kg (D), respectively, 1 h before administration of indomethacin. Nine rats were used in each group.



**Figure 2** Protective effect of irsogladine maleate on gastric mucosal lesions induced by indomethacin in rats. Each value represents the mean  $\pm$  SE for 9 rats. <sup>a</sup> $P < 0.05$  and <sup>b</sup> $P < 0.01$  vs control group.

#### Measurement of mucosal MPO levels

To estimate the degree of mucosal infiltration by neutrophils elicited by treatment with indomethacin, the MPO levels were measured in the gastric mucosa. Briefly, gastric mucosal samples were homogenized with a microhomogenizer in 1 mL of 10 mmol/L Tris (pH 7.4) containing 200 mmol/L NaCl, 5 mmol/L EDTA, 100 mL/L glycine, 1 mmol/L phenylmethanesufonyl fluoride, 1  $\mu$ g/mL leupeptin, and 28  $\mu$ g/mL aprotinin. The homogenate was centrifuged and the supernatant was retained for determination of MPO with an ELISA kit from Hycult Biotechnology (Uden, Netherlands) according to the manufacturer's instructions. The total protein in the supernatant was also determined as described above and the concentration of MPO was expressed as ng per gram of total protein.

#### Statistical analysis

The results are presented as mean  $\pm$  standard error of the mean (SE). The statistical significance of differences between normal and control groups was evaluated by the Aspin-Welch test, and the statistical significance of differences among the control and irsogladine maleate-treated groups was evaluated by the Williams test.  $P < 0.05$  was considered statistically significant. All tests were two-tailed, and performed with SAS version 8.2 (SAS Institute, Cary, NC, USA).

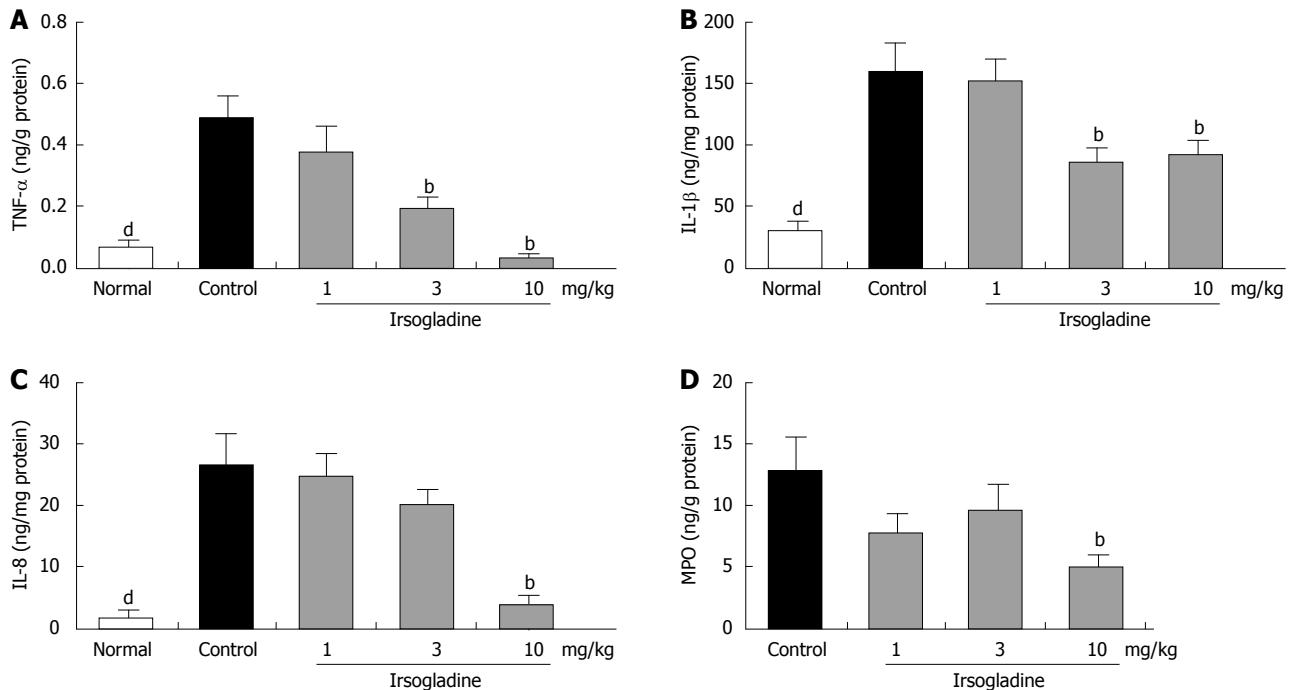
## RESULTS

#### Effect of irsogladine maleate on gastric mucosal injury induced by indomethacin

Four hours after oral administration of indomethacin, at a dose 48 mg/kg, gross linear hemorrhagic mucosal lesions were clearly observed in the glandular mucosa, and pretreatment with irsogladine maleate markedly reduced the number and severity of these lesions (Figure 1). Gastric mucosal injury was quantified under a dissecting microscope, and a gastric ulcer index of  $39.1 \pm 7.7$  mm was calculated (Figure 2). Pretreatment with irsogladine maleate reduced the gastric ulcer index in a dose-dependent manner to  $27.1 \pm 6.2$  mm at the dose of 1 mg/kg,  $18.3 \pm 7.2$  mm at the dose 3 mg/kg, and  $5.7 \pm 4.0$  mm at the dose of 10 mg/kg. In particular, irsogladine maleate at the dose of 3 mg/kg or 10 mg/kg significantly reduced the gastric ulcer index.

#### Effect of irsogladine maleate on TNF- $\alpha$ levels in gastric mucosa

Intragastric administration of indomethacin induced a marked increase in mucosal TNF- $\alpha$  concentrations. The



**Figure 3** Effect of irsogladine maleate on gastric mucosal TNF- $\alpha$  (A), IL-1 $\beta$  (B), IL-8 (C) and MPO (D) induced by indomethacin in rats. Each value represents the mean  $\pm$  SE for 9 rats,  $^bP < 0.01$  vs control group,  $^dP < 0.01$  vs normal group.

TNF- $\alpha$  level in the gastric mucosa of normal animals was  $0.070 \pm 0.014$  ng/g protein, which was significantly increased to  $0.490 \pm 0.072$  ng/g protein after intragastric administration of indomethacin (Figure 3A). Pretreatment with irsogladine maleate reduced the concentration of mucosal TNF- $\alpha$  in a dose-dependent manner to  $0.354 \pm 0.086$  ng/g protein at the dose of 1 mg/kg,  $0.186 \pm 0.042$  ng/g protein at the dose of 3 mg/kg, and  $0.045 \pm 0.005$  ng/g protein at the dose of 10 mg/kg, respectively. In particular, irsogladine maleate at the dose of 3 mg/kg or more significantly reduced the TNF- $\alpha$  levels in gastric mucosa.

#### **Effect of irsogladine maleate on IL-1 $\beta$ levels in gastric mucosa**

Intragastric administration of indomethacin induced a marked increase in mucosal IL-1 $\beta$  concentrations. The IL-1 $\beta$  level in the gastric mucosa of normal animals was  $36.1 \pm 8.1$  ng/g protein, which was significantly increased to  $160.2 \pm 19.8$  ng/g protein after intragastric administration of indomethacin (Figure 3B). Pretreatment with irsogladine maleate reduced the concentration of mucosal IL-1 $\beta$  to  $154.0 \pm 15.7$  ng/g protein at the dose of 1 mg/kg,  $85.4 \pm 15.4$  ng/g protein at the dose of 3 mg/kg, and  $93.0 \pm 17.4$  ng/g protein at the dose of 10 mg/kg, respectively. In particular, irsogladine maleate at the dose of 3 mg/kg or more significantly reduced the IL-1 $\beta$  levels in gastric mucosa.

#### **Effect of irsogladine maleate on IL-8 levels in gastric mucosa**

Intragastric administration of indomethacin induced a marked increase in mucosal IL-8 concentrations. The

IL-8 level in gastric mucosa of normal animals was  $1.1 \pm 0.5$  ng/g protein, which was significantly increased to  $27.0 \pm 6.6$  ng/g protein after intragastric administration of indomethacin (Figure 3C). Pretreatment with irsogladine maleate reduced the concentration of mucosal IL-8 in a dose-dependent manner to  $24.8 \pm 6.0$  ng/g protein at the dose of 1 mg/kg,  $19.9 \pm 5.9$  ng/g protein at the dose of 3 mg/kg, and  $2.8 \pm 0.6$  ng/g protein at the dose of 10 mg/kg, respectively. In particular, irsogladine maleate at the dose of 10 mg/kg significantly reduced the IL-8 levels in gastric mucosa.

#### **Effect of irsogladine maleate on MPO levels in gastric mucosa**

The MPO level in the gastric mucosa of indomethacin-treated animals was  $12.7 \pm 4.3$  ng/g protein (Figure 3D). Pretreatment with irsogladine maleate reduced the MPO concentration in gastric mucosa to  $7.0 \pm 1.6$  ng/g at the dose of 1 mg/kg,  $9.1 \pm 2.2$  ng/g at the dose of 3 mg/kg and  $4.9 \pm 0.8$  ng/g at the dose of 10 mg/kg, respectively. In particular, irsogladine maleate at the dose of 10 mg/kg significantly reduced the MPO levels in gastric mucosa.

## **DISCUSSION**

Though several studies have been published on the mechanisms underlying the mucosal protective effects of irsogladine maleate, an anti-ulcer drug often prescribed in Japan, on various gastrointestinal mucosal injuries<sup>[9-13]</sup>, the exact mechanisms by which this drug exerts its mucosal protective effects are not completely clear. In the present study, we investigated

the mechanisms underlying the gastroprotective effect of irsogladine maleate by using a rat experimental model of gastric injury elicited by indomethacin, focusing on the involvement of mucosal proinflammatory cytokine production and neutrophil infiltration into the gastric mucosa. We found that the concentrations of TNF- $\alpha$ , IL-1 $\beta$ , IL-8 and MPO in gastric mucosa of the rats increased concomitantly with the occurrence of gastric injury induced by a single intragastric dose of indomethacin. Pretreatment with irsogladine maleate markedly reduced the gastric injury with a concomitant decrease in the levels of these inflammatory factors.

Although the mechanisms of NSAID-induced gastric injury are not well understood, it is well accepted that both COX-dependent and independent mechanisms are involved in the pathogenesis of indomethacin-induced gastric injury. Mucosal proinflammatory cytokines, such as TNF- $\alpha$ , IL-1 $\beta$  and IL-8, are considered key inducers of gastric injury induced by NSAIDs like indomethacin<sup>[5,13]</sup>. For example, it is recognized that TNF- $\alpha$  may strongly promote inflammation and subsequent tissue destruction by recruiting leukocytes, particularly neutrophils and monocytes, through the induction of adhesion molecules on both leukocytes and vascular endothelial cells<sup>[17,18]</sup>. It is also known that TNF- $\alpha$  can induce the production of superoxide and proinflammatory cytokines by inflammatory cells<sup>[18,19]</sup>. In addition, intravenous administration of TNF- $\alpha$  induces gastric injury characterized by marked neutrophil infiltration into the gastric mucosa of rats<sup>[20]</sup>, while TNF- $\alpha$  levels in the gastric mucosa correlate well with the severity of gastric injury in rats<sup>[21-23]</sup>. It was reported that IL-1 $\beta$  and IL-8, as well as TNF- $\alpha$ , can promote inflammatory response and are involved in NSAID-induced gastric mucosal injury in humans and animals<sup>[17,24-26]</sup>.

In an attempt to clarify the mechanisms underlying the protective effects of irsogladine maleate on gastric mucosa, we evaluated its effects on the production of TNF- $\alpha$ , IL-1 $\beta$ , and IL-8 in gastric mucosa. We found that these cytokines were involved in the inflammatory response in a rat gastric mucosal injury model induced by indomethacin, and the concentrations of TNF- $\alpha$ , IL-1 $\beta$  and IL-8 in gastric mucosa increased concomitantly with the occurrence of gastric mucosal lesions, suggesting that the increased levels of these cytokines are closely associated with the occurrence and development of gastric mucosal lesions. Irsogladine maleate suppressed gastric mucosal injury at the dose of 3 mg/kg or higher. The doses of irsogladine maleate showing effective mucosal protection in this study are similar to those showing protection in previous studies of a variety of animal models of gastric injury<sup>[7-9,13]</sup>. Irsogladine maleate also significantly suppressed the increased levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-8 in gastric mucosa, indicating that the suppression of proinflammatory cytokine production in gastric mucosa by irsogladine maleate significantly contributes to its protective effect on gastric mucosa.

In addition to proinflammatory cytokines, the

accumulation and activation of neutrophils, which may lead to microcirculation disturbance and production of free radicals in gastric mucosa, are also important events in gastric injury induced by NSAIDs<sup>[6,27]</sup>. Studies on experimental models of gastric injury induced by NSAIDs have demonstrated that infiltration of neutrophils into the injured mucosa occurs in rats as early as 15-30 min after indomethacin administration<sup>[28]</sup>, and gastric injury can be ameliorated in animals by eliminating the neutrophils through the administration of antineutrophil antibodies<sup>[6]</sup>. Neutrophil infiltration into gastric mucosa is, therefore, considered a critical step in the development of gastric injury induced by NSAIDs. In the present study, irsogladine maleate significantly reduced the MPO levels in gastric mucosa, indicating a reduction in mucosal infiltration by neutrophils. The suppression of neutrophil infiltration into the gastric mucosa may, therefore, contribute to the protective effect of irsogladine maleate on gastric mucosa.

PDE4 inhibitors may exert their anti-inflammatory action on various inflammatory diseases by suppressing the production of proinflammatory cytokines and the expression of adhesion molecules by increasing the levels of intracellular cAMP<sup>[14-16]</sup>. In addition, the PDE4 inhibitor rolipram can ameliorate indomethacin-induced gastric injury in rats by inhibiting the production of TNF- $\alpha$ <sup>[29]</sup>. We and others have recently reported that irsogladine maleate prevents ischemia-reperfusion-induced gastric injury and indomethacin-induced small intestinal lesions in rats, probably by inhibiting PDE4<sup>[10,13]</sup>. It is, therefore, likely that irsogladine maleate exerts its protective effect on indomethacin-induced gastric injury by suppressing mucosal TNF- $\alpha$ , IL-1 $\beta$  and IL-8 production by neutrophils and/or macrophages through its inhibition of PDE4 in these inflammatory cells. The suppression of these proinflammatory cytokines by irsogladine maleate may in turn decrease the expression of adhesion molecules in both vascular endothelial cells and neutrophils, resulting in a reduction in mucosal infiltration by neutrophils with consequent prevention of tissue destruction. Irsogladine maleate also inhibits the *in vitro* production and release of superoxide from human neutrophils by inhibiting PDE4<sup>[12]</sup>. Therefore, it is likely that the protective effects of irsogladine maleate on gastric mucosa arise not only from its suppression of neutrophil infiltration through its inhibition of proinflammatory cytokine production and adhesion-molecule expression, but also from its direct suppression of neutrophil activation through its inhibition of PDE4.

In conclusion, irsogladine maleate exerts its mucosal protective effects on gastric injury by suppressing the acute inflammatory response and subsequent tissue destruction in gastric mucosa through its suppression of mucosal proinflammatory cytokine production and neutrophil infiltration as well as by directly inhibiting neutrophil activation. It is also likely that the suppression of proinflammatory cytokines or neutrophils by irsogladine maleate arises from its ability to inhibit PDE4.

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## COMMENTS

### Background

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely prescribed for their efficacy in the management of pain, fever and inflammation. However, NSAIDs are associated with significant risks of adverse gastrointestinal events, which considerably limit the use of these drugs. Although irsogladine maleate exhibits potent mucosal protective effects against indomethacin-induced gastrointestinal injuries in animals, its mechanisms of action are not fully understood.

### Research frontiers

We investigated the mechanisms underlying the protective effect of irsogladine maleate on gastric mucosa by using a rat experimental model of gastric injury elicited by indomethacin, focusing on the involvement of mucosal proinflammatory cytokine production and neutrophil infiltration into the gastric mucosa.

### Innovations and breakthroughs

The present study reported the effect of irsogladine maleate on mucosal proinflammatory cytokine production and neutrophil infiltration into the gastric mucosa in indomethacin-induced gastric injury.

### Applications

A single intragastric dose of irsogladine maleate could markedly reduce gastric injury with a concomitant decrease in the levels of inflammatory factors in indomethacin-treated rats, suggesting that irsogladine maleate can be applied to the treatment of patients with NSAID-induced gastric injury.

### Peer review

In this study, the authors examined the effects of irsogladine maleate on gastric mucosal injury and proinflammatory cytokine production and neutrophil infiltration (measured by MPO) using a rat model of gastric injury elicited by indomethacin. The authors found that irsogladine maleate could markedly reduce gastric injury in indomethacin-treated rats with a concomitant decrease in the gastric mucosal levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-8 and myeloperoxidase (MPO). The results of these straightforward experiments demonstrate that the protective effects of irsogladine are mediated by reducing neutrophil induction and inhibition of mucosal cytokine expression. This paper is well written and the data are novel.

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RAPID COMMUNICATION

# A anorectal fistula treatment with acellular extracellular matrix: A new technique

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**Author contributions:** Song WL performed research and wrote the paper; Wang ZJ designed and performed research; Zheng Y, Yang XQ, Peng YP performed research and analyzed data.

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## INTRODUCTION

Anorectal fistula is the chronic phase of anorectal infection and is characterized by chronic purulent drainage or cyclical pain associated with abscess reaccumulation followed by intermittent spontaneous decompression<sup>[1]</sup>. The goals of surgery for fistulainano are permanent healing and preservation of anal continence. Traditional surgical techniques, namely fistulotomy and seton technique, sever the internal anal sphincters and may damage the external anal sphincters. The recurrent rate of “lay-open” fistulotomy was reported to be 2%-9% with functional impairment ranging from 0%-17%<sup>[2-4]</sup>. The use of a seton has a recurrence rate of 0%-8%. Minor and major incontinence is 34%-64% and 2%-26%, respectively<sup>[5-10]</sup>.

Many different methods for treating anorectal fistulæ, particularly fistulæ in which fistulotomy are contraindicated, have been reported in recent years. Other alternative approaches are the application of fibrin glue and fistula plug. Since 1999, several studies on fibrin glue treatment of anal fistula have been published. The healing rate after debridement and fibrin glue injection is 14%-60%<sup>[11-13]</sup>. Incontinence may not be affected. Fistula plug, the latest technique for complex fistula-in-ano repair, has a reported failure rate of 13%. A success rate of 83% with a median follow-up of 12 mo is reported for high cryptoglandular anal fistulas, and the method has also been reported for a smaller group of Crohn fistulas<sup>[14,15]</sup>.

Our institution began repairing anorectal fistulæ using acellular extracellular matrix (AEM) in the past 1 yr. In our study, 30 patients with anorectal fistula were treated using acellular extracellular matrix. All of the 30 patients had successful closure of their fistula after a 7-14 d follow-up, giving an overall successful closure rate of 100% (30 of 30 patients). It can reduce pain, shorten disease course and protect anal function.

## Abstract

**AIM:** To investigate a new technique of the anorectal fistula treatment with acellular extracellular matrix (AEM).

**METHODS:** Thirty patients with anorectal fistula were treated with AEM. All fistula tracts and primary openings were identified using conventional fistula probe. All tracts were curetted with curet and irrigated with hydrogen peroxide and metronidazole. The AEM was pulled into the fistula tract from secondary to primary opening. The material was secured at the level of the primary opening. The excess AEM was trimmed at skin level at the secondary opening.

**RESULTS:** All of the 30 patients had successful closure of their fistula after a 7-14 d follow-up. The healing rate of anal fistula in treatment group was 100%. The ache time, healing time and anal deformation of treatment group were obviously superior to traditional surgical methods.

**CONCLUSION:** Using AEM anal fistula plug in treatment that causes the anorectal fistula is safe and successful in 100% of patients. It can reduce pain, shorten disease course and protect anal function.

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**Key words:** Acellular extracellular matrix; Anorectal fistula

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## MATERIALS AND METHODS

### **Patients**

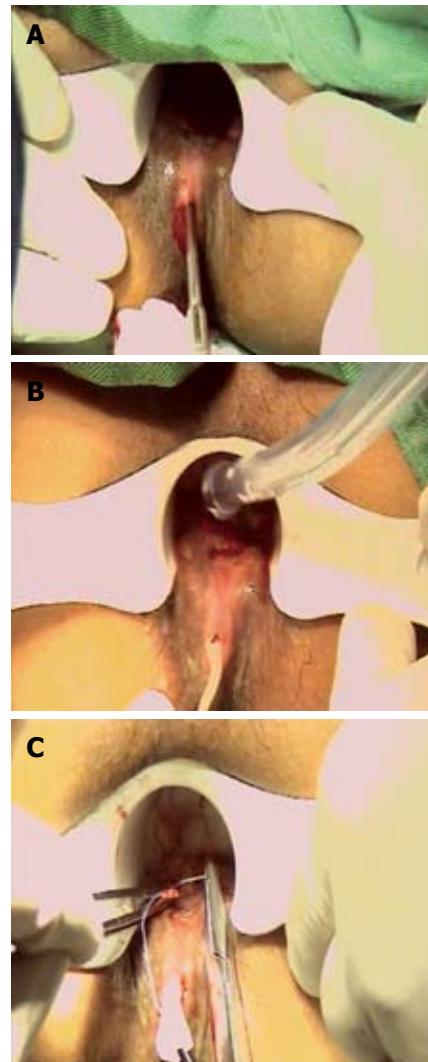
From January, 2007 to August, 2007, 30 patients with anorectal fistula were selected from ChaoYang Hospital of Capital Medicine University. They were treated with AEM (19 males, 11 females). All the patients had low anorectal fistula. Twenty-eight patients had simple anorectal fistula cases, and 2 patients had complicated anorectal fistula. their age was 18-72 years (mean, 38 years). All patients underwent mechanical bowel preparation the day before surgery, followed by 2 g of metronidazole by mouth the same evening. A broad-spectrum parenteral antibiotic was given to induce anesthesia. All procedures were performed under general anesthesia at a prone jackknife position.

### **Anal fistula acellular dermal matrix technique**

All operations were performed in the operating room. The primary and secondary fistula tract openings were identified. The fistula tract was then thoroughly cleaned with a blunt curette or gauze strip that was threaded through the tract (Figure 1A). All fistula tracts and primary openings were identified using a conventional fistula probe and/or hydrogen peroxide instillation<sup>[16,17]</sup>. All tracts were irrigated with hydrogen peroxide and metronidazole (Figure 1B). The AEM was cut out with a pair of scissors for three or four strips (Figure 2). Each primary opening was occluded by using a AEM anal fistula plug. The AEM was pulled into the fistula tract from secondary to primary the plug fitted snuggly into the tract (Figure 1C). The AEM material was secured at the level of the primary opening opening using a 2-0 vicryl, which was inserted deep to the internal sphincter muscle. The excess AEM was trimmed at skin level at the secondary opening. Care was taken to avoid complete closure of the secondary opening to allow drainage of material and to avoid a closed system. At the end of the procedure, the plug was completely buried within the fistula tract (Figure 1C). In the two cases of multiple separate fistules, the plug was cut into multiple strips to fit multiple fistula tracts. All patients were instructed to have a clear liquid diet for 48 h, avoid any strenuous activity; a broad-spectrum parenteral antibiotic was given. The status of fistula was determined at final follow-up. Success criteria were defined: closure of all secondary openings, absence of fistula drainage and abscess formation.

## RESULTS

Thirty patients were prospectively studied during an eight-month period. Twenty-seven patients had successful closure of their fistula tracts after a 7-14 d follow-up, but 3 patients had successful closure of their fistula tracts after 30 d follow-up. The mean healing time was 10.0 d (range, 7-14 d, 27/30). The mean operative time was 20 min (range, 15-25 min). All 28

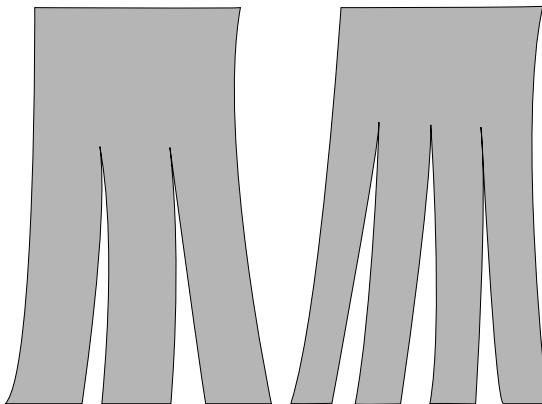


**Figure 1** Fistula tract thoroughly cleaned using a blunt curette (A), irrigated with hydrogen peroxide and etronidazole (B), pulled into the primary fistula opening until resistance(C). The AEM is secured at the level of the primary opening opening using a 2-0 vicryl. The excess of the AEM is trimmed at the level of the secondary opening. Care is taken to avoid complete closure of the secondary opening to allow free drainage of fluid and avoid a closed system.

patients with single fistula tracts had successful closure of their fistula tracts. These 2 patients with multiple tracts had successful closure of their fistula tracts too. The healing rate of anal fistula treatment group was 100%. There was no change of the continence status in all patients. There was no major post operative complication. Successful closure was not significantly associated with single fistula tracts or multiple fistula tracts.

## DISCUSSION

Traditional surgical techniques for anorectal fistula, include fistulotomy and seton techniques. Fistulotomy has been performed since ancient times. The outcome is generally acceptable. However, fistulotomy causes various degrees of anal sphincter injury<sup>[6,17-21]</sup>. The incontinence status is underestimated. The seton technique is to minimize incontinence, but only with



**Figure 2** AEM cut out with a knife for three or four strips.

moderate success. Recently, many techniques have been developed, such as endorectal advancement flap, anoderm island flap, excision and closure of internal opening, fibrin glue, and fistula plug. These techniques have less risk of anal incontinence, despite some recurrences. Several studies on fibrin glue treatment of anal fistula have been published since 1999, but, the healing rate after debridement and fibrin glue injection is 14%-60%<sup>[22-26]</sup>. Use of anal fistula plug for complex fistula-in-ano repair<sup>[27,28]</sup> has a reported success rate of 83%. The method has also been reported for a smaller group of Crohn fistulas<sup>[14,15]</sup>.

We used fibrin glue and fistula plug during the past year. Our institution began repairing all types of anorectal fistulae using the AEM to drain materials from human or animal skin tissue and to remove the composition of immunogenicity. This technique has been used in the field of burn and plastic surgery, stomatological surgery, tumor repaired surgery and urinology for a long time, and clinical results. In our study, the AEM was only used in low anorectal fistula, but could not be used in fistulas of cryptoglandular origin, fistules caused by Crohn's disease, recurrent fistules, and high anorectal fistules. Thirty patients with fistulae in ano were treated, 27 of them had successful closure of their fistula tracts after a 7-14 d follow-up. All the 28 patients with single fistula tracts had successful closure of their fistula tracts. Of them, 2 patients with multiple tracts had successful closure of their fistula tracts too. Giving an overall successful closure rate of 100% (30 of 30 patients), complications and recurrent case were encountered during the postoperative course, immune response and extensive fibrosis were not found in this study, suggesting that treatment of fistula with AEM can reduce pain, shorten disease course, protect anal function. The ache time, and healing time of the treatment group were obviously shorter compared with those achieved by using traditional surgical techniques<sup>[29]</sup>.

The AEM technique is based on sound principles. It is a new, simple, safe, less invasive and effective technique for closing anorectal fistules, and for avoiding the risks of anorectal incontinence. The early results are satisfactory.

## COMMENTS

### Background

Anorectal fistula is the chronic phase of anorectal infection. Traditional surgical techniques may damage the external anal sphincters. Many different methods for treating anorectal fistula have been reported in recent years. Other alternative approaches are the application of fibrin glue and fistula plug. However, the result of these approaches is uncertain.

### Research frontiers

The use of acellular extracellular matrix (AEM) in the anorectal fistula treatment was first study in the world.

### Innovations and breakthroughs

It is a new technique that is simple, safe, minimally invasive for closing anorectal fistules, and for avoiding the risks of anorectal incontinence. The early results are satisfactory.

### Applications

This technique can, reduce pain, shorten healing time, protect anal function. Therefore, it can be used in the treatment of fistulae.

### Peer review

This paper describes a new technique for the treatment of fistulae, which is of certain importance in clinical treatment of fistulae.

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RAPID COMMUNICATION

# Prokinetic effects of a ghrelin receptor agonist GHRP-6 in diabetic mice

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## Abstract

**AIM:** To investigate the effects of a ghrelin receptor agonist GHRP-6 on delayed gastrointestinal transit in alloxan-induced diabetic mice.

**METHODS:** A diabetic mouse model was established by intraperitoneal injection with alloxan. Mice were randomized into two main groups: normal mice and diabetic mice treated with GHRP-6 at doses of 0, 20, 50, 100 and 200 µg/kg ip. Gastric emptying (GE), intestinal transit (IT), and colonic transit (CT) were studied in mice after they had a phenol red meal following injection of GHRP-6. Based on the most effective GHRP-6 dosage, atropine was given at 1 mg/kg for 15 min before the GHRP-6 injection for each measurement. The mice in each group were sacrificed 20 min later and the percentages of GE, IT, and CT were calculated.

**RESULTS:** Percentages of GE, IT, and CT were significantly decreased in diabetic mice as compared to control mice. In the diabetic mice, GHRP-6 improved both GE and IT, but not CT. The most effective dose of GHRP-6 was 200 µg/kg and atropine blocked the prokinetic effects of GHRP-6 on GE and IT.

**CONCLUSION:** GHRP-6 accelerates delayed GE and IT, but has no effect on CT in diabetic mice. GHRP-6 may exert its prokinetic effects via the cholinergic pathway in the enteric nervous system, and therefore, has therapeutic potential for diabetic patients with delayed upper gastrointestinal transit.

## INTRODUCTION

Ghrelin is a peptide synthesized by endocrine cells of the gastric mucosa, initially identified in rodents<sup>[1]</sup>. The major actions of this recently discovered peptide include stimulation of growth hormone (GH) release<sup>[1-3]</sup>, regulation of appetite and nutrient ingestion<sup>[4-6]</sup>, and improvement of digestive motility<sup>[7-9]</sup>. When injected into mice<sup>[7,10]</sup>, rats<sup>[8]</sup>, human<sup>[9]</sup>, or dogs<sup>[11]</sup>, ghrelin accelerates gastric emptying of a liquid or solid meal.

Delayed gastrointestinal transit is a well-known diabetic complication, and may lead to uncomfortable gastrointestinal symptoms, such as frequent vomiting, emaciation, and unpredictable changes in blood glucose, all of which impair the quality of life of diabetic patients<sup>[12,13]</sup>. Gastrointestinal transit of solid or nutrient liquid meals is abnormally slow in approximately 50% of diabetic patients<sup>[12]</sup>. Delayed gastrointestinal transit may be associated with cardiac autonomic neuropathy, blood glucose concentration, and gastrointestinal symptoms<sup>[12]</sup>.

GHRP-6 is a peptide ghrelin receptor agonist, which has previously been reported to increase gastric emptying in normal rats<sup>[14]</sup>. Ghrelin has been shown to accelerate gastric emptying in animal models of postoperative ileus<sup>[15]</sup>, septic ileus<sup>[10]</sup>, burn-induced slow gastrointestinal transit<sup>[16]</sup> and diabetes mellitus<sup>[17,18]</sup>. In the present study, the prokinetic effect of GHRP-6 was investigated in a mouse model of diabetes mellitus.

## MATERIALS AND METHODS

### Chemicals

GHRP-6 was obtained from Tocris Cookson (Bristol, UK). Atropine sulphate, phenol red, and alloxan were purchased from Sigma (St Louis, MO).

### Diabetic mouse model

C57 mice (18–22 g) were provided by the Experimental Animal Center of Shanghai Academia Sinica. All procedures for the animal experiments were approved by the Medical Ethical Committee of Shanghai Jiaotong University. The mice were housed in stainless steel cages at a controlled temperature ( $22 \pm 2^\circ\text{C}$ ) with a 60%–65% relative humidity in a normal 12-h light and dark cycle. After exposure to a high-fat diet for 3 wk, the mice were fasted overnight with free access to water and injected intraperitoneally with alloxan (0.2 g/kg body weight) dissolved in a sterile normal saline solution. Seventy-two hours later, the fasting blood glucose level in the mice was determined using the glucose oxidase method with a glucose analyzer. Mice with a blood glucose level higher than 11.1 mmol/L were classified as diabetic mice (DB mice). The DB mice were continuously fed without control of blood glucose for 4 wk, and a model of DB mice was established for further investigations.

### Animal grouping for gastrointestinal transit studies

Gastric emptying, intestinal and colonic transit studies were then performed. For each study, mice were divided into two groups: a normal (control) group and a diabetic group. Mice in the diabetic group were treated with different doses of GHRP-6 (0, 20, 50, 100 and 200  $\mu\text{g}/\text{kg}$ ) given in a random order, with a total of six mice in each subgroup. A dose-response curve for GHRP-6 was obtained for the experiment. Based on the most effective dose of GHRP-6, another group of 6 mice were given atropine (1 mg/kg) injections 15 min before GHRP-6 injection.

### Gastric emptying

After a 12 h fast, the diabetic mice were injected with different doses of GHRP-6 (0, 20, 50, 100 and 200  $\mu\text{g}/\text{kg}$ ). After GHRP-6 injection, the mice received a gavage feeding (5 mg/kg body weight) of a phenol red test meal (0.5 g/L in 0.9% NaCl with 1.5% methylcellulose). Twenty minutes later, the mice were sacrificed. Their stomachs were clamped with a string above the lower oesophageal sphincter and a string beneath the pylorus to prevent leakage of phenol red. Gastric emptying was determined spectrophotometrically, according to the method previously described with certain slight modifications. The stomach of each individual mouse was resected just above the lower oesophageal sphincter and pyloric sphincter. Phenol red remained partly in the lumen of the stomach. The stomach and its contents were put into 5 mL of 0.1 mol/L NaOH. The stomach was minced. The samples containing the total amount of phenol red present in the stomach were further

diluted to 10 mL with 0.1 mol/L NaOH and left at room temperature for 1 h. Five milliliters of the supernatant was then centrifuged at 800 g for 20 min. The absorbance was read at a wavelength of 546 nm on a spectrophotometer (Shanghai Yixian Company, China), and the phenol red content in the stomach was calculated. Percentage of gastric emptying of the phenol red was calculated as [(infusion amount – remains)/infusion amount]  $\times 100\%$ .

### Intestinal and colonic transit

After an overnight fast, mice were given general anesthesia (2%–3% isoflurane inhalation) and underwent abdominal surgery. A small polyethylene tube was placed in the duodenum (or colon) *via* the stomach (or cecum), 0.5 cm distal to the pylorus (or ileocolic junction), fixed with sutures to the gut wall, and then tunneled through the abdominal wall subcutaneously to exit from the skin at the nape of the neck. Midline incisions were sutured, and mice were left to recover in separate cages. Food and water were provided abundantly. Three days later, after a 12 h fast, the mice were given different doses of GHRP-6 (0, 20, 50, 100 and 200  $\mu\text{g}/\text{kg}$ ) intraperitoneally. After GHRP-6 injection, a phenol red test meal at 5 mg/kg (0.5 g/L in 0.9% NaCl with 1.5% methylcellulose) was injected into the duodenum (or colon) *via* the implanted polyethylene tube. After 20 min, the mice were sacrificed. The distance of phenol red transit and the full length of the intestine or colon were calculated. Small intestine or colonic transit was assessed using the percentage ratio of phenol red transit over the full intestinal or colonic length.

### Statistical analysis

Statistical analysis of the data was conducted using one-way ANOVA for multiple comparisons. Data are expressed as mean  $\pm$  SE.  $P < 0.05$  was considered statistically significant.

## RESULTS

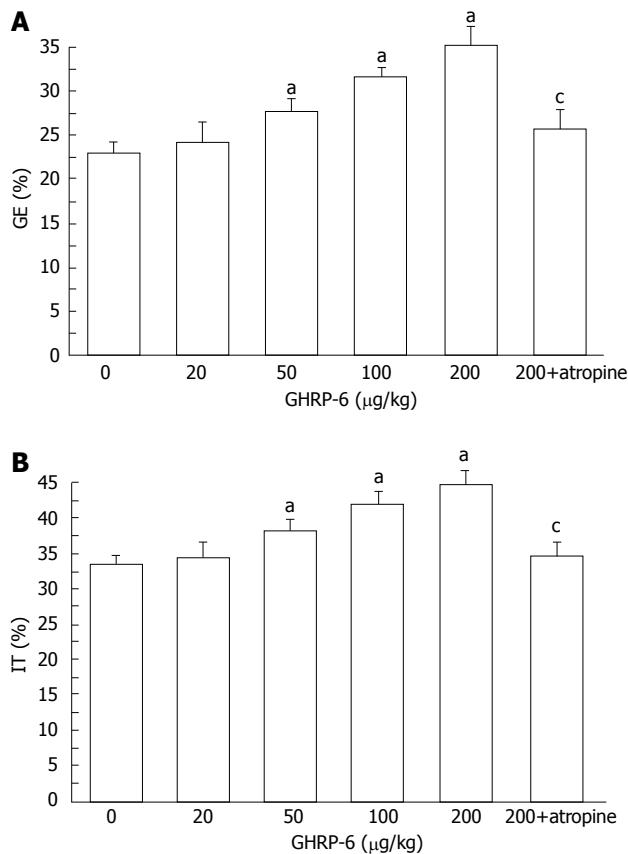
### Gastric emptying and intestinal and colonic transit in diabetic mice

Significantly delayed gastric emptying, intestinal and colonic transits were found in diabetic mice. Gastric emptying was significantly decreased in diabetic mice compared to normal mice ( $22.9\% \pm 1.4\% \text{ vs } 28.1\% \pm 1.3\%, P = 0.0216$ ).

Also, intestinal transit was significantly decreased in diabetic mice compared to normal mice ( $33.5\% \pm 1.2\% \text{ vs } 43.2\% \pm 1.9\%, P = 0.0132$ ). In addition, colonic transit was decreased in diabetic mice compared to normal mice ( $29.5\% \pm 1.9\% \text{ vs } 36.3\% \pm 1.6\%, P = 0.0148$ ).

### Effect of GHRP-6 on delayed gastric emptying in diabetic mice

GHRP-6 significantly accelerated gastric emptying in diabetic mice. The percentage of gastric emptying was



**Figure 1** GHRP-6 significantly increases gastric emptying (A) and intestinal transit (B) in diabetic mice. \* $P < 0.05$  vs control;  $^{\circ}P < 0.05$  vs 200 µg/kg GHRP-6 dose.

23.6%  $\pm$  1.9%, 26.9%  $\pm$  1.3%, 31.3%  $\pm$  0.9%, and 34.6%  $\pm$  1.5%, respectively, in the mice treated with 20, 50, 100, and 200 µg/kg GHRP-6. Except for the lowest dose, all of these doses normalized the rate of gastric emptying in diabetic mice ( $P = 0.0421$ ,  $P = 0.0324$  and  $P = 0.0103$ , respectively; Figure 1). We considered that the dosage of 200 µg/kg could most effectively increase the rate of gastric emptying.

#### Effect of GHRP-6 on delayed small intestinal transit in diabetic mice

GHRP-6 significantly accelerated intestinal transit in diabetic mice. The percentage of intestinal transit was 34.2%  $\pm$  1.9%, 39.1%  $\pm$  1.5%, 42.6%  $\pm$  1.7%, and 44.5%  $\pm$  1.8%, respectively, in the mice treated with 20, 50, 100, and 200 µg/kg GHRP-6. All of these doses, except for 20 µg/kg, normalized the delayed intestinal transit ( $P = 0.0321$ ,  $P = 0.0289$  and  $P = 0.0184$ , respectively; Figure 1). As above, the 200 µg/kg GHRP-6 dose was considered most effective in accelerating intestinal transit.

#### Effect of GHRP-6 on delayed colonic transit in diabetic mice

GHRP-6 had no effect on delayed colonic transit. The percentage of colonic transit was 30.8%  $\pm$  1.4%, 29.5%  $\pm$  1.7%, 30.3%  $\pm$  1.9%, and 31.2%  $\pm$  2.3%, respectively, in the mice treated with 20, 50, 100, and 200 µg/kg

GHRP-6. None of these doses was able to accelerate intestinal transit.

#### Effect of atropine on delayed gastric emptying and intestinal transit in diabetic mice

Atropine blocked the positive effects of 200 µg/kg GHRP-6 on gastric emptying and intestinal transit. The percentage of gastric emptying was significantly decreased from 34.6%  $\pm$  1.5% to 24.6%  $\pm$  1.8% ( $P = 0.0131$ , Figure 1). The small intestine transit was significantly decreased from 44.5%  $\pm$  1.8% to 35.4%  $\pm$  1.8% ( $P = 0.0145$ , Figure 1).

## DISCUSSION

In the present study, gastric emptying, intestinal and colonic transit were significantly delayed in the mice with alloxan-induced diabetes, which is consistent with previously reported findings<sup>[19,20]</sup>. Gastrointestinal motility disturbances including esophageal motor dysfunction, gastroparesis, constipation and diarrhea, are common in patients with diabetes mellitus. It has been reported that gastrointestinal transit is significantly slower in the diabetic animal model of human diabetes<sup>[21-23]</sup>. Inhibition of gastrointestinal motility has also been reported in humans with diabetes mellitus<sup>[24,25]</sup>. The pathogenesis of slow gastrointestinal transit in diabetes mellitus patients is not clear, but several mechanisms have been proposed<sup>[12]</sup>. Among them, autonomic neuropathy, a complication of long-standing diabetes mellitus, has been widely accepted as the culprit. This may lead to the absence of postprandial gastrointestinal response, a reflex that should present in healthy people<sup>[24]</sup>. Recent studies have shown that an acute change in blood glucose concentration also has a major effect on gastrointestinal motor function in healthy subjects<sup>[25]</sup>. In particular, acute hyperglycemia inhibits both the gastrointestinal and ascending components of peristaltic reflex. Poor glycemic control has the potential to cause delayed gastrointestinal transit in diabetic patients<sup>[26]</sup>. In our study, different doses of GHRP-6 were able to accelerate gastric emptying and intestinal transit in the diabetic mice, but had no effect on colonic transit, which is consistent with the results seen in animal models of postoperative ileus<sup>[15]</sup> and in burn-induced gastrointestinal delayed transit<sup>[16]</sup>. We believe that this result might be related to the distribution of ghrelin receptors in the gut<sup>[27,28]</sup>. The most effective dose of GHRP-6 for accelerating upper gastrointestinal transit was 200 µg/kg per mouse. Atropine blocked the GHRP-6 effect on gastric emptying and intestinal transit.

GHRP-6, possessing prokinetic characteristics, increases gastric emptying in healthy mice<sup>[29]</sup>. The mechanisms underlying the action of GHRP-6 seem to be neuron-dependent. *In vitro*, isolated strips of muscle fail to contract significantly when exposed to GHRP-6. *In vivo* the gastrokinetic effect of GHRP-6 in rats is abolished by atropine or vagotomy<sup>[14,29]</sup>. In our study, atropine blocked the effect of 200 µg/kg GHRP-6 on gastric emptying and intestinal transit, suggesting that

the prokinetic effect of GHRP-6 is mediated *via* the cholinergic pathway in the enteric nervous system.

It is reasonable to assume that GHRP-6 can be used as a potential drug for the treatment of diabetic patients with delayed gastrointestinal transit. Clinically, improvement in gastrointestinal transit facilitates enteral resuscitation, corrects blood glucose concentrations and reduces gastrointestinal symptoms in diabetic patients. In conclusion, GHRP-6 accelerates gastric emptying and intestinal transit in diabetic mice, an action that may be mediated *via* the cholinergic pathway in the enteric nerve system. GHRP-6 may have a therapeutic potential for diabetic patients with delayed upper gastrointestinal transit. The physiological role of GHRP-6 in the gastrointestinal tract remains to be identified, and its pharmacotherapeutic potential deserves to be further explored in diabetic patients suffering from delayed upper gastrointestinal transit.

## COMMENTS

### Background

Delayed gastrointestinal transit is common in patients with chronic diabetes and is always associated with impairments in quality of life and diabetic control. GHRP-6 is a potent prokinetic peptide. The effect of GHRP-6 on delayed gastrointestinal transit was studied in diabetic mice.

### Research frontiers

The effects of ghrelin on gastrointestinal motor activity and roles in motility regulation have been extensively studied. This study is the first to investigate the effects of ghrelin agonist GHRP-6 on diabetic mice with delayed gastrointestinal transit.

### Innovations and breakthroughs

GHRP-6 has been shown to accelerate gastric activity in postoperative and septic ileus animal models. However, it has not been studied in a diabetic animal model.

### Applications

According to animal experiments, GHRP-6 may be used as a potential therapeutic agent for the treatment of delayed gastrointestinal transit in diabetes.

### Peer review

This paper shows that GHRP-6 has an effect on gastric emptying and intestinal transit in diabetic mice, which may be mediated through the cholinergic pathways in the enteric nerve system. These results show that GHRP-6 can be used as a potential therapeutic drug for the treatment of delayed gastrointestinal transit in diabetes.

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RAPID COMMUNICATION

## OB glue paste technique for establishing nude mouse human gastric cancer orthotopic transplantation models

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Shi J, Wei PK, Zhang S, Qin ZF, Li J, Sun DZ, Xiao Y, Yu ZH, Lin HM, Zheng GJ, Su XM, Chen YL, Liu YF, Xu L. OB glue paste technique for establishing nude mouse human gastric cancer orthotopic transplantation models. *World J Gastroenterol* 2008; 14(30): 4800-4804 Available from: URL: <http://www.wjg-net.com/1007-9327/14/4800.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4800>

### Abstract

**AIM:** To establish nude mouse human gastric cancer orthotopic transplantation models using OB glue paste technique.

**METHODS:** Using OB glue paste technique, orthotopic transplantation models were established by implanting SGC-7901 and MKN-45 human gastric cancer cell strains into the gastric wall of nude mice. Biological features, growth of the implanted tumors, the success rate of transplantation and the rate of auto-metastasis of the two models were observed.

**RESULTS:** The success rates of orthotopic transplantation of the two models were 94.20% and 96%. The rates of hepatic metastasis, pulmonary metastasis, peritoneal metastasis, lymphocytic metastasis and splenic metastasis were 42.13% and 94.20%, 48.43% and 57.97%, 30.83% and 36.96%, 67.30% and 84.06%, and 59.75% and 10.53%, respectively. The occurrence of ascites was 47.80% and 36.96%.

**CONCLUSION:** OB glue paste technique is easy to follow. The biological behaviors of the nude mouse human gastric cancer orthotopic transplantation models established with this technique are similar to the natural processes of growth and metastasis of human gastric cancer, and, therefore, can be used as an ideal model for experimental research of proliferative metastasis of tumors.

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### INTRODUCTION

Establishment of human gastric cancer nude mouse transplantation models has undergone three representative stages<sup>[1-5]</sup>: subcutaneous transplantation of cell suspension<sup>[6]</sup>, gastric wall seeding of cell suspension<sup>[7-12]</sup>, and intra-gastric wall transplantation of histologically intact tissues<sup>[13-17]</sup>, of which the fresh tissue orthotopic transplantation model has two types in method: the “suture method”<sup>[18]</sup> and the “gastric bursa method”<sup>[19]</sup>. Enlightened by surgical use of OB glue, the present study used OB glue paste technique to implant SGC-7901 and MKN-45 human gastric cancer cell strains to the gastric wall of nude mice in an attempt to examine the biological features of proliferative metastasis of gastric cancer cells.

### MATERIALS AND METHODS

#### Animals

The study included 314 6-week BALB/C nude mice of both sexes weighing 18-20 g provided by Shanghai Experimental Animal Center of the Chinese Academy of Sciences (qualification No.: SCXK, Shanghai, 2004-0003). In this study, 166 cases of models for SGC-7901 and 148 cases for MKN-45 were established. The animals were raised in the said center in SPF experimental animal rooms (approval No.: SYXK, Shanghai, 2004-0011) with free access to water.

### Cell strains

SGC-7901 and MKN-45 cell strains were kind gifts from the laboratory of experimental pathology of Shanghai Institute of Tumor Research, CAS. Cancer cells were cultured in RPMI-1640 solution containing 10% bovine serum in a CO<sub>2</sub> thermostatic incubator and passaged routinely.

### Establishment and passage of subcutaneously transplanted tumor in nude mice

*In vitro* cancer cells were collected to the content of  $1 \times 10^7$ /mL. Each nude mouse was injected with 0.2 mL cancer cells under the cervical skin. When the implanted tumor grew to about 1cm diameter, it was removed out of the mouse. The tumor was cut into 1 mm × 1 mm × 2 mm pieces after scraping off the surrounding fibrous capsule, and implanted directly into the cervical skin of the nude mice. Each inter-mouse passage used two mice. The third generation subcutaneously transplanted tumor was used as the source of orthotopic transplantation in this study.

### Establishment of orthotopic transplantation models

The mice were purchased two days ahead of the experiment for environmental adaptation. The animals were fasted 12 h before operation and anesthetized with 0.4% pentobarbital sodium (60 mg/kg) intraperitoneally. The skin was sterilized routinely and a 1 cm incision was made along the left paramedian line to expose the peritoneum and gastric wall meticulously. The serous layer of the greater curvature of stomach where there are abundant blood vessels, was carefully ruptured with an injection needle until bleeding was visible, into which the tumor tissue was implanted. One to 2 drops of medical OB glue (Cyanoacrylate, medical OB 508 series for anastomosis, Guangzhou Bai Yun Medical Glue Co., Ltd., Batch No. 030703) were applied to seal the rupture. After the glue coagulated for about 10 s, the peritoneum was closed with No. 3 suture and the skin was closed with No. 1 suture.

### Sacrifice of animals and observation of metastasis of the transplanted tumor

When the mice were seen developing failing signs such as leanness, limited activity and listlessness, they were sacrificed by cervical dislocation and anatomized for comprehensive exploration of the chest and abdominal cavities and macroscopic observation of any transplanted tumor with regard to local growth, ascites, adjacent lymph nodes and distal organ metastasis. The transplanted tumor, enlarged lymph nodes, liver, spleen, pancreas and lungs were excised, and the specimens were fixed, sliced and stained for histopathologic observation under light and electron microscope. The tumor was weighed with an analytical balance and recorded. Four mice which died during the study were treated in the same manner without including into statistics, but for reference and later causative analysis.

### Preparation of chromosomal specimens of human gastric cancer cells

Part of the transplanted tumor tissues was sheared with aseptic technique and placed in serum-free 1640 medium for primary culture. When the cells grew vigorously and formed a single layer, the first generation passage was done, for which chromosomal specimens were prepared. The specimens were observed under an  $\times 40$  light microscope and photographed for chromosomal metakinesis of one cell with a microscopic camera.

## RESULTS

### Growth of the orthotopically transplanted tumor

One mouse died from excessive bleeding during establishment of the SGC-7901 orthotopic transplantation model. The skin suture fell off at about day 5 and the wound healed completely in a week. At week 3-4, 4 mm nodules were palpable in the upper abdomen, which grew large gradually by week 5-6 and became markedly large by week 8-10. At week 10 some of the tumors were even visible through the wall and as large as 10-20 mm in diameter. The surfaces of these tumors were nodular and hard in consistency. From week 11 on, the animals began presenting failing signs such as leanness, limited activity, listlessness and hypoactivity. One animal died. At week 12 the failing signs were more evident and severe, and tumors in some mice subcutaneously projected out, or the abdomens were bulky looking like a frog abdomen. The animals were sacrificed by cervical dislocation.

One animal in the MKN-45 orthotopic transplantation model died on the second day because of suture failure due to mutual fierce biting of the animals. The situation of the remaining animals was much the same as that of the SGC-7901 model. The only difference in the MKN-45 model was that the tumors grew faster in fewer days. By week 2, hard nodules about 4mm were palpable in both right and left upper abdomen; the tumors became large gradually by week 3-4 and grew to 10-15 mm in diameter by week 5-6; 3 animals died by week 6-7; and the failing signs became worse by week 8 when giant tumors of 15-20 mm in diameter were palpable. The animals were sacrificed by cervical dislocation.

### Invasion of the orthotopically transplanted tumor

A total of 164 cases of models with SGC-7901 were established in this study, including 159 cases of SGC-7901 orthotopic transplantation with a success rate of 97% (159/164). Gross anatomy revealed: the body of stomach was enlarged and the fundus was dilated; grayish fish meat like tumors were seen on the gastric wall; the tumor tissue was parenchymatous with vague margins and round or oval in shape. There were nodular processes on the surface, which infiltrated into the surrounding tissues and adhered with the mesentery, liver, spleen and peritoneum in varying degrees. There



**Figure 1** Macroscopic observation of orthotopically transplanted MKN-45 tumor.

was bloody ascites in some mice. The tumor sections were grayish, looking like fish meat and homogenous in consistency, on which there were abundant capillaries. In some large tumors, there were small necrotic patches in the center. The mean weight of the tumors was  $2.31 \pm 0.75$  g.

A total of 144 cases of models with MKN-45 were established, including 138 cases of orthotopic transplantation, with a success rate of 96% (138/144). Gross anatomic findings were much the same as those of the SGC-7901 model. The tumors were oval in shape, uneven and hard in consistency. There was a serous necrotic area in the center. The mean weight of the tumors was  $2.53 \pm 0.84$  g (Figure 1).

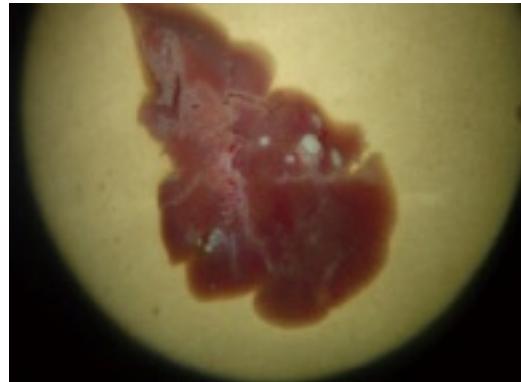
#### **Metastasis of the orthotopically transplanted tumor**

Intraperitoneal lymph nodes were enlarged in most mice of the SGC-7901 model. Involvement of the tumors was various. Several small grayish miliary nodules were seen in the liver of most tumor-bearing mice, which went into the hepatic parenchyma and were difficult to separate. Pyloric obstruction and bloody ascites were seen in some tumor-bearing mice, and pulmonary, splenic and peritoneal metastases were seen in other animals. The metastasis rates of the liver, lungs, peritoneum, lymph node and spleen were 42.02% (67/159), 48.43% (77/159), 30.82% (49/159), 67.30% (107/159) and 59.75% (95/159), respectively. The prevalence of ascites was 47.80% (76/159).

The situation in the MKN-45 model was similar to that of the SGC-7901 model, where the hepatic focus of metastasis was 2-3 mm (Figure 2). The lung surface was congested in some animals and transparent nodules about 1mm were visible. The metastasis rates of the liver, lungs, peritoneum, lymph node and spleen were 94.20% (130/138), 57.97% (80/138), 36.96% (51/138), 84.06% (116/138) and 10.86% (15/138), respectively. The prevalence of ascites was 31.88% (44/138).

#### **Histopathologic findings of the orthotopical transplantation models**

SGC-7901 gastric cancer tissue was adenocarcinoma of low differentiation. The tumor cells present as oval shape with large malformed nuclei, most of which were of pathologic mitosis with clear and multiple nucleoli.



**Figure 2** Hepatic metastatic focus of orthotopically transplanted MKN-45 tumor.

They were deranged like a nestle with rich sinusoids, in which filtration of lymphocytes was seen.

Histological sections of MKN-45 tumor body was also characterized by poorly differentiated adenocarcinoma, where cells were in patchy, nestle or streaky arrangement and shaped round, oval or irregular. Cell differentiation was poor, with large deep stained nuclei and a large nuclear-cytoplasmic ratio, where karyokinetic phase was seen. Fibrous connective tissue was seen in the mesenchyma, and infiltrative growth of the tumor tissue was seen in the gastric wall (Figure 3A).

The histological structure of the tumor metastatic foci in the liver and lungs was consistent with that of the orthotopic gastric tumor, mostly distributing around blood vessels of the lung parenchyma and hepatic sinusoid and growing infiltratively to the surrounding tissues. Large numbers of cancer cells were seen in the enlarged lymph nodes; they arranged closely, destroying almost all lymphatic structure. Cytohistological morphology of ascites smear was similar to that of the orthotopic tumor.

#### **Identification of cell chromosome of the orthotopically transplanted tumor**

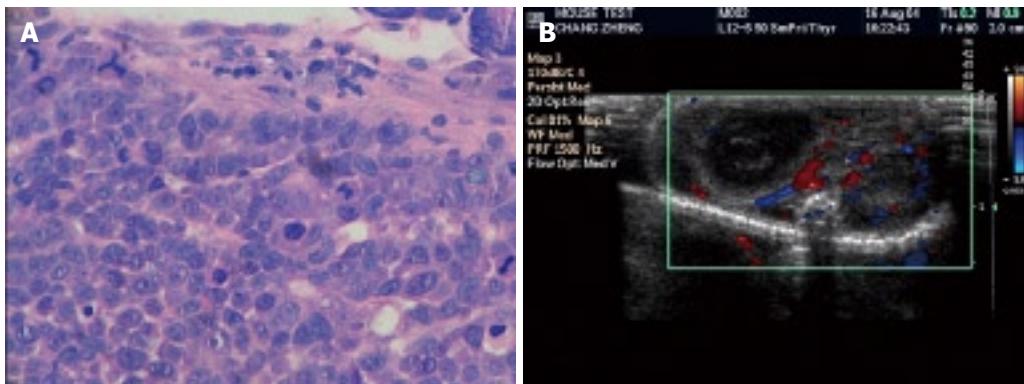
Under  $\times 40$  light microscope, no single chromosome in the metaphase was seen as a telocentric chromosome, indicating that it was a human chromosome. Distribution of the chromosomes was more than 46, indicating that they were malignant tumor cells.

#### **Color doppler imaging of the orthotopically transplanted tumor**

B-ultrasound detected low echo masses in the upper abdomen of the nude mice, which grew into the abdominal cavity (Figure 3B). Most blood flow of the tumors was in the periphery, or mixed blood flow was seen both inside and around the tumor. The course of vessels was irregular and the vessels were various in size and deranged, forming winding, fork-like and net-like blood flows. Blood flow was more abundant and faster in the periphery than in the center of the tumor (Figure 3B).

## **DISCUSSION**

Human gastric cancer fresh tissue orthotopic transplantation is the main means of model establishment. The



**Figure 3** A: Histological findings of orthotopically transplanted MKN-45 tumor; B: Ultrasound findings of orthotopically transplanted SGC-7901 tumor.

site of transplantation is usually between the serous layer and seromuscular layer of the greater curvature of stomach. The “suture method”<sup>[20]</sup> or the “gastric bursa method”<sup>[21-22]</sup> has many practical difficulties and disadvantages in establishing the model of transplantation, for example, the tumor tissue is easy to fall off postoperatively; manipulation is complex and needs relatively high skills; and there may be great blood loss during suturing, and mortality is relatively high. OB glue is biologic glue and has been used widely in surgery owing to its secure wound adhesion. We have had two-years experience with the use of “OB glue paste technique” to establish the human gastric cancer nude mouse orthotopic transplantation model since 2003, when the technique was first attempted in our department<sup>[23-30]</sup>. The present study used OB glue paste technique to establish two tumor strain orthotopic transplantation models. Observations showed that although different tumor strains grew at different rates, infiltrative growth and multi-organ metastases are common features of the two models, and these features are similar to the clinical presentation of invasive metastasis. Chromosomal identification also demonstrated that both orthotopic and metastatic tumors came from the human gastric cancer implanted. This technique is an ideal means of model establishment owing to its easier manipulation, shorter operating time, less blood loss, quicker postoperative recovery, and higher survival of experimental animals and avoidance of tumor falling off.

The success rate was not 100% in both cases. Anatomy of the mice that failed to bear tumors showed that there was local organ adhesion arising from manipulation, and part of the transplanted tumor tissues stop growing. With our experience, the following points are worthy of mention with regard to factors affecting the success rate: (1) The amount of glue should be appropriate, 1-2 drops are enough, too much glue would envelope the implant, halting its growth; (2) It is best to wait for 10 seconds or so before closing the abdomen so that the glue can coagulate sufficiently; or it may cause extensive adhesion of the surrounding tissues; (3) It is preferable to use the seromuscular layer near the antrum of the greater curvature, because rich blood flow there facilitates tumor growth and metastasis; (4) Rupture of

the seromuscular layer should not be too superficial, and bleeding is the hallmark. In addition, the tumor tissue to be implanted should be placed into the ruptured site before dropping OB glue. Smooth forceps can be used to push the rupture inward to form a denture before implanting the tumor tissues, if necessary; and (5) It is not preferable to leave too much suture outside the abdomen to avoid suturing failure due to fierce biting between animals.

## COMMENTS

### Background

Establishment of human gastric cancer nude mouse transplantation models has undergone three representative stages: subcutaneous transplantation of cell suspension, gastric wall seeding of cell suspension, and intra-gastric wall transplantation of histologically intact tissues. The fresh tissue orthotopic transplantation model has two types in method: the “suture method” and the “gastric bursa method”.

### Research frontiers

Human gastric cancer fresh tissue orthotopic transplantation is the main means of model establishment. But the “suture method” or the “gastric bursa method” transplantation has many practical difficulties and disadvantages in establishing the model. The “OB glue paste technique” was attempted to establish the human gastric cancer nude mouse orthotopic transplantation model.

### Innovations and breakthroughs

Observations showed that the “OB glue paste technique” is an ideal means of model establishment owing to its easier manipulation, shorter operating time, less blood loss, quicker postoperative recovery, higher survival of experimental animals and avoidance of tumor falling off.

### Applications

OB glue paste technique is easy to follow and can be used as an ideal model for experimental research of proliferative metastasis of tumors.

### Peer review

Shi et al established the nude mouse human gastric orthopedic transplantation model using OB glue paste technique. This is an interesting paper. However, several points should be addressed. If possible, the success rate of orthotopic transplantation of SGC-7901 and MKN-45 models should be indicated without using OB glue. If additional effect of OB glue was clarified, this paper would be more convincing.

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RAPID COMMUNICATION

# Risk factors for local recurrence of middle and lower rectal carcinoma after curative resection

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tumor infiltration ( $\chi^2 = 5.052, P = 0.168$ ), depth of tumor invasion ( $\chi^2 = 4.588, P = 0.101$ ), lymph node metastases ( $\chi^2 = 3.688, P = 0.055$ ) and TNM staging system ( $\chi^2 = 3.765, P = 0.152$ ). The local recurrence rate of middle and lower rectal carcinoma was 33.3% (4/12) in patients with positive circumferential resection margin and 6.8% (3/44) in those with negative circumferential resection margin. There was a significant difference between the two groups ( $\chi^2 = 6.061, P = 0.014$ ). Local recurrence of rectal carcinoma occurred in 6 of 36 patients (16.7%) with mesorectal metastasis, and in 1 of 20 patients (5.0%) without mesorectal metastasis. However, there was no significant difference between the two groups ( $\chi^2 = 1.600, P = 0.206$ ).

**CONCLUSION:** Family history, high CEA level, cancerous perforation, tumor differentiation, vessel cancerous emboli and circumferential resection margin status are the significant risk factors for local recurrence of middle and lower rectal carcinoma after curative resection. Local recurrence may be more frequent in patients with mesorectal metastasis than in patients without mesorectal metastasis.

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**Key words:** Middle and lower rectal carcinoma; Local recurrence; Circumferential resection margin; Mesorectal metastasis

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Wu ZY, Wan J, Zhao G, Peng L, Du JL, Yao Y, Liu QF, Lin HH. Risk factors for local recurrence of middle and lower rectal carcinoma after curative resection. *World J Gastroenterol* 2008; 14(30): 4805-4809 Available from: URL: <http://www.wjgnet.com/1007-9327/14/4805.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4805>

## INTRODUCTION

It is well known that local recurrence is of rectal carcinoma plays an important role in its prognosis<sup>[1-3]</sup>. However, local recurrence of rectal carcinoma occurs in about 4%-50% of patients even after radical resection of primary tumors and lymph nodes<sup>[4-8]</sup>. The risk factors

for local recurrence of rectal carcinoma remain unclear. Therefore, the aim of the current study was to explore the risk factors for local recurrence of middle and lower rectal carcinoma after curative resection. Specimens of middle and lower rectal carcinoma from 56 patients who underwent total mesorectal excision (TME) at the Department of General Surgery, Guangdong Provincial People's Hospital, from November, 2001 to July, 2003, were studied. A large slice technique was used to detect mesorectal metastasis and evaluate circumferential resection margin status. The relationship between mesorectal metastasis, local recurrence and circumferential resection margin status of rectal carcinoma was observed. The clinicopathologic characteristics of middle and lower rectal carcinoma were also evaluated.

## MATERIALS AND METHODS

### **Patients and specimens**

Specimens of middle and lower rectal carcinoma from 56 patients who underwent TME at the Department of General Surgery, Guangdong Provincial People's Hospital, from November, 2001 to July, 2003, were studied. There were 37 men and 19 women, ranging in age from 30 to 86 years, with a mean age of 60.5 years. None of these patients received preoperative chemotherapy or radiotherapy. Twenty-six patients had lower rectal carcinoma and 30 had middle rectal carcinoma. Tumors  $\geq 5$  cm and in  $< 5$  cm diameter were found in 18 and 38 patients, respectively. Low anterior resection was performed in 40 patients and abdominoperineal resection in 16 patients. TNM stages were as follows: stage I in 5 patients, stage II in 22 patients, and stage III in 29 patients. Poorly-differentiated carcinoma was observed in 14 patients, moderately-differentiated carcinoma in 37 patients, and well-differentiated carcinoma in 5 patients, respectively. A large slice technique was used to detect mesorectal metastasis and evaluate circumferential resection margin status. Two pathologists who were blinded to the clinicopathological data observed the specimens independently. If tumor cells were detected within 1 mm of circumferential margin, they were classified to have a positive circumferential resection margin as previously described<sup>[9-11]</sup>.

### **Statistical analysis**

Statistical analysis was performed by the Pearson  $\chi^2$  test to examine the association between local recurrence, circumferential resection margin status and mesorectal metastasis of rectal carcinoma. Clinicopathologic characteristics of the patients with middle and lower rectal carcinoma were also analyzed.  $P < 0.05$  was considered statistically significant.

## RESULTS

### **Correlation between local recurrence and clinicopathologic characteristics of patients with middle and lower rectal carcinoma**

Local recurrence of middle and lower rectal carcinoma

after curative resection was found in 7 of 56 patients (12.5%), which was significantly related with family history ( $\chi^2 = 3.929, P = 0.047$ ), high CEA level ( $\chi^2 = 4.964, P = 0.026$ ), cancerous perforation ( $\chi^2 = 8.503, P = 0.004$ ), tumor differentiation ( $\chi^2 = 9.315, P = 0.009$ ) and vessel cancerous emboli ( $\chi^2 = 11.879, P = 0.001$ ). In contrast, no significant correlation was found between local recurrence and other variables such as age ( $\chi^2 = 0.506, P = 0.477$ ) and gender ( $\chi^2 = 0.102, P = 0.749$ ), tumor diameter ( $\chi^2 = 0.421, P = 0.516$ ), tumor infiltration ( $\chi^2 = 5.052, P = 0.168$ ), depth of tumor invasion ( $\chi^2 = 4.588, P = 0.101$ ), lymph node metastases ( $\chi^2 = 3.688, P = 0.055$ ) and TNM staging system ( $\chi^2 = 3.765, P = 0.152$ ) (Table 1).

### **Correlation between circumferential resection margin status and local recurrence of middle and lower rectal carcinoma**

A positive circumferential resection margin of middle and lower rectal carcinoma was observed in 12 of 56 patients (21.4%). Local recurrence of middle and lower rectal carcinoma was found in 4 of 12 patients (33.3%) with a positive circumferential resection margin and in 3 of 44 patients (6.8%) with a negative circumferential resection margin. There was a significant difference between the two groups ( $\chi^2 = 6.061, P = 0.014$ ) (Table 1).

### **Correlation between mesorectal metastasis and local recurrence of middle and lower rectal carcinoma**

Mesorectal metastasis of middle and lower rectal carcinoma was detected in 36 of 56 patients (64.3%). The local recurrence rate of mesorectal metastasis was 16.7% (6 of 36 patients) and 5.0% (1 of 20 patients), respectively. However, there was no significant difference between the two groups ( $\chi^2 = 1.600, P = 0.206$ ) (Table 1).

## DISCUSSION

It is well known that middle and lower rectal carcinoma is one of the most common carcinomas in China. Local recurrence of middle and lower rectal carcinoma after curative resection has significant morbidity and mortality<sup>[4,12-15]</sup> and its recurrence rate varies from less than 4% to greater than 50%. Since TME was adopted as the standard treatment of patients with rectal carcinoma, a significant decrease in local recurrence and a trend to improve relative survival have been reported<sup>[16-18]</sup>. In our study, local recurrence of middle and lower rectal carcinoma occurred in 7 of 56 patients (12.5%) after TME, indicating that TME can significantly reduce the local recurrence rate of middle and lower rectal carcinomas.

The correlation between circumferential resection margin status and local recurrence of rectal carcinoma is still controversial<sup>[10,11,19-21]</sup>. Wibe *et al*<sup>[10]</sup> reported that a positive circumferential resection margin has a significant and major prognostic impact on the local recurrence rate of rectal carcinoma after TME. However, Luna-Perez *et al*<sup>[19]</sup> reported that circumferential resection margin involvement is not correlated significantly with

**Table 1** Local recurrence and circumferential resection margin status, mesorectal metastasis, and clinicopathologic characteristics of middle and lower rectal carcinoma

Clinicopathologic variable	Patients ( <i>n</i> )	Local recurrence		$\chi^2$	<i>P</i>
		Positive (%)	Negative (%)		
Gender					
Male	37	5 (13.5)	32 (86.5)		
Female	19	2 (10.5)	17 (89.5)	0.102	0.749
Age					
< 60 yr	25	4 (16.0)	21 (84.0)		
≥ 60 yr	31	3 (9.7)	28 (90.3)	0.506	0.477
Family history					
Yes	21	5 (23.8)	16 (76.2)		
No	35	2 (5.7)	33 (94.3)	3.929	0.047
CEA level					
High	26	6 (23.1)	20 (76.9)		
Normal	30	1 (3.3)	29 (96.7)	4.964	0.026
Cancerous perforation					
Yes	3	2 (33.3)	1 (66.7)		
No	53	5 (9.4)	48 (90.6)	8.503	0.004
Superficial diameter					
< 5 cm	38	4 (10.5)	34 (89.5)		
≥ 5 cm	18	3 (16.7)	15 (83.3)	0.421	0.516
Diameter of infiltration					
1/4	8	0 (0)	8 (100)		
1/2	16	1 (6.3)	15 (93.7)		
3/4	18	2 (11.1)	16 (88.9)		
4/4	14	4 (28.6)	10 (71.4)	5.052	0.168
Depth of invasion					
T <sub>1</sub>	6	0 (0)	6 (100)		
T <sub>2</sub>	23	1 (4.3)	22 (95.7)		
T <sub>3</sub>	27	6 (22.2)	21 (77.8)	4.588	0.101
Histologic differentiation					
Well	5	0 (0)	5 (100)		
Moderate	37	2 (5.4)	35 (94.6)		
Poorly	14	5 (35.7)	9 (64.3)	9.315	0.009
Lymph node metastasis					
Positive	29	6 (20.7)	23 (79.3)		
Negative	27	1 (3.7)	26 (96.3)	3.688	0.055
Vessel cancerous emboli					
Positive	12	5 (41.7)	7 (58.3)		
Negative	44	2 (4.5)	42 (95.5)	11.879	0.001
Circumferential resection margin					
Positive	12	4 (33.3)	8 (66.7)		
Negative	44	3 (6.8)	41 (93.2)	6.061	0.014
Mesorectal metastasis					
Positive	36	6 (16.7)	30 (83.3)		
Negative	20	1 (5.0)	19 (95.0)	1.6	0.206
TNM staging					
I	5	0 (0)	5 (100)		
II	22	1 (4.5)	21 (95.5)		
III	29	6 (20.7)	23 (79.3)	3.765	0.152

local recurrence of rectal adenocarcinoma ( $P = 0.33$ ). Hall *et al*<sup>[11]</sup> reported that the local recurrence rate of rectal carcinoma with a positive circumferential resection margin is 15% and 11% in those with a negative circumferential resection margin. The difference between the two groups was not significant. Our results demonstrate that circumferential resection margin involvement had a significant correlation with local recurrence of middle and low rectal carcinoma. Local recurrence was more frequently observed in rectal carcinomas with a positive circumferential resection margin (4 of 12 patients, 33.3%) than in those with a negative circumferential resection margin

(3 of 44 patients, 6.8%) ( $P = 0.014$ ), suggesting that circumferential resection margin status is an important predictor for the local recurrence of middle and low rectal carcinoma. It has been shown that residual mesorectal metastasis observed in rectal surgery may be the most important factor for local recurrence of rectal carcinoma<sup>[22,23]</sup>. In the current study, the local recurrence of rectal carcinoma was more frequent in patients with mesorectal metastasis than in those without mesorectal metastasis (16.7% vs 5.0%). However, there was no significant difference between the two groups ( $P = 0.206$ ). TME may be also a plausible explanation for the observation.

Sugihara *et al*<sup>[24]</sup> investigated the correlation between local recurrence and clinicopathologic characteristics of rectal carcinoma by multivariate analysis, and found that local recurrence of lower rectal cancer is significantly associated lymph node metastasis. It has been demonstrated that pathologic stages T and N are the significant predictors for the local recurrence of rectal carcinoma<sup>[25]</sup>. In the present study, local recurrence of poorly- and moderately- differentiated rectal carcinomas was found in 5 of 34 patients (35.7%) and in 2 of 37 patients (5.4%), respectively ( $P = 0.009$ ), while no local recurrence of well-differentiated rectal carcinoma was observed in any patients, suggesting that local recurrence of rectal carcinoma is significantly correlated with tumor differentiation. We also found that the local recurrence rate of rectal carcinoma was also correlated with the depth of tumor invasion. Local recurrence of  $T_3$  and  $T_2$  tumors was observed in 6 of 27 patients (22.2%) and in 1 of 23 patients (4.3%), respectively, while no local recurrence of  $T_1$  tumors was observed ( $P = 0.101$ ). Local recurrence of rectal carcinoma developed in 6 (20.7%) of the 29 patients with lymph node metastasis and in 1 (3.7%) of 27 patients without lymph node metastasis ( $P = 0.055$ ). These observations may be explained by the fact that the number of patients in our study was comparatively small. Further study with a larger sample size is needed.

Park *et al*<sup>[26]</sup> reported that change in perioperative serum CEA is a useful prognostic predictor for the occurrence of stage III rectal cancer and the survival of such patients. Oh *et al*<sup>[27]</sup> reported that vascular invasion is significantly associated with local recurrence of rectal cancer. Our results also demonstrate that local recurrence of rectal carcinoma had a significant correlation with high CEA level ( $P = 0.026$ ) and vessel cancerous emboli ( $P = 0.001$ ). We also found that family history and cancerous perforation were significantly correlated with local recurrence of rectal carcinoma ( $P < 0.05$ ).

In conclusion, extensive mesorectal excision and postoperative adjuvant chemotherapy should be used in the treatment of middle and lower rectal carcinoma.

## COMMENTS

### Background

It is well known that local recurrence is the most important prognostic factor for rectal carcinoma. However, local recurrence of rectal carcinoma occurs in about 4%-50% of patients even after radical resection of primary tumors and lymph nodes. The risk factors for local recurrence of rectal carcinoma remain unclear.

### Research frontiers

Since total mesorectal excision (TME) was adopted as the standard treatment of rectal carcinoma, a significant decrease in its local recurrence and a trend to improve the relative survival of rectal carcinoma patients have been reported. However, the local recurrence rate of rectal carcinoma is still high. The correlation between circumferential resection margin status and local recurrence of rectal carcinoma is still controversial.

### Innovations and breakthroughs

The aim of the current study was to explore the risk factors for local recurrence of middle and lower rectal carcinoma after curative resection. A large slice technique was used to detect its mesorectal metastasis and evaluate its circumferential resection margin status. The relationship between mesorectal

metastasis and circumferential resection margin status with local recurrence was identified. The clinicopathological characteristics of rectal carcinoma were described.

### Applications

Family history, high CEA level, cancerous perforation, tumor differentiation, vessel cancerous emboli and circumferential resection margin status are the significant risk factors of local recurrence of middle and lower rectal carcinoma after curative resection. Local recurrence of middle and lower rectal carcinoma may be more frequent observed in patients with mesorectal metastasis than in those without mesorectal metastasis.

### Terminology

Local recurrence of middle and lower rectal carcinoma was defined as any recurrence diagnosed or suspected in the pelvis (tumor bed, pelvic nodes, anastomosis, drain site, or perineum).

### Peer review

The authors explored the risk factors for local recurrence of middle and lower rectal carcinoma after curative resection, demonstrating that family history, high CEA level, cancerous perforation, tumor differentiation, vessel cancerous emboli and circumferential resection margin status are the significant risk factors for the local recurrence of middle and lower rectal carcinoma. The study was well designed. The data provided in the paper are interesting and valuable.

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RAPID COMMUNICATION

## Gastric cancer cell lines induced by trichostatin A

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**CONCLUSION:** TSA can induce cell apoptosis in BGC-823 and SGC-7901 cell lines. The expression of acetylated histone H3 might be correlated with apoptosis.

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**Key words:** BGC-823; SGC-7901; Trichostatin A; Apoptosis; Acetylated histone H3; Gastric cancer

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## Abstract

**AIM:** To explore the effect of trichostatin A (TSA) on apoptosis and acetylated histone H3 levels in gastric cancer cell lines BGC-823 and SGC-7901.

**METHODS:** The effect of TSA on growth inhibition and apoptosis was examined by MTT, fluorescence microscopy and PI single-labeled flow cytometry. The acetylated histone H3 level was detected by Western blot.

**RESULTS:** TSA induced apoptosis in gastric cancer cell lines BGC-823 and SGC-7901 was in a dose and time-dependent manner. Apoptotic cells varied significantly between TSA treated groups (37.5 ng/mL 72 h for BGC-823 cell line and 75 ng/mL 72 h for SGC-7901 cell line) and control group ( $0.85 \pm 0.14$  vs  $1.14 \pm 0.07$ ,  $P = 0.02$ ;  $0.94 \pm 0.07$  vs  $1.15 \pm 0.06$ ,  $P = 0.02$ ). Morphologic changes of apoptosis, including nuclear chromatin condensation and fluorescence strength, were observed under fluorescence microscopy. TSA treatment in BGC-823 and SGC-7901 cell lines obviously induced cell apoptosis, which was demonstrated by the increased percentage of sub-G1 phase cells, the reduction of G1-phase cells and the increase of apoptosis rates in flow cytometric analysis. The result of Western blot showed that the expression of acetylated histone H3 increased in BGC-823 and SGC-7901 TSA treatment groups as compared with the control group.

## INTRODUCTION

Gastric cancer is the second most common cancer worldwide<sup>[1]</sup>. It is often not detected until an advanced stage; consequently, the 5-year survival rates are low (10%-20%). Owing to local invasion and metastasis, radiation therapy or chemotherapy does not significantly increase the length or improve the quality of life of patients with advanced gastric cancer. Therefore, there is growing interest in the development of novel neoadjuvant and adjuvant treatment modalities.

It is widely accepted that histone acetylation is essential to establish a transcriptional competent state of chromatin<sup>[2-4]</sup>. The reversible (de)acetylation of the N-terminal histone tails by specific histone acetylases and deacetylases (HDAC) is involved in the regulation of gene expression. Dysfunction of histone acetylases and HDACs are associated with different types of cancer<sup>[5-8]</sup>. Various HDAC inhibitors (HDACIs) have been described to induce cell cycle arrest, differentiation, and apoptosis in cell lines<sup>[9-12]</sup>. Many of these have potent antitumor activities *in vivo*<sup>[13]</sup>. One of the most effective and best studied HDAC inhibitors is trichostatin A (TSA). The crystallographic analysis of TSA and a histone deacetylase homologue indicates that TSA interacts reversibly with the HDAC catalytic site preventing binding of the substrate<sup>[13]</sup>. Considering that HDAC inhibitors are able to induce apoptosis in different cell types<sup>[14-16]</sup>, we intend to know their potential

to induce apoptosis in gastric cancer cell lines BGC-823 and SGC-7901. We also studied the effect of TSA-induced acetylated histone H3 level on these cell lines.

## MATERIALS AND METHODS

### Reagents and antibodies

Stock solutions of TSA (Sigma-Aldrich, USA) in ethanol were stored at -20°C. Dimethylthiazole diphenyl tetrazolium bromide (MTT), propidium iodide (PI, Beijing Zhongshan Golden Bridge Biotechnology, China) and Hoechst 33342 (Sigma-Aldrich) were used. Antibody against GAPDH was purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, USA). Anti-acetyl-histone H3 (rabbit polyclonal IgG) and Goat Anti-Rabbit IgG, HRP-conjugate were purchased from Upstate Biotechnology (Upstate Biotechnology, USA).

### Cell culture and treatment

Human gastric epithelial cell lines BGC-823 and SGC-7901 were obtained from Institute of Tumor Research of Heilongjiang. BGC-823 cells and SGC-7901 cells were cultured and maintained in RPMI 1640, supplemented with fetal bovine serum 10% (v/v), penicillin 100 IU/mL, and streptomycin 100 µg/mL in a humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C.

### MTT assay

MTT assay was used to obtain the number of living cells in the sample. Cells were seeded on 96-well plates at a predetermined optimal cell density to ensure exponential growth in the duration of the assay. After a 24 h preincubation, growth medium was replaced with experimental medium containing the appropriate drug or control. Six duplicate wells were set up for each sample, and cells untreated with drug served as control. Treatment was conducted at 12, 24, 48 and 72 h with final TSA concentrations of 37.5, 75, 150, 300 and 600 ng/mL, respectively. After incubation, 10 µL MTT (6 g/L, Sigma) was added to each well and the incubation was continued for 4 h at 37°C. After removal of the medium, MTT stabilization solution (dimethylsulphoxide: ethanol = 1:1) was added to each well, and shaken for 10 min until all crystals were dissolved. Then, optical density was detected in a microplate reader at 550 nm wavelength using an ELISA reader. The negative control well without cells was used as zero point of absorbance. Each assay was performed in triplicate.

### Detection of chromatin condensation

Chromatin condensation was detected by nuclear staining with Hoechst 33342. BGC-823 and SGC-7901 cells were collected by centrifugation (500 × g for 5 min at 4°C) and washed twice with PBS. Cells were fixed in 10% formaldehyde and stored at 4°C. For analysis, cells were washed in PBS, then Hoechst 33342 (5 mg/L) was directly added to the medium by gently shaking at 4°C for 5 min. Stained nuclei were visualized under a Zeiss Axiophot fluorescence microscope at

400 × magnification with an excitation wavelength of 355-366 nm and an emission wavelength of 465-480 nm. Four independent replicates were used. In this way, apoptotic BGC-823 and SGC-7901 cells were stained brightly blue because of their chromatin condensation, while normal BGC-823 and SGC-7901 cells were evenly stained slightly blue.

### Cell cycle and apoptosis assays

BGC-823 and SGC-7901 cells were treated as indicated. Floating and adherent cells were collected by centrifugation (500 × g for 5 min at 4°C) and washed twice with PBS. Cells were fixed in 90% ethanol and stored at -20°C. For analysis, cells were washed in PBS and stained by suspension in PI (50 mg/L) containing RNase A (2 mg/L) for 30 min at 4°C. Stained cells were analyzed on a FACSscan (Becton-Dickinson, Heidelberg, Germany).

### Western blotting

Cells treated as indicated were harvested in 5 mL of medium, pelleted by centrifugation (1000 × g for 5 min at 4°C), then washed twice with ice-cold PBS and lysed in ice-cold HEPES buffer [HEPES (pH 7.5) 50 mmol/L, NaCl 10 mmol/L, MgCl<sub>2</sub> 5 mmol/L, EDTA 1 mmol/L, glycerol 110% (v/v), Triton X-100 1% (v/v), a cocktail of protease inhibitors, and 1 mg/L TSA on ice for 30 min. The lysates were clarified by centrifugation (15000 × g for 10 min at 4°C) and the supernatants then either analyzed immediately or stored at -80°C. Equivalent amounts of protein (50 µg) from total cell lysates were resolved by SDS-PAGE using precast 12% Bis-Tris gradient gels and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked overnight at 4°C in blocking buffer [nonfat dried milk 5% (v/v), NaCl 150 mmol/L, Tris (pH 8.0) 10 mmol/L and 0.05% Tween 20 (v/v)]. Proteins were detected by incubation with primary antibodies at appropriate dilutions in blocking buffer overnight at 4°C. Unbound antibody was removed by washing with Tris-buffered saline (pH 7.2) containing 0.5% Tween 20 (TBS-T). The membrane was then incubated at room temperature with horseradish peroxidase-conjugated secondary antibody. After extensive washing with TBS-T, bands were visualized by enhanced chemiluminescence followed by exposure to autoradiography.

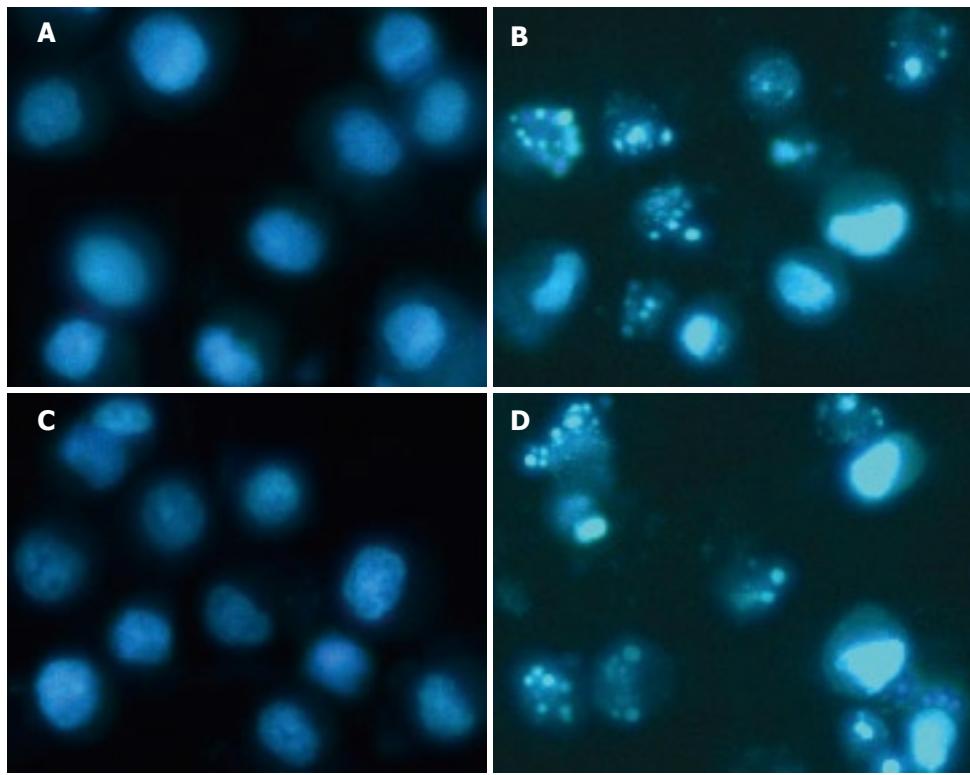
## RESULTS

### TSA inhibited the proliferation of BGC-823 and SGC-7901 cells

TSA inhibited cellular proliferation and survival in BGC-823 and SGC-7901 cell lines. It resulted in a significant decrease in the cell population of BGC-823 and SGC-7901 compared with control, following treatment with TSA. Inhibition of TSA was dependent on the dose and incubation time (Tables 1 and 2).

### TSA induced apoptosis of BGC-823 and SGC-7901 cells

To investigate the effects of TSA induced cytotoxicity,



**Figure 1** TSA (37.5 ng/mL per 72 h in BGC-823 cells and 75 ng/mL per 72 h in SGC-7901 cells) induced apoptosis (Hoechst 33342 staining,  $\times 400$ ). **A:** No typical apoptotic BGC-823 cells were observed in control group; **B:** BGC-823 cells treated with TSA (37.5 ng/mL per 72 h) showed the typical apoptotic nuclear morphology; **C:** No typical apoptotic SGC-7901 cells were observed in control group; **D:** SGC-7901 cells treated with TSA (75 ng/mL for 72 h) showed the typical apoptotic nuclear morphology.

**Table 1 Cell proliferation of BGC-823 and SGC-7901 cells incubated with various concentrations of TSA for 72 h (mean  $\pm$  SD)**

TSA	1	2	3	4	5	Control
BGC-823	$0.81 \pm 0.09^1$	$0.75 \pm 0.14^1$	$0.71 \pm 0.10^1$	$0.14 \pm 0.02^2$	$0.14 \pm 0.03^2$	$1.12 \pm 0.06$
SGC-7901	$1.11 \pm 0.04$	$0.87 \pm 0.04^1$	$0.58 \pm 0.09^1$	$0.23 \pm 0.06^2$	$0.21 \pm 0.04^2$	$1.16 \pm 0.07$

<sup>1</sup>P = 0.02 vs control; <sup>2</sup>P = 0.005 vs control. 1: 37.5 ng/mL; 2: 75 ng/mL; 3: 150 ng/mL; 4: 300 ng/mL; 5: 600 ng/mL.

**Table 2 Cell proliferation of BGC-823 (37.5 ng/mL) and SGC-7901 cells (75 ng/mL) for 12, 24, 48 and 72 h (mean  $\pm$  SD)**

TSA	12	24	48	72	Control
BGC-823	$1.13 \pm 0.12$	$1.13 \pm 0.04$	$1.11 \pm 0.02$	$0.85 \pm 0.14^1$	$1.14 \pm 0.07$
SGC-7901	$1.14 \pm 0.21$	$1.07 \pm 0.07$	$1.01 \pm 0.17$	$0.94 \pm 0.07^1$	$1.15 \pm 0.06$

<sup>1</sup>P = 0.02 vs control.

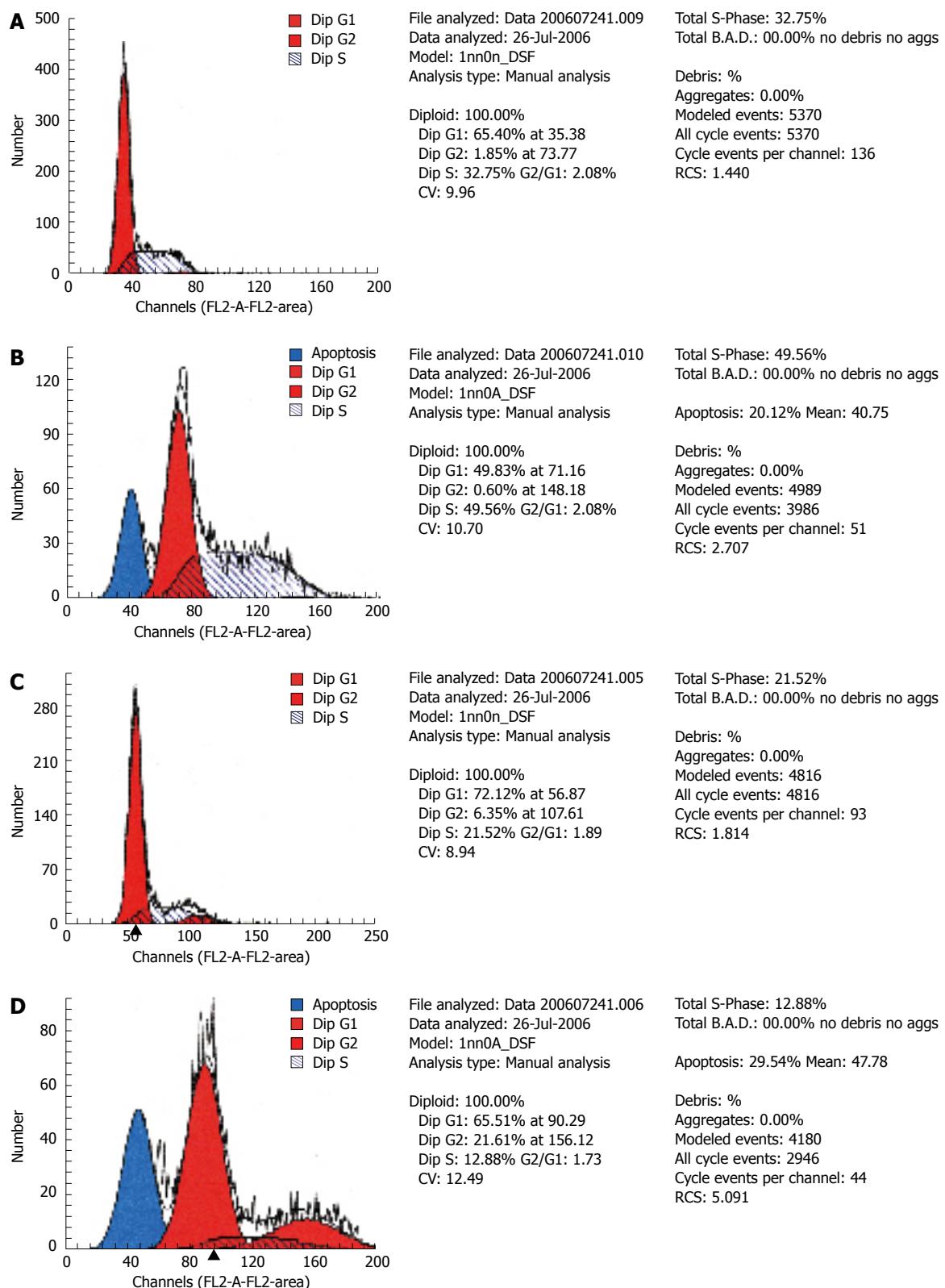
morphologic changes of apoptosis were observed under fluorescence microscope. At 72 h, cells treated with or without TSA (37.5 ng/mL in BGC-823 and 75 ng/mL in SGC-7901) were stained by Hoechst 33342, a classical way of identifying apoptotic cells, to observe nuclei morphology. The result indicated that nuclei of most BGC-823 and SGC-7901 cells treated with TSA were stained highly condensed, bright nucleus; while the cells in control group were stained average slightly blue (Figure 1).

TSA treatment (37.5 ng/mL per 72 h) sensitively induced apoptosis of BGC-823 cells, which was

demonstrated by the raised percentage of sub-G1 phase cells, the increase of apoptosis rates (20.12%) in flow cytometry (Figure 2). Cell cycle effects were examined by FACS analysis. The reduction of G1-phase cells (65.40%-49.83%) and the increment of S-phase cells (32.75%-49.56%) were observed in BGC-823 cells (Figure 2A, B). TSA induced apoptosis in SGC-7901 cells. Upon treatment with TSA (75 ng/mL) for 72 h in SGC-7901 cells, the reduction of G1-phase cells (72.12%-65.51%), S-phase cells (21.52%-12.88%) and the increment of G2-phase cells (6.35%-21.61%), and apoptosis rates (29.54%) were observed by FACS analysis (Figure 2C, D).

#### **TSA regulated the level of acetylated histone H3 in BGC-823 and SGC-7901 cell lines**

Western blot analysis was used to detect the level of acetylated histone H3 of BGC-823 cells and SGC-7901 cells treated with TSA (37.5 ng/mL per 48 h in BGC-823 cells and 75 ng/mL per 48 h in SGC-7901 cells). It was



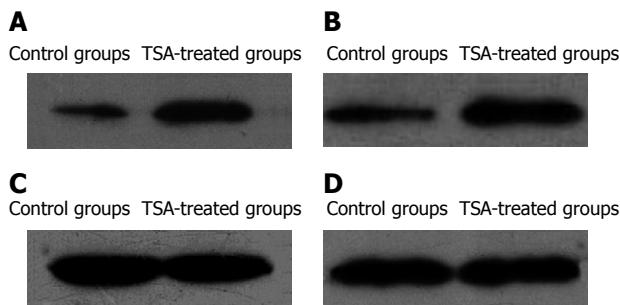
**Figure 2** Effect of TSA on the cell cycle and apoptosis as analyzed by FACS. **A:** Control of BGC-823 cells; **B:** Treatment with 37.5 ng/mL TSA for 72 h in BGC-823 cells; **C:** Control of SGC-7901 cells; **D:** Treatment with 75 ng/mL TSA for 72 h in SGC-7901 cells.

shown that there was an increase of acetylated histone H3 level in TSA treated cells (Figure 3).

## DISCUSSION

Histones are small-sized and basic-charged proteins

essential for chromatin folding. Posttranslational modifications such as acetylation, methylation, and phosphorylation have been suggested to be involved in the regulation of gene expression, cell division, nucleosome assembly, and DNA repair processes *via* alterations in the nucleosome architecture<sup>[17]</sup>. To



**Figure 3** Cell lysates were analyzed by Western blots using anti-GAPDH and anti-acetyl-histone H3 antibody. **A:** BGC-823 cells acetylated histone H3 (17 kDa); **B:** SGC-7901 cell acetylated histone H3 (17 kDa); **C:** BGC-823 cells GAPDH (loading control, 37 kDa); **D:** SGC-7901 cells GAPDH (loading control, 37 kDa).

date, acetylation is the best understood among these modifications. Chromatin structure and, thereby, transcription is controlled by the level of acetylation of histones, which is determined by the balance between histone acetyl transferase (HAT) activity and histone deacetylase (HDAC) activity. While proteins with HAT activity have been demonstrated to function as transcriptional coactivators, proteins with HDAC activity induce transcriptional repression<sup>[18]</sup>. Therefore, acetylation of histones seems to be predominantly implicated in the regulation of gene transcription due to nucleosome remodeling. The aberrant utilization of HDACs is believed to be a contributing factor in carcinogenesis.

HDAC inhibitors are members of a new class of agents able to regulate gene expression by modulating chromatin structure. HDAC inhibitors have been known to induce differentiation, growth arrest, and apoptosis in cancer cells<sup>[15,19,20]</sup>. TSA, an antifungal antibiotic with cytostatic and differentiating properties in mammalian cell culture, is a potent and specific inhibitor of HDAC activity. X-ray crystallographic studies showed that TSA has a tubular structure with a zinc atom at its base and TSA fits into this structure with the hydroxamic moiety of the inhibitor binding to the zinc. Numerous studies have reported that TSA can induce apoptosis in cancer cells<sup>[21-23]</sup>.

Moderately and poorly differentiated gastric adenocarcinomas are the main types of gastric cancer in China. The rate of each was 71.0% and 58.7% in early gastric cancer and advanced gastric cancer, respectively. The cell lines BGC-823 and SGC-7901 were derived from human gastric adenocarcinoma. The stem cells in tumor tissue have infinite proliferating ability, and can form colonies *in vitro*. The BGC-823 cell line is a poorly-differentiated human gastric adenocarcinoma cell line. SGC-7901 cell was a moderately-differentiated human stomach adenocarcinoma cell line with mutant p53. Here, we examined the effect of TSA on BGC-823 and SGC-7901 cells.

We found that low concentrations of TSA can significantly reduce the growth of BGC-823 and SGC-7901 cells, and the effects of TSA on BGC-823 and SGC-7901 cells are dose and time-dependent. The

mechanism leading to these effects remained unknown. Accumulating evidence suggests that inhibition of HDAC activity leads to relaxation of the structure of chromatin associated with a specific set of programmed genes. The relaxed chromatin structure allows these genes to be expressed, which, in turn, arrests tumor cell growth<sup>[13,24]</sup>. This suggests that induction of histone hyperacetylation by HDAC inhibitors is responsible for the antiproliferative activity through selective induction of genes that play important roles in the cell cycle and cell morphology<sup>[25]</sup>. It has been shown that reversible acetylation of lysine on histone H3 plays an important role in regulating gene transcription<sup>[26]</sup>. Our findings indicate that acetylated histone H3 expression levels increased in BGC-823 and SGC-7901 cells following TSA treatment. Increased acetylated histone H3 expression levels in BGC-823 and SGC-7901 cells may be an important event in mediating the apoptosis of BGC-823 and SGC-7901 cells induced by TSA. These findings generate the necessity to investigate the mechanism of TSA in the treatment of BGC-823 and SGC-7901 cells. Therefore, we suppose that HDAC inhibitors cause acetylated histones to accumulate in tumor tissues, and this accumulation can be used as a trigger of the biologic activity of the HDAC inhibitors.

In summary, in this study we showed that TSA can induce apoptosis in gastric cancer cell lines BGC-823 and SGC-7901. We demonstrated that the expression of acetylated histone H3 might be correlated with apoptosis. Further work will be necessary to explore additional mechanisms that lead to induction of apoptosis.

## COMMENTS

### Background

Histones are small-sized and basic-charged proteins essential for chromatin folding. The acetylation state of histones is reversibly regulated by histone acetyltransferase (HAT) and deacetylase (HDAC). An imbalance of this reaction leads to an aberrant behavior of the cells in morphology, cell cycle, differentiation, and carcinogenesis. HDAC is especially known to play an important role in carcinogenesis.

### Research frontiers

There is increasing evidence that HDAC inhibitors are effective therapeutic agents in the treatment of a variety of cancers refractory to conventional anticancer agents. Several structurally diverse HDAC inhibitors, such as trichostatin A (TSA), amicropic acid, or butyrate, have been identified and their *in vitro* activity in transformed cells makes them promising agents for cancer therapy. Although extensive studies have been done, roles of HDAC inhibitor in gastric cancer are still unclear.

### Innovations and breakthroughs

This study investigates the effects of the HDAC inhibitor TSA on gastric cancer cell lines BGC-823 and SGC-7901. The results demonstrate that low concentrations of TSA can significantly reduce the growth of BGC-823 and SGC-7901 cells and the effects of TSA on BGC-823 and SGC-7901 cells are dose and time-dependent. These findings indicate that acetylated histone H3 expression levels increased in BGC-823 and SGC-7901 cells following TSA treatment.

### Applications

It can be seen from this paper that the HDAC inhibitor, TSA, can induce apoptosis of BGC-823 and SGC-7901 cells. It suggests that HDAC is a promising target for the development of anticancer drugs for gastric cancer.

### Peer review

This paper examined the role of TSA in gastric cancer cell lines BGC-823

and SGC-7901. The results show that TSA can induce apoptosis of BGC-823 and SGC-7901 cells. And the expression of acetylated histone H3 might be correlated with apoptosis. It suggests that TSA has a potential against gastric cancer.

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RAPID COMMUNICATION

## Diversity of *Helicobacter pylori* isolates in expression of antigens and induction of antibodies

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of IgG antibodies against UreB, HpaA, VacA, CagA, NapA, FlaA and FlaB were 100%, 87.4%, 43%, 71.5%, 89.4%, 84.8% and 79.5%, respectively. Furthermore, the expression frequencies of VacA and NapA were found to be relative to the severity of gastric diseases ( $P = 0.016$  and  $P < 0.0001$ , respectively).

**CONCLUSION:** UreB antigen is the top option of developing genetically engineered vaccine against *H pylori* followed by NapA or HpaA.

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**Key words:** *Helicobacter pylori*; Major protein antigens; Expression frequency; Antibody levels

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### Abstract

**AIM:** To obtain evidence for selection of antigens used in genetically engineered vaccine against *Helicobacter pylori* (*H pylori*).

**METHODS:** Enzyme linked immunoabsorbent assay (ELISA) was established on the basis of recombinant protein antigens rUreB, rHpaA, rVacA, rCagA1, rNapA, rFlaA and rFlaB of *H pylori* to detect expression rates of the antigens in bacterial isolates as well as positive rates of the antibodies in sera from *H pylori*-infected patients. PCR was applied to the detection of carrying rates of the genes encoding antigens in the isolates.

**RESULTS:** The outputs of rUreB, rHpaA, rVacA, rCagA1, rNapA, rFlaA and rFlaB were approximately 35%, 32%, 15%, 23%, 56%, 25% and 20% of the total bacterial proteins, respectively. One hundred and fifty-one strains of *H pylori* were isolated from 347 biopsy specimens of chronic gastritis, peptic ulcer or gastric adenocarcinoma, with a positive rate of 43.5%. All of the isolates expressed UreB, HpaA, FlaA and FlaB while 52.3%, 92.1% and 93.4% of the isolates expressed VacA, CagA and NapA, respectively. In the sera of 151 *H pylori*-infected patients, the positive rates

### INTRODUCTION

In 1983, for the first time, Barry J. Marshall and J. Robin Warren successfully isolated *Helicobacter pylori* (*H pylori*) from the gastric mucosa of patients with chronic active gastritis or peptic ulcer<sup>[1,2]</sup>, for which they were awarded the Nobel Prize in Physiology or Medicine in 2005. Thereafter, lots of findings have confirmed that *H pylori* is strongly associated with chronic gastritis, peptic ulcer<sup>[3-5]</sup>, gastric adenocarcinoma, mucosa-associated lymphoid tissue (MALT) lymphoma and primary gastric non-Hodgkin's lymphoma<sup>[6,7]</sup>. Vaccination is by far the most effective and economical method in infection prevention, though the vaccine against *H pylori* is scarcely touched up to date, which is probably due to the culture of *H pylori* characterized by enriched media, slow growth and incubation in a microaerophilic environment, plus difficulty in storage, etc. Thus, it may be a cost-effective strategy in developing a genetically engineered vaccine against *H pylori*.

The selection for antigenic targets is critical in the design of a genetically engineered vaccine against *H pylori*. The best ascertained antigens out of the outer membrane protein antigens are UreB and HpaA followed by VacA, CagA, NapA, FlaA and FlaB<sup>[8-13]</sup>. Tomb reported all the genomic sequences of *H pylori* in 1997<sup>[14]</sup>, and in our previous study, these genomic prokaryotic expression systems were constructed.

The expression rates of outer membrane protein antigens are known to be diverse in the same bacterial isolates from various area, and so are the levels of immune response to different protein antigens, all of which will ultimately affect the selection of vaccine antigens. In order to develop effective genetically engineered vaccines, the encoding gene of the antigen should be widely located at the genome of clinical strains. The gene sequence should be conservative, the natural expression rate should also be high, and the immunogenicity of expression products should be potent. Our research focused on the carrying rates and expression level of 7 genes of *H pylori* from the clinical *H pylori* in China, including rUreB, rHpaA, rVacA, rCagA1, rNapA, rFlaA and rFlaB. Meanwhile, the levels of specific antibodies against the 7 antigens in serum were measured. This research will further our understanding of the diversity of carrying rates of the encoding genes, the expression levels and immunogenicity of the antigens, and provide evidence for the selection of antigens employed in genetically engineered vaccines against *H pylori*.

## MATERIALS AND METHODS

### Materials

Three hundred and forty-seven biopsy specimens and serum samples were collected gastroscopically from patients with chronic gastritis, peptic ulcer or gastric adenocarcinoma in hospitals between January, 2005 and December, 2006, and 35 serum samples were collected from healthy children. All the patients were not treated with antibiotics, anti-inflammatory agents or antacids within a month prior to the sample collections. *H pylori* NCTC11637 strain, 10 clinical strains of *Bacillus coli* and 10 *Staphylococcus aureus* strains were offered by Department of Pathogenic Biology, Zhejiang University School of Medicine. Urease test kit was from Sanqiang BioChem Co., Ltd., Fujian Province, China. Oxidase test kit was from Hangzhou Tianhe Microorganism Reagent Co., Ltd., Zhejiang Province, China. Gel image analysis system was from Bio-Rad, and Ni-NTA affinity column was from BioColor. Rabbit anti-*H pylori* antibody was from DAKO. HRP-labeling goat anti-rabbit IgG and HRP-labeling goat anti-human IgG were from Jackson ImmunoResearch. The primer and PCR SuperMix High Fidelity were from TaKaRa Biotechnology, Dalian, China.

### Isolation and identification of bacteria

Each biopsied specimen from the patients was divided

into two parts. One was detected by urease test to check whether the urease test is positive. If it was positive, the other was then used to isolate *H pylori* as previously described<sup>[15]</sup>. Biopsy specimens were homogenized with a tissue grinder and inoculated onto Columbia agar plates supplemented with 8% defibrinated sheep blood, 5 mg/L TMP, 10 mg/L vancomycin, 2500 U/L bacilliosporin B and 2.5 mg/L amphotericin B. The plates were incubated at 37°C under microaerophilic conditions (5% O<sub>2</sub>, 10% CO<sub>2</sub>, 85% N<sub>2</sub>) for 3-5 d. Isolates were identified as *H pylori* with Gram-staining. The positive strains were stored in Brucella broth at -70°C.

### Expression, purification and identification of recombinant protein antigens

The gene clones of ureB, hpaA, vacA, cagA, napA, flaA and flaB were from *H pylori* NCTC11637 strain. The plasmids pET32a or pET42a and an *Escherichia coli* (*E.coli*) BL21DE3 (presented by the Department of Pathogenic Biology, Zhejiang University School of Medicine) were used as the expression system and host cells, respectively. The expression system of recombinant antigens was inoculated in LB medium with 0.5 mmol/L IPTG and shaken at 250 r/min and cultured at 30°C for 4-5 h. The expression of rUreB (569 aa, about 63 kDa), rHpaA (260 aa, about 29 kDa), rVacA (747 aa, about 82 kDa), rCagA1 (716 aa, about 79 kDa), rNapA (144 aa, about 16 kDa), rFlaA (510 aa, about 56 kDa) and rFlaB (514 aa, about 57 kDa) were detected with SDS-PAGE and gel image analysis system. These recombinant protein antigens were purified by Ni-NTA affinity column. The purity quotient of recombinant protein antigens was detected with SDS-PAGE. The recombinant antigens were identified by Western blot. Rabbit anti-*H pylori* were used as the first antibody and HRP-labeling goat anti-rabbit IgG as the second antibody. The density of extracted proteins was determined by ultraviolet spectrophotometry<sup>[16]</sup>.

### Preparation of recombinant protein antiserum

Each of these recombinant proteins was respectively mixed with Freund's complete adjuvant, and thereafter administered into the New Zealand rabbits for immunization with multiple dorsal ID, four times, once every two weeks. Rabbit serum was collected by cardiac puncture two weeks after the final immunization. The titer of antiserum was determined by double immunodiffusion.

### Detection of specific serum antibodies

The purified rUreB, rHpaA, rVacA, rCagA1, rNapA, rFlaA and rFlaB were diluted to 20 µg/mL with carbonic acid buffer (pH 9.6), coated in a 96-well plate with 0.1 mL of the dilution per well overnight. Based on the result of the preliminary experiment, serum samples from patients with chronic gastritis, peptic ulcer or gastric adenocarcinoma, and those from healthy people (1:200 dilution in both) were used as the first antibody, and HRP-labeling goat anti-human IgG (1:4000 dilution)

**Table 1 Major information relative to PCR for amplifying 7 genes of *H pylori***

Target genes	Primer sequences (5'-3')	Reference	Tm (°C)	Products (bp)
<i>ureB</i>	F: CGTCGGCAATAGCTGCCATAGT R: GTAGGTCTGCTACTGAAGCCTTA	[18]	55	463
<i>hpaA</i>	F: CTTTAGGTGCGAGCGTGGTG R: AATTCTTGGCGTCTTTGATAA	[19]	54	716
<i>vacA</i>	F: ATGAAATACAACAAACACACCG R: CAACCTCCATCAATCTACTGGA	[20]	54	337
<i>CagA</i>	F: ATAAGCTAAATTAGACAACCTTGAGCGA R: TTAGAATAATCAACAAAC ATCACGCCAT	[21]	56	297
<i>napA</i>	F: TCGGATCGTGTGTTATG R: GATCGTCCGCTAAAGTTAC	[22]	50	344
<i>flaA</i>	F: GCTTTTCAGGTCAATAC R: GTAGCGATACGAACCTGA	[13]	52	524
<i>flaB</i>	F: GATAATACCAATATCGC R: CTCATCCATCGCTTATC	[13]	52	241

as the second antibody. The ELISA result of the serum sample from a patient was considered positive if the optical density at 490 nm ( $A_{490}$ ) was over the mean  $\pm$  3SD of negative serum sample<sup>[17]</sup>.

#### Expression levels of target proteins on clinical strains

*H pylori* NCTC11637 strain, clinical strains of *H pylori*, 10 *Bacillus coli* strains and 10 *Staphylococcus aureus* strains were ultrasonically broken up and centrifuged at 1000 r/min to obtain supernatant, which was later determined by ultraviolet spectrophotometry<sup>[16]</sup>. The proteins of *H pylori*, *Bacillus coli* and *Staphylococcus aureus* were diluted to 50 µg/mL with carbonic acid buffer (pH 9.6), coated in a 96-well plate with 0.1 mL of the dilution per well overnight. Based on the result of the preliminary experiment, the self-prepared rabbit antiserum against the recombinant protein (1:200 or 1:400 dilution) was used as the first antibody, and HRP-labeling goat antiserum against rabbit IgG (1:3000 dilution) as the second antibody, respectively. The ELISA result of an ultrasonic supernatant sample of *H pylori* was considered positive if the  $A_{490}$  value was over the mean  $\pm$  SD of 20 ultrasonic supernatant samples at the same protein concentration of *Bacillus coli* and *Staphylococcus aureus*<sup>[17]</sup>.

#### Detection of target genes

The genes of clinical *H pylori* *ureB*, *hpaA*, *vacA*, *cagA*, *napA*, *flaA* and *flaB* were detected by PCR. Each reaction volume was 50 µL with an amplification of 35 cycles. Both the upstream and downstream primer concentrations were 20 pmol. Primer sequences, main reaction values and the product are shown in Table 1<sup>[13,18-22]</sup>. The PCR products were identified with 2% agarose gel staining using the fluorescent dye ethidium bromide.

#### Comparison of positive target gene rates and their products with serum antibodies

The positive rates of target genes and their products of clinical *H pylori*, as well as specific antibodies against target antigens in patients' sera were compared, which

would benefit the comprehension of the diversity of positive rates of target genes, the expression levels and immunogenicity of their antigens.

#### Comparison of positive *H pylori* infection rates with expression rates of virulent factors in different gastric diseases

*HpaA*, *FlaA* and *FlaB* are structural proteins essential to the outer membrane of *H pylori*, while *UreB*, *VacA*, *CagA* and *NapA* are virulent factors of functional proteins. Accordingly, the positive *H pylori* infection rates were compared with the expression rates of virulent factors and the severity of gastric diseases.

#### Statistical analysis

Data were analyzed with SPSS10.0 statistical software. The positive ratio was performed by chi square test.  $P < 0.05$  was considered statistically significant.

## RESULTS

#### Positive *H pylori* infection rates

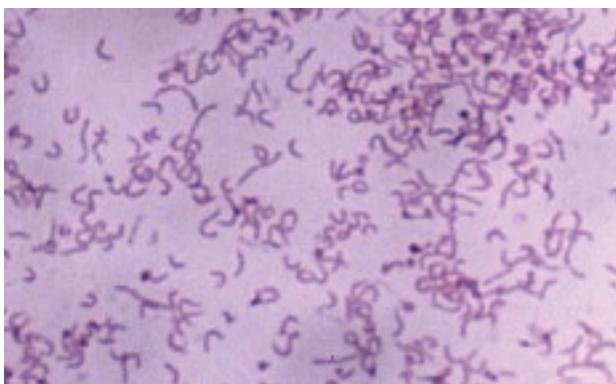
One hundred and fifty-one strains of *H pylori* were successfully isolated from 347 biopsy specimens of chronic gastritis, peptic ulcer or gastric adenocarcinoma with a positive rate of 43.5% (Figure 1). Of the 151 *H pylori*-infected patients, 102 were diagnosed to have chronic gastritis (38 with superficial gastritis, 52 with active gastritis and 12 with atrophic gastritis), and 43 patients were diagnosed to have peptic ulceration (16 with gastrectosis, 18 with duodenobulbar ulcer and 9 with complex ulcer), and 6 patients with gastric adenocarcinoma.

#### Expression of target recombinant proteins and detection of purified recombinant proteins and their antigens

The expression rate of *rUreB*, *rHpaA*, *rVacA*, *rCagA1*, *rNapA*, *rFlaA* and *rFlaB* was approximately 35%, 32%, 15%, 23%, 56%, 25% and 20%, respectively. The purified recombinant proteins were shown as a single band detected by SDS-PAGE (Figure 2). The recombinant protein antigens could be recognized specifically by the rabbit antiserum against the whole cells of *H pylori*, and the results were shown to be positive by Western Blot analysis (Figure 3).

**Table 2** Comparison between positive rates of 7 genes and their products of *H pylori* and serum antibodies

Target gene (positive/negative)	Positive rate (%)	Target antigen (positive/negative)	Positive rate (%)	Target antibody (positive/negative)	Positive rate (%)
<i>ureB</i> (151/0)	100	UreB (151/0)	100	Anti-UreB (151/0)	100
<i>hpaA</i> (151/0)	100	HpaA (151/0)	100	Anti-HpaA (132/19)	87.4
<i>vacA</i> (151/0)	100	VacA (79/72)	52.3	Anti-VacA (65/86)	43.0
<i>CagA</i> (146/5)	96.7	CagA (139/12)	92.1	Anti-CagA (108/43)	71.5
<i>napA</i> (148/3)	98.0	NapA (141/10)	93.4	Anti-NapA (135/16)	89.4
<i>flaA</i> (151/0)	100	FlaA (151/0)	100	Anti-FlaA (128/23)	84.8
<i>flaB</i> (151/0)	100	FlaB (151/0)	100	Anti-FlaB (120/31)	79.5

Figure 1 *H pylori* isolated from a patients' biopsy (Gram-staining,  $\times 1000$ ).

### Titer of rabbit antiserum against target recombinant proteins

All the rUreB, rHpaA, rVacA, rCagA1, rNapA, rFlaA and rFlaB could efficiently induce the rabbits to produce specific antibodies, and the titer of antisera was 1:8, 1:8, 1:4, 1:4, 1:8, 1:4 and 1:4, respectively.

### Positive rates of specific serum antibodies

The  $A_{490}$  (mean  $\pm$  SD) of the negative control sera from the 35 healthy children was  $0.23 \pm 0.05$ , and the  $A_{490}$  of the positive standard was 0.38. In the sera from 151 *H pylori*-infected patients, the positive rate of IgG antibodies against UreB, HpaA, VacA, CagA, NapA, FlaA and FlaB was 100% (151/151), 87.4% (132/151), 43% (65/151), 71.5% (108/151), 89.4% (135/151), 84.8% (128/151) and 79.5% (120/151), respectively. The total range of the  $A_{490}$  was 0.27-2.05.

### Positive rates of target protein antigens of clinical *H pylori* strains

The  $A_{490}$  (mean  $\pm$  SD) of the negative control proteins from 10 *Bacillus coli* strains and 10 *Staphylococcus aureus* strains was  $0.21 \pm 0.03$ , and the  $A_{490}$  of the positive standard was 0.30. UreB, HpaA, FlaA and FlaB were expressed in 100% of the isolates (151/151), while VacA, CagA and NapA were expressed in 52.3% (79/151), 92.1% (139/151) and 93.4% (141/151) of the isolates. The total range of the  $A_{490}$  was 0.33-1.97.

### Positive rates of target genes of clinical *H pylori* strains

In the 151 clinical *H pylori* strains, the PCR products showed that the positive rate of ureB, hpaA, vacA, flaA

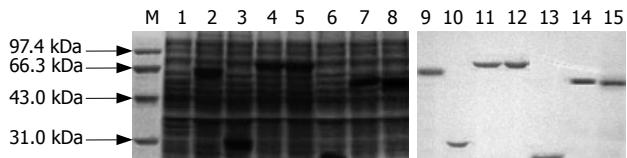


Figure 2 Expression and purification of target recombinant proteins. M: Marker (BioColor); Lane 1: Blank expression vector; Lanes 2-8 and 9-15: rUreB, rHpaA, rVacA, rCagA1, rNapA, rFlaA and rFlaB, respectively.

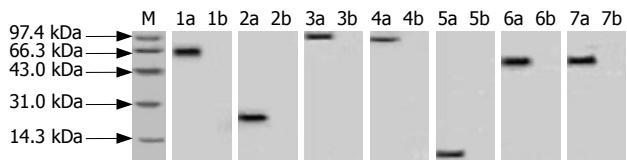


Figure 3 Detection results of target recombinant proteins by Western blot assay. M: Marker (BioColor); Lanes 1a-7a: rUreB, rHpaA, rVacA, rCagA1, rNapA, rFlaA, rFlaB, respectively; Lanes 1b-7b: blank controls.

and flaB genes was 100%, respectively, while the positive rate of CagA and napA genes was 96.7% (146/151) and 98% (148/151), respectively. The representative amplified product of each gene is shown in Figure 4.

### Positive rates of target genes, expression products and specific antibodies

In the 151 clinical strains of *H pylori*, the positive rates of ureB, hpaA, vacA, cagA, napA, flaA and flaB genes and their expression products as well as the specific corresponding antibodies are listed in Table 2. The positive rate of ureB gene and its expression product was completely coincident with that of the antibody. The positive rate of antibody against CagA was obviously lower than the positive rate and expression rate of cagA. The expression rate of vacA gene and the positive rate of corresponding antibody were obviously lower than the positive rate of vacA gene. There was a sequential decrease in the positive rate of the remaining genes, expression products and specific antibodies.

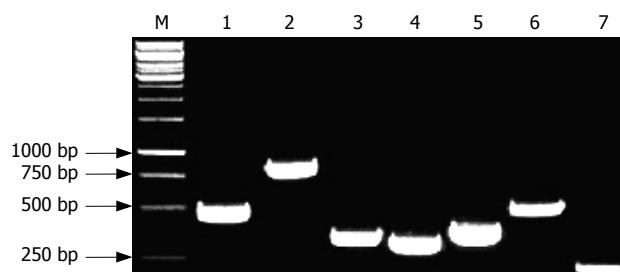
### Correlation between positive *H pylori* infection rates and expression rates of virulent factors in different gastric diseases

There was no statistically significant difference in the positive rates of *H pylori* isolated from the specimens of different gastric diseases. The expression rate of VacA

**Table 3** Correlation between positive *H pylori* infection rates and virulent factor expression in different gastric diseases

Disease	Patient	Positive number/rate of bacteria, n/%	Positive number/rate of virulence factor, n/%			
			UreB	VacA	CagA	NapA
Chronic gastritis	240	102/42.5	102/100	47/46.1 <sup>a</sup>	93/91.2	94/92.2
Surface gastritis	93	38/40.9	38/100	13/34.2	34/89.5	36/94.7 <sup>d</sup>
Active gastritis	120	52/43.3	52/100	30/59.6 <sup>c</sup>	48/92.3	50/96.2 <sup>d</sup>
Atrophic gastritis	27	12/44.4	12/100	4/33.3	11/91.7	8/66.7
Peptic ulcer	92	43/46.7	43/100	29/67.4	40/93.0	41/95.4
Stomach	35	16/45.7	16/100	11/68.9	15/93.8	15/93.8
Duodenum	39	18/46.2	18/100	11/61.1	16/88.9	17/94.4
Complexity	18	9/50.0	9/100	7/77.8	9/100	9/100
Gastric adenocarcinoma	15	6/40.0	6/100	3/50.0	6/100	6/100

<sup>a</sup>P < 0.05 vs peptic ulcer, <sup>c</sup>P < 0.05 vs active gastritis, <sup>d</sup>P < 0.01 vs atrophic gastritis.



**Figure 4** PCR products of 7 genes of *H pylori*. M: Marker (BioColor); Lanes 1-7: ureB, hpaA, vacA, CagA, napA, flaA and flaB genes of *H pylori* NCTC 11637, respectively.

gene in *H pylori* isolated from specimens of chronic gastritis was lower than that isolated from specimens of peptic ulcer ( $\chi^2 = 5.55$ ,  $P = 0.019$ ). The expression rate of VacA gene in superficial and atrophic gastritis was lower than that in active gastritis ( $\chi^2 = 5.76$ ,  $P = 0.016$ ). The expression rate of NapA gene in *H pylori* isolated from atrophic gastritis (66.7%) was lower than that in *H pylori* isolated from superficial and active gastritis (94.7%, 96.2%;  $\chi^2 = 6.81$ ,  $P = 0.009$ ;  $\chi^2 = 9.98$ ,  $P = 0.002$ ). There was no statistically significant difference in the expression rate of UreB and CagA genes and in their virulence factors in the specimens from different gastric diseases (Table 3).

## DISCUSSION

It was reported that there are many protective antigens against *H pylori*, such as UreB, HpaA, VacA, CagA, NapA, FlaA, FlaB and heat shock protein (HSP)<sup>[8-13,23]</sup>. Due to cross-antigens against HSP in different sibling bacteria<sup>[23]</sup>, 7 kinds of protective antigens of *H pylori* except for HSP were selected in our research. In order to develop effective genetically engineered vaccines, the encoding gene of the antigen should be widely located at the genome of clinical strains. The gene sequence should be conservative, the natural expression rate should also be high, and the immunogenicity of expression products should be potent. In addition, the high expression rate of recombinant proteins is also one of the most important problems to be solved in preparing a genetically engineered vaccine. Thus, it is essential

to understand the above-mentioned characteristics of protein antigens of *H pylori*.

The biological characteristics of bacteria and other microbes exhibit an obvious diversity in different regions, mainly manifested as different dominant serological types or genotypes according to their different antigenicity, toxicity and drug resistance. So far, the gene orders of ureB, hpaA, napA, flaA and flaB have been confirmed as highly conservative, while the gene order of vacA can mutate moderately between signal peptide zone (S) and intermedial zone (M), and the mutations of CagA gene order are mainly located at the 3' terminal repeat<sup>[8,9,12,13,15]</sup>. Our research focused on the carrying rates and expression level of the 7 kinds of genes of *H pylori* from the clinical *H pylori* in China. The PCR products showed the carrying rate of ureB, hpaA, vacA, flaA and flaB genes was 100%, respectively, while the carrying rate of cagA and napA genes was 96.7% and 98%, respectively. However, the expression rates of these genes were significantly different. As shown above, all the strains (100%) could express UreB, HpaA, FlaA and FlaB, and more than 90% strains (92.1% and 93.4%) could express CagA and NapA, but the expression rate of VacA was only 52.3%. While the unique epitoxin of *H pylori*, VacA, has always been the selective antigen against gene vaccine<sup>[11,24]</sup>, our study demonstrated that VacA was not the ideal antigen on the grounds that the carrying rate (100%) was not consistent with the expression rate (approximately 50%) of the gene. It was reported that the activity of vacuolus toxin is high if the vacA genotype is s1/m1, and moderate or low if the vacA genotype is s1/m2<sup>[25]</sup>. The expression rate of vacA gene was low in our study, which is relevant to the vacA genotype s1a/m2, because the vacA genotype of 55.1% *H pylori* strains is a low virulent s1a/m2 in China<sup>[15]</sup>. Almost 100% of the *H pylori* strains could carry napA gene, while the expression rate of NapA is only 60%<sup>[12]</sup>. In our research, the expression rate of napA gene was high (98%), which might be related to the protein expression of diverse epidemic strains in different regions.

In vaccination, antibodies mainly play their preventive function, while cell-mediated immunity generally eliminates the pathogenic microorganisms which invade

the human body. Because of their high level and long duration in all antibodies, IgG antibodies are regarded as the most important antibodies, thus IgG antibodies against UreB, HpaA, VacA, CagA, NapA, FlaA and FlaB in serum samples were detected in our study. The results show that the positive rate of IgG antibodies against UreB, NapA, HpaA and FlaA was 100%, 89.4%, 87.4% and 84.8%, respectively, while the positive rate of IgG antibodies against FlaB, CagA and VacA was 79.5%, 71.5% and 43%, respectively. The positive rate of IgG antibodies against HpaA, CagA, FlaA and FlaB was 71.5%-87.4%, while the carrying rate (96.7%-100%) and expression rate (92.1%-100%) of these genes were higher. This discrepancy might be related to the weak antigenicity and low natural expression of these proteins, presumably due to the insufficient exposure of these proteins to the surface of bacteria. For example, HpaA is usually called an adhesin of *H pylori*, and in fact, it is a tunica vaginalis protein expressed in the flagellae of *H pylori*<sup>[9]</sup>. FlaA and FlaB expressed in the flagella are subunits of flagellin<sup>[13]</sup>. As an intein of *H pylori*, CagA is a non-exocrine protein and only when *H pylori* has contact with host cells, does it act on the target cells through type III secretory system<sup>[10,11]</sup>. However, the high expression rate of rNapA, rUreB and rHpaA and the low expression rate of the other 4 kinds of proteins would consequently affect the selection of genetically engineered vaccines against *H pylori*.

Moreover, the relationship between the infection rate of *H pylori*, virulence factors and various gastropathies was also taken into account in our research. As mentioned above, VacA and NapA are the most important virulence factors. The former can induce vacuolar degeneration of target cells, while the latter can activate leukocytes to evoke inflammatory reactions. Urease (Ure) is the key colonization factor for *H pylori*, which is considered closely related to the virulence for its resistance to the acid environment. CagA is not directly cytotoxic, though it is the most important component of cag pathogenicity island (CPI), and thus also regarded as a reference index to bacterial virulence<sup>[26]</sup>. No statistically significant difference in the positive rate of *H pylori* isolated the specimens of different gastric diseases was found in our study. All of the strains could express UreB, and the expression rate of cagA gene was not statistically significant in the specimens of different gastric diseases. However, the expression rate of vacA gene in *H pylori* isolated from specimens of chronic gastritis was lower than that isolated from specimens of peptic ulcer ( $P = 0.019$ ), and its expression rate in superficial and atrophic gastritis was lower than that in active gastritis ( $P = 0.016$ ). The expression rate of NapA gene in *H pylori* isolated from specimens of atrophic gastritis was lower than that in *H pylori* isolated from specimens of superficial gastritis and active gastritis ( $P < 0.0001$ ). These results suggest that different gastritis might not be related to the infection rate of *H pylori* but related to the virulence of VacA and NapA.

In conclusion, according to the expression rate of the 7 kinds of genes, the positive rates of

serum antibodies, quantities of the recombinant proteins, UreB is the top option for antigens in developing genetically engineered vaccines against *H pylori* followed by NapA and HpaA.

## COMMENTS

### Background

Lots of findings have confirmed that *Helicobacter pylori* (*H pylori*) is strongly associated with chronic gastritis, peptic ulcer, gastric adenocarcinoma, mucosa-associated lymphoid tissue (MALT) lymphoma and primary gastric non-Hodgkin's lymphoma. Vaccination is by far the most effective and economical method in infection prevention, though the vaccine against *H pylori* is scarcely touched up to date. So, it is a very important task to search the vaccine against *H pylori* nowadays.

### Research frontiers

The vaccine against *H pylori* is scarcely touched nowadays, probably due to the culture of *H pylori* characterized by enriched media, slow growth and incubation in a microaerophilic environment, plus difficulty in storage, etc. Thus, it may be a cost-effective strategy in developing a genetically engineered vaccine against *H pylori*. The selection of antigenic targets is critical in the design of a genetically engineered vaccine against *H pylori*.

### Innovations and breakthroughs

In this study, the expression rate of antigens in bacterial isolates and the positive rate of antigenic antibodies in sera from *H pylori*-infected patients were detected by ELISA according to the recombinant protein antigens rUreB, rHpaA, rVacA, rCagA1, rNapA, rFlaA and rFlaB of *H pylori*. Meanwhile, PCR was performed to detect the carrying rate of genes encoding antigens in the isolates. Our research focused on the carrying rate and expression level of 7 kinds of genes of *H pylori* from the clinical *H pylori* in China.

### Applications

The experimental results of this study indicate that UreB is the first choice of the 7 kinds of recombinant protein antigens in developing a genetically engineered vaccine against *H pylori* followed by NapA or HpaA. Our results also indicate that different diseases are no correlated to the *H pylori* infection rate but to the virulence of *H pylori* strains. These results provide additional evidence for selection of antigens used in genetically engineered vaccines against *H pylori*.

### Peer review

It is an interesting paper with almost novel findings which may help develop *H pylori* vaccines.

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CASE REPORT

# Diagnostic and therapeutic role of endoscopic retrograde cholangiopancreatography in biliary rhabdomyosarcoma

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## INTRODUCTION

Biliary rhabdomyosarcoma (BRMS) is an uncommon cause of recurrent jaundice and conjugated hyperbilirubinemia in children. Because its presentation may mimic that of a choledochal cyst, the correct diagnosis is frequently made intraoperatively at a planned choledochal cystectomy. However, identification of this entity prior to surgery is important because early surgery may carry excess morbidity and mortality. We present a patient with BRMS in whom therapeutic endoscopy was used successfully as an adjunct to chemotherapy and radiotherapy.

## CASE REPORT

A 3-year-old African American boy was referred to our center with a 1-wk history of scleral icterus, pruritus and light-colored diarrhea. His medical history included a self-limited episode of scleral icterus accompanied by diarrhea 1 mo earlier. His family history was unremarkable. Examination revealed a well-developed, healthy-appearing child. His abdomen was non-distended, the liver was soft and palpable 6 cm beneath the right costal margin without splenomegaly.

Laboratory investigations showed serum aspartate aminotransferase (AST) 391 U/L (20-60 U/L), alanine aminotransferase (ALT) 269 U/L (5-45 U/L), alkaline phosphatase (AP) 2 759 U/L (145-320 U/L), gamma-glutamyl transferase (GGT) 704 U/L (6-19 U/L), unconjugated bilirubin (UCB) 0.5 mg/dL (< 1 mg/dL), conjugated bilirubin (CB) 1.3 mg/dL (< 0.35 mg/dL) and international normalized ratio (INR) 0.9 (0.9-1.1). Hepatitis A, B and C serologies and stool studies were negative; amylase and lipase were normal.

Abdominal ultrasound revealed dilated intrahepatic and extrahepatic bile ducts and a dilated gallbladder without stones, which was suspicious for a choledochal cyst. Abdominal magnetic resonance imaging (MRI) showed an enhancing soft-tissue mass (5.8 cm × 2 cm × 1.5 cm) within the common bile duct (CBD)

## Abstract

Biliary rhabdomyosarcoma (BRMS) is an uncommon childhood malignancy which has been managed surgically. We present a case of a 3-year-old boy with BRMS, in whom endoscopic retrograde cholangiopancreatography (ERCP) was successfully used both diagnostically and therapeutically, thus obviating the need for surgery and its attendant risks of morbidity and mortality. We conclude that ERCP is an effective alternative to surgery for BRMS in some patients.

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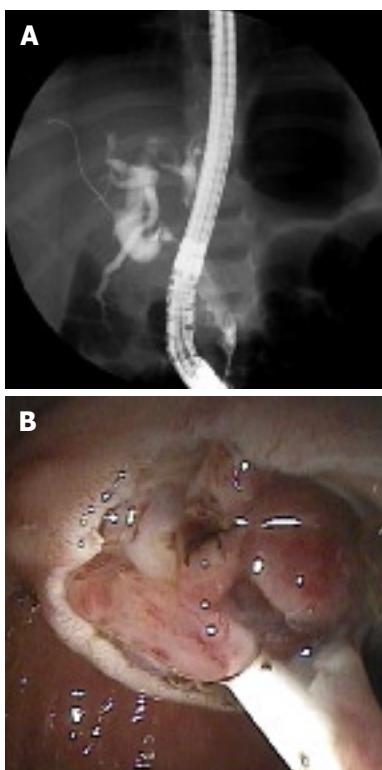
**Key words:** Pancreaticobiliary; Malignancy; Pediatric; Endoscopic retrograde cholangiopancreatography; Jaundice

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Himes RW, Rajman I, Finegold MJ, Russell HV, Fishman DS. Diagnostic and therapeutic role of endoscopic retrograde

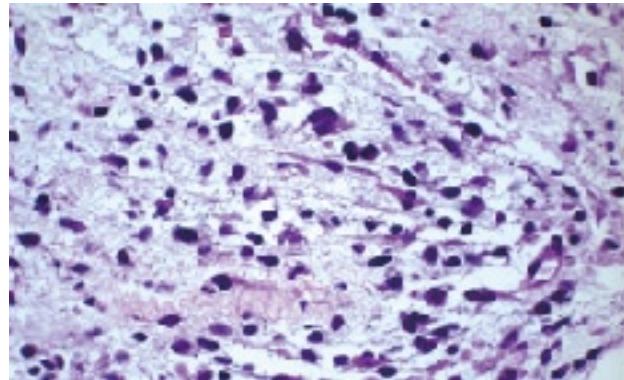


**Figure 1** Contrast enhanced abdominal MRI shows a crescent shaped enhancing soft-tissue density within a dilated CBD.

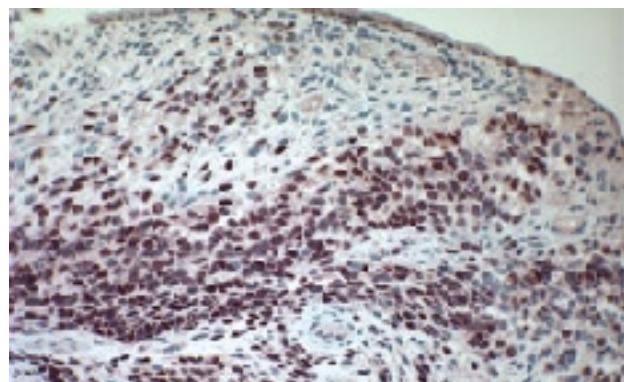


**Figure 2** **A:** ERCP demonstrates filling defects along the dilated CBD and a diffusely dilated intrahepatic and extrahepatic biliary tree; **B:** Intraductal tumor protruding from the ampulla post-sphincterotomy. A 7-french biliary stent was placed for decompression.

suggestive of BRMS (Figure 1). No intra-abdominal lymphadenopathy was present. The patient underwent (ERCP) to obtain tissue for diagnostic confirmation and for placement of a biliary stent. Filling defects were present on opacification of the biliary tree (Figure 2A) and sphincterotomy of the boggy ampulla revealed a protuberant fleshy mass (Figure 2B). Histology showed poorly differentiated round to stellate-shaped neoplastic cells in a myxoid stroma consistent with rhabdomyosarcoma of the botryoid subtype (Figure 3). Immunohistochemical stains for myogenin (Figure 4) and MyoD1 were positive. Chest computed tomography, whole body bone scan and bone marrow examination were unremarkable and a venous access device was



**Figure 3** A few rhabdomyosarcoma cells have eosinophilic cytoplasm suggesting skeletal muscle differentiation but most are very undifferentiated in a myxoid stroma (HE, x 400).



**Figure 4** Immunostain for myogenin reveals almost 100% positivity in neoplastic cell nuclei. The growth of this tumor beneath biliary epithelium (top) is typical of botryoid rhabdomyosarcoma (x 250).

placed to commence chemotherapy with vincristine, actinomycin-D and cyclophosphamide.

After 12 wk of chemotherapy the patient was anicteric and the tumor was undetectable by MRI, so the biliary stent was removed. At this endoscopy a repeat biopsy was obtained at the tumor bed which was histologically consistent with BRMS. Based on persistent disease, a radiation dose of 50.4 Gy was subsequently delivered to the tumor bed. The patient has completed therapy without significant complications.

## DISCUSSION

Although it is the most common biliary tumor in children, BRMS comprises only about 1% of all rhabdomyosarcomas<sup>[1-3]</sup>. BRMS usually arises in the CBD, but may originate from anywhere along the biliary tree. Positive regional lymph nodes or distant metastases were present at diagnosis in nearly 25% of children in one series<sup>[4]</sup>; and hepatic, pulmonary, peritoneal and bone metastases have been described.

Median age at presentation is 3 years and males are affected more often than females<sup>[1]</sup>. Presenting signs and symptoms include jaundice, scleral icterus, pruritus, acholic stools and abdominal pain or distention. Fever,

vomiting, diarrhea, malaise and anorexia occur less frequently.

Laboratory evaluation of patients reveals AP and gamma glutamyl-transferase elevation out of proportion to transaminases and a variable increase in CB depending on the extent of biliary obstruction. As in our patient, imaging shows a dilated biliary tree and may demonstrate an obstructive soft-tissue density.

Treatment protocols developed by the Soft Tissue Sarcoma Committee of the Children's Oncology Group, formerly the Intergroup Rhabdomyosarcoma Study Group (IRSG), stratify patients according to the site and size of the tumor, histologic subtype, degree of surgical resection and presence or absence of nodal disease or distant metastases. Although early case series advocated primary surgical resection together with chemotherapy and radiotherapy, a review by Spunt and colleagues<sup>[4]</sup> described the clinical features and outcomes of 25 children with BRMS and questioned the necessity of aggressive surgical excision for patients with tumors in this primary site. In their series, only six patients underwent gross tumor resections, of which, only two resulted in negative margins. In spite of residual tumor burden, however, 92% of patients who survived post-operatively remained disease-free. Moreover, they found that complications were more frequent in patients who underwent primary resection, these included ascending cholangitis, peritonitis, small bowel obstruction and peptic ulcer disease. The estimated five-year survival for

all patients with BRMS in this study was 66%; patients with local/regional disease fared better with 78% survival at five years<sup>[4]</sup>.

BRMS is a rare malignancy of childhood that presents with clinical and radiographic evidence that can mimic choledochal cysts. With highly effective chemotherapy, ERCP is an important diagnostic and therapeutic modality that may obviate the need for early and aggressive surgery in patients with BRMS.

## ACKNOWLEDGMENTS

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CASE REPORT

## Pancreatic transection from blunt trauma associated with vascular and biliary lesions: A case report

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**Author contributions:** Baiocchi GL conceived of the study and drafted the manuscript; Tiberio GAM and Gardani M participated in the design of the study; Gheza F and Cantù M performed the clinical research; Portolani N and Giulini SM performed the intervention and participated in the design of the study; all authors read and approved the final manuscript.

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Baiocchi GL, Tiberio GAM, Gheza F, Gardani M, Cantù M, Portolani N, Giulini SM. Pancreatic transection from blunt trauma associated with vascular and biliary lesions: A case report. *World J Gastroenterol* 2008; 14(30): 4826-4829 Available from: URL: <http://www.wjgnet.com/1007-9327/14/4826.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4826>

### Abstract

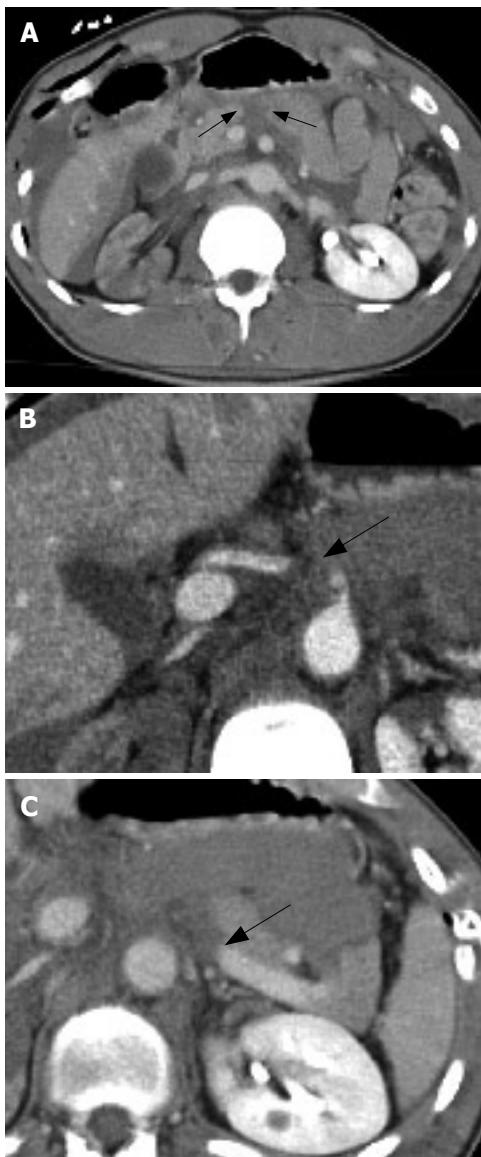
Major injuries of the pancreas may result in considerable morbidity and mortality when associated with vascular and visceral injuries. In such cases, a right diagnosis and a prompt surgical intervention are necessary to give a chance to the patient. We herein describe a case of blunt abdominal trauma in a 29-year-old man whose pancreatic rupture was associated with hepatic artery, splenic vein and extrahepatic bile duct damage. Immediate surgery was performed after computer tomography (CT), the haemorrhagic lesions dictate the emergency transfer to the operating room. Spleno-pancreatic resection was done with reconstruction of the hepatic artery, ligation of the splenic vein and a Roux-en-Y bilio-jejunial diversion. The early post-operative course was complicated by stenosis of the arterial reconstruction, which was treated by endovascular angioplasty followed by percutaneous drainage of symptomatic pseudocyst, rest and antibiotics. Finally, the patient was discharged and was alive without clinical problems at the time when we wrote this case report. The present case underlines the clinical relevance of vascular and visceral injuries associated with pancreatic trauma and the problems arising in the diagnostic evaluation and the surgical strategy of complex multiple visceral and vascular lesions in blunt abdominal trauma.

### INTRODUCTION

Isolated pancreatic injury occurs in less than 5% of major blunt abdominal traumas due to its retroperitoneal location<sup>[1]</sup>. In the majority of patients, pancreatic trauma is associated with major thoraco-abdominal lesions, which are the main factors influencing its clinical evolution in the acute phase. On the contrary, in the subsequent period, its mortality is related to septic complications and subsequent pancreatic or biliary disruption.

Few retrospective series of blunt pancreatic trauma, including more than 30 patients, have been published<sup>[2-6]</sup>. Its postoperative morbidity ranges from 42%<sup>[3]</sup> to 62%<sup>[2]</sup>, and its mortality is as high as 18.1%<sup>[6]</sup>. Age, severity of injury, amylase level, abdominal pain, injury severity score (ISS), presence of associated lesions, duration of shock, unrecognised diagnosis, delay in treatment and postoperative sepsis are the factors that significantly influence its outcome. Cases of multiple visceral and vascular lesions in the pancreatic area are not reported in the literature, presumably because the gravity of such situations does not allow the patient to survive.

We herein describe a case of blunt abdominal trauma causing pancreatic rupture associated with multiple arterial, venous, and biliary lesions, and discussed the diagnostic and therapeutic approach to such a rare case of multiple severe traumatic lesions.



**Figure 1** CT scan shows pancreatic transection between the head and body (A), thrombosis of the hepatic artery at its origin from the celiac trunk (B) and rupture/thrombosis of the splenic vein (C).

## CASE REPORT

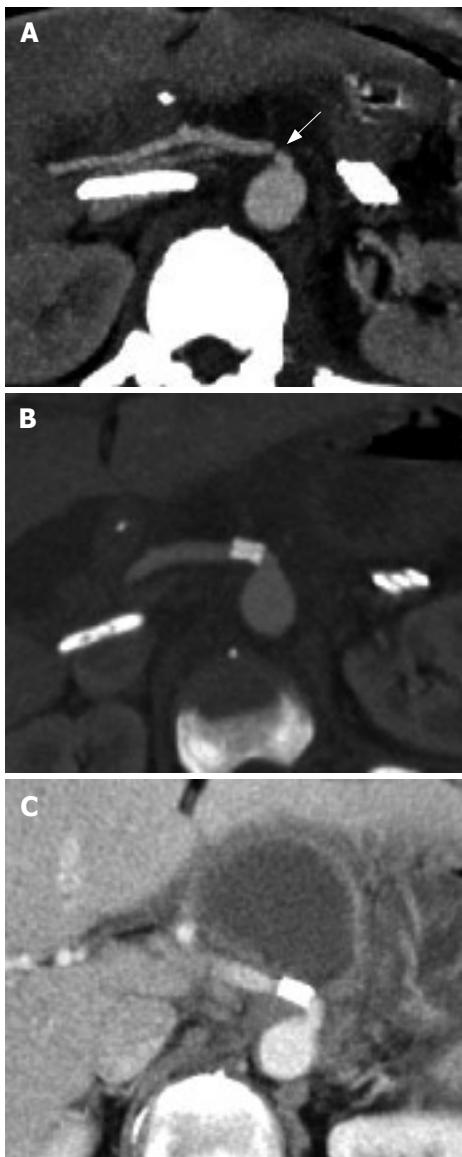
A 29-year-old male sustained a blunt abdominal trauma crushed by a carriage at work. At first observation he was in stable hemodynamic conditions, but complained of abdominal and back pain. Initial radiologic evaluation (chest and pelvis X-ray, and abdominal US) detected a right pneumothorax, bilateral rib fractures and a small amount of free blood in the abdominal cavity. Thoraco-abdominal computer tomography (CT) scan confirmed the presence of hemoperitoneum and revealed hepatic and spleen contusions, pancreatic transection at the junction between head and body (Figure 1A), thrombosis of the hepatic artery at its origin from the celiac trunk (Figure 1B), and thrombosis of the splenic vein (Figure 1C), as well as contusion of the right renal artery with no renal perfusion and fracture of D12 and L1. ISS was 34<sup>[7]</sup>. An immediate laparotomy was performed with free blood and bile found in the peritoneal cavity. After

the gastro-colic ligament was cut open, the pancreatic transaction and contusion of the common hepatic artery at its origin were confirmed with their continuity maintained by adventitia, while intima and media layers were partitioned with consequent occlusion of the artery. Splenic vein was completely transected at its confluence with the mesenterico-portal axis. Removal of the retropancreatic hematoma induced bleeding, stopped at first by digital compression and then by clamping the splenic vein and tangentially suturing the spleno-portal confluence. A complete interruption of the choledocus was demonstrated, his distal stump was ligated and the proximal one was clamped. The intra operative Doppler evaluation demonstrated that the right kidney was perfused with normal flow in the renal artery. The arterial blood supply to the liver was maintained by the inverted flow into the gastro-duodenal artery, but no pulsation could be appreciated at porta hepatis. Thus, a distal spleno-pancreatectomy was performed. After a short resection of the dissected tract, the common hepatic artery was reconstructed by end-to-end direct anastomosis to the celiac artery. Cholecystectomy and hepatico-jejunal anastomosis completed the operation. In the first postoperative period, ALT abd AST increased up to 154 U/L and 121 U/L, respectively. Postoperative CT scan showed stenosis of the celiac-hepatic anastomosis (Figure 2A), requiring operative angiography with stenting of the hepatic origin. A further CT control demonstrated the good results of the procedure (Figure 2B). Prophylactic octreotide acetate, 300 mg/d, was given after the intervention, as previously described<sup>[8]</sup>, and serum lipases were in the normal range. The patient was finally discharged on day 27 after operation.

Three months later, the patient complained of abdominal pain and dyspepsia, fever and leucocytosis. Abdominal US and CT scan showed a pancreatic pseudocyst (8 cm in diameter) in the epigastric region, between the residual pancreas, the posterior part of the stomach and the hepatic artery (Figure 2C), requiring percutaneous drainage, rest and antibiotic therapy. A significant reduction in size of the pseudocyst was achieved and the patient was discharged. At the 3-year control, the patient had normal quality of life, laboratory tests and abdominal US.

## DISCUSSION

Blunt pancreatic trauma is frequently combined with other organ injuries, which may cause early death of the patient<sup>[2-6]</sup>. The case reported in the present paper is of particular significance for the presence of multiple severe lesions involving vital structures in the pancreatic region, as a consequence of a violent trauma focused in this area, substantially sparing the rest of the patient's body. In our knowledge, this is the first reported case of contemporary traumatic disruption of the pancreatic neck, choledocus, spleno-portal confluence and common hepatic artery, probably because a patient so severely damaged is unlikely to survive to the required diagnostic



**Figure 2** CT scan 12 h after operation showing severe stenosis of hepatic artery-ceeliac reconstruction (**A**), good result of the procedure confirmed by CT after endovascular treatment (**B**), and a symptomatic pseudocyst in the epigastric region three months later (**C**) which was successfully treated by percutaneous drainage, rest and antibiotics.

and therapeutic time. In a paper analysing over 300 cases of traumatic vascular injury, the overall mortality was 54%, increasing up to 73% when 3 vessels were involved and 100% when 4 vessels were involved<sup>[9]</sup>. Patients affected by associated vascular and pancreatic injuries were even more rarely described (only 3 out of 101 cases analysed by Bradley and Coll)<sup>[6]</sup>.

The good outcome of the presented case is in our opinion due to the spontaneous partial haemostasis, but also to the prompt intervention, whose indication was dictated by the contrast-enhanced CT scan. The limits of this examination in defining the presence and gravity of pancreas trauma are well known. It was reported that the sensitivity of pancreatic rupture is 42.9%<sup>[6,10,11]</sup> and its low accuracy is particularly critical in defining the damage of the main pancreatic duct, the single most important factor both for surgical indication and choice

of the best surgical strategy<sup>[12-14]</sup>. In our case, CT allowed to exclude thoracic lesions and to focus on the pancreatic region, demonstrating the pancreatic body fracture and the presence of associated vascular damage. At the same time, choledochus fracture was not demonstrated, a wrong diagnosis of right renal artery thrombosis was suggested and also the hepatic artery and the splenic vein thrombosis were not clearly evaluated. This is in agreement with the literature, which reported that the mean injury grade resulting from CT is significantly lower than that resulting from surgical exploration<sup>[11]</sup>.

To avoid this limit of CT, endoscopic retrograde cholangio-pancreatography has been proposed to allow a more accurate identification of the biliary and Wirsung duct discontinuity, the superiority of which over CT scan in the evaluation of such structures is well documented<sup>[5]</sup>. However, in emergency situations, endoscopic examination is rarely considered since it requires a significant loss of time and is uselessly invasive, particularly if a surgical intervention has already proved to be necessary. In the reported case, the demonstrated vascular lesions dictated the necessity of immediate laparotomy, due to either the haemorrhagic risk or the ischemic damage to the liver. Thus, CT scan, even though it may be considered not completely accurate, is very useful for the correct management of this case.

These considerations shift the problem of complete diagnostic evaluation to the operating table, namely surgical exploration must be meticulous and systematic to demonstrate, define and treat the lesions associated with a pancreatic rupture. Undervaluation of the associated lesions is the most important negative prognostic factor for pancreatic trauma. By sectioning the gastrocolic ligament and the lesser omentum and completely mobilizing the right colon with the hepatic flexure and the right part of the transverse colon, or by performing a wide Kocher manoeuvre, a complete control of the structures potentially involved in pancreatic trauma is obtained. Surgical exploration can be completed by intraoperative instrumental evaluations such as cholangiography, endoscopy and doppler ultrasound, especially in cases in which preoperative imaging has been lacking or not exhaustive. In the presented case, the only employed intraoperative diagnostic tool was Doppler spectral analysis of the hepatic and portal flow. Evident lesions of the choledochus and pancreatic duct could be observed at inspection and irrigation. A vascular lesion may be really difficult to ascertain by simple exploration. However, after a complete transection, the haemorrhage can spontaneously stop and a complete thrombosis may be undiagnosed by a simple palpation. Intraoperative Doppler scan finally allows evaluation of the flow dynamics of suspected vascular lesions. In our case, it enabled us to demonstrate the preservation of a normal mesenterico-portal flow, the absence of residual stenosis of the mesenterico-portal axis after its suture and to document the collateral arterial flow at the hepatic hilum maintained by the gastroduodenal artery in presence of a contusion with short dissection occluding

the common hepatic artery. In case of a post-traumatic hepatic artery thrombosis, in the context of a complex trauma, reconstruction of this artery can be considered not necessary if a compensating inverted gastroduodenal artery flow is demonstrated. Reconstruction of a damaged hepatic artery is difficult, time-consuming and not rarely ineffective. However, when a bilio-digestive anastomosis has to be performed, a normal arterial flow is needed for the optimal perfusion of the biliary anastomotic stump. Intraoperative evaluation of the arterial collateral liver vascularisation may be misleading, lacking of quantitative determination. For these reasons, we did prefer to reconstruct the celiac-hepatic artery continuity by an end-to-end direct anastomosis, that later has proved to be technically imperfect and to require a post-operative percutaneous angioplasty with stenting. Our effort to maintain the continuity of the hepatic artery was justified by the transitory post-operative rise of hepatic enzymes, which returned to the normal range after stenting of the anastomosis. Moreover, a case of unrecognised hepatic artery dissection, having as a consequence a fatal fulminate hepatic failure, was recently reported<sup>[15]</sup>. Alternative techniques of revascularization by means of a prosthetic conduit should be avoided if possible, because septic complications are frequent in the postoperative course.

With regard to the biliary reconstruction, hepatico-jejunal anastomosis is preferable to a direct end-to-end reconstruction, as it allows a wide resection of the contused choledocus and anastomosis on a healthy tissue without tension<sup>[16]</sup>.

For the treatment of pancreatic transection, a number of techniques have been proposed, as an alternative to the distal pancreatectomy, accounting for 30% of the operations for pancreatic injuries<sup>[17]</sup>. With the aim to preserve endocrine pancreatic function, the distal pancreas can be anatomised to a Roux-en-Y jejunal loop, or to the stomach<sup>[18]</sup>. In presence of multiple lesions associated with the pancreatic injury, the strategy should be guided by the principle of obtaining repair of the lesions by a more simple and safe technical solution, reducing the risk of postoperative complications. Distal pancreatectomy with splenectomy, or whenever possible with splenic preservation, has a complication rate of 22.2%, lower than that of distal pancreas preserving procedures<sup>[2]</sup>. Moreover, postoperative exocrine and endocrine insufficiency is quite rare when the cephalic portion of the gland is preserved. The risk of developing proximal pancreatic stump fistula, with eventual pseudocyst formation, is independent of the treatment of the distal portion of the pancreas.

Our case demonstrates that if the haemorrhage can be spontaneously and temporarily controlled, giving to the surgeon the time strictly necessary to obtain the basilar diagnostic indications and to perform laparotomy, a systematic exploration and a surgical strategy inspired

by the principle of doing all but just what is necessary to save the patient, allow to obtain both immediate survival and long-term good quality of life.

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CASE REPORT

## Unexpected discovery of 2 cases of hepatocyte nuclear factor 1 $\alpha$ -mutated infracentimetric adenomatosis

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### INTRODUCTION

Hepatocellular adenomas (HCAs) are found as unique or multiple entities. The term adenomatosis is used<sup>[1]</sup>, if the number of HCAs is greater than ten; specific etiological factors, including glycogenosis or patients taking male hormones, were initially excluded from the definition<sup>[1]</sup>, as well as women that previously were but are no longer taking oral contraceptives<sup>[2]</sup>. Adenomatosis is suspected if multiple nodules are observed on ultrasound performed in various circumstances: pain, discomfort, mass, shock related to bleeding, hepatocellular carcinoma (HCC), or by chance<sup>[3]</sup>.

HCAs belong to various categories, as shown by molecular testing of hepatocyte nuclear factor 1 $\alpha$  (HNF1 $\alpha$ )<sup>[4-6]</sup>, and  $\beta$ -catenin<sup>[5,6]</sup>, and by the expression of members of the acute phase inflammatory response [serum amyloid A (SAA), and C-reactive protein (CRP)] at both the mRNA and protein levels<sup>[7]</sup>. We used genotype/phenotype classification to identify four groups of HCA: HNF1 $\alpha$ -mutated,  $\beta$ -catenin-mutated, inflammatory HCA and HCA without known mutation. Some inflammatory adenomas are also  $\beta$ -catenin-mutated. This classification also applies to adenomatosis. However, the number of cases of adenomatosis studied in the series used for classification is less than the number of cases of single or multiple HCA.

We report two cases in which the diagnosis of adenomatosis was unexpected, based on radiological and clinical grounds, and for whom molecular and immunohistochemistry testing revealed HNF1 $\alpha$ -mutations.

### CASE REPORT

#### Case one

A 54-year-old woman was admitted to our surgical department in September, 2004, due to the discovery of hyperechoic infracentimetric nodules, within the context

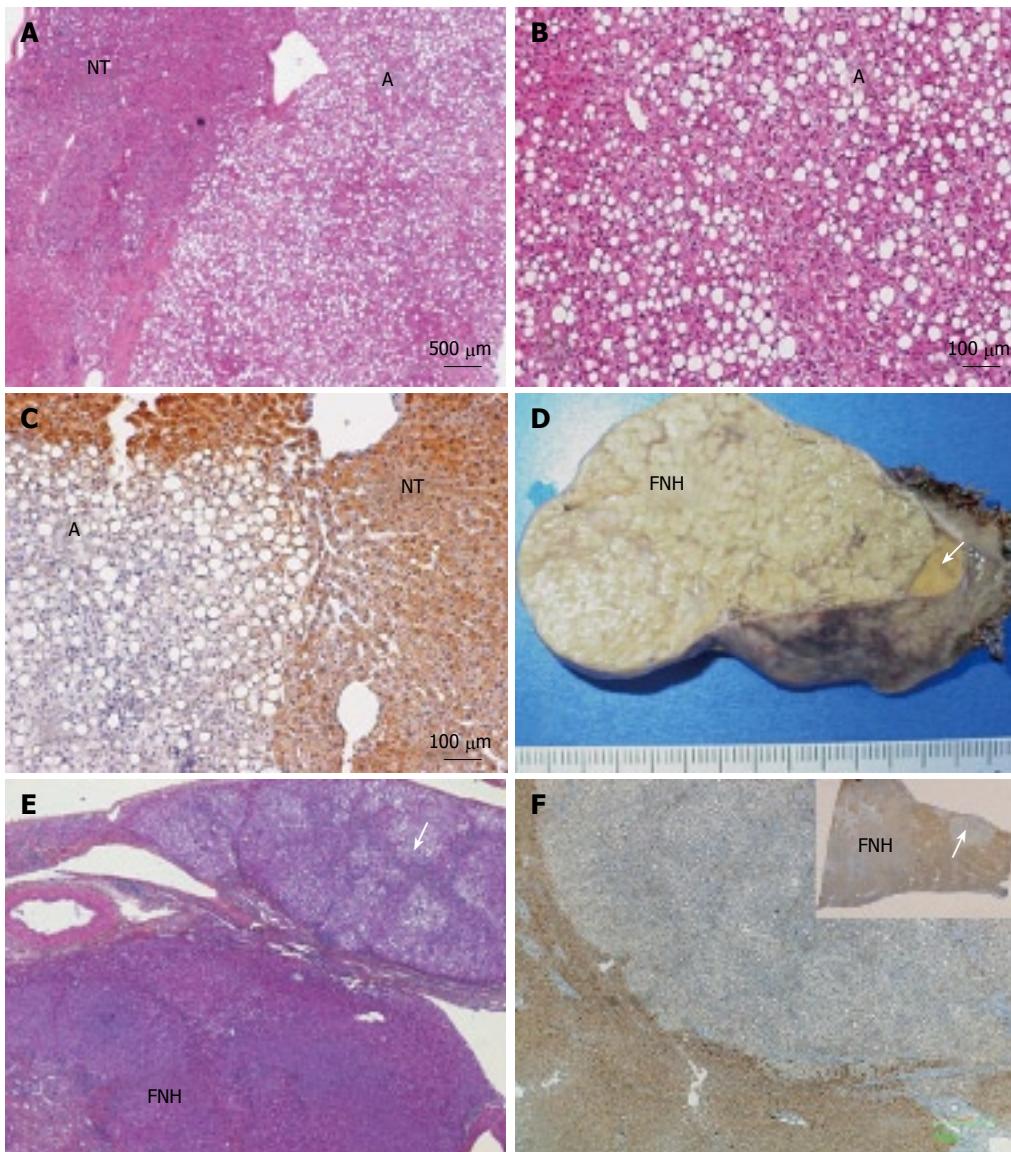
### Abstract

We present 2 cases of hepatocyte nuclear factor 1 $\alpha$  (HNF1 $\alpha$ )-mutated adenomatosis, discovered for reasons unrelated to this disease, and identified using immunohistochemical methods. These new tools may further our understanding of the link between adenomas/adenomatosis subtypes and their complications, and their association with other abnormalities.

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**Key words:** Hepatocellular adenoma; Adenomatosis; Hepatocyte nuclear factor 1; Hepatocyte nuclear factor 1 $\alpha$  mutation;  $\beta$ -catenin mutation; Focal nodular hyperplasia

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**Figure 1** Case 1- HE staining for steatotic adenoma showing non-encapsulated nodule (A, B) and non-tumoral liver (NT) (A), LFABP immunostaining for steatotic or non steatotic tumoral hepatocytes showing no expression of LFABP (C); Case 2- a small yellowish nodule (arrow) adjacent to a typical focal nodular hyperplasia (FNH) (D), HE staining for the small nodule showing a steatotic adenoma adjacent to the FNH (E) and another small steatotic microadenoma (arrows) in the non-tumoral liver (F).

of malignancy. Her past history was remarkable: she had a haemangioma of the bulbar area for over 20 years, a cerebellar meningioma discovered later and surgically removed in June, 1999, and a choroidal melanoma treated with proton therapy in February, 2004. At the time of those diagnoses, liver nodules were not visible on ultrasound. She had 2 children and took the pill for a very short period of time (less than 1 year prior to consulting our clinic). She weighed 70 kg and was 163 cm high. Physical examination revealed a cerebellar syndrome. Several punctiform angiomas were present on the thorax and limbs. Liver function tests were normal, with the exception of 39 IU/L GGT (normal < 34 IU/L). Her blood glucose was 7.1 mmol/L (normal < 6.1 mmol/L). She had a family history of non-insulin-dependant diabetes (father and mother of the father). Magnetic resonance imaging confirmed the presence of several infra-centimetric liver nodules, but the results

were inconclusive in relation to their nature. A liver biopsy was proposed under laparoscopic guidance to confirm the suspected diagnosis of melanoma metastasis. Multiple tan nodules were observed on the surface of the liver by the surgeon. A surgical biopsy (1.5 cm × 0.8 cm) containing two yellowish nodules (0.8 mm), and a histological examination of the tissue ruled out melanoma metastasis.

Both small liver nodules were non-encapsulated and showed a benign steatotic hepatocytic proliferation, intermingled with thin-walled isolated arteries and veins (Figure 1A and B). Cytokeratin (CK) 19 was negative and very few progenitor-like cells were visible on CK7 immunostaining (not shown). Therefore, the most probable diagnosis of these small nodules was HCA. Also, a complete absence of liver fatty acid-binding protein (LFABP), in contrast to normal expression detected in hepatocytes of the surrounding liver, favoured

a diagnosis of HNF1 $\alpha$ -mutated HCA (Figure 1C). The non-tumoral liver was limited to a thin band of tissue containing mildly enlarged fibrotic portal tracts.

### Case two

A 34-year-old woman visited our surgical clinic in February, 2004, for consultation. Two liver nodules were discovered by chance (one in segment III: 40 mm  $\times$  35 mm and one in segment VI: 20 mm  $\times$  20 mm) and there were at least two additional infracentimetric nodules. The two larger nodules were described as characteristic focal nodular hyperplasia (FNH), but no final diagnosis of the smaller nodules was made. The patient had been taking oral contraceptives for 12 years. However, she was no longer taking them in the last 2 years prior to consultation at our clinic. Her family history was complex (two maternal uncles had a cerebral vascular accident at a young age, with at least one of their children having an aneurysm resulting in a death, another uncle died after cardiac surgery, her mother and two maternal aunts also had breast cancer). Blood tests, including liver function tests, were normal. An operation was ruled out, and instead the patient was monitored. She came back 6 mo later complaining of epigastric pain. The size of the largest nodule increased (60 mm  $\times$  40 mm). Segmentectomy III and VI were performed under coelioscope. The surgeon observed multiple tan nodules on the surface of the liver. The two large nodules were macro and microscopically typical FNH. Three other small nodules were also clearly visible on the resected specimen, one of which was adjacent to the FNH (Figure 1D).

Routine examination of the nodules (cases 1 and 2) consisted of HE, Masson's trichrome, and reticulin stains, as well as CK7, CK19, and a smooth muscle actin (SMA), LFABP, serum amyloid A, glutamine synthetase (GS) and  $\beta$ -catenin immunostains<sup>[7]</sup>.

All the three small nodules were steatotic, with mild ductular reaction and inflammation, as well as a few entrapped portal tracts at the periphery. It was, thus, not possible to distinguish clearly between small, incomplete, so-called FNH-like or pre-FNH<sup>[8,9]</sup> and adenomas (Figure 1E). In case 1, the complete absence of LFABP in the nodule favoured a diagnosis of HNF1 $\alpha$ -mutated HCA, whereas the LFABP immunostaining was normal both in non-tumoral livers and in adjacent FNH (Figure 1F).

SAA, GS and  $\beta$ -catenin immunostaining were negative in both cases (data not shown).

## DISCUSSION

The above two cases lead to several comments.

Such cases of adenomatosis would have been totally ignored, if for specific reasons (fear of metastasis in case 1, and surgery for a growing and painful FNH in case 2) the surface of the liver had not been observed. This is indeed true for any benign lesion without clinical or biological manifestations, including FNH,

HCA, haemangioma, etc. Thus, the true prevalence of adenomatosis is impossible to calculate, as long as we do not have the tools to recognize clinically-, biologically- and radiologically-silent HCA, particularly microadenomas. We usually ignore the outcome of such cases, but it is likely that such small nodules will remain silent, particularly in patients that are no longer on oral contraception, which was the case in our two patients.

One can argue that the presence of multiple nodules on surface of the liver does not definitely prove that all nodules are microadenomas, this is particularly true for case 2, a case associated with FNH. The possibility of multiple FNH associated with occasional adenomas cannot be excluded. However, in our experience we have never observed such a case, whereas the presence of FNH is not a rare event in adenomatosis<sup>[10]</sup>.

As a result of advances made in molecular biology and immunohistochemistry, there has been much progress in the field of HCA. In particular with genotype/phenotype correlations, it is now possible, on routine pathological examinations, to identify typical cases of HNF1 $\alpha$ -mutated or inflammatory adenomas with a greater certainty. HNF1 $\alpha$  HCA is characterized by their fat distribution<sup>[6]</sup>.

We can easily and rapidly classify 80% of adenomas with the use of immunohistochemistry, including SAA, LFABP, GS and  $\beta$ -catenin, the results of which correlate well with molecular studies<sup>[6]</sup>. The genotype/phenotype classification of HCA using immunohistochemical methods, particularly  $\beta$ -catenin immunostaining coupled to GS, is particularly important for detecting patients at risk of developing HCC, as recently reported<sup>[4,6,7,11]</sup>. Immunostains are also important for identifying atypical nodules and small sized-resected nodules and have also been particularly used to differentiate micro adenomas from other types of nodules, especially pre-FNH<sup>[8,10,12]</sup> if fibrosis or some bile ductules are present.

The next step is to validate immunohistochemical methods on liver biopsy. To better interpret the results, it is mandatory to compare the data obtained in the non tumoral liver. Non tumoral livers express LFABP, but not SAA. GS is expressed only around hepatic veins.

The term adenomatosis has frequently been used in cases with more than 10 nodules<sup>[11]</sup>. If nodules are visible on surface of the liver, the diagnosis is usually easy, irrespective of the number of larger nodules detected by imaging techniques. Adenomatosis may also be suspected, if microadenomas are present on the resected specimen containing one or several HCAs from the non-tumoral liver<sup>[13]</sup>. In our opinion, the term adenomatosis has been used incorrectly, if defined on the basis of there being more than 3 lesions on imaging<sup>[13]</sup>, without the presence of nodules visible on the liver surface or microadenomas discovered by the pathologist on the resected specimen from the non-tumoral liver. In our experience, adenomatosis is associated with HNF1 $\alpha$ -mutations (90% being somatic) in most cases<sup>[7]</sup>.

Patients with adenomas/adenomatosis may have other organ abnormalities or diseases that may or may not be related to the pathogenesis of adenomas. In

1989, Wanless *et al*<sup>[14]</sup> noticed that patients with diseases, including meningioma, astrocytoma, telangiectasia of the brain, berry aneurysm, had what he called "telangiectatic FNH" more often (TFNH). Genotype/phenotype analysis<sup>[4,6,7]</sup> has revealed that TFNH are indeed inflammatory/telangiectatic adenomas<sup>[15,16]</sup>. Curiously enough in case 1, meningioma and telangiectasia of the brain were not associated with inflammatory/telangiectatic adenomatosis, but with HNF1 $\alpha$ -mutated adenomatosis.

With this new HCA classification at hand and the knowledge that adenomas/adenomatosis can be associated with various factors, including FNH<sup>[10]</sup>, diabetes with or without familial form of adenomas/adenomatosis<sup>[17-20]</sup>, polycystic ovaries<sup>[21]</sup>, and obesity/NASH<sup>[2,22]</sup>, it is of interest to collect clinical, biological and radiological data, which should not only be related to the liver but also be related to other organs (such as the brain, skin, thyroid, ovaries, kidneys, pancreas *etc*) to understand if there is a link or not between these various types of abnormalities.

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CASE REPORT

## Cerebral and pulmonary embolisms after transcatheter arterial chemoembolization for hepatocellular carcinoma

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### Abstract

A cerebral lipiodol embolism is an extremely rare complication of transcatheter arterial chemoembolization for hepatocellular carcinoma. We present a case of cerebral lipiodol embolism that occurred after the third arterial chemoembolization, report the clinical and radiological findings, and review the medical literature.

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**Key words:** Transcatheter arterial chemoembolization; Cerebral embolism; Complication; Hepatocellular carcinoma; Lipiodol

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### INTRODUCTION

Hepatocellular carcinoma (HCC) is a common malignancy in Asia<sup>[1,2]</sup>. Many patients with HCC present with advanced stage disease when first diagnosed. There are a variety of different therapeutic modalities used to treat advanced HCC. Transcatheter arterial chemoembolization (TACE) is used to confine tumors to the liver, in addition to shrinking their size, limiting the progression to vascular invasion, and decreasing the risk that viable tumor will embolize systemically, during hepatic manipulation at transplant hepatectomy<sup>[3-5]</sup>. Although TACE is an invasive procedure associated with several potential complications, it is a procedure that has been adopted worldwide and is the mainstay of treatment of patients with advanced HCC<sup>[6]</sup>.

To date, the literature on embolic injury to the brain, associated with iodized oil after TACE, includes only four reports<sup>[7-10]</sup>. Here we report our experience with a patient who had HCC and a cerebral lipiodol embolism (CLE) after TACE and review the previous literature.

### CASE REPORT

A 62-year-old woman with advanced HCC was admitted to the hospital for her third course of TACE. Six months previously, she was diagnosed with HCC. A 15 cm × 12 cm, well demarcated and exophytic growing tumor, at the right hepatic lobe without portal invasion, was initially identified. The exophytic HCC growth was contiguous to the diaphragm (Figure 1). Although serum alpha-fetoprotein (4514 ng/mL) was very elevated, metastatic evidence to the regional lymph nodes and bone marrow was not detected. The patient was initially treated with TACE and had a second course six months later.

On physical examination, a non-tender smooth surfaced mass was palpated at the right upper abdomen. Laboratory data (normal values in parentheses) on admission revealed a white blood cell count of  $5.95 \times 10^9/L$  (normal,  $4 \times 10^9-10 \times 10^9$ ), a platelet count of  $292 \times 10^9/L$  (normal,  $150 \times 10^9-450 \times 10^9$ ), and hypoalbuminemia, 3.6 g/dL (normal, 3.8-5.1). The hemoglobin was low, 10.0 g/dL (normal, 12.0-18.0). The coagulation time, liver function studies and renal function



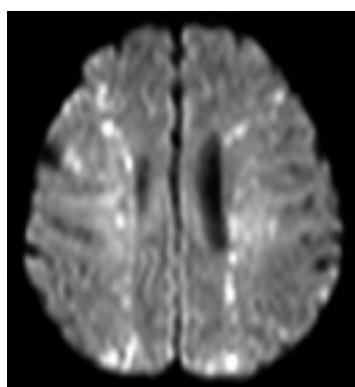
**Figure 1** Enhanced abdominal CT scan obtained after second chemoembolization shows a 15 cm × 12 cm capsulated HCC with necrotic change occupying the right hepatic lobe. The right dome of mass revealed heterogenous enhancement adjacent to the diaphragm, suggesting a viable portion (arrow).



**Figure 2** A brain CT scan without contrast obtained 7 h after chemoembolization shows multiple increased attenuated lesions in both cerebral hemisphere, consistent with deposition of iodized oil.



**Figure 3** A chest CT scan obtained 7 h after chemoembolization shows lipiodol oil dense depositions (arrow) at right basal lungs.



**Figure 4** Diffusion-weighted MR images obtained 48 h after TACE shows multiple hyperattenuating and hypertensive punctate-patchy lesions in both cerebral hemispheres.

were within normal range. Serological studies for hepatitis B were positive for HBsAg and HBeAb. The serum DNA level of hepatitis B virus was 64 400 copies/mL.

There was no evidence of cirrhosis. The distal subtraction angiography (DSA) revealed the HCC was supplied from the right inferior phrenic artery and right hepatic artery. A third course of TACE was performed via two arteries using a mixture of 50 mg adriamycin and 30 mL lipiodol as well as gelatin sponge particles (Gelform; Upjohn, MI, USA).

During the procedure, patient had no complaints, and the vital signs remained stable. Immediately after the procedure, the patients' level of consciousness deteriorated; in addition, she had breathing difficulty. The arterial blood gas analysis showed a PaO<sub>2</sub> of 55.7 mmHg, consistent with hypoxemia. A computed tomography (CT) scan of the brain, without contrast, 7 h after the TACE procedure revealed multiple lesions of increased attenuation in the cerebral cortex, basal ganglia, thalamus, and cerebellum (Figure 2).

At the same time, a CT of the chest revealed hyperattenuated images at the right lung base (Figure 3). Forty-eight hours after the procedure, magnetic resonance imaging (MRI) of the brain was performed. Diffusion-weighted (DW) images demonstrated disseminated hyperattenuated and hyper-intense punctate-patchy lesions in the cerebrum and cerebellum (Figure 4). The laboratory data was not remarkable except for a

leukocytosis. The chest X-ray showed a right pleural effusion and diffuse parenchymal infiltration one day post TACE. Echocardiography revealed no atrial septal defect or other intracardiac shunt. Three weeks later, a follow up brain CT scan revealed complete resolution of the lesions. The patients' level of consciousness gradually had improved. The neurological symptoms recovered completely by discharge 6 wk later.

## DISCUSSION

TACE is an invasive procedure associated with several potential complications, including the postembolization syndrome, septicemia, hepatic insufficiency, liver abscess formation, intrahepatic biloma, embolization of extrahepatic organs, cholecystitis, tumor rupture, multiple intrahepatic aneurysms, gastrointestinal mucosal lesions, variceal bleeding, and iatrogenic dissection or perforation of the vessel<sup>[11]</sup>. However, a CLE, following TACE in patients with HCC, is an extremely rare complication<sup>[7-10]</sup>.

The symptoms associated with CLE are variable and include visual loss, headache, motor dysfunction, and mental status changes; the severity of such symptoms varies with the site of lipiodol deposition. Occasionally, patients present with disorientation and hypoxia when the lungs are involved in lipiodol embolization (Table 1).

It is known that CLE is associated with a right-to-left shunt, and infusion of a large dose of lipiodol<sup>[12,13]</sup>. Right-to-left shunt is often undetectable on routine examination, including chest or abdominal CT, DSA,

**Table 1 Clinical characteristics of seven patients with CLE at initial presentation**

Age	Sex	Symptoms	ABGA (PaO <sub>2</sub> mmHg)	Shunt of evidence	Course of TACE (was occurred CLE)	Reference
52	Male	Headache Mental change Motor weakness	74.7	(-)	2	8
58	Male	Visual loss Headache Chest pain Shortness of breath	70.2	(-)	1	8
56	Male	Dyspnea	66	ND	3	8
81	Female	Asleep, Tachypnea Motor weakness	ND	ND	1.2	9
76	Male	Mental change Hypoxia	Hypoxia	ND	16	7
70	Female	Mental change Disorientation	ND	(-)	1	10
62	Female	Asleep	56.6	(-)	3	Present case

ND: Not described.

and routine echocardiography. Evidence of a shunt has not been reported in previous studies (Table 2). It is not necessary to routinely examine for detection of shunt prior to the TACE. But, if a condition is present that increases the risk of a shunt<sup>[14]</sup>, further evaluation to detect the shunt is indicated.

In most prior cases of CLE including present case, the HCC was a large tumor<sup>[7,9,10]</sup>. Determination of the optimal dose of lipiodol is of critical importance. The lipiodol dose is determined by a variety of factors including the blood supply of the tumor, the tumor size, the patients' condition, procedure tolerance, catheter position, and liver function reserve<sup>[15,16]</sup>. Generally, it is known that lipiodol dose should not exceed 15 mL to 20 mL in order to prevent the risk of an extrahepatic embolism<sup>[13]</sup>. However, there were prior reported cases of CLE that had appropriate doses of lipiodol<sup>[8-10]</sup>. Moreover, there was a study that recommended the use of a high dose iodized oil (more than 20 mL) TACE for patients with large HCC<sup>[17]</sup>. Therefore, all procedures must be individualized.

Another risk factor of a CLE is the communication between inferior phrenic artery (IPA) and the pulmonary artery; this might occur with adhesive pleura or tumor invasion<sup>[10,11]</sup>. In the previous reports, including present case, the vessels used for the lipiodol infusion described for four of seven cases. Among the four cases, there were three where lipiodol was infused into the right IPA<sup>[7,9]</sup>. In addition, in one of the four cases, where the IPA was not used, the lipiodol was infused into the right renal capsular artery (RCA), which might have

**Table 2 Outcomes and associated risk factors for TACE in the patients with CLE**

Doses of lipiodol (mL)	Tumor size	Pulmonary embolism	Injection artery of lipiodol	Recovery time (wk)	Reference
35	ND	(+)	ND	3	8
8	ND	(+)	ND	3	8
ND	ND	(+)	ND	2	8
15	10 cm × 14 cm	(+)	RIPA, RMA	2	9
ND	Large	(+)	RIPA Hepatic proper artery Epicoledocal artery	6	7
12	Large	(-)	RCA, RHA, MHA	Death	10
30	10 cm × 15 cm	(+)	RIPA, RHA	6	Present case

ND: Not described; RCA: Renal capsular artery; RHA: Right hepatic artery; MHA: Middle hepatic artery; RIPA: Right inferior phrenic artery; RMA: Right mammary artery.

been anastomosed to the IPA. Evidence of pulmonary embolism was noted in six of seven cases.

Therefore, we think that right IPA to pulmonary artery shunt is also a potential route for right-to-left shunt.

In the present case, because we performed echocardiography and DSA, we confirmed the absence of intracardiac and intratumoral shunts. The communication between IPA and pulmonary vessels occurred via adherent pleura and tumor recurrence. Moreover, a large dose of lipiodol was used and, therefore, the patient had a greater risk for a CLE.

As previous reports recommended<sup>[10]</sup>, the total dose of lipiodol should not exceed 20 mL and injection of lipiodol via the IPA should be used with caution during TACE procedure.

To prevent a CLE, an individualized plan of therapy including the lipiodol dose, evaluation for a shunt and choice of the vessels used for the lipiodol infusion prior to TACE are important considerations.

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CASE REPORT

# Lobulated inflammatory myoglandular polyp in the ascending colon observed by magnifying endoscopy and treated with endoscopic polypectomy

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Author contributions: Kanzaki H and Hirasaki S contributed equally to this work; Kanzaki H, Hirasaki S, Okuda M, Kudo K and Suzuki S were involved in the care of the patient; Kanzaki H and Hirasaki S performed the endoscopic resection; Hirasaki S and Kanzaki H wrote the paper.

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## INTRODUCTION

Inflammatory myoglandular polyp (IMGP) is characterized by inflammatory granulation tissue in the lamina propria<sup>[1]</sup>, proliferation of smooth muscle<sup>[2]</sup>, and hyperplastic glands with variable cystic change<sup>[3-7]</sup>. Only a small number of cases have been reported, and its pathogenesis and natural history remain unclear<sup>[8-12]</sup>. Herein, we describe a relatively rare case of lobulated-type IMGP in the ascending colon causing hematochezia. We also report the magnifying endoscopy findings of this IMGP.

## Abstract

The patient was a 33-year-old man with hematochezia. Colonoscopy revealed a lobulated peduncular polyp with bleeding, about 40 mm in diameter, in the ascending colon. The polyp had both red and white components and a mosaic pattern. Magnifying observation revealed a red rugged surface component, and smooth white nodules with enlarged round or oval crypt openings. Endoscopic polypectomy was performed. Histological examination of the specimen revealed inflammatory granulation tissue in the lamina propria, proliferation of smooth muscle, and hyperplastic glands with cystic change. This polyp was diagnosed as inflammatory myoglandular polyp (IMGP). Lobulated-type IMGP in the ascending colon is rare.

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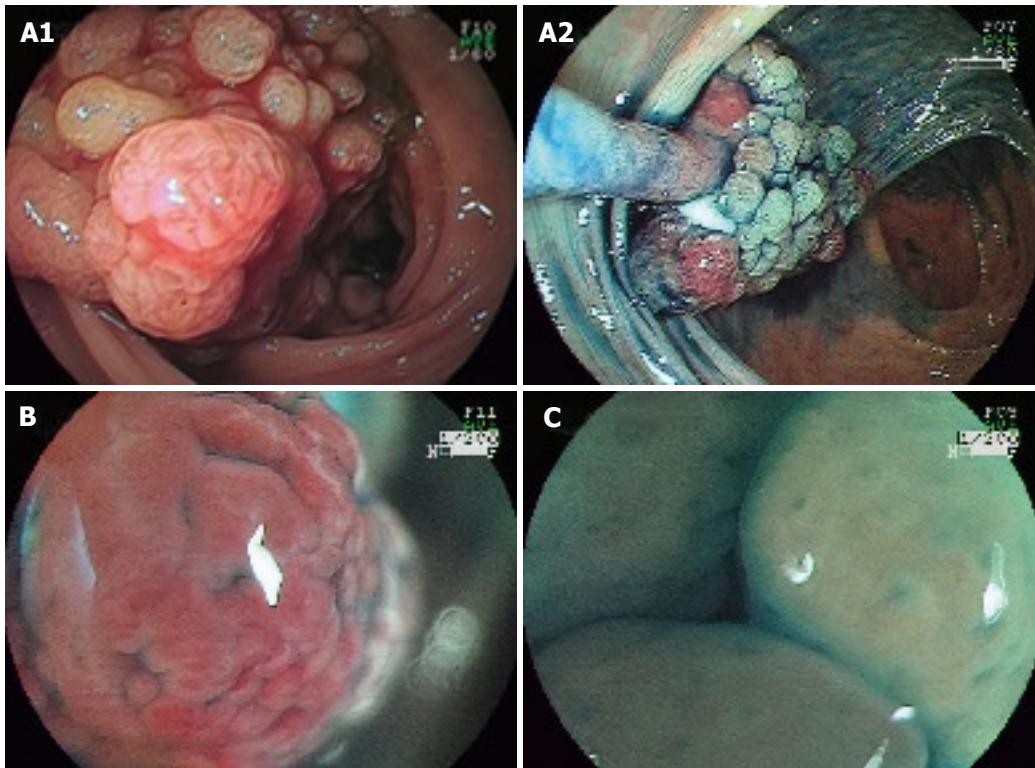
**Key words:** Inflammatory polyp; Colonoscopy; Magnifying endoscopy; Endoscopic polypectomy; Hematochezia

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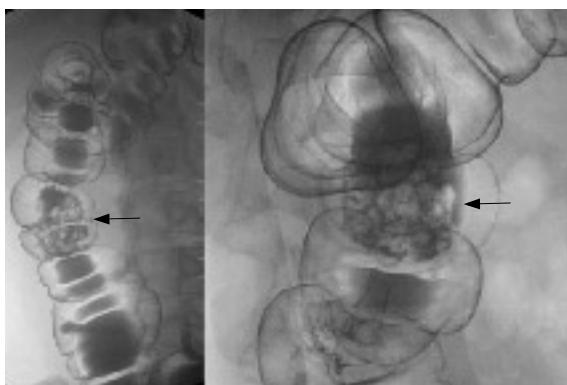
Kanzaki H, Hirasaki S, Okuda M, Kudo K, Suzuki S. Lobulated inflammatory myoglandular polyp in the ascending colon observed by magnifying endoscopy and treated with endoscopic polypectomy. *World J Gastroenterol* 2008; 14(30): 4838-4840 Available from: URL: <http://www.wjnet.com>

## CASE REPORT

A 33-year-old man presented with the symptom of hematochezia. He was diagnosed with chronic renal failure and underwent hemodialysis 5 years ago. His body temperature was 36.7°C, blood pressure was 148/82 mmHg, and radial pulse rate was 70 beats/min and regular. He had anemia. Laboratory tests showed hemoglobin concentration of 8.0 g/dL [normal range (NR): 12-16 g/dL], a red blood cell count of  $325 \times 10^4/\mu\text{L}$  (NR:  $380-500 \times 10^4/\mu\text{L}$ ), a white blood cell count of 8400/ $\mu\text{L}$ , and a platelet count of  $28.8 \times 10^4/\mu\text{L}$ . The levels of hepatic and biliary enzymes, such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP),  $\gamma$ -glutamyltranspeptidase ( $\gamma$ -GTP), and lactate dehydrogenase (LDH), were normal. On renal function tests, the blood urea nitrogen and creatinine levels were 44.9 mg/dL (NR: 8-20 mg/dL) and 14.1 (NR: 0.5-1.3 mg/dL), respectively. Colonoscopy revealed a lobulated peduncular polyp with bleeding, about 40 mm in diameter, in the ascending colon (Figure 1A1). The polyp had both red and white components and a mosaic pattern (Figure 1A2). Magnifying observation (EC-450ZH, Fujinon Toshiba ES Systems) revealed a red, slightly rugged surface component without normal mucosal structure (Figure 1B), and smooth white nodules with enlarged round or oval crypt openings (Figure 1C). We speculated that this polyp was non-neoplastic. It was suspected to



**Figure 1** Endoscopy showing a lobulated, peduncular polyp with bleeding, about 40 mm in diameter, in the ascending colon with both red and white components and a mosaic pattern observed in the polyp (A), magnifying endoscopy revealing a rugged surface of red component without normal mucosal structure ( $\times 50$ ) (B), and aggregated smooth nodules with enlarged round or oval crypt openings in the white component ( $\times 50$ ) (C).

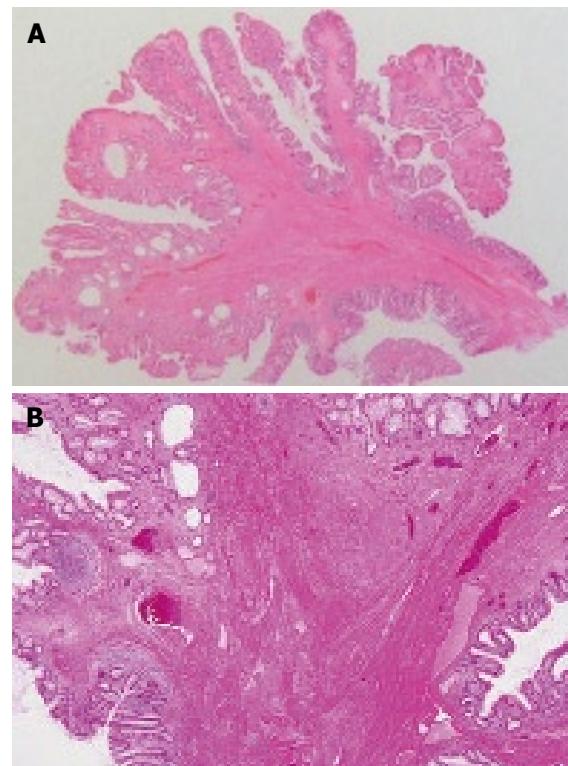


**Figure 2** Double-contrast radiograph of the ascending colon showing an about 40 mm lobulated peduncular polyp (arrow).

be an inflammatory polyp from endoscopic findings. An air contrast barium enema also revealed a pedunculated, lobulated polyp in the ascending colon (Figure 2). Endoscopic polypectomy was performed. Histological examination of the specimen revealed inflammatory granulation tissue in the lamina propria, proliferation of smooth muscle, and hyperplastic glands with cystic change (Figure 3). The lesion was diagnosed as IMGP. After endoscopic polypectomy, the symptom of hematochezia was resolved.

## DISCUSSION

IMGP is a non-neoplastic colorectal polyp, first described by Nakamura *et al*<sup>[1]</sup>. IMGP is solitary,



**Figure 3** Microscopy of the polypectomy specimen showing a stalked polyp containing numerous cystically dilated glands on the cross section under lower-power view (HE,  $\times 4$ ) (A) and inflammatory granulation tissue in the lamina propria mucosae and proliferation of smooth muscle (HE,  $\times 50$ ) (B).

pedunculated and rarely covered by a fibrin cap, and

follows a benign course. Also, IMGP has no association with inflammatory bowel diseases and is located not only in the rectosigmoid, but also in the descending and transverse colon<sup>[5]</sup>.

Only a small number of IMGP cases have been reported. According to Fujino *et al*<sup>[8]</sup>, a review of the literature revealed 48 cases of IMGP in the large intestine up to 2001. However, recent advances in diagnostic techniques have enabled us to identify small and asymptomatic polyps, and reports on IMGP of the colon have been increasing<sup>[9,10]</sup>. Fujino *et al*<sup>[8]</sup> described that the macroscopic appearance was the pedunculated type in 83.3% of cases. In that report, the sites of IMGP in the large intestine were studied and 47 of 48 cases (97.9%) had lesions in the rectum to transverse colon. Thus, IMGPs of the large intestine are predominantly in the distal colon<sup>[8,9]</sup>.

IMGPs in the colon are usually asymptomatic and often detected incidentally on barium enema or endoscopy<sup>[8-10]</sup>. Another review of the literature revealed that the main clinical feature of colorectal IMGPs is hematochezia<sup>[10,11]</sup>. Endoscopic characteristic findings are as follows: (1) pedunculated or semipedunculated, (2) red and (3) smooth, spherical and hyperemic surface with patchy mucous exudation and erosion<sup>[8,9]</sup>. IMGPs should be distinguished from other colorectal polyps such as inflammatory fibroid polyps (IFP)<sup>[10,13]</sup>, Peutz-Jegher-type polyps or juvenile polyps<sup>[10,11]</sup>. However, the correct endoscopic diagnosis of colorectal IMGP can seldom be made. Endoscopic findings of IMGP are similar to those of IFP and juvenile polyp. The final diagnosis of colonic IMGP depends on the pathological findings of endoscopic mucosal resection (EMR) or endoscopic polypectomy specimens. Harada *et al*<sup>[7]</sup> reviewed the literature and described that 4 of 40 IMGPs (10%) were lobulated. The present case was rare because IMGP located in the ascending colon and the radiographic and endoscopic findings of the polyp were the lobulated type.

Moriyama *et al*<sup>[9]</sup> reported magnifying endoscopic findings of 5 IMGPs in 2003. They described that magnifying observation revealed a slightly rugged surface consisting of aggregated smooth nodules with enlarged round or oval crypt openings. In the present case, magnifying endoscopic findings were the same as Moriyama's description. However, this polyp was unique because the lesion had red and white components and a mosaic pattern. There have been few reports on magnifying endoscopic findings of IMGP. To clarify the characteristic magnifying endoscopic findings of IMGP, we should accumulate and analyze many cases of IMGP.

As to therapy, IMGP of the large intestine can best be removed endoscopically, because it is thought to be clinically and histologically benign. Most Japanese cases are treated with polypectomy or EMR<sup>[5,7-10]</sup>. Endoscopic or surgical treatment is necessary if gastrointestinal bleeding<sup>[9]</sup> or colonic intussusception occurs. Local excision of the polyp is curative. Kayhan *et al*<sup>[12]</sup> reported a case of large IMGP (> 6 cm in diameter), which was too large for endoscopic removal, and treated with surgical resection. We consider that the percentage of

colonic IMGP patients who undergo surgical resection will decrease and endoscopic resection will increase in the future because of advances in diagnostic techniques such as improved endoscopic images and the discovery of asymptomatic small IMGPs.

In conclusion, we reported a case of IMGP in the ascending colon causing hematochezia. IMGP should generally be taken into consideration as a differential diagnosis of peduncular polyp of the colon. IMGP of the large intestine is not fatal and patients remain asymptomatic in their daily lives except for gastrointestinal bleeding or bowel obstruction. Therefore, it is likely that there will be many latent patients with IMGP in the future. Endoscopists should be aware of IMGP endoscopic characteristics, although lobulated type-IMGP in the ascending colon is rare. Further studies on the magnifying endoscopy findings of IMGP are certainly required.

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CASE REPORT

## Laparoscopic cystogastrostomy for the treatment of pancreatic pseudocysts: A case report

Qin-Song Sheng, Da-Zhi Chen, Ren Lang, Zhong-Kui Jin, Dong-Dong Han, Li-Xin Li, Yong-Jiu Yang, Ping Li, Fei Pan, Dong Zhang, Zhao-Wei Qu, Qiang He

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Sheng QS, Chen DZ, Lang R, Jin ZK, Han DD, Li LX, Yang YJ, Li P, Pan F, Zhang D, Qu ZW, He Q. Laparoscopic cystogastrostomy for the treatment of pancreatic pseudocysts: A case report. *World J Gastroenterol* 2008; 14(30): 4841-4843 Available from: URL: <http://www.wjnet.com/1007-9327/14/4841.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4841>

### INTRODUCTION

Pancreatic pseudocysts (PPs), common sequelae of acute or chronic pancreatitis and trauma, are fluid collections arising in or adjacent to the pancreas enclosed by a wall of fibrous granulation tissue, but lacking a true epithelial lining. Interventions indicated for symptomatic, large ( $> 6$  cm in diameter), complicated and persistent ( $> 6$  wk) PP<sup>[1]</sup>, include percutaneous, endoscopic or surgical approaches<sup>[2]</sup>. With the advent of minimally invasive techniques such as cystogastrostomy, cystojejunostomy and cystoduodenostomy<sup>[3]</sup>, laparoscopy plays a great role in the management of PP. Moreover, laparoscopic cystogastrostomy has been described as a safe and efficacious alternative to open drainage of PP in adults<sup>[1,2]</sup>.

We report, in this paper, a case of a patient with a pancreatic pseudocyst caused by acute pancreatitis, who underwent intragastric laparoscopic cystogastrostomy.

### CASE REPORT

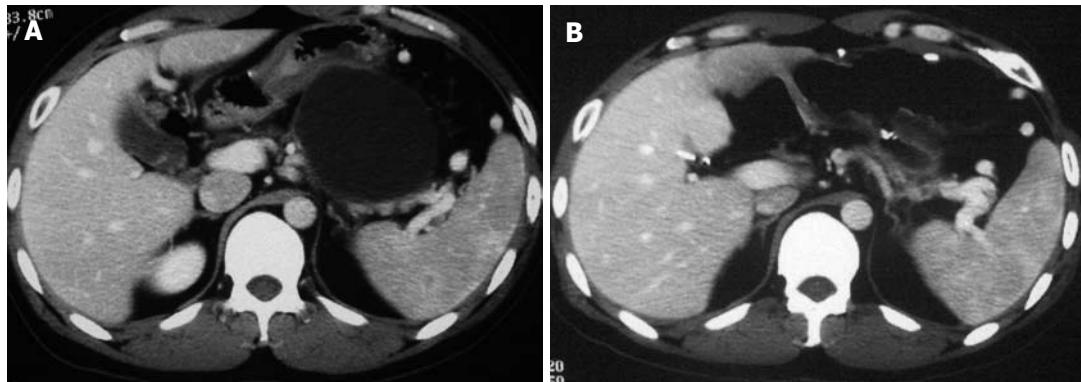
A 30-year-old male patient, complaining of epigastric pain and postprandial distension, was admitted to our hospital. He developed acute pancreatitis 10 mo ago. Regular B type ultrasound and computed tomography (CT) showed a pseudocyst at the body of the pancreas. A recent CT scan (Figure 1) demonstrated a 7.5 cm × 6.0 cm mass with its anterior wall closely contacted with the posterior wall of the stomach, as well as splenic vein compression and splenomegaly.

### Abstract

Pancreatic pseudocysts (PPs) are collections of pancreatic secretions that are lined by fibrous tissues and may contain necrotic debris or blood. The interventions including percutaneous, endoscopic or surgical approaches are based on the size, location, symptoms and complications of a pseudocyst. With the availability of advanced imaging systems and cameras, better hemostatic equipments and excellent laparoscopic techniques, most pseudocysts can be found and managed by laparoscopy. We describe a case of a 30-year-old male patient with a pancreatic pseudocyst amenable to laparoscopic cystogastrostomy. An incision was made through the anterior gastric wall to expose the posterior gastric wall in close contact with the pseudocyst using an ultrasonically activated scalpel. Then, another incision was made for cystogastrostomy to obtain complete and unobstructed drainage. The patient recovered well after operation and was symptom-free during a 6-mo follow-up, suggesting that laparoscopic cystogastrostomy is a safe and effective alternative to open cystogastrostomy for minimally invasive management of PP.

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**Key words:** Pancreatic pseudocyst; Laparoscopic



**Figure 1** **A:** Pre-operative CT scan shows a 7.5 cm X 6.0 cm pseudocyst, is in close contact with the posterior wall of the stomach. In addition, splenic vein compression and splenomegaly are also observed. **B:** Post-operative CT scan reveals effective drainage of the pseudocyst and dramatic relief of splenic vein compression.

The patient underwent laparoscopic cystogastrostomy. In brief, after general anesthesia, a 10-mm port was placed subumbilically for laparoscopy, two 5-mm working ports were placed in the left subcostal area and a 5-mm port was placed in the subxiphoid. Laparoscopic ultrasound showed that a pseudocyst was firmly adhered to the posterior wall of the stomach. An ultrasonically activated scalpel was used to create a 5 cm anterior gastrostomy at the maximal displacement site of the stomach. A laparoscopic needle was introduced to confirm the location of the pseudocyst and to sample fluid. Then, the scalpel was used to create a cystogastrostomy opening approximately 4 cm in size between the adherent posterior and anterior gastric walls of the pseudocyst. After the needle entered the pseudocyst, more than 500 mL of fluid contents was aspirated. Electrocautery diathermy and titanium clips were used to achieve hemostasis at the bleeding sites in the cystogastrostomy. The anterior gastrostomy was closed using a linear stapler. A nasojejunal tube was left in place at the end of the procedure which took 150 min. Histologic analysis revealed a typical pancreatic pseudocyst.

After the operation, the patient was given parenteral nutrition and enteral nutrition *via* a nasojejunal tube, and recovered well without complications. The nasojejunal tube was removed on the 8th postoperative day and the patient was discharged on the 11th postoperative day. A repeat CT scan (Figure 1B) before discharge revealed effective drainage of the pseudocyst and dramatic relief of splenic vein compression. The patient was free of symptoms and signs of recurrent pseudocyst during a 6-mo follow-up.

## DISCUSSION

Pancreatic pseudocysts occur in 2%-10% of patients after acute pancreatitis and in about 10%-30% of patients after chronic pancreatitis<sup>[4]</sup>, and can be treated with different procedures. In general, spontaneous regression of small asymptomatic PPs may be observed in 30%-60% of acute pancreatitis patients<sup>[5]</sup>. Conservative management with bowel rest and parenteral

nutrition increases the likelihood of spontaneous regression<sup>[6]</sup>. However, a large number of patients with PPs need interventions. Factors determining the route and time of intervention include (1) location, size and persistence of the cyst, (2) maturity of the cyst wall when the patient presents with symptoms, (3) presence or absence of complications, (4) availability of local expertise and experience<sup>[2]</sup>. Generally, indications for intervention of PPs include > 6 cm in diameter, > 6 wk in persistence, symptoms (including epigastric pain, nausea, vomiting, biliary obstruction, and duodenal obstruction), complications (including infection, hemorrhage, rupture) and matured wall<sup>[1,2,7]</sup>.

Intervention options for treatment include percutaneous, endoscopic, and surgical procedures. Percutaneous drainage of PPs is a procedure of choice for infected and obstructed pseudocysts with an immature wall. It is also used in situations where definitive internal drainage could not be done<sup>[2]</sup>. However, this procedure seems to have a high risk of recurrence or development of pancreatic percutaneous fistula<sup>[8]</sup>. Furthermore, percutaneous drainage is inadequate in many cases because of thick viscous contents, which may cause luminal obstruction of drainage catheters<sup>[9]</sup>. Endoscopic drainage in the presence of endoscopic ultrasound (EUS) is an important procedure in the management of pseudocysts, especially cysts indenting the stomach or duodenum and in the absence of necrotic tissue<sup>[2]</sup>. However, endoscopic drainage is associated with a high rate of technical failure, cyst recurrence, infection, bleeding, stent blockage, and inadequate drainage. Aljarabah *et al*<sup>[1]</sup> hold that endoscopic drainage is more suitable for chronic PPs within the head and body of the gland, whereas acute PPs, particularly those that complicate necrotizing pancreatitis, are best managed with laparoscopic surgery where expertise is available. Laparoscopic drainage of mature PPs is minimally invasive and offers definitive drainage. Over the past years, laparoscopic surgery of the pancreas has increasingly emerged as a procedure in the treatment of PPs<sup>[3]</sup>. Laparoscopic procedures for pancreatic pseudocysts include pancreatic cystogastrostomy, cystoduodenostomy, and cystojejunostomy. Barragan *et al*<sup>[10]</sup> compared the laparoscopic procedures by analyzing their advantages and

disadvantages, and concluded that when the pancreatic cyst is located in close contact with the posterior wall of the stomach, it is best drained with the anterior procedure.

According to the history of acute pancreatitis and the location, size, and persistence of the pseudocyst in our case, percutaneous procedure and EUS-guided drainage were not suitable, and laparoscopic cystogastrostomy was performed via the anterior procedure. Laparoscopy was carried out using a 4-port technique. Because the pseudocyst shared a common wall, a cystogastrostomy, 4 cm in size, was created directly using an ultrasonic scalpel. The superior visualization provided by laparoscopy afforded the ability to obtain hemostasis efficiently, and the adequate incision helped establish effective drainage, thus preventing recurrence of the pseudocyst. The patient recovered well after the operation and was free of symptoms after 6 mo of follow-up.

Laparoscopic drainage, a minimally invasive technique, can definitively drain mature PPs. Our patient was treated with laparoscopic procedures without any untoward effects. Although our experience is limited, our minimally invasive procedure appears to be safe and effective. Laparoscopic cystogastrostomy should be considered a choice of treatment in the management of PPs.

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## Meetings

### Events Calendar 2008-2009

FALK SYMPOSIA 2008  
January 24-25, Frankfurt, Germany  
Falk Workshop: Perspectives in Liver Transplantation

International Gastroenterological Congresses 2008  
February 14-16, Paris, France  
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies  
[www.easl.ch/hepatitis-conference](http://www.easl.ch/hepatitis-conference)

February 14-17, Berlin, Germany  
8<sup>th</sup> International Conference on New Trends in Immunosuppression and Immunotherapy  
[www.kenes.com/immuno](http://www.kenes.com/immuno)

February 28, Lyon, France  
3<sup>rd</sup> Congress of ECCO - the European Crohn's and Colitis Organisation Inflammatory Bowel Diseases 2008  
[www.ecco-ibd.eu](http://www.ecco-ibd.eu)

February 29, Québec, Canada  
Canadian Association of Gastroenterology  
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK  
British Society of Gastroenterology Annual Meeting  
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China  
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea  
Asian Pacific Association for the Study of the Liver  
18<sup>th</sup> Conference of APASL: New Horizons in Hepatology  
[www.apaslseoul2008.org](http://www.apaslseoul2008.org)

March 29-April 1, Shanghai, China  
Shanghai-Hong Kong International Liver Congress  
[www.livercongress.org](http://www.livercongress.org)

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco  
OESO 9<sup>th</sup> World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas  
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA  
SAGES 2008 Annual Meeting - part of Surgical Spring Week  
[www.sages.org/08program/html/](http://www.sages.org/08program/html/)

April 18-22, Buenos Aires, Argentina  
9<sup>th</sup> World Congress of the International Hepato-Pancreato Biliary Association  
Association for the Study of the Liver  
[www.ca-ihpba.com.ar](http://www.ca-ihpba.com.ar)

April 23-27, Milan, Italy  
43<sup>rd</sup> Annual Meeting of the European Association for the Study of the Liver  
[www.easl.ch](http://www.easl.ch)

May 2-3, Budapest, Hungary  
Falk Symposium 164: Intestinal

### Disorders

May 18-21, San Diego, California, USA  
Digestive Disease Week 2008

May 21-22, California, USA  
ASGE Annual Postgraduate Course Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion  
E-mail: education@sgo.org

June 4-7, Helsinki, Finland  
The 39<sup>th</sup> Nordic Meeting of Gastroenterology  
[www.congrex.com/ngc2008](http://www.congrex.com/ngc2008)

June 5-8, Sitges (Barcelona), Spain  
Semana de las Enfermedades Digestivas  
E-mail: sepd@sepdi.es

June 6-8, Prague, Czech Republic  
3<sup>rd</sup> Annual European Meeting: Perspectives in Inflammatory Bowel Diseases  
E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey  
ESGAR 2008 19<sup>th</sup> Annual Meeting and Postgraduate Course  
E-mail: fca@netvisao.pt

June 11-13, Stockholm, Sweden  
16<sup>th</sup> International Congress of the European Association for Endoscopic Surgery  
E-mail: info@aes-eur.org

June 13-14, Amsterdam, Netherlands  
Falk Symposium 165: XX International Bile Acid Meeting, Bile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic  
Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management  
E-mail: idca2008@guantar.cz

June 25-28, Barcelona, Spain  
10<sup>th</sup> World Congress on Gastrointestinal Cancer  
Imedex and ESMO  
E-mail: meetings@imedex.com

June 25-28, Lodz, Poland  
Joint Meeting of the European Pancreatic Club (EPC) and the International Association of Pancreatology (IAP)  
E-mail: office@epc-iap2008.org  
[www.e-p-c.org](http://www.e-p-c.org)  
[www.pancreatology.org](http://www.pancreatology.org)

June 26-28, Bratislava, Slovakia  
5<sup>th</sup> Central European Gastroenterology Meeting  
[www.ceurgem2008.cz](http://www.ceurgem2008.cz)

July 9-12, Paris, France  
ILTS 14<sup>th</sup> Annual International Congress  
[www.ilts.org](http://www.ilts.org)

September 10-13, Budapest, Hungary  
11<sup>th</sup> World Congress of the International Society for Diseases of the Esophagus  
E-mail: isde@isde.net

September 13-16, New Delhi, India  
Asia Pacific Digestive Week  
E-mail: apdw@apdw2008.net

APDW 2008  
September 13-16, New Delhi, Indian Organized: Indian Society of Gastroenterology

III FALK GASTRO-CONFERENCE

September 17, Mainz, Germany  
Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany  
Falk Symposium 166: GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic  
Prague Hepatology Meeting 2008  
[www.czech-hepatology.cz/phm2008](http://www.czech-hepatology.cz/phm2008)

September 20-21, Mainz, Germany  
Falk Symposium 167: Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France  
Third Annual Meeting  
European Society of Coloproctology  
[www.escp.eu.com](http://www.escp.eu.com)



October 8-11, Istanbul, Turkey  
18<sup>th</sup> World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists  
E-mail: orkun.sahin@serenas.com.tr

October 18-22, Vienna, Austria  
16<sup>th</sup> United European Gastroenterology Week  
[www.negf.org](http://www.negf.org)  
[www.acv.at](http://www.acv.at)

October 22-25, Minnesota, USA  
Anstralian Gastroenterology Week 2008  
E-mail: gesa@gesa.org.au

October 22-25, Brisbane, Australia  
71<sup>st</sup> Annual Colon and Rectal Surgery Conference  
E-mail: info@colonrectalcourse.org

October 31-November 4, Moscone West Convention Center, San Francisco, CA  
59<sup>th</sup> AASLD Annual Meeting and Postgraduate Course  
The Liver Meeting Information: [www.aasld.org](http://www.aasld.org)

November 6-9, Lucerne, Switzerland  
Neurogastroenterology & Motility Joint International Meeting 2008  
E-mail: ngm2008@mci-group.com  
[www.ngm2008.com](http://www.ngm2008.com)

November 12, Santiago de Chile, Chile  
Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

November 28-29, Cairo, Egypt  
1<sup>st</sup> Hepatology and Gastroenterology Post Graduate Course  
[www.egyptgastrohep.com](http://www.egyptgastrohep.com)

December 7-9, Seoul, Korea  
6<sup>th</sup> International Meeting Hepatocellular Carcinoma: Eastern and Western Experiences  
E-mail: sglee@amc.seoul.kr

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[www.falkfoundation.de](http://www.falkfoundation.de)

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January 18-19, March 28-29, June 6-7, October 3-4

N.O.T.E.S  
April 3-5, November 27-29  
Laparoscopic Digestive Surgery

June 27-28, November 7-8  
Laparoscopic Colorectal Surgery

July 3-5  
Interventional GI Endoscopy Techniques  
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E-mail: [info@eits.fr](mailto:info@eits.fr)

International Gastroenterological Congresses 2009  
March 23-26, Glasgow, Scotland  
Meeting of the British Society of Gastroenterology (BSG)  
E-mail: [bsg@mailbox.ulcc.ac.uk](mailto:bsg@mailbox.ulcc.ac.uk)

May 17-20, Denver, Colorado, USA  
Digestive Disease Week 2009

November 21-25, London, UK  
Gastro 2009 UEGW/World Congress of Gastroenterology  
[www.gastro2009.org](http://www.gastro2009.org)



### Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London, The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.



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### GENERAL INFORMATION

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- 2 Lin GZ, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixudiarrhoea. *Shijie Huaren Xiaohua Zaishi* 1999; **7**: 285-287

In press

- 3 Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

- 5 Vallancien G, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

Volume with supplement

- 7 Geraud G, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment

of migraine and in comparison with sumatriptan. *Headache* 2002; 42 Suppl 2: S93-99 [PMID: 12028325]

*Issue with no volume*

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (401): 230-238 [PMID: 12151900]

*No volume or issue*

- 9 Outreach: Bringing HIV-positive individuals into care. *HRS A Careaction* 2002; 1-6 [PMID: 12154804]

## Books

*Personal author(s)*

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

*Chapter in a book (list all authors)*

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

*Author(s) and editor(s)*

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

*Conference proceedings*

- 13 **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

*Conference paper*

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

**Electronic journal** (list all authors)

- 15 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/eid.htm>

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[1]Passed away on October 20, 2007

[2]Passed away on June 11, 2007



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EDITORIAL

# Cytomegalovirus infection after liver transplantation: Current concepts and challenges

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## Abstract

Cytomegalovirus (CMV) is a common viral pathogen that influences the outcome of liver transplantation. In addition to the direct effects of CMV syndrome and tissue-invasive diseases, CMV is associated with an increased predisposition to acute and chronic allograft rejection, accelerated hepatitis C recurrence, and other opportunistic infections, as well as reduced overall patient and allograft survival. Risk factors for CMV disease are often interrelated, and include CMV D+/R-serostatus, acute rejection, female gender, age, use of high-dose mycophenolate mofetil and prednisone, and the overall state of immunity. In addition to the role of CMV-specific CD4+ and CD8+ T lymphocytes, there are data to suggest that functionality of the innate immune system contributes to CMV disease pathogenesis. In one study, liver transplant recipients with a specific polymorphism in innate immune molecules known as Toll-like receptors were more likely to develop higher levels of CMV replication and clinical disease. Because of the direct and indirect adverse effects of CMV disease, its prevention, whether through antiviral prophylaxis or preemptive therapy, is an essential component in improving the outcome of liver transplantation. In the majority of transplant centers, antiviral prophylaxis is the preferred strategy over preemptive therapy for the prevention of CMV disease in CMV-seronegative recipients of liver allografts from CMV-seropositive donors (D+/R-). However, the major drawback of antiviral prophylaxis is the occurrence of delayed-onset primary CMV disease. In several prospective and retrospective studies, the incidence of delayed-onset primary CMV disease ranged from 16% to 47% of CMV D+/R- liver transplant recipients.

Current data suggests that delayed-onset CMV disease is associated with increased mortality after liver transplantation. Therefore, optimized strategies for prevention and novel drugs with unique modes of action are needed. Currently, a randomized controlled clinical trial is being performed comparing the efficacy and safety of maribavir, a novel benzimidazole riboside, and oral ganciclovir as prophylaxis against primary CMV disease in liver transplant recipients. The treatment of CMV disease consists mainly of intravenous (IV) ganciclovir, and if feasible, a reduction in the degree of immunosuppression. A recent controlled clinical trial demonstrated that valganciclovir is as effective and safe as IV ganciclovir for the treatment of CMV disease in solid organ (including liver) transplant recipients. In this article, the author reviews the current state and the future perspectives of prevention and treatment of CMV disease after liver transplantation.

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**Key words:** Cytomegalovirus; Outcome; Hepatitis; Transplantation; Valganciclovir; Maribavir; Prophylaxis; Treatment

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## INTRODUCTION

Throughout the four decades that have elapsed since the first successful liver transplantation in 1967, cytomegalovirus (CMV) has remained the single most common viral pathogen influencing the outcome of this procedure. Infection with CMV is not only a very common complication after liver transplantation but it also contributes significantly to the morbidity and mortality, both by direct and indirect mechanisms<sup>[1,2]</sup>.

CMV is a ubiquitous herpes virus that infects 60%-100% of humans<sup>[1,2]</sup>, with primary CMV infection occurring most commonly during the first 2 decades of life. If immunocompetent, the infected individuals are mostly asymptomatic or may present with a benign febrile infectious mononucleosis-like illness. However, in individuals with compromised immunity, such as liver transplant recipients, clinical disease with high morbidity may develop and, in some cases, may lead to death<sup>[1,2]</sup>.

Facilitated by its ability to evade the immune system, infection with CMV results in a state of latency in several host cells<sup>[1,2]</sup>. Consequently, these cellular sites of viral latency become reservoirs of reactivation during periods of stress and cytokine release, and serve as vehicles for transmission to susceptible hosts. Both these scenarios are operational in liver transplant recipients, wherein the pharmacologic-induced impairment of immune response to "endogenously reactivated" or "allograft-transmitted" CMV leads to febrile and tissue-invasive diseases<sup>[1,2]</sup>. Because of the lack of a pre-existing CMV-specific immunity, CMV-seronegative recipients of liver allografts from CMV-seropositive donors (CMV D+/R-) are at the highest risk of CMV disease and its complications<sup>[3-5]</sup>.

This article reviews the current concepts and challenges in the management of CMV after liver transplantation. Historical aspects of the disease are discussed to emphasize the remarkable improvements that have been achieved over the past several years. Conversely, ongoing issues of delayed-onset and drug-resistant CMV disease are discussed in detail, to highlight future perspectives in terms of CMV disease prevention and treatment.

## CLINICAL IMPACT OF CMV IN LIVER TRANSPLANTATION

### Direct CMV effects

The clinical illness caused by CMV commonly manifests as fever, bone marrow suppression, and organ-invasive diseases (Table 1)<sup>[1]</sup>. These direct clinical effects are traditionally classified as CMV syndrome (fever with myelosuppression) and tissue-invasive CMV disease, which most often involves the gastrointestinal tract (in the form of CMV gastritis, esophagitis, enteritis, and colitis), although virtually any organ system may be involved<sup>[6]</sup>. Infection of the liver (i.e., CMV hepatitis) is especially common in liver transplant recipients (compared to other solid organ transplant recipients), and this may manifest with symptoms indistinguishable from acute allograft rejection<sup>[7]</sup>. The availability of sensitive tests for the rapid detection of CMV in the blood may obviate the need for liver biopsy to differentiate the CMV infection from rejection. However, in many cases, a liver biopsy is needed to differentiate or to demonstrate the co-existence of CMV disease and allograft rejection.

In the absence of effective antiviral prophylaxis, the

**Table 1 Direct and indirect clinical effects of CMV after solid organ transplantation**

Direct effects	Indirect effects
CMV syndrome	Acute allograft rejection
Fever	
Myelosuppression	
Malaise	
Tissue-invasive CMV disease <sup>1</sup>	Chronic allograft rejection
Gastrointestinal disease (colitis, esophagitis, gastritis, enteritis)	Vanishing bile duct syndrome
Hepatitis	
Pneumonitis	Chronic ductopenic rejection
CNS disease	Hepatitis C virus recurrence
Retinitis	Allograft hepatitis, fibrosis and allograft failure
	Opportunistic and other infections
Mortality	Fungal superinfection
	Nocardiosis
	Bacterial superinfection
	Epstein-Barr virus and PTLD
	HHV-6 and HHV-7 infections
	Vascular thrombosis
	Mortality

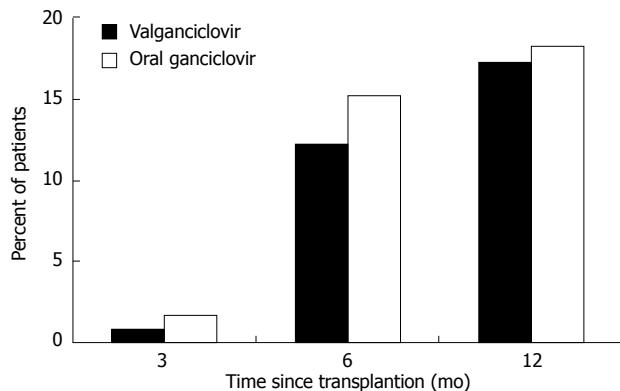
<sup>1</sup>Any organ system may be affected by CMV.

**Table 2 Estimated incidence of CMV disease during the first 12 mo after liver transplantation**

	Use of anti-CMV prophylaxis	
	Yes <sup>1</sup>	No
CMV D+/R-	12%-30%	44%-65%
CMV D+/R+	2.7%	18.2%
CMV D-/R+	3.9%	7.9%
CMV D-/R-	0	0
All patients	4.8%	18%-29%

D: Donor; R: Recipient. <sup>1</sup>Most cases occur as delayed-onset CMV disease. CMV disease occurs rarely during prophylaxis with oral valganciclovir. Data adapted from<sup>[4,5,7]</sup>.

direct effects of CMV occur most commonly during the first 3 mo after liver transplantation<sup>[6]</sup>. Overall, it is estimated that 18%-29% of all liver transplant recipients will develop CMV disease (Table 2)<sup>[4,5,8-10]</sup>. However, this incidence varies widely depending upon donor and recipient CMV serologic status; it may be as high as 44%-65% in CMV D+/R-, or as low as 8%-19% in CMV-seropositive liver transplant recipients (CMV R+)<sup>[4,8,10]</sup>. The incidence is markedly reduced in liver transplant recipients who receive prophylaxis with 3 mo of valganciclovir and oral ganciclovir. Recent studies have reported CMV disease rates of 12%-30% in high-risk CMV D+/R-, and < 10% in CMV R+ liver transplant recipients who received 3 mo of antiviral prophylaxis<sup>[3,4,8,10-12]</sup>. In individuals who received antiviral prophylaxis, CMV disease occurred 3 mo to 6 mo after completing antiviral prophylaxis; hence, the term "delayed-onset (also termed late-onset) CMV disease" (Figure 1)<sup>[3]</sup>.



**Figure 1** Time to the onset of CMV disease in solid organ transplant recipients who received 3 mo of oral ganciclovir or valganciclovir prophylaxis. Data obtained from the study by Paya et al<sup>[3]</sup>.

### Indirect CMV effects

The clinical impact of CMV extends beyond the direct effect of the virus. Numerous indirect outcomes, believed to be mediated by the ability of the virus to modulate the immune system have been reported (Table 1)<sup>[1,2]</sup>. CMV is known to be a potent up-regulator of alloantigens, thereby increasing the risk of acute rejection and chronic allograft dysfunction<sup>[13]</sup>. CMV is associated with vanishing bile duct syndrome and ductopenic rejection, leading to chronic cholestasis and eventually allograft failure<sup>[14-16]</sup>. Several studies have reported a higher incidence of vascular and hepatic artery thrombosis in liver transplant recipients with CMV disease, an effect that is believed to result from CMV infection of the vascular endothelial cells<sup>[17,18]</sup>. The immunomodulatory effects of CMV have also been blamed for the higher predisposition to other opportunistic infections including fungi, other viruses, and bacteria such as *Nocardia* sp.<sup>[19,20]</sup>. CMV-infected transplant recipients are more likely to develop Epstein-Barr virus-associated post transplant lymphoproliferative disorders (PTLDs), or develop co-infections with other viruses such as human herpes virus (HHV)-6 and HHV-7<sup>[19,21]</sup>. A well-described interaction between members of the beta-herpes group of viruses has been described, exemplified by the observation that reactivation of HHV-6 and HHV-7 is associated with an increased predisposition to CMV disease after liver transplantation<sup>[22-24]</sup>. In a similar manner, there is a significant association between CMV and hepatitis C virus<sup>[25-30]</sup>, manifested by an accelerated course of HCV recurrence in patients who develop CMV infection after liver transplantation. In our analysis of 92 HCV-infected liver transplant recipients, there was a four-fold higher risk of allograft failure and mortality in patients with CMV infection and disease<sup>[28,30]</sup>. Three years after liver transplantation, 48% patients who developed CMV disease had allograft loss or had died, compared to 35% patients with asymptomatic CMV infection, and 17% in those who did not develop CMV infection<sup>[28,30]</sup>.

### Impact on mortality

CMV infection is an independent predictor of mortality

**Table 3** Selected traditional and novel factors associated with the increased risk of CMV disease after liver transplantation

Traditional factors	Recently identified factors
CMV D+/R- > CMV R+	Toll-like receptor gene polymorphism
Allograft rejection	Mannose binding lectin deficiency
High viral replication	Chemokine and cytokine defects (IL-10, MCP-1, CCR5)
Mycophenolate mofetil	Deficiency in CMV-specific CD4+ T cells
Muromonab-CD3	Deficiency in CMV-specific CD8+ T cells
Anti-thymocyte globulin	Expression of immune evasion genes
Alemtuzumab	Programmed cell death 1 expression
HHV-6	
HHV-7	
Renal insufficiency	
Others <sup>1</sup>	

<sup>1</sup>Others factors include re-transplantation, volume of blood transfusion, sepsis and factors associated with high tumor necrosis factor- $\alpha$  secretion<sup>[14,11,13,21,39-42,77,89-93]</sup>.

after solid organ transplantation, by mechanisms which may be direct, indirect or immunomodulatory<sup>[19,31,32]</sup>. CMV was a major cause of mortality after liver transplantation prior to the availability of intravenous (IV) and oral ganciclovir. Several recent meta-analyses have demonstrated that the use of anti-CMV drugs, either through antiviral prophylaxis or preemptive therapy, have led to significant reduction in the overall mortality after solid organ transplantation<sup>[19,33-35]</sup>. However, despite much improvement in outcome, there is emerging data to suggest that even in the contemporary era, with widespread use of antiviral prophylaxis, development of delayed onset CMV disease remains a common problem, and importantly, is associated with significantly increased risk of mortality after liver transplantation<sup>[32]</sup>. An analysis of 437 liver transplant recipients demonstrated that CMV disease occurred in 37 patients (8.5%), and its occurrence was independently associated with a 5-fold increased risk of all-cause mortality, and an 11-fold increased risk of infection-related mortality after liver transplantation<sup>[32]</sup>. The other significant and independent predictors of mortality in this study included the need for pre-liver transplant hemodialysis, a higher model for end-stage liver disease (MELD) score, and post-transplant occurrence of bacterial and fungal infections<sup>[32]</sup>.

## RISK FACTORS FOR CMV DISEASE AFTER LIVER TRANSPLANTATION

### Lack of pre-existing CMV-specific immunity

The most common predisposing factor for the occurrence of CMV disease after liver transplantation is the lack of an effective CMV-specific immunity<sup>[4,19]</sup>. As a result, CMV D+/R- are at the highest risk of CMV disease<sup>[4,19]</sup>, while CMV R+ patients have a modest risk and CMV D-/R- have the lowest risk of CMV disease after liver transplantation (Table 3).

### Drug-induced immunodeficiency

The use of highly potent pharmacologic immuno-

suppression severely impairs the ability of liver transplant recipients to mount an effective immune response against reactivating CMV, thereby predisposing to increased risk of CMV disease<sup>[4,19]</sup>. The severity of immune dysfunction is particularly intense with lymphocyte-depleting drugs such as muromonab-CD3 (OKT3) and anti-thymocyte globulin<sup>[36,37]</sup>. More recently, the use of alemtuzumab has been found to be associated with higher risk of CMV disease<sup>[38]</sup>. Drugs used for maintenance immunosuppression have also been associated with CMV disease, particularly high doses of mycophenolate mofetil<sup>[30,39]</sup>. It is very likely that immunosuppressive drugs not only predispose to CMV disease, but the net state of combined pharmacologic immunosuppression increases the risk of CMV disease after liver transplantation<sup>[1,2,19]</sup>.

### **Defects in innate and CMV-specific cell-mediated immunity**

The appreciation of the role of the immune system in controlling CMV led to recent observations that inherent defects in immunity, such as mutations in the innate immunity-associated genes, increased the risk of CMV disease after liver transplantation (Table 3). In a study of 92 liver transplant recipients, a genetic polymorphism in the Toll-like receptor (TLR)-2 gene, which resulted from the substitution of arginine to glutamine at position 753 in the protein-receptor, was significantly associated with a higher degree of CMV replication and a higher incidence of CMV disease<sup>[40]</sup>. TLR2 is a pattern recognition receptor expressed in innate immune cells, and its function is to sense the glycoprotein B of CMV, thereby signaling the immune cells to produce antiviral peptides and other cytokines<sup>[40]</sup>. Our *in vitro* data suggests that this specific genetic polymorphism causes an impairment of cellular recognition of CMV by TLR2-expressing cells<sup>[40]</sup>.

Likewise, the CMV-specific T cell compartment is necessary for adequate control of CMV after liver transplantation<sup>[41]</sup>, although a recent study indicated that CMV-specific T cells may not necessarily predict the risk after liver transplantation<sup>[41]</sup>. There are ongoing studies in this field that may further clarify the prognostic role of CMV-specific T cell assays in stratifying CMV disease risk after liver transplantation.

Other immune measures, such as programmed death-1 expression<sup>[42]</sup> and immune evasion genes<sup>[43]</sup> have also been assessed as prognostic indicators of CMV disease after liver transplantation. In one study, programmed death-1 receptor up-regulation was significantly associated with incipient and overt CMV disease and with CMV viremia<sup>[42]</sup>.

### **Allograft rejection**

Allograft rejection *per se* is one of the most potent inducers of CMV reactivation, and thus considered a significant risk factor for CMV disease after liver transplantation<sup>[12]</sup>. Cytokines released during acute rejection, particularly tumor necrosis factor- $\alpha$ <sup>[44]</sup>, are

potent transactivators of latent CMV<sup>[45]</sup>, as demonstrated in animal models<sup>[46]</sup>. Moreover, therapy for allograft rejection with the intensification of immunosuppressive regimen further increases the risk of CMV disease both by enhancing its reactivation and by impairing the ability to generate effective cell-mediated immunity against replicating CMV<sup>[47]</sup>. Conversely, CMV induces allo-stimulation and increases the risk of allograft rejection, thereby creating a bidirectional relationship between CMV and allograft rejection<sup>[13]</sup>.

### **Virus-to-virus interactions**

Virus-virus interaction may influence the risk of CMV disease after liver transplantation<sup>[21,22,26-30]</sup>. Reactivation of HHV-6 has been shown to predispose to an increased incidence of CMV disease after liver transplantation<sup>[21,22,24]</sup>. In a study on 247 patients, HHV-6 seroconversion was an independent marker of CMV disease after liver transplantation. Likewise, HCV-infected liver transplant recipients also have a higher incidence of CMV disease<sup>[48]</sup>, although our data in the era of valganciclovir prophylaxis has refuted this observation<sup>[25]</sup>.

### **Degree of viral replication**

The risk of CMV disease after liver transplantation is associated, in direct proportion, with the degree of CMV replication, which is partly a function of over-immunosuppression<sup>[8,23,49,50]</sup>. In one study, a viral load of 1-2860 CMV copies/ $10^6$  peripheral blood mononuclear cells (PBMC) increased CMV disease risk by nine-fold, while viral loads  $> 2860/10^6$  PBMC increased the risk by 50-fold<sup>[8]</sup>.

### **Other factors**

Other factors associated with CMV disease after transplantation include cold ischemia time, bacterial and fungal infections and sepsis, the amount of blood loss, fulminant hepatic failure as the indication for transplantation, age, female gender, Hispanic race, and renal insufficiency<sup>[2,3,19,51]</sup>. It is likely that other factors that have not yet been identified may also be influence the risk of CMV disease after liver transplantation.

## **PREVENTION OF CMV DISEASE AFTER LIVER TRANSPLANTATION**

Because of the adverse effects of CMV on transplant outcome, its prevention is key to management of such patients<sup>[19]</sup>. Over the years, the pharmacologic agents used for CMV prevention have evolved, from the use of acyclovir<sup>[52]</sup> and immunoglobulins<sup>[53]</sup> to IV and oral ganciclovir<sup>[4]</sup> and more recently, valganciclovir<sup>[5]</sup>. There are two major strategies for CMV disease prevention after liver transplantation: (1) preemptive therapy (wherein CMV reactivation is aggressively monitored by sensitive assays and upon detection, antiviral therapy is administered preemptively to prevent its progression to clinical disease); and (2) antiviral prophylaxis (wherein

antiviral drugs such as ganciclovir and valganciclovir are administered to patients at risk of CMV disease after liver transplantation<sup>[19]</sup>. Both strategies are highly effective in preventing CMV disease after liver transplantation<sup>[4,5,54-57]</sup>. However, antiviral prophylaxis is generally regarded as a more efficient approach and is used by the majority of transplant centers in preventing primary CMV disease in high-risk CMV D+/R- liver transplant recipients<sup>[4,8,54]</sup>. Indeed, the current American Society of Transplantation recommendation is to use antiviral prophylaxis in all CMV D+/R- liver (and other solid organ transplant) recipients<sup>[58]</sup>. Moreover, primary antiviral prophylaxis has the added benefit of reduction in bacterial and fungal opportunistic infections and mortality<sup>[33,34]</sup>.

### **Preemptive therapy**

The basic principle of preemptive therapy is to detect the presence of CMV reactivation prior to the onset of clinical symptoms, so that antiviral drugs are administered early in order to halt the progression of asymptomatic infection to full-blown clinical disease<sup>[50,54,55,57,59]</sup>. The success of this approach relies on patient compliance with CMV surveillance<sup>[60]</sup>, availability of highly sensitive CMV assay that predicts the risk of disease<sup>[61]</sup>, and early administration of antiviral drugs such as IV ganciclovir and oral valganciclovir<sup>[9,55,59]</sup>. With the advance in molecular diagnostic microbiology, including the availability of polymerase chain reaction (PCR), it is now possible to employ successfully preemptive therapy in liver transplant recipients (reviewed in<sup>[61]</sup>). Several studies have reported the success of IV or oral ganciclovir and valganciclovir in the preemptive treatment of CMV reactivation in liver transplant recipients, including high-risk CMV D+/R-patients<sup>[56,59]</sup>. However, some studies have indicated that preemptive therapy may not be completely effective in CMV D+/R- liver transplant recipients since the replication kinetics of CMV in immune-deficient individuals is so rapid<sup>[49]</sup> that it may result in clinical illness prior to CMV detection with once a week PCR assay<sup>[8,54]</sup>. Indeed, in our clinical experience, nearly 25% of CMV D+/R- liver transplant recipients who developed CMV disease were not identified early by a protocol-based weekly CMV PCR assay<sup>[8,54]</sup>. Accordingly, the current guideline from the AST does not recommend preemptive approach in CMV D+/R- liver transplant recipients<sup>[58]</sup>. However, this approach is recommended, and is highly effective, in CMV-seropositive liver transplant recipients. Reassuringly, clinical trials have demonstrated the efficacy of preemptive therapy in CMV disease prevention<sup>[54-56,59]</sup>. Three meta-analyses that collectively analyzed data from prospective clinical trials confirmed the efficacy and benefits of preemptive therapy in the prevention of CMV disease<sup>[34,35,62]</sup>. When conducted properly, preemptive therapy, with the use of oral ganciclovir, IV ganciclovir, or valganciclovir resulted in reduction of CMV disease by about 70%<sup>[34,35,62]</sup>. Moreover, preemptive therapy is not associated with late onset CMV disease (unlike with antiviral prophylaxis,

as discussed below)<sup>[55,59]</sup>. Currently, valganciclovir is the most commonly used drug for preemptive therapy, and in one study, was demonstrated to be as effective in terms of clinical and virologic response, when compared with IV ganciclovir<sup>[55,59]</sup>. In addition, preemptive therapy may be beneficial in reducing the indirect effects of CMV. In one study, the incidence of major opportunistic infections, bacteremia, bacterial infection, HCV recurrence, and rejection were not significantly different between liver transplant patients who received preemptive therapy and those who did not have CMV reactivation<sup>[63]</sup>.

### **Antiviral prophylaxis**

Several clinical trials have demonstrated that antiviral prophylaxis is highly effective in preventing the direct, and possibly the indirect effects of CMV after liver transplantation<sup>[4,5]</sup>. Recent meta-analyses have highlighted the clinical benefits<sup>[34,35,62]</sup>. Compared to placebo or no treatment, patients who received antiviral prophylaxis had lower incidence of CMV disease (58%-80% reduction) and CMV infection (about 40% reduction)<sup>[62]</sup>. In one meta-analysis, a 25% reduction in the incidence of acute allograft rejection was also observed<sup>[34]</sup>. In two studies, a reduction in all-cause mortality was also observed<sup>[34,62]</sup>, mainly due to a decline in CMV-related death<sup>[62]</sup>. A reduction in the incidence of other herpes viruses, bacterial, and protozoal infections was also observed<sup>[62]</sup>. Indeed, a survey of several transplant centers showed a general preference for antiviral prophylaxis over preemptive therapy in the prevention of CMV disease in CMV D+/R- and R+ liver transplant recipients.

### **Acyclovir prophylaxis**

The use of acyclovir as anti-CMV prophylaxis after liver transplantation has been supplanted by ganciclovir (and valganciclovir) because of the superior efficacy of the latter drugs in CMV disease prevention. In a study on 143 liver transplant recipients, CMV infection developed in 61% patients who received 3 mo of high-dose oral acyclovir compared to 24% patients who received 14 d of IV ganciclovir followed by 3 mo of acyclovir ( $P < 0.001$ )<sup>[64]</sup>. In a second study, 57% and 23% patients in the acyclovir group compared to 37% and 11% patients in the ganciclovir-acyclovir group developed CMV infection and disease, respectively<sup>[52]</sup>. In a third randomized controlled trial on 250 liver transplant recipients, CMV infection and disease occurred in 38% and 10% of patients in the acyclovir group, respectively, compared to 5% and 1% in the ganciclovir group, respectively<sup>[65]</sup>.

### **Ganciclovir prophylaxis**

The current data indicates that ganciclovir-based regimen is more effective (compared to acyclovir and immunoglobulins) in reducing the incidence of CMV after liver transplantation. In one study, the administration of IV ganciclovir for 90-100 d reduced the incidence of CMV disease in CMV D+/R- liver

transplant recipients to 5.4% (compared to 40% in patients who received < 7 wk of prophylaxis)<sup>[65]</sup>. The major drawback to IV ganciclovir was the need for long-term IV access and the risk of thrombosis, phlebitis, and line-associated infections<sup>[37,66]</sup>. Subsequently, oral ganciclovir became available, and in a landmark randomized trial that compared the drug with placebo, oral ganciclovir for 98 d reduced significantly the 6-mo incidence of CMV infection (51.5% vs 24.5%;  $P < 0.001$ ), and CMV disease (19% vs 5%;  $P < 0.001$ ) in liver transplant recipients<sup>[4]</sup>, including CMV D+/R-patients (44% vs 15%,  $P = 0.02$ ) and patients who received antilymphocyte antibodies (33% vs 5%;  $P = 0.002$ )<sup>[4]</sup>. Among CMV R+ liver transplant recipients, oral ganciclovir for 12 wk reduced the incidence of CMV disease to 1% (compared to 7% in patients who received acyclovir)<sup>[67]</sup>. These studies were in support of the United States FDA approval of oral ganciclovir prophylaxis for the prevention of CMV disease in liver transplant recipients. Oral ganciclovir, however, is poorly absorbed, and its oral administration results in low systemic ganciclovir levels<sup>[68]</sup>. This factor has been implicated in the emergence of ganciclovir-resistant CMV in certain clinical settings<sup>[69,70]</sup>, such as high-risk CMV D+/R- patients, and those receiving potent immunosuppressive regimens.

### **Valganciclovir prophylaxis**

Valganciclovir, a valine ester of ganciclovir, which results in enhanced absorption, resulting in systemic drug levels that are comparable to IV ganciclovir<sup>[68,71]</sup>. Pharmacokinetic studies indicate that a 900 mg dose of valganciclovir achieves a similar daily area under the concentration time curve (AUC<sub>24</sub>) as an IV dose of 5 mg/kg of ganciclovir<sup>[68]</sup>. The role of valganciclovir in the prevention of CMV disease after liver transplantation was evaluated in a multicenter randomized non-inferiority clinical trial that compared it with oral ganciclovir in a cohort of 364 CMV D+/R- solid organ transplant (including liver) recipients (Figure 1)<sup>[5]</sup>. Overall, the 6-mo incidence of CMV disease was 12% and 15% in the valganciclovir and oral ganciclovir groups, respectively<sup>[5]</sup>. Follow-up at one year, demonstrated that the incidence of protocol-defined CMV disease in all patients was 17.2% and 18.4% with valganciclovir and oral ganciclovir, respectively<sup>[5]</sup> (Notably, the incidence of investigator-determined CMV disease cases was about 28% and 30%, respectively).

However, in 177 liver transplant recipients who participated in the clinical trial, the incidence of CMV disease was 19% in the valganciclovir group as opposed to only 12% in the ganciclovir group<sup>[5]</sup>. There was also a higher incidence of tissue-invasive CMV disease in the valganciclovir group. While the clinical trial was not designed to determine differences between the transplanted organs, these results raised skepticism about the efficacy of valganciclovir prophylaxis after liver transplantation. As a result of these findings, valganciclovir prophylaxis did not gain approval from the US-FDA for prophylaxis against CMV disease after

liver transplantation (valganciclovir received approval for prevention of CMV disease in heart, kidney, and pancreas recipients). Although not FDA-approved for prophylaxis in liver transplant recipients, valganciclovir is the most widely used drug for the prevention of CMV disease after liver transplantation<sup>[72]</sup>.

The efficacy of valganciclovir (and oral ganciclovir) prophylaxis is undermined by the emergence of late-onset CMV disease (Figure 1). In a retrospective study on 203 liver transplant recipients who received valganciclovir 900 mg daily for 3 to 6 mo, the overall incidence of CMV disease was 14%<sup>[73]</sup>. The incidence varied among the different CMV serogroups (16% in D+/R+ group; 7% in D-/R+ group; and 26% in D+/R-group)<sup>[73]</sup>. These findings illustrate that the burden of delayed-onset CMV disease remains high particularly in the CMV D+/R- group<sup>[5]</sup>. In our analysis of 67 CMV D+/R- liver transplant recipients who received 3 mo of oral ganciclovir and valganciclovir prophylaxis, the two year incidence of CMV disease was 29%<sup>[3]</sup>. The incidence of delayed-onset CMV disease was not significantly different between patients who received oral ganciclovir or valganciclovir (22% vs 28%;  $P = 0.63$ )<sup>[3]</sup>.

### **Maribavir prophylaxis (investigational)**

The search for anti-CMV strategies continues to evolve with the recent entry of maribavir into clinical trials. Maribavir, a novel benzimidazole riboside compound that inhibits viral DNA assembly and egress of viral capsids<sup>[74]</sup>, is now undergoing clinical trials for the prevention of primary CMV disease after liver transplantation<sup>[75,76]</sup>. Because it has a unique mechanism of action that is distinct from ganciclovir, foscarnet, and cidofovir (all of which act to inhibit CMV DNA polymerase), maribavir is expected to expand the therapeutic armamentarium against CMV<sup>[75]</sup>. So far, it does not show cross-resistance with the currently available drugs. Therefore, it has a good potential as an alternative drug for the treatment of ganciclovir-resistant CMV. In addition, maribavir provides a more favorable toxicity profile compared to foscarnet and cidofovir, both of which are highly nephrotoxic. In preliminary studies conducted in allogeneic bone marrow transplant recipients, maribavir was found to be safe and did not have myelosuppressive effects. In terms of efficacy, when compared with placebo, maribavir showed significant reduction in CMV viremia<sup>[76]</sup>. The ongoing comparative multicenter trial of maribavir and oral ganciclovir in liver transplant recipients will likely complete enrollment in 2009. In this multi-center international randomized trial, the incidence of CMV disease will be compared between patients randomized to oral maribavir, and the currently approved standard oral ganciclovir.

### **The challenge of delayed- and late-onset CMV disease**

With the success of a 3-mo anti-CMV prophylaxis program (in terms of the almost complete elimination of CMV disease in individuals who are actively taking antiviral drugs), the challenge of delayed- and late-onset CMV disease has emerged. Indeed, in many

high-risk CMV D+/R- individuals, the use of antiviral prophylaxis has only delayed the onset of CMV disease to 3-6 mo after liver transplantation<sup>[3-5,12]</sup>. In one of these retrospective studies, CMV disease occurred in 14 of 54 (26%) CMV D+/R- liver transplant recipients who received valganciclovir for at least 3 mo<sup>[73]</sup>. Our clinical data suggests that, while no breakthrough CMV disease occurred during the 3 mo of oral ganciclovir or valganciclovir prophylaxis, 29% of CMV D+/R- liver transplant recipients developed delayed-onset primary CMV disease<sup>[3]</sup>. Thus, one out of every four CMV D+/R-liver transplant recipients will develop CMV disease after cessation of antiviral prophylaxis<sup>[3]</sup>. Delayed-onset CMV disease commonly presents as CMV syndrome, with fever and bone marrow suppression<sup>[3]</sup>. In less than half of the patients, CMV manifested as tissue-invasive disease, and frequently affected the gastrointestinal tract<sup>[3]</sup>. Factors such as age<sup>[3]</sup>, female gender<sup>[3,77]</sup>, renal dysfunction<sup>[77]</sup>, and allograft rejection<sup>[12]</sup> predisposed to the development of delayed-onset primary CMV disease<sup>[3,12,77,78]</sup>. Delayed-onset CMV disease appears to be clinically less severe, although it is associated with significant mortality after liver transplantation<sup>[32]</sup>. Therefore, a better method for CMV prevention is needed among CMV D+/R- liver transplant recipients.

Currently, there is an ongoing effort (in kidney transplant recipients only) to assess the efficacy and safety of 3 mo *vs* 6 mo of valganciclovir prophylaxis. Foreshadowing what may be expected from this trial, a recent single center study on 68 CMV D+/R- kidney transplant recipients demonstrated a significantly lower incidence of CMV disease in patients who received 24 wk compared to 12 wk of oral ganciclovir prophylaxis (7% *vs* 31%, respectively)<sup>[79]</sup>. If this practice is proven safe and effective, it may eventually be adopted in the liver transplant field. There are concerns regarding ganciclovir resistance, drug toxicity, and cost with such a prolonged prophylactic approach. In addition, the long-term drug toxicity of ganciclovir-based regimen is not known. In animal studies, ganciclovir has been shown to be mutagenic, teratogenic, carcinogenic, and has caused aspermatogenesis, although the clinical relevance of these findings in humans is unclear<sup>[68]</sup>.

Another strategy that is gaining interest is an aggressive effort to minimize immunosuppression, including the use of prednisone-free regimens. In one Kidney and Pancreas Transplant Program, the incidence of CMV disease was markedly reduced in patients receiving a steroid-free immunosuppressive regimen<sup>[80]</sup>. Many liver transplant programs (including ours) have adapted this approach, and have minimized immunosuppression gradually so that patients are maintained on tacrolimus monotherapy beyond the 4th mo after liver transplantation. In a retrospective analysis, we observed a higher incidence of CMV disease among transplant recipients who were still receiving mycophenolate mofetil and prednisone at the time they discontinue antiviral prophylaxis. The major consequence of this approach, however, is the risk of allograft rejection when the level of immunosuppression

is reduced to levels lower than necessary for the prevention of allo-stimulation<sup>[13]</sup>.

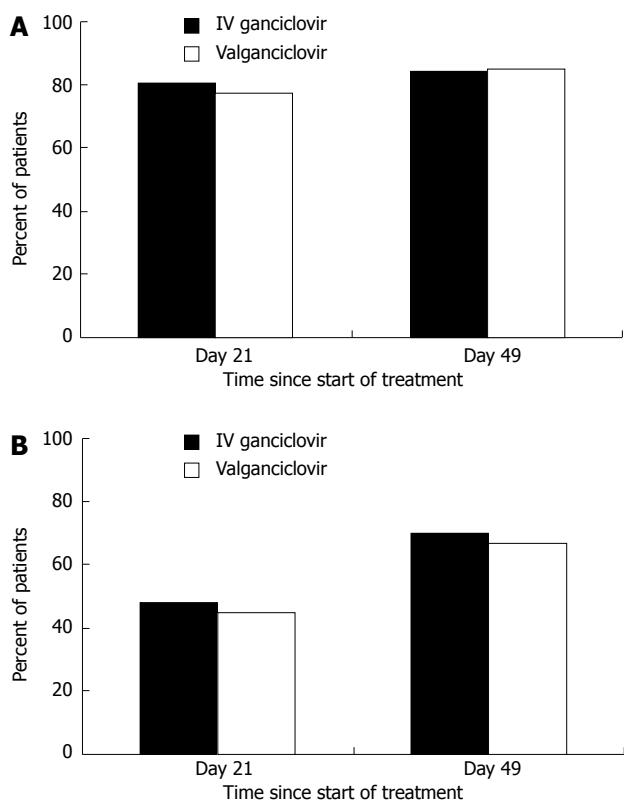
## TREATMENT OF CMV DISEASE AFTER LIVER TRANSPLANTATION

The current recommendation for antiviral treatment of CMV disease after liver transplantation is IV ganciclovir<sup>[58,66,81]</sup>. Equally important is the reduction in the degree of pharmacologic immunosuppression<sup>[19]</sup>. Oral ganciclovir should not be used for the treatment of active CMV disease because of its poor bioavailability<sup>[19]</sup>. Valganciclovir, a prodrug of ganciclovir that provides high systemic ganciclovir concentrations<sup>[71]</sup>, has now made it possible for oral treatment of CMV disease<sup>[68,81]</sup>. Indeed, in AIDS patients, valganciclovir is approved as induction and maintenance treatment of CMV retinitis<sup>[82]</sup>. There is good clinical data to support the use of valganciclovir for the treatment of CMV after solid organ transplantation<sup>[81]</sup>. Viral kinetic studies showed comparable viral decay between IV ganciclovir and valganciclovir<sup>[50]</sup>. In a recent study, 321 solid organ (including liver) transplant recipients with non-severe CMV disease were randomized to valganciclovir or IV ganciclovir for a fixed 21-d course, followed by valganciclovir maintenance treatment for 4 wk; the proportion of patients with viral eradication at 21 and 49 d were comparable in the IV ganciclovir and valganciclovir groups (Figure 2)<sup>[81]</sup>. The overall time to viral eradication was 21 d with valganciclovir and 19 d with IV ganciclovir<sup>[81]</sup>. The calculated viral decay was 11.5 d with valganciclovir and 10.4 d with IV ganciclovir<sup>[81]</sup>. Likewise, clinical resolution was not different between the two groups. It was noted that patients enrolled in this trial were mostly CMV-seropositive, the majority were kidney recipients, and patients with severe CMV disease were excluded. Despite these limitations, this pivotal trial now supports the use of valganciclovir for oral treatment of CMV disease, at least in selected transplant patients<sup>[81]</sup>. In many instances, valganciclovir is used as a step-down treatment when the clinical symptoms have resolved after an initial induction treatment with IV ganciclovir.

The duration of treatment of CMV disease should be individualized<sup>[58,83]</sup>. The persistence of the virus at the end of therapy (by polymerase chain reaction [PCR] or pp65 antigenemia) is associated with a higher risk of clinical relapse<sup>[84]</sup>. It is now generally accepted that multiple (at least two) weekly negative CMV PCR results should be obtained before antiviral therapy is discontinued. Although this may be true for non-tissue invasive CMV syndromes, the utility of such an approach may not necessarily apply to tissue-invasive disease, which may manifest as “compartmentalized disease”<sup>[19]</sup>.

### ***The challenge of treating compartmentalized CMV disease***

Compartmentalized CMV disease refers to clinical



**Figure 2** The proportion of solid organ transplant patients with resolution of clinical symptoms (A) and viremia eradication (B) at day 21 and 49 following the start of valganciclovir or IV ganciclovir treatment of CMV disease. Data obtained from the study by Asberg *et al*<sup>[81]</sup>.

syndromes wherein the virus is detected in the affected tissues but is minimally detectable or undetectable in the blood<sup>[19]</sup>. In the current era, gastrointestinal CMV disease (in the form of gastritis, esophagitis, enteritis, colitis) constitutes the vast majority of tissue-invasive patients<sup>[3,19]</sup>, and in a number of cases, this type of CMV disease is “compartmentalized.” Such a clinical presentation is reminiscent of CMV retinitis, a very rare manifestation of tissue-invasive CMV disease after transplantation, that is often not accompanied by viremia<sup>[82,85]</sup>. This dilemma brings to the forefront the limitation of viral load monitoring in assessing duration of treatment. In our clinical practice, it is not uncommon to have negative blood PCR assay even when there is histologic evidence of tissue invasion. Accordingly, it has become a more common practice to perform colonoscopy or upper endoscopy to document clearance of gastrointestinal CMV disease prior to discontinuation of therapy. Our anecdotal experience however indicates that this may not be necessary in mild to moderate disease as long as sufficient therapy is provided.

### The challenge of treating ganciclovir-resistant CMV disease

Ganciclovir-resistant CMV is now emerging as an important complication of prolonged antiviral drug use after transplantation<sup>[2,19,70]</sup>. Currently, ganciclovir-resistant CMV is very rarely seen in liver transplant recipients (it is more common after kidney-pancreas and

lung transplantation). Unlike lung and kidney-pancreas transplant recipients who have rates as high as 9% and 13%, respectively, the estimated incidence of ganciclovir resistant CMV after liver transplantation is < 0.5%<sup>[70,86]</sup>. Several studies have identified risk factors for ganciclovir-resistant CMV<sup>[2,19,70]</sup>, including CMV D+/R- status, high levels of viral replication, potent immunosuppressive therapy, and suboptimal ganciclovir levels. The vast majority of drug-resistant cases involve the selection of viral strains with UL97 (kinase) mutation<sup>[2,19,70,75,87]</sup>. UL97 mutation generally confers resistance to ganciclovir, although in some cases, a concomitant UL54 mutation (CMV DNA polymerase) is also observed, in which case, cross-resistance with cidofovir and/or foscarnet is likely. As noted, no cross-resistance has been observed with the investigational drug, maribavir.

Drug-resistant CMV is associated with significant morbidity and mortality, and there is a very limited number of antiviral drugs (which are often toxic) available for treatment<sup>[86]</sup>. Drug-resistant CMV should be suspected when viral load or antigenemia rises or does not decline to undetectable levels despite IV ganciclovir treatment. The diagnosis is confirmed by genetic analysis to demonstrate mutational changes in UL97 and UL54 genes encoding for kinase and polymerase, respectively<sup>[70,86]</sup>. In our retrospective study of 225 CMV D+/R- solid organ transplant recipients who received 3 mo of valganciclovir prophylaxis, CMV disease occurred in 65 patients (29%), including four (8%) caused by drug-resistant CMV, judged by the failure of the viral load to decline to undetectable levels while on IV ganciclovir treatment<sup>[70,88]</sup>. In our cohort, one liver transplant recipient was clinically suspected to have ganciclovir-resistant strain, although the genotypic assay failed to document any mutations<sup>[88]</sup>. The treatment of ganciclovir-resistant CMV should be guided by genotypic analysis. In patients where foscarnet or cidofovir was used, nephrotoxicity was a major adverse effect<sup>[88]</sup>. Other potential drugs for the treatment of multi-drug resistant CMV include the off-label use of immunoglobulins and leflunomide, although data supporting their use are only anecdotal<sup>[19]</sup>. The potential clinical utility of maribavir in the treatment of resistant CMV is highly anticipated<sup>[74-76,87]</sup>.

### CONCLUSION

Remarkable advances in molecular diagnostics and therapeutics has led to marked reduction in the incidence and severity of CMV disease after liver transplantation, and a parallel decline in the associated morbidity and mortality. However, despite these improvements, CMV remains a common infectious complication and continues to negatively influence the outcome of liver transplantation. In addition to viral factors and pharmacologic immunosuppression, the role of innate and adaptive immune deficiencies is being recognized in the pathogenesis of CMV disease after liver transplantation. Such novel findings should provide additional avenues and opportunities for

improving our management strategies. Prevention of CMV with antiviral prophylaxis and preemptive therapy is effective, although a well-controlled trial assessing these two strategies in a head-to-head comparison is yet to be conducted after liver transplantation. Currently, valganciclovir prophylaxis is the most common approach for the prevention of CMV disease in CMV D+/R- and R+ liver transplant recipients. The availability of predictive diagnostic tests has paved the way for the successful use of preemptive therapy in preventing the progression of CMV reactivation to clinical disease even in high-risk liver transplant patients. IV ganciclovir remains the standard of treatment for established CMV disease, although valganciclovir has now been shown to be equally effective in the treatment of mild to moderate CMV diseases. The duration of treatment should be individualized, depending upon clinical and laboratory parameters such as the decline of CMV load in the blood as measured by rapid and sensitive molecular testing. In this context, it is generally recommended that treatment should be continued until all evidence of active infection, such as positive CMV viral load, has resolved. Ganciclovir-resistant CMV and compartmentalized tissue-invasive disease (most commonly with gastrointestinal CMV disease) are emerging challenges to the management of CMV after liver transplantation. These, together with the common occurrence of late-onset CMV disease in high-risk patients, should serve as catalysts to the ongoing search for the optimal preventive strategy for CMV disease after liver transplantation.

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## Cytokine orchestration in post-operative peritoneal adhesion formation

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### Abstract

Peritoneal adhesions are a near inevitable occurrence after laparotomy and a major cause of both patient and physician misery. To date, clinical attempts at their amelioration have concentrated on manipulating the physical factors that affect their development despite a wealth of experimental data elucidating the molecular mechanisms that underlie their initiation, development and maturation. However, the advent of targeted, specific anti-cytokine agents as directed therapy for inflammatory and neoplastic conditions raises the prospect of a new era for anti-adhesion strategies. To harness this potential will require considerable cross-disciplinary collaboration and that surgeon-scientists propel themselves to the forefront of this emerging field.

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**Key words:** Postoperative peritoneal adhesion formation; Cytokines; Vascular endothelial growth factor

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### INTRODUCTION

Post-operative peritoneal adhesion formation remains a considerable source of patient and physician frustration and a significant burden on hospital resources<sup>[1]</sup>. As the commonest cause of small bowel obstruction in patients who have previously undergone laparotomy, adhesions account for 40% of all cases of intestinal obstruction and 60%-70% of those affecting the small bowel. After a first such clinical episode, 53% of patients will go on to develop a second relapse, and 83% of these will have chronic symptoms<sup>[2]</sup>. Some 14% of those who manifest overt adhesive intestinal obstruction do so within 2 years of their initial surgery, with 2.6% requiring operative adhesiolysis for its relief<sup>[3]</sup>. Furthermore, approximately 20% of patients developing adhesional bowel obstruction do so at a remove of more than ten years after their index operation<sup>[4]</sup>. Post-operative adhesions are also a common cofactor in female infertility in those with prior laparotomy<sup>[5,6]</sup> and they add markedly to the technical complexity of any repeat abdominal operation. By doing so, they give rise to considerable surgeon frustration<sup>[7]</sup> and a heightened risk of patient morbidity<sup>[8]</sup>.

For all these reasons, this iatrogenic complication weighs heavily on the balance books of health care providers. Indeed, in overall costs, the financial cost due to adhesion-related morbidity approximates the expenditure required for the surgical management of gastric or rectal cancer<sup>[9]</sup> and this is then further compounded by the cost of medicolegal claims and settlements. Finally, the considerable number of bed-days consumed by the sequelae and treatment of post-operative adhesions (indeed in Finland, adhesion-related admissions exceeds the number of bed-days appropriated to varicose vein surgery) also reinforces the urgency for developing effective means of adhesion abrogation.

Unfortunately, however, clinical strategies and therapies aimed at controlling or alleviating adhesion formation have been largely inadequate in their address of both ongoing human suffering<sup>[10]</sup> and economic cost<sup>[11]</sup>. To date these attempts have mostly concentrated on employing physical means to align<sup>[12-14]</sup> or separate<sup>[15]</sup> adjacent loops of bowel in the early post-operative period (so that any configuration of interloop bands is either organised or hindered respectively) or have focused on manipulating peritoneal fibrinolytic mechanisms<sup>[16-18]</sup>.

## CYTOKINE ORCHESTRATION IN POST-OPERATIVE ADHESION FORMATION

Adhesions however represent a form of secondary wound healing. Therefore the mesothelial tissue response to injury (occurring either directly due to handling and dissection or indirectly due to desiccation, cooling or relative ischaemia at sites both adjacent to and distant from the actual operative site) is initiated locally and thence both propagated and orchestrated by cytokine signaling. Although systemic<sup>[19]</sup> and genetic elements<sup>[20]</sup> may also influence the severity of the cascade and factors such as bacterial contamination can potentiate it<sup>[21]</sup>, interruption or manipulation of key cellular processes early in the response cascade would seem likely to markedly diminish all downstream events including the ultimate fibrotic endpoint. Furthermore, the increasing sophistication of anti-cytokine therapies now allows single components of complex cellular processes to be specifically targeted. In addition, potentially efficacious agents have already been proved both safe and useful in the management of anti-neoplastic<sup>[22]</sup> and anti-inflammatory conditions<sup>[23]</sup>. Therefore a new era in the approach to adhesion amelioration may be in the offing.

## SPECIFIC TARGETTING OF SELECTED CYTOKINES

There has of course been a vast array of cytokines and chemokines implicated in the initiation, development and maturation of abdominal adhesions after laparotomy (Table 1) and therefore it may initially appear forbidding to try and narrow the therapeutic target most likely to lead to unopposed benefit. Tumor necrosis factor was one of the earliest cytokines investigated and certainly seems to represent one important factor. However its recent elucidation as a key mediator of the bacterial response to infection seems to mitigate against using monoclonal antibodies (already commercially available) to abrogate this cytokine early after intestinal operation<sup>[24]</sup>. Equally, the variability of action depending on the relative proportions of its isoforms and the central role it plays in wound healing would also seem to deter use of directed therapy against transforming growth factor-beta. Of the remaining candidate targets the majority only really have a slender evidence base to support their selection from out of the general post-operative molecular milieu. The one exception, at present, would seem to be vascular endothelial growth factor (VEGF).

Although this important signaling protein is best known as a potent angiogenic cytokine (and indeed may be proposed as having a role in the process of adhesion growth through the induction of new blood vessels into areas of operative tissue injury<sup>[25]</sup>), VEGF is now also well established as being directly involved in restorative tissue processes, including early inflammatory responses, as well as wound repair and remodeling *via* effecting fibroblast function<sup>[26]</sup>. Furthermore, the central role of VEGF in facilitating increased vascular permeability

(essential for the early proinflammatory response to injury) as well as the subsequent deposition of the fibrin-rich matrix necessary for subsequent cellular migration and proliferation<sup>[27,28]</sup> would seem to make it a prime putative agent in the formation of peritoneal adhesions. It is not surprising therefore that VEGF has been consistently positively implicated (albeit non-selectively) in this process<sup>[29]</sup>. The realization that peritoneal mast cells both constitutively and inducibly express this cytokine<sup>[30,31]</sup> further suggests an intriguing link given that these cells are known also to be central to adhesion formation<sup>[32]</sup>. However, it may well be that rather than through direct secretion, mast cells effect the threshold concentration of this cytokine by exciting the egress of neutrophils and monocytes from the circulation into the peritoneum and that it is these cells that instead then contribute most to regional VEGF levels.

Regardless of its exact cellular origin, VEGF seems to represent an ideal target as its levels correlate with adhesion formation in animal models with its regulation (either positively<sup>[19]</sup> or negatively<sup>[32]</sup>) affecting the degree to which they form after peritoneal operations. The clinical success and safety of VEGF neutralization by a specific monoclonal antibody in the treatment of malignant diseases<sup>[33]</sup> adds further impetus to the need to try its pharmacological manipulation as an anti-adhesion strategy particularly as selective therapeutic targeting of the cytokine does not seem to disrupt operative wound healing in a clinically important fashion<sup>[34]</sup>.

## DETERMINATION OF CLINICAL EFFICACY

Clinical evidence of efficacy of anti-adhesion therapies is notoriously difficult to attain as second look-laparotomy to assess distribution and intensity of peritoneal reaction is not ethically justifiable (although may be possible in the case of certain gynecological procedures<sup>[35]</sup>). Additionally, the mere presence of adhesions, even if extensive, does not necessarily correlate with the incidence and severity of subsequent symptomatic episodes and long-term follow-up is required to determine the full-extent of the problems arising. These challenges are not however insurmountable as have been shown by those who advance the cause of bioactive substances<sup>[36,37]</sup> and the difficulties that would be encountered in establishing a progressing and adequately powered multi coated blinded study would be markedly outweighed by the huge benefit to patients of many differing specialties. With regard to monoclonal antibody therapies in particular, there now exists the opportunity to piggy-back on the human safety testing performed on this class of drug in alternative settings. While pursuit of molecular mechanisms for adhesion amelioration will undoubtedly still be expensive<sup>[38]</sup>, the cost incurred by the management of adhesion-related morbidity<sup>[39,40]</sup> economically justifies considerable investment in any potential means of their attenuation.

## CONCLUSION

There have long been a multitude of groups proposing

**Table 1** Overview of literature to date regarding cytokine orchestration in postoperative adhesion formation. Included in the list are cytokines, chemokines, and proteases as well as trigger enzymes

Cytokine <sup>Ref</sup>	Mechanism investigated	In vitro/vivo	Species	Experimental model	Effect on adhesion formation
Heparin-binding growth factor <sup>[41]</sup>	Macrophage and neutrophil omental migration	In vivo	Mouse	(1) Partial hepatectomy (2) Omental adherence	Exacerbated by Midkine- omental inflammation reduced
HGF <sup>[42]</sup>	Mesothelial cell proliferation and migration	Both	Rat	Cecal abrasion	Exacerbated by local HGF gene transfer
IFN-γ, HGF <sup>[43]</sup>	Natural killer T cell activity	Both	Mouse	Cecal cauterization	Attenuated by HGF
IL-1 <sup>[44]</sup>	Nonspecific inflammation	In vivo	Rat	Cecal abrasion	Exacerbated by IL-1
IL-1, TNF <sup>[45]</sup>	Proinflammatory markers	In vivo	Human	Adhesion samples	IL-1 & TNF-α associated with adhesion
IL-1, IL-6, TNF-α <sup>[46]</sup>	Cellular mediation	In vitro	Human	Peritoneal fluid sampling	Adhesions associated with IL-6 and IL-1
IL-10 <sup>[47]</sup>	Natural antiinflammatory	In vivo	Mouse	Peritoneal injury	Attenuated by IL-10 but no effect with IL-10 mAb. No association with IL-10 levels
IL-10 <sup>[48]</sup>	Immunosuppression	In vivo	Mouse	Peritoneal injury	Attenuated by IL-10
IL-1b, TNF-α, TGF-β1, IL-10, IFNg, GM-CSF <sup>[49]</sup>	Inflammatory	In vitro	Human	Peritoneal fluid sampling	Only IFN-γ and TGF-β1 associated with adhesion formation. No association found with other cytokines.
IL-6 <sup>[50]</sup>	Early proinflammatory effects	In vivo	Rat	Cecal abrasion with C <sub>2</sub> H <sub>5</sub> OH	Exacerbated by IL-6, attenuated by monoclonal Ab to IL-6
PAF <sup>[51]</sup>	Early inflammatory mediators	In vivo	Rat	Uterine horn abrasion	Adhesions and IL-6 levels attenuated by Lexipafant (PAF antagonist)
Substance P <sup>[52]</sup>	Substance P mediation	In vivo	Rat	Peritoneal ischaemic buttons	Substance P and TGF-β1 as well as ICAM-1 and VCAM-1 increased
TGF <sup>[53]</sup>	TGF isoforms	In vivo	Mouse	Serosal abrasion and apposition	Exacerbated by TGF-β3, attenuated by combined TGF-β1 and TGF-β2 mAB
TGF-β <sup>[54]</sup>	TGF-β regulation of extracellular matrix	In vitro	Human	Human fibroblast culture	Dichloroacetic acid inhibited fibronectin and collagen type III expression
TGF-β <sup>[55]</sup>	Chemoattraction	In vitro	Rat	Cecal abrasion	TGF-β mRNA increased by trauma
TGF-β <sup>[56]</sup>	Mast cells	In vivo	Hamster	Uterine horn abrasion	Exacerbated by chymase inhibitor
TGF-β <sup>[57]</sup>	Chemoattraction	In vivo	Rat	Uterine horn abrasion	Exacerbated by TGF-β
TGF-β <sup>[58]</sup>	Mast cells	In vitro	Human	Cell culture	TGF-β and tryptase increased collagen
TGF-β <sup>[59]</sup>	Peritoneal repair	In vivo	Rat	Uterine horn abrasion	No antiadhesion effect of anti-TGF mAb
TGF-β <sup>[60]</sup>	Immunosuppression	In vivo	Rat	Small bowel transplant	Adhesions attenuated by tacrolimus
TGF-β <sup>[61]</sup>	Mast cells	In vivo	Rat	Uterus scraping	TGF-β increased by trauma, adhesions attenuated by chymase inhibition
TGF-β <sup>[62]</sup>	Cellular effects of Tisseel	In vitro	Human	Cell culture	Fibroblasts TGF-β reduced
TGF-β, MMP-9, TIMP-1 <sup>[63]</sup>	Matrix factors	In vivo	Human	Sampled peritoneal fluid	Adhesion assor with reduced MMP-9 but elevated MMP-9/TIMP-1 ratio
TGF-β/MDF <sup>[64]</sup>	Carboxymethylcellulose sponge	In vivo	Rat	Cecal denudation & apposition	Effect of sponge independent to cytokine release (barrier function)
TGF-β1 <sup>[65]</sup>	Chemoattraction	In vitro	Human	Cell culture	TGF-β1 increased in scar tissue
TGF-β1 <sup>[66]</sup>	Extracellular matrix	In vivo	Mouse	Cecal abrasion	Exacerbated by haploid insufficiency
TGF-β1 <sup>[67]</sup>	Fibrinolysis	In vitro	Human	Biopsy sampling	Attenuated by TGF-β1 overexpression
TGF-β1 <sup>[68]</sup>	Peritonitis	In vivo	Rat	Cecal ligation and puncture	Peritonitis upregulates TGF-β1 expression
TGF-β1 <sup>[69]</sup>	Mitogenicity of macrophages & fibroblasts	In vivo	Rat	Small Bowel transection and re-anastomosis	Adhesions and TGF-1 levels attenuated by ACE inhibition
TGF-β1, MMP1&2, TPA, TIMP-1 <sup>[70]</sup>	Cellular effects of seprafilm	In vitro	Human	Human fibroblast & mesothelial cell culture	No cytokine effect induced by Seprafilm (barrier effect important)
TGF-β1, TGF-β2 <sup>[71]</sup>	Basal expression	In vitro	Human	Biopsy sampling	Sit-specific TGF-β1 & TGF-β3 expression
TGF-β1 <sup>[72]</sup>	Cellular effects of changtong	In vivo	Rat/rabbit	Cecal abrasion	TGF-β reduced in rats
TNF, IL-1, IL-6 <sup>[73]</sup>	Effects of gloves and powders	In vivo	Rat	Cecal abrasion	Adhesions increased by glove powder
TNF-α <sup>[74]</sup>	Proinflammatory effects of TNF-α	In vivo	Rat	Cecal abrasion	Adhesion formation attenuated by infliximab but no histological effect
TNF-α, IL-1 <sup>[75]</sup>	Proinflammatory markers	In vivo	Rat	Cecal abrasion or small bowel resection	TNF-α appears a good biological marker for adhesion formation
TNF-α, IL-1 <sup>[76]</sup>	Immunosuppression	In vivo	Rat	Cecal abrasion	Adhesion formation attenuated by mAbs to IL1 and IL-1/TNF-α
TNF-α, IL-6 <sup>[77]</sup>	Proinflammatory mediators	In vitro	Mouse	Murine macrophages	Adhesion formation attenuated by hyaluronic acid and dexamethasone
TNF-α, MMP <sup>[78]</sup>	Mesothelium reaction to peritoneal injury	In vivo	Rat	Peritoneal wounding	No effect of MMP & TACE inhibition, TNF-α may not be adhesiogenic
TNF-α, TGF-β1 <sup>[79]</sup>	PROACT to injured peritoneum	In vivo	Human	Tissue sampling	TNF-α and TGF-β reduced by heating
VEGF <sup>[80]</sup>	Angiogenesis	In vivo	Rat	Uterus-peritoneal scrub	Associated by angiogenesis
VEGF <sup>[29,32]</sup>	Vascular permeability	In vivo	Mouse	Peritoneal injury	Adhesions attenuated by Antiserum and monoclonal antibody
VEGF, basic-FGF <sup>[25]</sup>	Fibrovascular band formation	In vivo	Human	Adhesion samples	VEGF in endothelial cells associated with adhesion formation

VEGF, IL-6 <sup>[21]</sup>	Bacterial Translocation	Both	Mouse	Caecal abrasion & suture	Adhesions attenuated by rBPI
VEGF, PIGF <sup>[81]</sup>	Pnenumoperitoneum	In vivo	Mouse	Lap. uterine horn model	Exacerbated by VEGF and CO <sub>2</sub>
CCL 1-CCR 8 <sup>[83]</sup>	Specific recruitment of peritoneal macrophages	Both	Mouse	Peritoneal ischaemic button & colitis-associated peritoneal adhesions	Unaffected by CCR8 gene deficiency and antiCCL1-neutralizing antibody
CD 28 T cell costimulatory pathway <sup>[84]</sup>	CD28 T cell costimulatory pathway/Inhibitor programmed death-1 pathway	Both	Mouse	Caecal abrasion	Exacerbated by CD28 T Cell costimulatory pathway but unaffected by death-1 pathway
Interferon-inducible protein-10 <sup>[85]</sup>	Regulates influxing neutrophils, monocytes and lymphocytes	In vivo	Mouse	Peritoneal side wall injury	
Broad spectrum of chemokines <sup>[86]</sup>	Broad spectrum chemokine inhibitor NR58-3.14.3	In vivo	Mouse	Peritoneal traumatization	Adhesions significantly attenuated
MCP-1 <sup>[87]</sup>	Fibroblast and mononuclear cell chemotaxis	In vivo	Mouse	Peritoneal injury	Attenuated by MCP-1 antibody
MCP-1 <sup>[88]</sup>	Fibroblast and mononuclear cell chemotaxis	In vivo	Human	Cell culture	
MCP-1 <sup>[89]</sup>	Fibroblast and mononuclear cell chemotaxis	In vivo	Human	Cell culture	
T cells, IL-17, CXC MPI-2/ CXCL8, CXCL1 <sup>[90]</sup>	CD4+ T cells	In vivo	Mouse	Caecal abrasion	Unaffected by anti-IL-17 antibodies

HGF: Hepatocyte growth factor; IFN- $\gamma$ : Interferon-gamma; IL: Interleukin; TNF- $\alpha$ : Tumour necrosis factor-alpha; TGF- $\beta$ : Transforming growth factor-beta; GM-CSF: Granulocyte macrophage colony stimulating factor; PAF: Platelet activating factor; MMP: Matrix metalloproteinase; TIMP: Tissue inhibitor of metalloproteinase; MDF: Macrophage deactivating factor; TPA: Tissue plasminogen activator; VEGF: Vascular endothelial growth factor; FGF: Fibroblast growth factor; PIGF: Placental growth factor; MCP: Monocyte chemotactic protein.

novel, potential therapies for the attenuation of adhesion formation at a preclinical level- the onus now though is on leading surgeon-scientists to corral their endeavour and progress their preclinical expertise into the clinical setting. For a start, the most likely candidate cytokine must be agreed (in our mind VEGF would seem the most apposite) and the most appropriate means of affecting its activity (whether directly<sup>[52]</sup> or indirectly<sup>[21]</sup>) selected. Furthermore industry interest will need to be stimulated for its support for Phase II and III trials as well as for the subsequent manufacture and marketing processes is crucial. Above all, though it must be realized that the timing for a concerted attempt to prove that molecular manipulation of post-operative peritoneal formation has never been better.

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# Narrow-band imaging optical chromocolonoscopy: Advantages and limitations

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*in vivo* visualization of vascular structures, but further study assessing reproducibility and effectiveness in the colorectum is ongoing at various medical centers.

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**Key words:** Narrow-band imaging; Colonoscopy; Sequential system; Non-sequential system; Polyps; Chromoendoscopy

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## Abstract

Narrow-band imaging (NBI) is an innovative optical technology that modifies the center wavelength and bandwidth of an endoscope's light into narrow-band illumination of  $415 \pm 30$  nm. NBI markedly improves capillary pattern contrast and is an *in vivo* method for visualizing microvessel morphological changes in superficial neoplastic lesions. The scientific basis for NBI is that short wavelength light falls within the hemoglobin absorption band, thereby facilitating clearer visualization of vascular structures. Several studies have reported advantages and limitations of NBI colonoscopy in the colorectum. One difficulty in evaluating results, however, has been non-standardization of NBI systems (Sequential and non-sequential). Utilization of NBI technology has been increasing worldwide, but accurate pit pattern analysis and sufficient skill in magnifying colonoscopy are basic fundamentals required for proficiency in NBI diagnosis of colorectal lesions. Modern optical technology without proper image interpretation wastes resources, confuses untrained endoscopists and delays inter-institutional validation studies. Training in the principles of "optical image-enhanced endoscopy" is needed to close the gap between technological advancements and their clinical usefulness. Currently available evidence indicates that NBI constitutes an effective and reliable alternative to chromocolonoscopy for

## INTRODUCTION

In 1971, Folkman proposed that all tumor growth was angiogenesis-dependent. This was the foundation for the development of angiogenic research and helped to stimulate investigation that is now being pursued by scientists in many different fields worldwide<sup>[1]</sup>. New blood vessel creation favors a transition from hyperplasia to neoplasia (i.e., the passage from a state of cellular multiplication to a state of uncontrolled proliferation characteristic of tumor cells)<sup>[2]</sup>.

An *in vivo* means for visualizing angiogenesis or microvessel morphological changes in superficial neoplasms would constitute a promising method for the diagnosis of early gastrointestinal tumors. Narrow-band imaging (NBI) is an innovative optical technology developed in Japan that modifies the center wavelength and bandwidth of an endoscope's light into a narrow-band illumination of  $415 \pm 30$  nm. By utilizing this narrow spectrum, contrast in the capillary pattern of the superficial layer is markedly improved<sup>[3]</sup>, thereby facilitating clearer visualization of vascular structures during gastrointestinal endoscopy<sup>[4]</sup>.

The first clinical study of the NBI system for the diagnosis of gastrointestinal tumors was reported by Sano *et al*<sup>[5]</sup> in 2001. Their promising observations resulted in the first pilot colorectal study in which the NBI system demonstrated better vascular pattern

visualization than conventional colonoscopy in the diagnosis of colorectal polyps<sup>[6]</sup>. These early studies opened the way for subsequently using NBI in the diagnosis of pre-malignant and malignant lesions of the hypo-pharynx, esophagus and stomach<sup>[4,7,8]</sup>.

This review focuses on the current advantages and limitations of using the NBI system in the diagnosis of colorectal lesions.

## SCIENTIFIC BASIS FOR NBI

Video endoscopes use white light from a xenon source for illumination. In order to understand the reflectance spectrum of any tissue, both the scattering process and absorption must be taken into account. Based on the Monte Carlo simulation, several investigations into the mechanism of scattering from tissue structures have determined that the penetration depth of the light depends on the wavelength. The depth of penetration into the gastrointestinal tract mucosa is superficial for the blue band, intermediate for the green band and deep for the red band (penetration depth range: 0.15 to 0.30 mm). As a result, NBI systems use optical filters for green and blue sequential illumination and narrow the bandwidth of spectral transmittance<sup>[9,10]</sup> (Figure 1).

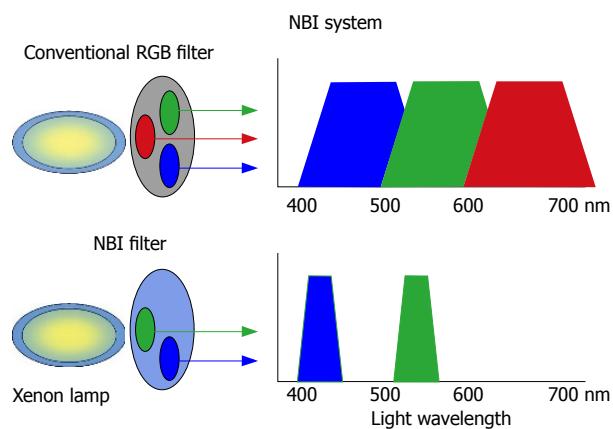
The scientific basis for the NBI system is that light with a short wavelength falls within the hemoglobin absorption band, so that blood vessels may be more clearly seen due to sufficient contrast<sup>[6]</sup>.

## IMAGE RECONSTRUCTION FROM REFLECTED LIGHT

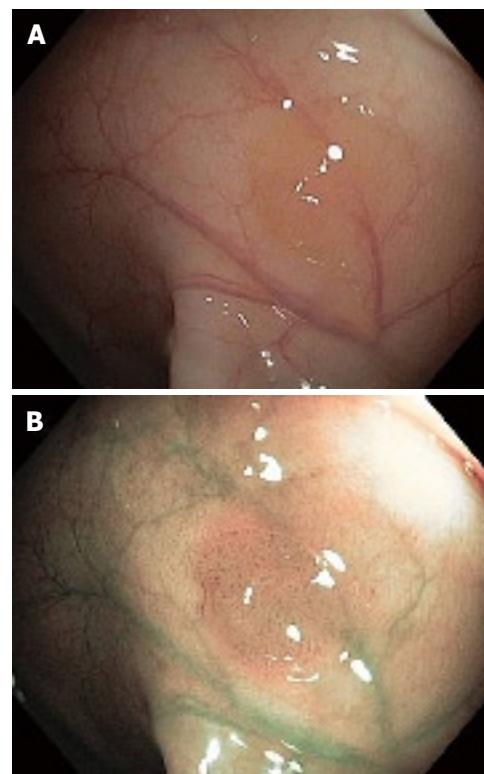
Two different types of NBI systems are used to reconstruct images from the reflected light. The non-sequential system (Exera II), also referred to as the "color chip system", uses a color charge coupled device (CCD) in which pixels are selectively assigned to specific wavelength ranges. The CCD captures the full range of the white light and transfers it in a single step to the processor in order to reconstruct natural color on the video monitor (Figure 2).

In contrast, the sequential system (Lucera Spectrum) uses a monochrome CCD in which pixels are not selectively attributed to specific colors, but transferred sequentially in the RGB bands to the processor. A rotating RGB interference filter is interposed after the white light source and the mucosa is illuminated alternately in each of the three RGB bands<sup>[11]</sup> (Figure 3).

Although the concept and basic design is the same for both the NBI sequential and non-sequential systems, a difference in color images exists due to differences in the color spectral characteristics of the RGB rotary filters used in the Lucera Spectrum and the color CCD used in the Exera II. There is considerable potential for further development, however, by improving NBI technology in the non-sequential endoscopic video system.



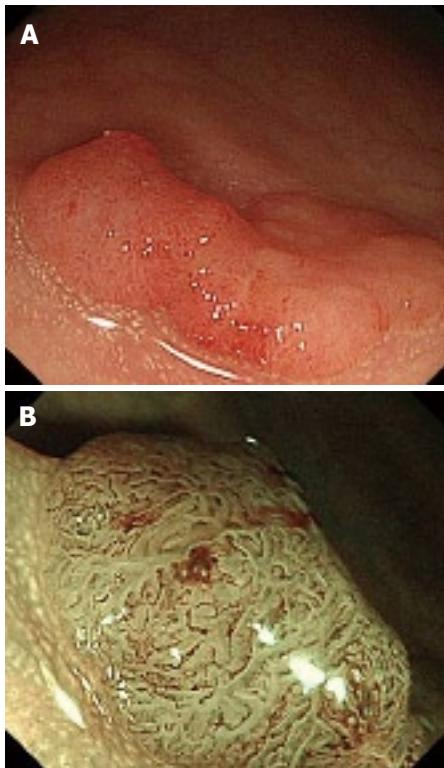
**Figure 1** NBI system. Different from the conventional RGB filter, the NBI filter consists of two narrow bands ( $415 \pm 30$  nm and  $540 \pm 30$  nm, respectively) that make it possible to observe clearly superficial vascular patterns for clinical evaluation.



**Figure 2** NBI colonoscopy image with non-sequential system. **A:** Conventional view of an I<sub>s</sub> polyp, 12 mm in diameter located in the sigmoid colon; **B:** NBI view clearly showing the superficial meshed vascular pattern on the polyp's surface indicating an adenomatous polyp.

## ARE YIELDS OF SMALL AND FLAT ADENOMAS HIGHER WITH NBI?

An interesting Japanese study involving 48 patients in which conventional white light colonoscopy was first performed followed later by blind NBI colonoscopy on the same patients found that the total number of neoplastic lesions detected by NBI was significantly higher than the total number of neoplastic lesions detected using conventional colonoscopy ( $P = 0.02$ ). Based on macroscopic appearance, location and tumor



**Figure 3** NBI colonoscopy image with sequential system. **A:** Conventional view of an IIa polyp, 20 mm in diameter located in the rectum; **B:** Meshed capillary vessels are clearly seen using magnifying NBI as dark brown areas diagnosing an intramucosal cancer.

size, flat lesions < 5 mm located in the right colon in particular were more frequently diagnosed using NBI<sup>[12]</sup>.

Although no Western study has as yet validated those Japanese results, a recent report indicated that adenomas were detected more frequently in the NBI group (23%) than in the control group (17%), but the difference was not statistically significant ( $P = 0.129$ )<sup>[13]</sup>. In contrast, it has also been recently reported that NBI did not result in better detection of adenomas. In that particular study, a colonoscopist with a known high detection rate using white light colonoscopy conducted patient examinations with high-definition colonoscopes using either white light or NBI<sup>[14]</sup>.

The fact that differences still exist between Japan and Western countries demonstrates that prospective studies are needed to determine which of these early reports are valid.

## NBI FOR NON-NEOPLASTIC AND NEOPLASTIC LESIONS

For lesions < 10 mm, it is generally accepted that hyperplastic polyps and other non-neoplastic colorectal lesions do not require endoscopic treatment because they are benign and have no malignant potential<sup>[15,16]</sup>. In contrast, adenomatous polyps should be removed to prevent progression of the adenoma-carcinoma sequence<sup>[17]</sup>.

Magnified chromocolonoscopy (MCC) has been

presented as the best means for *in vivo* selective management of colorectal polyps<sup>[18,19]</sup> and it is suggested that colorectal polyps should not be treated only on the basis of polyp size, but also with respect to the underlying histological characteristics observed during MCC<sup>[20]</sup>. The NBI system has been proposed for optical image-enhanced endoscopy because it features a simple one-touch button for changing from white light to NBI and does not require indigo carmine dye spraying.

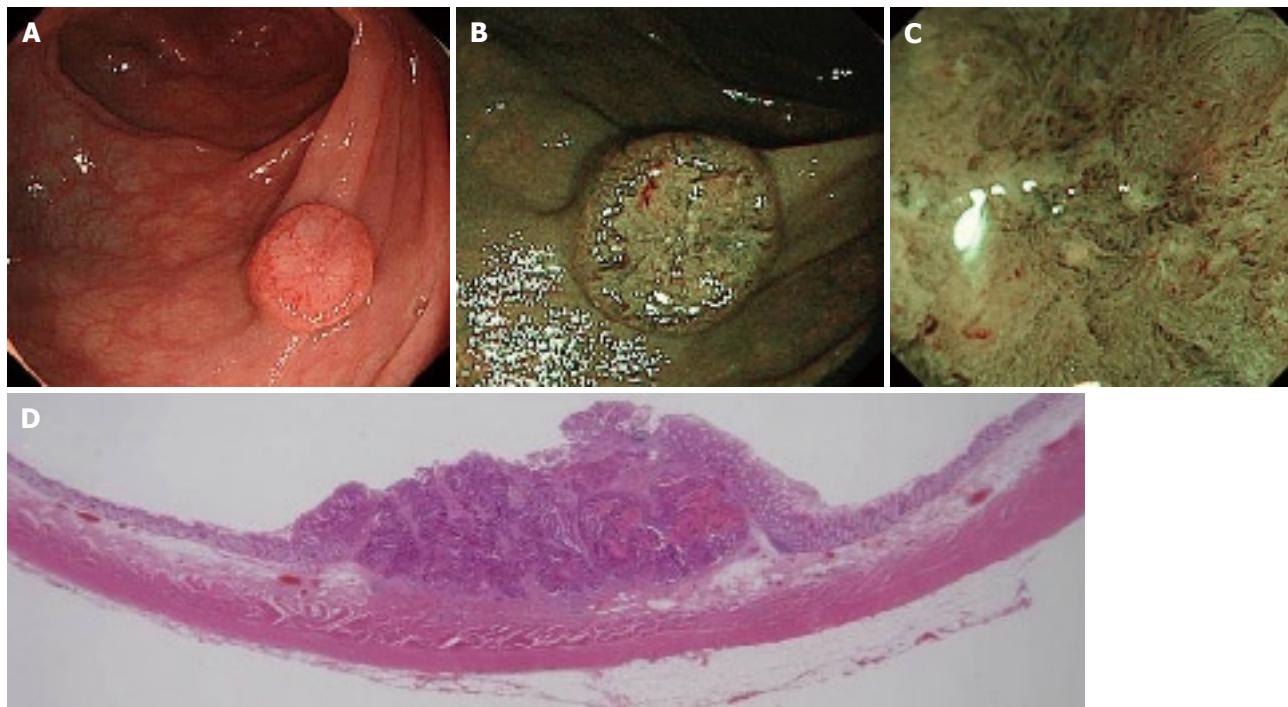
An early study of an NBI prototype used for differentiating non-neoplastic from neoplastic lesions in 34 patients with 43 lesions reported better visualization of the mucosal vascular network and lesion compared to conventional endoscopy. Chromocolonoscopy and NBI both had a sensitivity of 100% and a specificity of 75%<sup>[6]</sup>. Thereafter, the effectiveness of conventional colonoscopy, chromoendoscopy and the NBI system in distinguishing between non-neoplastic and neoplastic colonic polyps was assessed in 78 patients with 110 lesions. No significant difference existed between the NBI system and chromoendoscopy, but the sensitivity, specificity and accuracy of conventional colonoscopy were significantly lower (82.9%, 80.0% and 81.8%, respectively) compared to both chromoendoscopy and the NBI system (95.7%, 87.5% and 92.7%, respectively)<sup>[21]</sup>.

More recently, a classification of colorectal polyps based on the presence or absence of superficial meshed capillary vessels and their diameter, observed under NBI (CP type I - III) was proposed in Japan by Sano *et al*<sup>[22]</sup>. Although a promising and exciting alternative to differentiate the nature of colorectal polyps, Western prospective studies, however, are needed for its standardization worldwide.

## NBI FOR INVASIVE AND NON-INVASIVE COLORECTAL CANCER

There is growing evidence to support the theory that lesions with submucosal (sm) invasion < 1000 µm (sm1) without lympho-vascular invasion or a poorly differentiated component do not involve lymph node metastases<sup>[23]</sup>. In Japan, analysis of the pit pattern types proposed by Kudo *et al*<sup>[24]</sup> has been proven effective in predicting the level of sm invasion. In practice, however, limitations have been reported using the V<sub>1</sub> pit pattern to discriminate between mucosal (m), slight submucosal (sm1) and, deep submucosal (sm2) or deeper invasion<sup>[25]</sup>. The invasive pattern proposed by Fujii *et al*<sup>[26-28]</sup> (distorted and irregular crypts and a demarcated area) has also been reported to be effective in predicting sm2.

One promising area for NBI is in the accurate estimation of invasive depth for early colorectal cancers. Hirata *et al*<sup>[29]</sup> analyzed 148 colorectal lesions and recently reported a high degree of correspondence between pit pattern analysis by NBI and chromoendoscopy although the correspondence between MCC and NBI in evaluating the V<sub>1</sub> pit pattern of 48 early carcinomas



**Figure 4** NBI image of colorectal cancer. **A:** Conventional view of an IIa + IIc lesion, 12 mm in size, located in the transverse colon; **B:** NBI view shows a well demarcated area and meshed capillary vessels clearly visible characterized by thick diameter, branching and curtal irregularity; **C:** Magnifying NBI view additionally shows the presence of a nearly avascular or loose microvascular area due to histological desmoplastic changes in the stromal tissue, suggesting deep submucosal invasion; **D:** Histopathological analysis revealed an adenocarcinoma invading deeply into the submucosa (2500  $\mu$ m) with lymphovascular invasion.

was only 78%. Diagnosis using the type V pit pattern was possible by also evaluating various capillary features including vessel diameter, irregularity and the capillary network observed during NBI and not by relying solely on the pit pattern.

Two other promising studies on predicting the depth of invasion of early colorectal cancer by analyzing the microvascular architecture were published recently. Using NBI with magnification, Fukuzawa *et al*<sup>[30]</sup> observed several microvascular architecture characteristics in 61 early colorectal lesions (m-sm1: 37; sm2-3: 24). Univariate analysis showed that wide caliber, irregular caliber, tortuosity, irregularity, short length and non-dense arrangement were significantly more frequent in sm2-3 lesions compared to m-sm1 lesions ( $P < 0.001$ ). Multivariate analysis, however, revealed that irregularity and non-dense arrangement were the remaining independent factors<sup>[30]</sup> (Figure 4). Horimatsu *et al*<sup>[31]</sup> analyzed the presence of “meshed brown capillary vessels” in 27 colorectal lesions (m-sm1: 12; sm2: 15) also using NBI colonoscopy with magnification. The overall diagnostic accuracy, sensitivity, and specificity of microvessel density and the lack of uniformity in microvessel diameters for distinguishing between sm1 and sm2 lesions was 82.4% (14/17), 93.3% (14/15) and 75.0% (9/12), respectively<sup>[31]</sup> (Figure 4).

## DETECTION OF DYSPLASTIC AREAS IN ULCERATIVE COLITIS

Although patients with longstanding ulcerative

colitis are at increased risk of developing colorectal cancer, endoscopic detection of early neoplasia is difficult because these lesions can be subtle and even macroscopically invisible at times. A laborious protocol has been proposed involving not only target biopsies from suspicious lesions, but also two to four random biopsies taken every 10 cm of the colon<sup>[32]</sup>. MCC has emerged as the best method currently available for identifying dysplastic lesions in an inflammatory bowel disease setting<sup>[33,34]</sup>.

In terms of NBI research, the limitations of the first NBI prototype were recently shown in a prospective randomized crossover study of 42 patients with longstanding ulcerative colitis. In that study, the sensitivity of NBI for the detection of neoplasia was merely comparable to conventional colonoscopy although a larger number of suspicious lesions were found during NBI colonoscopy<sup>[35]</sup>. A more positive report on the effectiveness of a third generation NBI prototype plus magnification indicated that just as NBI reveals fine superficial blood vessels whose diameters and densities are increased in neoplastic lesions compared with normal mucosa, dysplastic lesions observed using NBI also have a darker capillary vascular pattern compared with normal mucosa<sup>[36]</sup>.

## WILL CONVENTIONAL CHROMOCOLONOSCOPY BE REPLACED BY NBI?

It is still too early to answer this question. In Japan,

chromocolonoscopy has demonstrated its effectiveness in the differentiation between adenomatous and hyperplastic polyps and is a promising method for distinguishing superficial from deep submucosal cancers, but it is regarded as an inconvenient and difficult procedure in Western countries<sup>[37]</sup>. Indigo carmine dye spraying is inexpensive and differs in practice from the NBI system in that it does not target superficial vascular patterns, but instead accentuates lesion contours and highlights the pit pattern of colonic crypts<sup>[25]</sup>. It is interesting to note that indigo carmine dye spraying is not recommended before an NBI examination because it might obscure blood vessel visualization.

In contrast, NBI even without magnification when using the non-sequential system provides accurate definition of vascular vessels throughout the entire colonic mucosa and more clearly defines the borders of a lesion without the necessity of using dye spraying. The recently developed NBI system requires an expensive new processor, however, so the cost-benefit issue requires further analysis<sup>[38]</sup>. In addition, the diagnostic accuracy of NBI is affected by the learning curve associated with this new methodology and extra time may be needed to perform the examination.

## USELESS TECHNOLOGY IN UNQUALIFIED HANDS

The acquisition and use of NBI technology is increasing in many countries, but it should be emphasized that accurate analysis of the pit pattern types and familiarity with MCC are basic fundamentals necessary to become proficient in NBI diagnosis of colorectal lesions. Modern optical technology without proper image interpretation wastes valuable resources, can cause confusion for inadequately trained endoscopists and may result in the delay of inter-institutional validation studies. Training general endoscopists in the principles and applications of optical image-enhanced endoscopy as practiced in Japan (i.e., stereomicroscopy, conventional chromoendoscopy, magnifying endoscopy and pit pattern analysis)<sup>[20,24-26]</sup> in approved centers by qualified experts will be required to narrow and, hopefully, close the existing gap between the latest advancements in optical technology and their clinical usefulness.

## CONCLUSION

Several studies have previously reported on the advantages and limitations of NBI optical image-enhanced colonoscopy in the diagnosis of colorectal diseases. One difficulty in evaluating the results, however, has been non-standardization of the NBI systems and prototypes used in the research. Despite this shortcoming, there seems to be considerable potential for further development by improving NBI technology in the non-sequential endoscopic video system by modifying the characteristics of the interference filters.

In practice, the latest technological advancements

incorporated into third generation NBI prototypes appear to offer a clear advantage over conventional chromocolonoscopy. Additional validation studies are needed, however, to confirm the effectiveness of NBI for screening colonoscopy, identification of adenomatous polyps, determining depth of invasion of early colorectal cancers, evaluating free margins after endoscopic resection and detection of dysplastic lesion in an inflammatory bowel disease setting.

A number of other questions remain unsolved that deserve additional examinations including whether NBI is less time-consuming, its cost effectiveness, whether magnification is absolutely required and whether the NBI system should completely replace chromocolonoscopy. Further studies assessing these issues are ongoing at various medical centers worldwide.

At the present time, NBI constitutes an effective and reliable alternative to chromocolonoscopy for *in vivo* visualization of vascular structures. Due to widespread incorporation of NBI technology outside Japan, however, there is an increasing need to train general endoscopists in the basic principles and applications of advanced optical image-enhanced endoscopy.

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# Low intensity ultrasound-induced apoptosis in human gastric carcinoma cells

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Several new proteins appeared after US irradiation and were up or down regulated more than 2 times. Some heat shock proteins (HSPs) were found to be associated with the signal process simulating the apoptosis of cells.

**CONCLUSION:** Low intensity US could induce apoptosis in human gastric carcinoma cells. US-induced apoptosis is related to US intensity/culture time. US-induced apoptosis may be caspases-dependent and endoplasmic reticulum (ER) stress-triggered apoptosis may also contribute to it. Proteomic experimental system is useful in finding the protein alteration in carcinoma cells after US irradiation, helping to develop a new cancer therapy.

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**Key words:** SGC-7901 human gastric carcinoma cells; Low intensity ultrasound; Apoptosis; Caspases-dependent; Proteomics

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## INTRODUCTION

Gastric carcinoma is the second commonest cause of cancer-related deaths worldwide. Although surgical removal of the stomach is the only curative treatment in clinical practice, more studies revealed that chemotherapy and radiation therapy given after surgery could improve the chance of a cure for many patients. To improve the survival rate, new therapeutic methods should be developed.

In non-surgical cancer treatment, induction of

apoptosis is a preferred mode of killing cancer cells<sup>[1,2]</sup>. Several investigators have reported that US irradiation could induce apoptosis in human leukemia cell lines<sup>[3-7]</sup>, including K562, HL-60, Nalm-6 and U937. It was also reported that apoptosis of some solid carcinoma cells, such as human ovarian carcinomas cells<sup>[8]</sup>, SMMC-7721 cells (data not shown) and Walker 256 carcinosarcoma cells<sup>[9]</sup>, are triggered by US irradiation. Different from high intensity focused ultrasound (HIFU) which can thermally ablate tissues *via* hyperthermia in different carcinomas<sup>[10-12]</sup>, low intensity ultrasound (US) defined as therapeutic US, with a relatively lower intensity than HIFU, has a great potential in apoptosis therapy for cancer and can be relatively easily applied<sup>[8,13]</sup>. So it is suggested that low intensity US-induced apoptosis in tumor cells could probably offer a new approach to elimination of cancer<sup>[4]</sup>. To our knowledge, US-induced apoptosis in gastric carcinoma has not yet been reported. Compared with human leukemia cell lines, low intensity US-induced apoptosis in solid carcinoma cells or tissues is still in the process of investigation. The mechanism has also not yet been elucidated.

The aim of this study was to prove low-intensity US-induced apoptosis in human SGC-7901 gastric carcinoma cells and investigate the effects of US intensity and culture time after US irradiation on US-induced apoptosis. Comparative proteomic analysis was performed to examine the alteration in protein profile of gastric carcinoma cells induced by US irradiation. The potential molecular mechanism was suggested *via* the protein functional analysis.

## MATERIALS AND METHODS

### Chemicals and materials

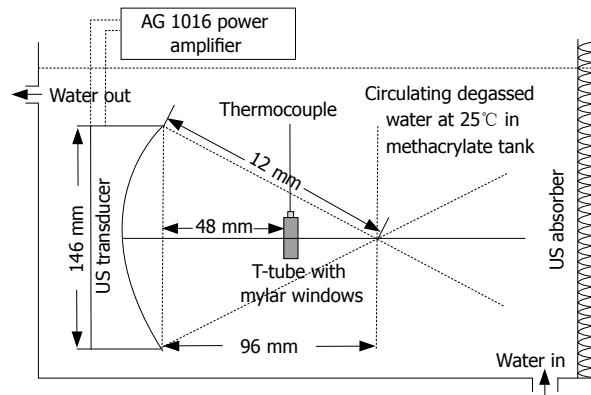
Fluorescein isothiocyanate (FITC)-Annexin V/propidium iodide (PI) kit was purchased from Jingmei Biotechnology Company, China. Immobilized pH gradient (IPG) strips (pH3-10, nonlinear, 13 cm) and IPG buffer (pH3-10, nonlinear) were purchased from Pharmacia, Amersham Biotech. Dithiothreitol (DTT), PMSF and CHAPS were purchased from Sigma Company (USA). All the buffers were made using high purity MilliQ water. Elite ESP flow cytometer was purchased from Coulter Electronics Hialeah, FL. IPGphor isoelectric focusing equipment, Hoefer SE 600 vertical chambers, electrophoresis apparatus, Image Master 2D Elite 4.01 software were purchased from Pharmacia, Amersham Biotech. Voyager-DE MALDI-TOF MS was a product from Applied Biosystems (USA).

### SGC-7901 cell line cultures

SGC-7901 human gastric carcinoma cells (Center of Laboratory Animals of the Forth Military Medical University) were cultured in RPMI 1640 culture medium in a water-saturated atmosphere with 50 mL/L CO<sub>2</sub> at 37°C.

### US irradiation set

The US irradiation system is shown in Figure 1. It consists



**Figure 1** US irradiation system.

of a AWG 2021 arbitrary waveform generator (Sony Tektronix Co., Tokyo, Japan), a AG 1016 power amplifier (T&C Power conversion, Inc., Rochester, NY, USA) and a transducer designed as a semispherical ring with an aperture 146 mm and a radius of curvature 120 mm (Imasonic, Besancon, France), a 750 mm × 750 mm × 500 mm polymethyl methacrylate tank filled with degassed water with 10-cm high water above the transducer. The central frequency is 1.2 MHz. Temperature of water in the tank was controlled at 25°C by circulating. Cell samples were carried in a specially designed inverted "T" tube as previously described<sup>[6,9]</sup>.

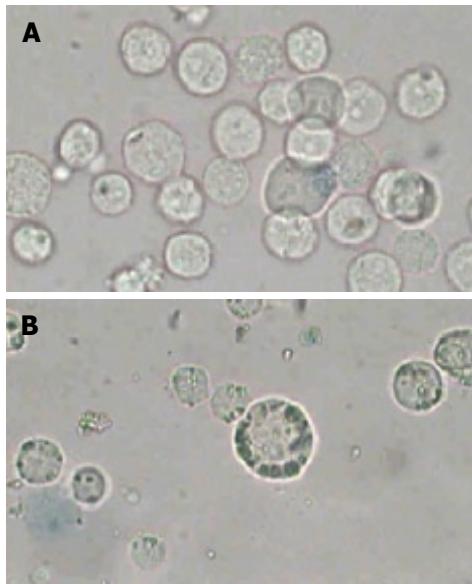
### US treatment and sample collection

The cell samples were divided into ten groups and each treatment was given in three replicates. The first group was used as the control that imitated the whole process with no irradiation. The 2-5 groups were irradiated for 10 min at the intensity of 3.0 W/cm<sup>2</sup>, 6.0 W/cm<sup>2</sup>, 9.0 W/cm<sup>2</sup> and 12.0 W/cm<sup>2</sup> ( $I_{SPT,A}$ ), respectively, and then incubated at 37°C in a humidified air containing 50 mL/L CO<sub>2</sub>. Apoptosis detection and microscopy observation were performed in each sample. Remained cells were washed 3 times with cold PBS, centrifuged at 1500 × g for 10 min to get cell pellets, then frozen quickly in liquid nitrogen and stored in a -80°C ultra low temperature freezer (LegaciTM Refregeration system, REVCO Technologies) for subsequent 2D PAGE analysis. The whole process was performed in sterile condition.

To investigate the relationship between culture time after US irradiation and cell apoptosis, the 6-10 groups were irradiated at the intensity of 12.0 W/cm<sup>2</sup> and incubated at 37°C in a humidified air containing 50 mL/L CO<sub>2</sub> for 0, 6, 12, 18 and 24 h, respectively. The following morphologic observation and apoptosis detection were performed as described above.

### Morphological observation and flow cytometry (FCM) examination

The morphologic changes in apoptotic and necrosis cells could be examined under microscope and detected by analysis of a light scatter signal by FCM<sup>[14]</sup>.



**Figure 2** Morphologic characteristics of SGC-7901 cells before and after US irradiation.

SGC-7901 cells were stained with trypan blue and then observed under microscope (Olympus BX40) to detect their structure change after US irradiation. Furthermore, the cells were analyzed with FCM to detect phosphatidylserine (PS) exposure on the cell membrane as a typical marker of early apoptosis.

#### Two-dimensional electrophoresis (2DE) analysis

Proteins were extracted from control and irradiated cells, respectively, with the freeze-thaw lysis method. Then, the protein solution (containing 200 µg proteins) was loaded to perform isoelectric focusing and second dimension separation as previously described<sup>[15]</sup>. Gels were silver stained.

2D gel images of control and irradiated cells were acquired using a Sharp JX-330 scanner, compared and matched *via* Image Master 2D Elite software. New protein spots and proteins expressing 2 times higher or lower were selected for the following identification based on MALDI-TOF-MS.

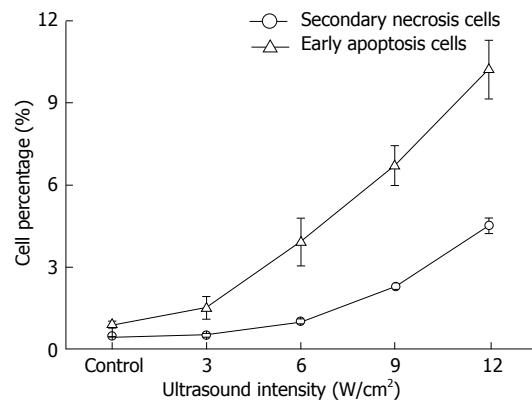
#### Identification of proteins using MALDI-TOF-MS

The selected protein spots were excised from the gel and in-gel digestion with trypsin was performed. The extracted enzymolyzed peptides were identified by peptide mass fingerprinting based on MALDI-TOF-MS (Voyager-DETM), analyzed using data explore, and matched in ProFound-peptide mapping and SWISS-PROT.

## RESULTS

#### Morphologic observation

The morphologic observations showed that the distinct morphologic characteristics of apoptotic cells, such as condensation of nucleus, chromatin margination (Figure 2B) were found after the cells were irradiated at 12.0 W/cm<sup>2</sup> for 10 min and incubated for 12 h



**Figure 3** Percentage of early apoptotic cells and secondary necrosis cells in control and US-irradiated SGC-7901 cells at different US intensities.

after irradiation. Simultaneously, the characteristics of apoptotic cells were not observed in the control samples incubated at 37°C in a humidified air containing 50 mL/L CO<sub>2</sub> (Figure 2A). However, the morphologic observation could not unambiguously give the quantity information about the proportion of cells in different physiological states, such as viable cells, apoptotic cells and secondary necrotic cells. So a further analysis was performed for the detection of functional characteristics such as exclusion of PI or plasma membrane integrity or such dyes as Annexin V-FITC.

#### Apoptosis assay

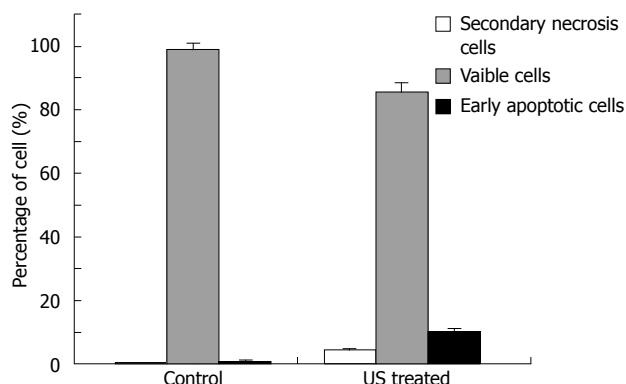
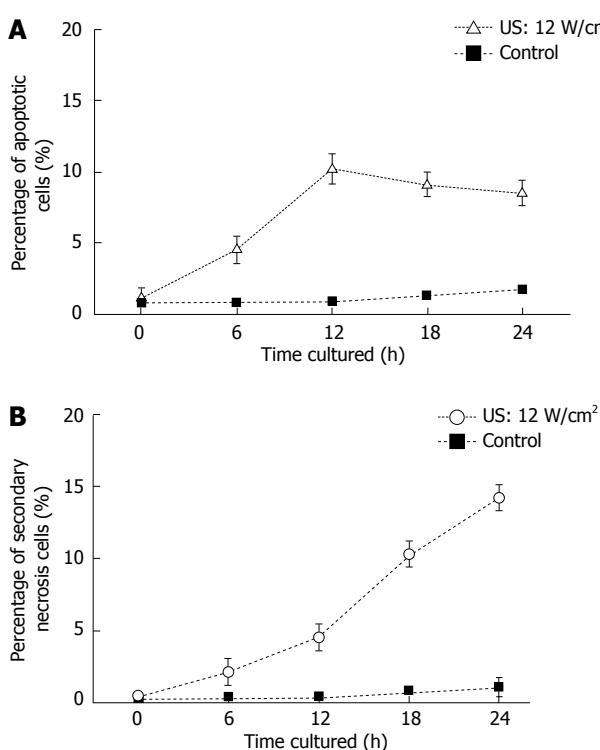
FCM with double staining of FITC-Annexin V/PI was performed to detect PS expression on the cell membrane as an end point of early apoptosis<sup>[16]</sup>, showing the exact percentage of cells in different styles, including viable cells (Annexin V/PI), early apoptotic cells (Annexin V<sup>+</sup>/PI), and secondary necrosis (Annexin V<sup>+</sup>/PI<sup>+</sup>).

The relationship between cell apoptosis and US intensity/culture time after US irradiation was investigated. The results show that the percentage of early apoptosis and secondary necrosis in the US irradiated cells increased with the increased US intensity (Figure 3). In the mean time, the percentage of viable cells decreased after US irradiation. The percentage of early apoptosis (incubated for 12 h after irradiation at the intensity of 12.0 W/cm<sup>2</sup> ( $I_{SPTA}$ ) for 10 min) increased more than 10% compared with the control cells cultured at 37°C in a humidified air containing 50 mL/L CO<sub>2</sub> (Figure 4), suggesting that apoptosis of cells can be induced by US.

US induced early apoptosis (Figure 5A) and secondary necrosis (Figure 5B) as a function of culture time after US irradiation, are determined by FCM with double staining of FITC-Annexin V/PI. Human SGC-7901 gastric carcinoma cells were irradiated by US at the intensity of 12.0 W/cm<sup>2</sup> for 10 min in this study. Apoptotic cells increased with the increased culture time after US irradiation and reached its maximum at 12 h. Then, the percentage of apoptotic cells gradually decreased. However, the secondary necrosis cells kept increasing. The percentage of viable cells kept decreasing after US irradiation.

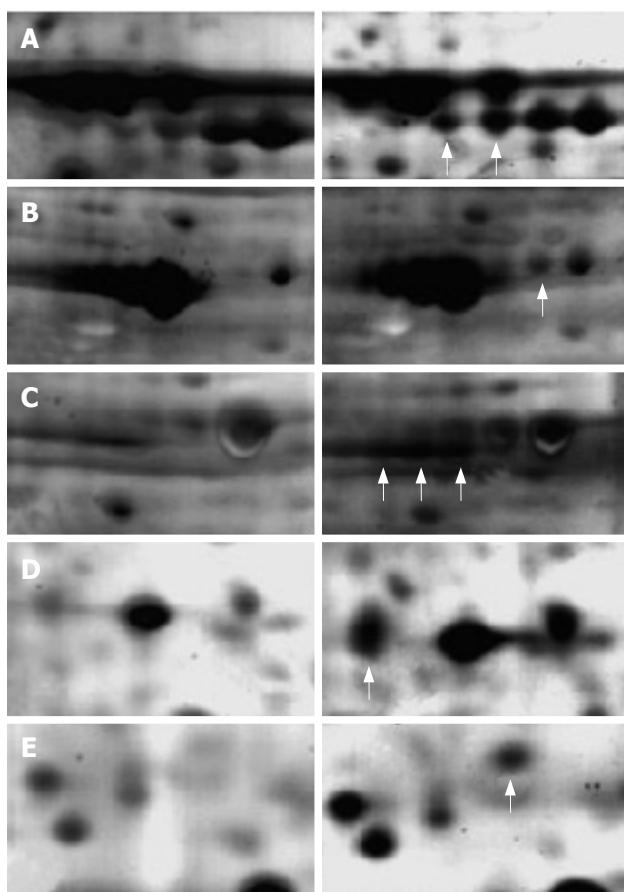
**Table 1** Identified information about proteins related to US-induced apoptosis

Swiss-Prot No.	Protein name	Theo. pI/Mw	Exp. pI/Mw	Protein function
P11021	GRP78, Bip	5.2/71.0	5.1/74.4	ER-stress associated
P38646	HSP70 (Heat shock 70 kDa protein)	5.9/73.7	5.8/72.1	Activation of pre-caspase 3
P10809	CH60 (60 kDa HSP)	5.7/61.2	5.5/58.9	Tumor suppressor and pro-apoptotic protein
P27797	Calreticulin precursor	4.3/48.2	4.5/52.8	ER-stress triggered apoptosis
P35232	Prohibitin	5.6/29.9	5.4/31.3	Inhibit the tumor cell growth

**Figure 4** Percentage of viable cells, early apoptotic cells and secondary necrosis cells in the control and US-irradiated SGC-7901 cells.**Figure 5** US-induced early apoptosis (A) and secondary necrosis (B) as a function of culture time after US irradiation, determined by FCM.

## 2DE analysis

A total of 1137 protein spots were successfully separated from the human gastric carcinoma SGC-7901 cells, 798 of which had a good match with the proteins in the 2D map of US irradiated cells. The unmatched protein spots amounted to 339. Some of the matched proteins had obviously different expressing characteristics (more than 2 times), indicating that they are obviously up-regulated or down-regulated after US irradiation. The protein expression between control cells (left) and US irradiated cells (right) is also shown in Figure 6.

**Figure 6** Magnified features of protein spots with different expressing characteristics after US irradiation. Arrow points to the differently expressed protein spots. A: Bip; B: HSP70; C: CH60; D: Calreticulin; E: Prohibitin.

or down-regulated after US irradiation. The protein expression between control cells (left) and US irradiated cells (right) is also shown in Figure 6.

## Identification of proteins by MALDI-TOF-MS

Proteins were identified by peptide mass fingerprinting based on MALDI-TOF-MS. The identified information about the proteins shown in Figure 6 is summarized in Table 1. The expression of these proteins increased more than 2 times after US irradiation. However, 4 proteins were unmatched although with good spectra. Further identification of them is needed via the tandem MS/MS.

## DISCUSSION

In the present study, we first demonstrated that low intensity US could induce early apoptosis and secondary

necrosis in gastric carcinoma cells. US irradiation could trigger apoptosis in both carcinoma and normal cells. However, a series of investigations showed that malignant cells are much more susceptible to US exposure and more prone to being killed than normal cells<sup>[17,18]</sup>. Lagneaux *et al*<sup>[4]</sup> also demonstrated that carcinoma cells are sensitive to ultrasonic treatment with a high percentage of apoptotic cells observed 5 h after treatment, whereas ultrasonic treatment has no effect on normal cells isolated from leukemic patients. This characteristic of carcinoma cells gives the chance to develop US irradiation as a new therapeutic approach to cancer treatment. Furthermore, during the US irradiation, focused US oriented by targeted multi-functional US contrast agents (UCA), could aim at the targeted region, decreasing side-effects on normal tissues around it. In addition, Gene regulation of target cells may be utilized in modifying cellular response to US treatment, thus increasing the sensitivity of diseased cells while making normal cells resistant to the side effects<sup>[19]</sup>. Therefore, US-induced apoptosis, as a novel cancer therapy, is of practical importance.

Low intensity US-induced apoptosis in gastric carcinoma cells may be an early event that increased with time in this study. The culture time for apoptotic cells to reach their maximum apoptosis is 12 h. Another study on US-induced apoptosis of human hepatocarcinoma cells performed in our laboratory (data not shown) showed that the culture time is 6 h after treatment. This discrepancy may be due to the different sensitivities of carcinoma cells to apoptosis-inducing factors. Similar results have also been reported by other investigators<sup>[5,20]</sup>, suggesting that the sensitivity of carcinoma cells to US irradiation is related to the time when apoptotic cells reach their maximum.

Apoptosis is always a special cellular response to stress stimuli, such as exposure to chemotherapeutic drugs, oxidative stress, free radicals, X-rays, ultraviolet radiation and shear stress. US irradiation belongs to the physical stress factor. Ashush *et al*<sup>[3]</sup> first reported that apoptosis is induced by high-intensity pulsed US in human leukemia cell lines, Lagneaux and Meulenaer demonstrated that low intensity US could also trigger apoptosis in human leukemia cell lines and low intensity US-induced apoptosis in tumor cells could probably offer a new approach to the elimination of cancer<sup>[4]</sup>. Feril *et al*<sup>[6]</sup> also pointed out that US without increasing its temperature and time can increase hyperthermia-induced apoptosis. It was reported that early apoptosis and necrosis induced by US are mainly caused by inertial cavitation<sup>[5-7]</sup>. During US cavitation, collapsing bubbles could create drastic conditions in an extremely short time, with a temperature of 2000 to 5000 K and a pressure up to 1800 atm inside it. Shear forces, jets and shock waves are also produced outside the bubbles. The inertial cavitation also induces free radical formation *via* the thermal dissociation of water into ·OH and ·H radicals. Any kind of these stress factors from US cavitation might cause apoptosis of tumor

cells. Apoptosis may be triggered as a response to cell membrane and DNA damage induced by inertial cavitation or *via* the action of residual hydrogen peroxide<sup>[21,22]</sup>. It is further proved when introduction of dissolved gases or UCA, Optison™ and YM454 to the system enhanced the US-induced cell killing *in vitro*<sup>[5,7]</sup>. Moreover, intracellular ROS generated from mitochondria rather than from extracellular ROS (directly produced by inertial cavitation in the medium), are involved in the regulation of apoptosis induced by US<sup>[23]</sup>. However, Lagneaux and Meulenaer claimed that US-induced apoptosis is probably related to the oxidative stress<sup>[4]</sup>.

To investigate the molecular mechanism of US-induced apoptosis in carcinoma cells, comparative proteomic analysis is a novel method that was introduced in this study. Because the biologic processes are directly executed by proteins that are dynamically modified and processed at multiple levels during or after sonication, the alteration in protein profile can reflect the status of differentiation and physiological conditions of cells. Protein functional analysis can also help reveal the molecular signal processes triggered by US irradiation in tumor cells or tissues.

Apoptosis is well-known to require the activation of caspases, a group of enzymes involved in apoptotic cascade events<sup>[24]</sup>. Apoptotic stimulus can activate apoptosis-related proteins to enter mitochondria, then induce mitochondria membrane to form pores to release molecules into cytosol, such as cytochrome C (CytC). The released molecules can activate caspase-9, which cleaves procaspase-3 to caspase-3, finally inducing apoptosis<sup>[25,26]</sup>. The activity of different caspases was not detected in this study, but some proteins, such as CH60, related to the activation of caspases were up-regulated in the cells after sonication. CH60 is regarded as a protective protein induced by the response process of mammalian cells under stress, which promotes apoptosis by helping maturation of pro-caspase-3<sup>[27,28]</sup>. Pro-caspase-3 is the key enzyme involved in the caspases-dependent apoptotic process, indicating that the US-induced apoptosis may be caspases-dependent. It is known that members of the heat shock protein (HSP) family function at multiple points in the apoptotic signal pathway. Sometimes, due to their cytoprotective role, they inhibit the apoptotic response by inhibiting the key steps in the apoptotic process. However, in other times, they serve as chaperones of a key signaling protein or directly promote apoptosis. Vykhodtseva reported that focused US sonication close to the thermal threshold exposures could induce apoptosis, suggesting that the mechanism leading to apoptosis is the production of HSPs<sup>[29]</sup>. In this study, CH60, HSP70 and Bip were found belonging to the HSP family. They participate in the apoptosis induced *via* different ways. Bip and calreticulin participate in the press-induced reactions on the endoplasmic reticulum (ER), both of them are pivotal molecules mediating ER-initiated apoptosis involved in ER stress. Prohibitin is an antiproliferative

protein and functions as a tumor suppressor. Commonly, it is involved in blocking DNA synthesis and negative regulation of cell proliferation. In US-induced apoptosis of tumor cells, the increased expression of prohibitin would help inhibit the growth of tumor cells.

In summary, low intensity US can induce apoptosis in human gastric carcinoma cells. The percentage of early apoptosis and secondary necrosis in the US-irradiated cells are increased with increased US intensity; Moreover, apoptotic cells increase with increased culture time after US irradiation and reach their maximum at about 12 h. Group of HSPs participates in the US-induced apoptosis. US-induced apoptosis is caspases-dependent, and ER stress-triggered apoptosis may also contribute to it. Moreover, experimental system can also be introduced into the research of bio-effects caused by other physical and chemical factors on tumor cells, which would be significant in investigating tumor cell apoptotic mechanism and cancer therapy.

## COMMENTS

### Background

Low intensity ultrasound (US) irradiation could trigger apoptosis in carcinoma cells, and therefore has distinct potential as a technique for cancer therapy. To investigate US-induced apoptosis in different carcinoma cells is significant to better understand its mechanism and develop a new therapeutic approach.

### Research frontiers

The present study demonstrated low intensity US-induced apoptosis in gastric carcinoma cells. Based on protein functional analysis, the molecular mechanism of US-induced apoptosis is suggested.

### Innovations and breakthroughs

Some previous studies have proved that US can induce apoptosis in human leukemia cells and solid carcinoma cells. The present study first demonstrated that low intensity US could induce early apoptosis and secondary necrosis in gastric carcinoma cells. The effects of US intensity and culture time after treatment on US-induced apoptosis are revealed. The potential molecular mechanism of US-induced apoptosis is suggested via comparative proteomic analysis.

### Applications

The present study would help to make better analysis of US-induced apoptosis in gastric carcinoma cells, and promote new therapeutic schemes. Further investigation of its mechanism would supply more useful information about US-induced apoptosis in solid carcinoma cells.

### Peer review

The authors demonstrated that low intensity US could induce early apoptosis and secondary necrosis in gastric cell line SGC-7901. As suggested by the authors, low intensity US irradiation may help to develop new cancer therapies. Therefore, this study may provide important information about gastric cancer.

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## COLORECTAL CANCER

# Prognostic significance of BMP and activin membrane-bound inhibitor in colorectal cancer

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**RESULTS:** Immunohistochemical analysis revealed that BAMBI expression was observed in 148 (80.8%), and strong BAMBI expression was observed in 46% of the CRCs. Strong BAMBI expression was positively correlated with histological type, depth of invasion, lymph node metastases, and tumor node metastasis (TNM) stage ( $P < 0.05$ ). Clear associations were found between BAMBI and  $\beta$ -catenin ( $P = 0.035$ ) and p53 ( $P = 0.049$ ) expression. In curatively resected CRC, 5-year recurrence-free survival was 51.9% ( $P = 0.037$ ) for strong BAMBI expression compared to 79.8% for weak BAMBI expression. In the Cox's multivariate analysis, lymph node metastases (RR 6.685;  $P < 0.001$ ) and depth of invasion (RR 14.0;  $P = 0.013$ ) were significant indicators for recurrence, and strong BAMBI expression (RR 2.26;  $P = 0.057$ ) tended to be significant.

**CONCLUSION:** BAMBI was linked to a potentially aggressive tumor phenotype and predicted tumor recurrence and cancer-related death in CRC. BAMBI expression might be applicable in the routine clinical setting of CRC.

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**Key words:** BMP and activin membrane-bound inhibitor; Colorectal cancer; Transforming growth factor-beta signal; Prognosis; Wnt signal

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## INTRODUCTION

Activation of the Wnt/ $\beta$ -catenin pathway is a critical process in the malignant transformation of colonic

## Abstract

**AIM:** To investigate the clinical significance of BMP and activin membrane-bound inhibitor (BAMBI) which is a pseudoreceptor of transforming growth factor-beta (TGF- $\beta$ ) type I receptors and acts as a negative regulator of TGF- $\beta$  signaling and expression aberrantly elevated in colorectal cancers (CRCs). We studied BAMBI expression in CRCs.

**METHODS:** We studied BAMBI expression in 183 surgically resected CRCs by immunochemical and immunoblotting analyses using a generated monoclonal anti-BAMBI antibody. Commercially available anti- $\beta$ -catenin and anti-p53 antibodies were also applied for immunochemical analyses as a comparison control.

epithelium<sup>[1-5]</sup>. Wnt signaling promotes the stabilization and accumulation of β-catenin, which in turn interacts with the T cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors and activates the transcription of downstream genes such as *c-Myc*, *cyclin D1*, and *Axin2*<sup>[6-10]</sup>. Constitutive activation of β-catenin-TCF-mediated transcription is believed to be a critical step in the tumorigenesis of various cancers<sup>[11-13]</sup>. Conversely, the transforming growth factor-beta (TGF-β) pathway participates in tumor suppressor activities such as growth inhibition and apoptosis, and this negative regulation is lost during colorectal carcinogenesis, with mutations in the type II receptor, SMAD2, and SMAD4<sup>[14-17]</sup>. TGF-β also mediates tumor-promoting effects such as growth stimulation, as well as increases in motility, invasion, and metastasis through either differential effects on tumor and stromal cells or a fundamental alteration in the TGF-β responsiveness of the tumor cells themselves<sup>[18-20]</sup>.

The TGF-β and Wnt/β-catenin signaling pathways cross talk to regulate tumor biology<sup>[20]</sup>. Axin, a negative regulator of the Wnt pathway, activates TGF-β signaling through Smad3 binding<sup>[21]</sup>, while TCF/LEF interacts directly with Smad3 to regulate the expression of their target genes<sup>[22]</sup>. It is also known that β-catenin and TCF/LEF both interact directly with Smad4 to regulate target genes during development<sup>[23]</sup>.

BMP and activin membrane-bound inhibitor (BAMBI) is a transmembrane glycoprotein induced by BMP signaling<sup>[24]</sup> that is related to TGF-β-type I receptors, but lacks an intracellular kinase domain<sup>[25]</sup>. BAMBI inhibits TGF-β signaling by forming a heterodimer with TGF-β-type II receptors<sup>[25]</sup>. Previously, we found that both Wnt/β-catenin signaling and TGF-β signaling activate transcription of *BAMBI* and that *BAMBI* expression is aberrantly elevated in most colorectal cancers (CRCs)<sup>[26]</sup>. To analyze the clinical significance of BAMBI, we studied its expression in CRC using immunohistochemical staining. We show that BAMBI overexpression is correlated with aggressive tumor phenotypes and predicts tumor recurrence and cancer-related death in CRC. BAMBI may be usable as a target for diagnostic and antibody medicine.

## MATERIALS AND METHODS

### Materials

Colorectal tumor tissues were obtained from 183 consecutive patients who underwent to surgical resection between January 1995 and July 2006 at Gunma University Hospital. All of the patients underwent to radical colorectal resection intended to obtain clear pathological margins and regional lymphadenectomy, even in Stage IV. The clinicopathological features of the patients are shown in Table 1. The subjects were 115 males and 68 females with a mean age of 66 years (range 27-95). Of these, 113 were colon and 70 were rectal cancers. The tumor tissues were staged pathologically according to the American Joint Committee on Cancer

**Table 1 Baseline characteristics and clinicopathological classification**

Characteristic	Category	Data
Age	Mean (yr)	66
	Range	27-95
Gender	Male	n = 115 62.8%
	Female	n = 68 37.2%
Location <sup>1</sup>	Right side	61 33.3
	Left side	52 28.4
	Rectum	70 38.3
Tumor size (mm)	Mean	45.1
	Range	10-110
	≤ 39	74 40.4
	40-59	62 33.9
	≥ 60	47 25.7
Depth of invasion <sup>2</sup>	T0	7 3.8
	T1	18 9.9
	T2	22 12.0
	T3	129 70.5
	T4	7 3.8
Histology <sup>3</sup>	G1	51 27.9
	G2	116 63.4
	G3	16 8.7
TNM stage	I	40 21.8
	II	51 27.9
	III	53 29.0
	IV	39 21.3

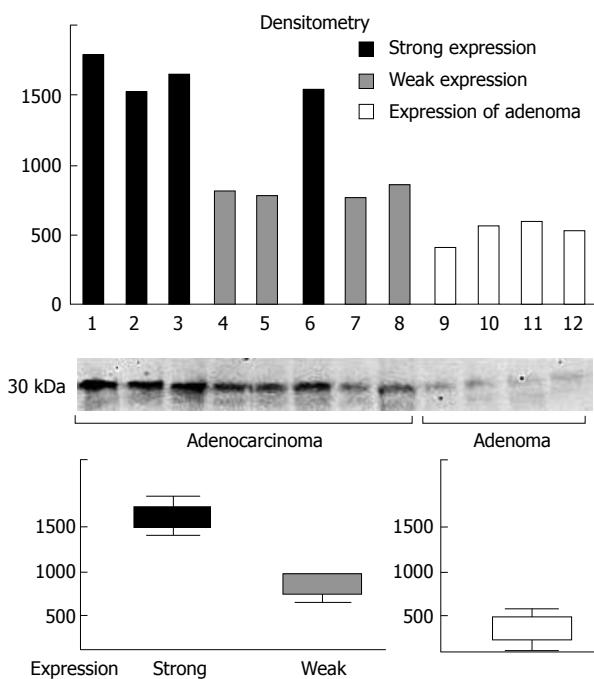
<sup>1</sup>The colon was divided into the right and left sides at the splenic flexure; <sup>2</sup>According to the TNM classification; <sup>3</sup>G1: Well-differentiated adenocarcinoma; G2: Moderately differentiated adenocarcinoma; G3: Poorly differentiated adenocarcinoma.

Tumor-Node-Metastasis (TNM) classification. The tumors were categorized according to the World Health Organization (WHO) classification as well differentiated (G1; 51 cases, 27.9%), moderate (G2; 116 cases, 63.4%), and poor (G3; 16 cases, 8.7%).

Anti-BAMBI antibodies were generated by immunizing mice with a fragment comprising either amino acids 45-147 or 177-241. Known methods were used to prepare the anti-BAMBI antibody, collect antibody-producing cells, obtain cell fusion, select and clone hybridomas, collect and purify monoclonal antibodies<sup>[26]</sup>. Twenty-six mouse monoclonal anti-BAMBI antibodies were generated at the first screening. To check the utility of immunostaining for paraffin sections, these monoclonal antibodies were further screened using various immunochemistry methods, using formalin-fixed and paraffin-embedded colon cancers. Finally, one monoclonal antibody was selected and its specificity was confirmed in an absorbed test using excess GST-BAMBI protein and in the immunoblotting analysis<sup>[26]</sup>.

### Methods

Frozen samples (25 CRCs and 5 tubular adenomas were picked at random) kept at -80°C were thawed, cut into small pieces, and homogenized in SDS lysis buffer (Sigma-Aldrich, St. Louis, MO). The homogenate was then centrifuged at 10 000 g for 15 min at 4°C, and the protein concentration of the supernatant was estimated using the BCA protein assay kit (Pierce, Rockford, IL).



**Figure 1** Immunoblotting analysis using anti-BAMBI monoclonal antibody detected BAMBI as a 30 kDa protein in all of the CRC and tubular adenoma specimens. No. 1 to 3 and 6: Strong expression; No. 4, 5, 7 and 8: Weak expression. The levels of BAMBI expression in CRCs were higher than those in tubular adenomas (lanes No. 9 to 12). The quantitative analysis using densitometry of the bands of BAMBI protein revealed that the band intensity in the weak and strong expression groups in CRCs were about two and three times higher than that in tubular adenomas, respectively.

Twenty micrograms of protein from each supernatant was electrophoresed on 7.5% SDS polyacrylamide gel under reducing conditions and electrotransferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). After treatment with blocking solution, the membrane was incubated with anti-BAMBI antibody (dilution 1:1000) overnight at 4°C. The membrane was then treated with goat anti-mouse IgG conjugated with horseradish peroxidase (Dako, Carpinteria, CA, dilution 1:200 000) for 1 h at room temperature, and the enhanced chemiluminescence kit (ECL Advance detection kit, Amersham, Piscataway, NJ) was used for development according to the manufacturer's instructions. The luminescence image was captured and digitized using Lumi Analyst 3.0v (Boehringer Mannheim GmbH, Germany), and the integrated densities of each band were quantified using densitometry software (ImageJ 1.37v, National Institutes of Health, Bethesda, MD). To examine the equality of protein loading, the total amount of protein loaded in each lane was examined by staining with Coomassie blue.

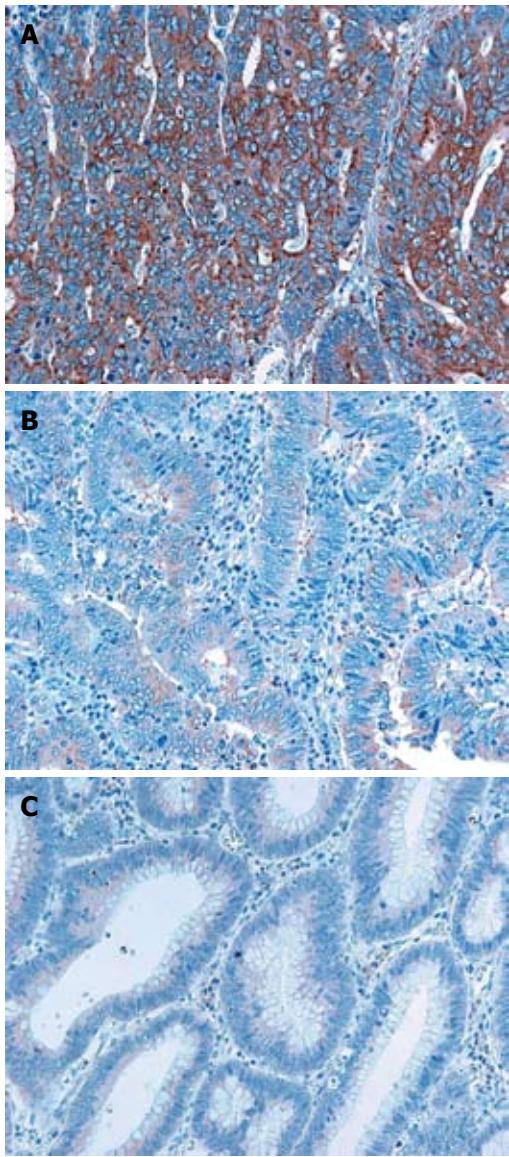
For the immunohistochemical study, 3 μm thick sections were cut from formalin-fixed, paraffin-embedded tissue blocks containing representative tumor histology. The sections were de-waxed in xylene, rehydrated with graded ethanol, and subjected to an antigen retrieval step. For antigen retrieval, the slides for β-catenin and p53 were placed in 0.01 mol/L citrate buffer, 20% ZnSO<sub>4</sub> solution, and heated in a microwave processor (Energy Beam Science, East Granby, CT) at

95°C for 20 min. They were then cooled and rinsed in 0.01 mol/L phosphate-buffered saline (PBS), pH 7.4. The antigen retrieval was not performed on the immunohistochemistry for BAMBI. The slides were then reduced with nonspecific antibody binding by incubating the sections with 10% normal serum in PBS for 30 min at room temperature. After decanting the excess serum, the sections were incubated overnight at 4°C with each monoclonal antibody for BAMBI (1:2000 dilution), β-catenin (1:1600 dilution), and p53 (1:50 dilution) in humidified chambers. After washing thoroughly with PBS, the sections were set in a Ventana Nexus Automated Stainer (Ventana Medical Systems, Tucson, AZ) for immunostaining. The automated protocol was performed according to the manufacturer's instructions, which were based on the labeled streptavidin–biotin method and used the I-VIEW DAB universal kit (Ventana Medical Systems); the kit included a universal biotinylated IgG secondary antibody (anti-mouse), avidin horseradish peroxidase, DAB, 0.03% H<sub>2</sub>O<sub>2</sub>, and 0.5% CuSO<sub>4</sub>. After immunostaining, the sections were lightly counterstained with hematoxylin, and then mounted in a carousel inside the staining module and run to completion. To standardize and confirm the immunohistochemical evaluation of BAMBI, 26 samples of colon carcinoma with tubular adenoma were collected and were tentatively subjected to immunohistochemistry using paraffin sections. Immunohistochemical staining intensity was categorized blindly by two pathologists (T.N, S.S.). Most tubular adenomas showed weak immunoreactivity compared to the cancer component.

Therefore, diffuse BAMBI immunoreactivity, which was observed in the cytoplasm and cell membrane, but not in the nucleus, was evaluated as weak expression if the positivity was similar to that of tubular adenomas. If the BAMBI immunoreactivity was far stronger than that of tubular adenomas, it was evaluated as strong expression (Figures 1 and 2). No BAMBI immunoreactivity was evaluated as negative.

The intensity of nuclear β-catenin immunostaining was graded semiquantitatively into four categories: negative, weakly positive, moderately positive, and strongly positive<sup>[27]</sup>. This evaluation was grouped into two categories: low expression (negative and weakly positive) and high expression (moderately to strongly positive). To evaluate the p53 immunostaining, the nuclear immunoreactivity for p53 was counted in five random tumor areas at high magnification ( $\times 400$ ), and the average percentage of these positive cells was calculated. The p53 immunoreactivity was evaluated as negative or positive expression if the percentage of nuclear positivity was less than or more than 70%, respectively<sup>[28]</sup>.

Statistical analysis was performed using SPSS 11.5 software (SPSS Japan, Tokyo, Japan). The Mann-Whitney U-test, Kruskal-Wallis test, and Spearman's coefficient of rank correlation were used to examine the correlation of BAMBI with other protein and pathological factors. Recurrence-free and overall survival curves were generated using the Kaplan-Meier method,



**Figure 2** Examples of the immunostaining of BAMBI at strong expression (A), weak expression (B) levels, and expression of adenoma (C) ( $\times 200$ ). The immunohistochemical expression level correctly reflected the expression level of BAMBI protein in CRC tissues.

and the log-rank test was used to compare the curves. Deaths without recurrence were included as censored observations. The relative risk and confidence limits were estimated for each variable using the Cox univariate model, using the most suitable prognostic category as the referent group. A multivariate Cox proportional hazard model was also developed using stepwise regression (forward selection) with predictive variables. The limits for entry and removal were both  $P < 0.5$ . Statistical significance was set at  $P < 0.05$ .

## RESULTS

### Immunoblotting analysis

Immunoblotting analysis using anti-BAMBI monoclonal antibody detected BAMBI as a 30-kDa protein in all of the CRC and tubular adenoma specimens. The levels of BAMBI expression in CRCs were higher than those

in tubular adenomas (Figure 1). By contrast, no specific band was detected in normal colonic epithelium (data not shown). The quantitative analysis of the bands revealed that the band intensity was divided into two groups: strong expression and weak expression was three and two times higher than that of tubular adenoma, respectively (Figure 1). Furthermore, CRC tissues with strong expression in immunoblotting were strongly positive for BAMBI immunohistochemistry, whereas other CRC tissues with weak expression revealed weak immunoreactivity to BAMBI (Figure 2). These results indicate that the immunohistochemical expression level correctly reflected the level of BAMBI protein expressed in CRC tissues.

Immunohistochemically, no BAMBI expression was detected in normal colonic epithelium. In contrast, diffuse BAMBI immunoreactivity was observed in the cytoplasm and cell membrane of tubular adenoma and CRC cells (Figure 3). We observed BAMBI immunoreactivity in 148 (80.8%) of 183 CRC tissues, and weak and strong BAMBI expression was observed in 80 (54.1%) and 68 (45.9%) CRCs, respectively.

Nuclear  $\beta$ -catenin immunostaining was not observed in normal colonic mucosa. Nuclear  $\beta$ -catenin immunoreactivity was observed in 154 (84.1%) of 183 CRCs. Nuclear accumulation of p53 was observed in 74 (40.4%) of 183 CRCs (data not shown).

### Relationship between BAMBI expression and clinicopathological factors

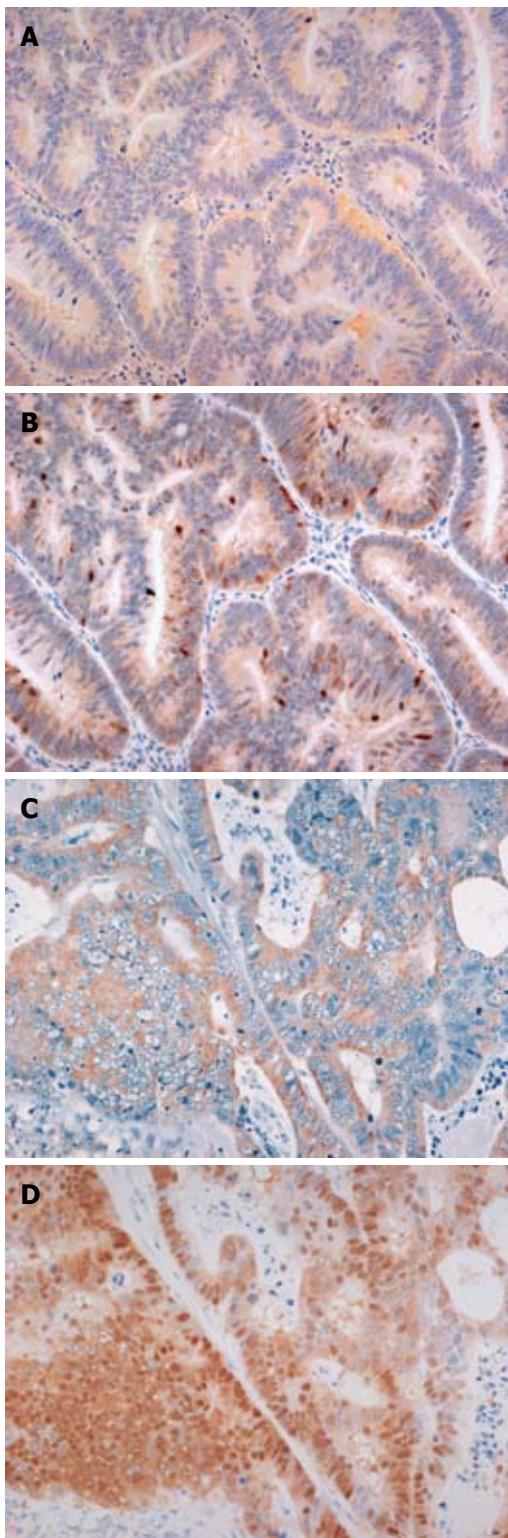
Strong immunohistochemical expression of BAMBI was positively correlated with histological type ( $P = 0.023$ ), depth of invasion ( $P = 0.021$ ), the presence or absence of lymph node metastases ( $P = 0.035$ ), and TNM staging ( $P < 0.001$ ), whereas no correlations were found with gender, age, location, or tumor size (data not shown).

### Correlation between BAMBI and $\beta$ -catenin/p53 expression

We evaluated the correlation of immunohistochemical BAMBI expression with immunohistochemical  $\beta$ -catenin and p53 expression. Strong expression of BAMBI was significantly associated with  $\beta$ -catenin ( $P = 0.035$ ) and p53 ( $P = 0.049$ ) expression (Table 2).

### Recurrence-free and overall survival according to BAMBI expression

The Stage IV patients who underwent to macroscopic resection of metastasis with no residual disease were excluded from the recurrence-free and overall survival groups and multivariate analysis. The patients who died from non-cancer-related causes were excluded from the recurrence-free survival and overall survival groups. In all, 157 of 183 patients were analyzed with a mean follow-up period of 33.7 mo (range, 2-137). In the background analysis, histological type ( $P = 0.043$ ) and TNM staging ( $P = 0.035$ ) were significant in strong BAMBI expression compared to weak BAMBI expression. The tumor depth ( $P = 0.054$ ) tended to be



**Figure 3** BAMBI and  $\beta$ -catenin expression in adenoma and cancer ( $\times 200$ ). Adjacent sections of an adenoma showing weak BAMBI expression in the cytoplasm and cell membrane (A) and weak nuclear localization of  $\beta$ -catenin (B); adjacent sections of a tumor showing strong BAMBI expression in the cytoplasm and cell membrane (C) and strong nuclear localization of  $\beta$ -catenin (D).

significant in strong BAMBI expression (Table 3). The cancer recurred in 30 patients, and 20 patients died of cancer-related causes.

No significant differences were observed in the recurrence-free and overall survival at each stage

**Table 2 Correlation between BAMBI expression and  $\beta$ -catenin/p53 expression**

		BAMBI expression <sup>1</sup>		$P^2$
		Weak (n)	Strong (n)	
$\beta$ -catenin <sup>3</sup>	Low	Negative	8	6
		Weak	19	10
	High	Moderate	24	14
		Strong	29	38
p53 <sup>3</sup>	Negative		55	36
	Positive		25	3
		80 (54.1%)	68 (45.9%)	

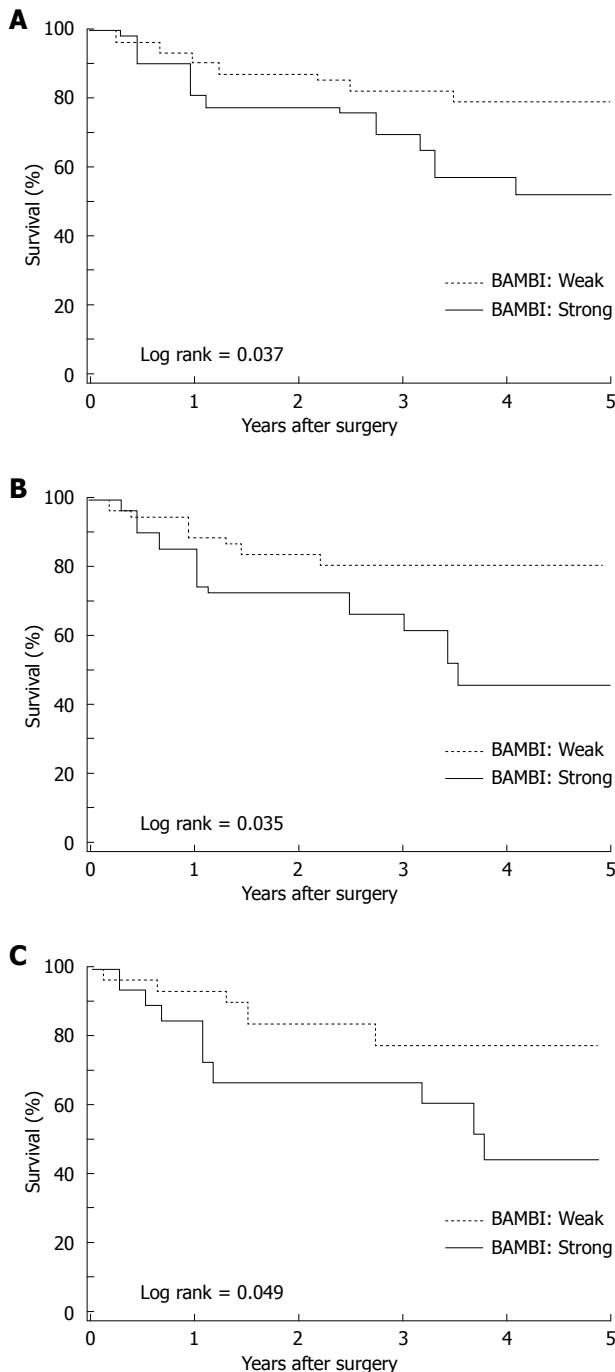
<sup>1</sup>BAMBI expression was scored as described in the Methods; <sup>2</sup>Mann-Whitney test;  $P < 0.05$  was considered statistically significant; <sup>3</sup> $\beta$ -Catenin/p53 expression were scored as described in the Methods.

**Table 3 BAMBI expression in human colorectal carcinomas after curative surgery: Correlations with clinicopathological parameters**

		BAMBI expression				$P^1$
		Weak n	Weak %	Strong n	Strong %	
Gender	Male	41	26.1	11	7.1	NS
	Female	69	43.9	36	22.9	
Age	27-59	60	38.2	24	15.4	NS
	60-95	50	31.8	23	14.6	
Location	Right side	37	23.6	12	7.6	NS
	Left side	27	17.2	16	10.2	
	Rectum	46	29.3	19	12.1	
Tumor size (mm)	$\leq 39$	50	31.8	19	12.1	NS
	40-59	35	22.3	15	9.6	
	$\geq 60$	25	15.9	13	8.3	
Depth of invasion	Tis, T1, T2	38	24.2	9	5.7	0.054
	T3, T4	72	45.9	38	24.2	
Histology	G1	36	22.9	9	5.7	0.043
	G2	67	42.7	32	20.4	
	G3	7	4.5	6	3.8	
LN metastases <sup>2</sup>	No	59	37.6	22	14.0	NS
	Yes	51	32.5	25	15.9	
TNM stage	I	33	21.0	7	4.5	NS
	II	35	22.3	16	10.2	
	III	35	22.3	18	11.4	
	IV	7	4.5	6	3.8	
Metastatic site	Liver	2	28.6	5	83.3	NS
	Yes	5	71.4	1	16.7	
Lung	No	6	85.7	6	100	NS
	Yes	1	14.3	0	0	
Distant lymph nodes	No	7	100	6	100	NS
	Yes	0	0	0	0	

<sup>1</sup>Kruskal-Wallis or Mann-Whitney test;  $P < 0.05$  was considered statistically significant; <sup>2</sup>Lymph node (LN) metastases. NS: Not significant.

according to the BAMBI expression level. However, the 5-year recurrence-free survival rate differed significantly ( $P = 0.037$ ); it was 51.9% with strong BAMBI expression compared to 79.8% for weak BAMBI expression (Figure 4A). The 5-year overall survival rate was 61.0% for strong BAMBI expression and 75.0% for weak BAMBI expression ( $P = 0.495$ ). No significant differences were detected in recurrence-free survival according to  $\beta$ -catenin (low vs high: 77.2% vs 65.9%,  $P = 0.109$ ) and p53 (negative vs positive: 75.8% vs 61.1%,  $P = 0.109$ ).



**Figure 4** **A:** The 5-year recurrence-free survival rate differed significantly ( $P = 0.037$ ) and was 51.9% for strong BAMBI expression compared to 79.8% for weak BAMBI expression; **B:** The impact of the coexpression of BAMBI and  $\beta$ -catenin on the recurrence-free survival with high  $\beta$ -catenin expression, the 5-year recurrence-free survival was 46.9% for strong BAMBI expression compared to 80.8% for weak BAMBI expression ( $P = 0.035$ ); **C:** The impact of the coexpression of BAMBI and p53 on the recurrence-free survival with positive p53 expression, the 5-year recurrence-free survival rate was 43.4% for strong BAMBI expression compared to 78.2% for weak BAMBI expression ( $P = 0.049$ ).

0.417) expression.

Furthermore, we evaluated the impact of the coexpression of BAMBI,  $\beta$ -catenin, and p53 on recurrence-free survival. With high  $\beta$ -catenin expression, the 5-year recurrence-free survival was 46.9% for strong BAMBI expression compared to 80.8% for

weak BAMBI expression ( $P = 0.035$ ; Figure 4B). Weak  $\beta$ -catenin expression did not affect the survival rate according to the BAMBI expression level (weak vs strong: 75.0% vs 79.2%,  $P = 0.6294$ ). With positive p53 expression, the 5-year recurrence-free survival rate was 43.4% for the strong BAMBI expression group compared to 78.2% for the weak BAMBI expression group ( $P = 0.049$ ; Figure 4C). With negative p53 expression, the 5-year recurrence-free survival rate was 62.4% with strong BAMBI expression versus 80.8% for weak BAMBI expression ( $P = 0.582$ ).

#### Cox's proportional hazards regression models

The relative risk and confidence limits for recurrence-free survival were estimated for each variable using the Cox univariate model. TNM staging (relative risk 4.63; 95% CI 2.84-7.56;  $P < 0.001$ ), presence or absence of lymph node metastases (relative risk 8.937; 95% CI 3.10-25.77;  $P < 0.001$ ), depth of invasion (relative risk 14.77; 95% CI 2.01-108.55;  $P = 0.008$ ), and strong BAMBI expression (relative risk 2.109; 95% CI 1.029-4.323;  $P = 0.041$ ) were significant. In the multivariate Cox proportional hazard model, TNM stage (relative risk 4.35; 95% CI 2.65-7.14;  $P < 0.001$ ) was the only significant indicator for recurrence. According to Spearman's coefficient of rank correlation, BAMBI expression was significantly correlated with TNM staging ( $P = 0.038$ ) and the nuclear expression of  $\beta$ -catenin ( $P = 0.01$ ). In the model excluding TNM stage and the nuclear expression of  $\beta$ -catenin, lymph node metastases (relative risk 6.685; 95% CI 2.24-19.93;  $P < 0.001$ ), depth of invasion (relative risk 14.01; 95% CI 1.74-112.69;  $P = 0.013$ ), and strong BAMBI expression (relative risk 2.26; 95% CI 0.95-5.38;  $P = 0.057$ ) were significant indicators of recurrence (Table 4).

## DISCUSSION

BAMBI was expressed in 80% of CRC tumors, and strong BAMBI expression was observed in 46% of CRC tumors. The region with BAMBI expression matched the region of  $\beta$ -catenin expression, and the expression of BAMBI and  $\beta$ -catenin were correlated, consistent with the fact that  $\beta$ -catenin is responsible for the aberrant expression of BAMBI<sup>[26]</sup>. In addition, the association of BAMBI expression and p53 expression is consistent with a previous report that p53 induces Siah-1, which mediates  $\beta$ -catenin degradation<sup>[29]</sup>. The overexpression of BAMBI inhibits the response of tumor cells to TGF- $\beta$  signaling, and interferes with TGF- $\beta$ -mediated growth arrest *in vitro*<sup>[25,26]</sup>. These results indicate that BAMBI expression is one of the critical early genetic events in CRC tumorigenesis.

Strong BAMBI expression was more frequently associated with deep tumor penetration, lymph node metastases, and advanced TNM stage. Patients with strong BAMBI expression had shorter recurrence-free and overall survival, and strong BAMBI expression was an independent factor predicting a lower recurrence-free survival in the multivariate analysis. The nuclear

**Table 4** Relative risks of prognosis according to proportional-hazards regression models

Characteristics	Category	Univariate analysis			Multivariate analysis		
		Hazard ratio	P	95% CI	Hazard ratio	P	95% CI
Depth of invasion	Tis, T1, T2/T3, T4	14.773	0.008	2.011-108.548	14.008	0.013	1.741-112.694
LN metastases	Yes/No	8.937	< 0.001	3.100-25.768	6.685	< 0.001	2.242-19.927
TNM stage	0/ I / II / III / IV	4.633	< 0.001	2.838-7.563			
BAMBI	Weak/Strong	2.109	0.041	1.029-4.324	2.265	0.057	0.952-5.385
β-Catenin	Low/High	1.944	0.108	0.864-4.377			
p53	Negative/Positive	1.702	0.147	0.830-3.489	0.937	0.871	0.427-2.057

accumulation of β-catenin contributes to the transactivation of target genes, which encode regulators of differentiation and effectors supporting invasion and metastasis (CD44, laminin-52, matrix metalloproteinase MMP-7 and MT1-MMP) and angiogenesis (vascular endothelial growth factor), among others<sup>[5]</sup>. In fact, the overexpression of nuclear β-catenin is reported to be an independent predictor of short patient survival time<sup>[30-33]</sup>. Inconsistent with these reports, we showed that nuclear β-catenin accumulation alone does not affect survival and is not a prognostic factor in the univariate and multivariate analyses. However, we found that patients with both strong BAMBI expression and the overexpression of β-catenin have poorer survival compared to those without β-catenin expression. Smad activation or expression is lost in approximately 10% of CRC occurrences, and these patients have a poor prognosis because of its association with advanced disease and the presence of lymph node metastases at diagnosis<sup>[34]</sup>. BAMBI expression allows tumor cells to escape from TGF-β signaling and activate oncogenic processes such as growth stimulation, increases in motility, and invasion. Therefore, BAMBI plays an important role in the invasiveness and metastatic potential of colon cancers through cross-talk with the Wnt and TGF-β signaling pathways.

Although the available data on the correlation of p53 status and the prognosis of colorectal cancers are controversial<sup>[35]</sup>, we found that p53 expression status did not influence the recurrence-free and overall survival and was not a prognostic factor in the univariate and multivariate analyses. Nevertheless, patients with both strong BAMBI expression and p53 overexpression had lower survival compared to those without p53 expression. We speculate that target genes induced by BAMBI overexpression may complement or cooperate with those induced by p53 inactivation and thereby contribute to poorer survival.

Although CRC prognosis is stage and grade dependent, cancers with similar clinicopathological features may have major differences in outcome. A large proportion of patients with Stage II disease and some with Stage III disease can be cured by surgery alone and do not derive any benefit from adjuvant therapy<sup>[36]</sup>. Therefore, the identification of robust molecular prognostic markers to supplement conventional pathological staging systems is highly desirable. Several molecular markers are promising prognostic predictors for the outcome in CRC, including deleted in colorectal

cancer (DCC), microsatellite instability (MSI), and loss of heterozygosity at 18q<sup>[37,38]</sup>. CRC patients with strong BAMBI expression had more aggressive disease and their recurrence-free survival was markedly shorter. In contrast, patients with weak BAMBI expression had less aggressive disease and significantly longer recurrence-free survival. Strong BAMBI expression was tending to be an independent molecular predictor for survival secondary to lymph node metastasis and depth of tumor in the Cox hazard model. Therefore, strong BAMBI expression might be applicable in clinical practice to predict recurrence in addition to lymph node metastasis and depth of tumor.

In clinical scenarios, targeting the TGF-β signaling pathway for chemoprevention and the treatment of human cancers should decrease or abrogate TGF-β signaling, particularly for advanced or metastatic disease<sup>[20]</sup>. Given that the TGF-β signaling pathway has an important role in tumor-induced immunosuppression<sup>[39]</sup>, inhibitors of this pathway may be used to improve natural immunosurveillance of tumor cells or to enhance the effectiveness of active or passive immunotherapy strategies. Our findings suggest that the development of a new active monoclonal anti-BAMBI antibody may offer a great improvement in survival of CRC patients and might also serve as a diagnostic tool for CRC prognosis.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

In generally, BMP and activin membrane-bound inhibitor (BAMBI) expression is aberrantly elevated in most colorectal cancers (CRCs). However, few studies are reported on BAMBI expression in colorectal tissue. To analyze the clinical significance of BAMBI, authors studied its expression in CRC using immunohistochemical staining. They show that BAMBI overexpression is correlated with aggressive tumor phenotypes and predicts tumor recurrence and cancer-related death in CRC. This study was to map BAMBI expression in colorectal tissue and analyze the relationship between BAMBI expression and CRC prognosis.

### Research frontiers

To analyze the clinical significance of BAMBI, authors studied its expression in CRC using immunohistochemical staining. They show that BAMBI overexpression is correlated with aggressive tumor phenotypes and predicts tumor recurrence and cancer-related death in CRC. BAMBI may be usable as a

target for diagnostic and antibody medicine.

### Innovations and breakthroughs

The results of this study show that BAMBI expression plays a role in the pathogenesis of colorectal cancer.

### Applications

The expression level of BAMBI plays an important role in the pathogenesis of colorectal cancer. The development of a new active monoclonal anti-BAMBI antibody may offer a great improvement in survival of CRC patients and might also serve as a diagnostic tool for CRC prognosis.

### Peer review

This is an extremely well written and researched paper. It discovers yet another marker of prognosis for CRC.

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CLINICAL RESEARCH

# Irritable bowel syndrome subtypes differ in body awareness, psychological symptoms and biochemical stress markers

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**CONCLUSION:** IBS subtypes showed different profiles in body awareness, somatic and psychological symptoms and in biochemical variables. D-IBS differed compared to the other groups by lowered body awareness, less psychological symptoms and a higher sense of coherence and elevated C-peptide values. C-IBS and A-IBS subtypes suffered more from depression and anxiety, associated with a lower quality of life. These differences may be important and will be taken into account in our treatment of these patients.

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**Key words:** Irritable bowel syndrome subtypes; Physiotherapy; Body awareness; Stress; Biochemistry

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## Abstract

**AIM:** To elucidate the differences in somatic, psychological and biochemical pattern between the subtypes of irritable bowel syndrome (IBS).

**METHODS:** Eighty IBS patients, 30 diarrhoea predominant (D-IBS), 16 constipation predominant (C-IBS) and 34 alternating IBS (A-IBS) underwent physiotherapeutic examinations for dysfunctions in body movements and awareness and were compared to an apparently healthy control group (AHC). All groups answered questionnaires for gastrointestinal and psychological symptoms. Biochemical variables were analysed in blood.

**RESULTS:** The D-IBS group showed less body awareness, less psychological symptoms, a more normal sense of coherence and psychosocial rating as well as higher C-peptide values. C-IBS had a higher degree of body dysfunction and psychological symptoms, as well as the lowest sense of coherence compared to controls and D-IBS. They also demonstrated the most elevated prolactin levels. A-IBS had the lowest degree of body disturbance, deteriorated quality of life and affected biochemical pattern. All subtypes had higher pain scores compared to controls. In addition they all had significantly increased triglycerides and elevated morning cortisol levels, however, without statistical significance compared with the controls.

## INTRODUCTION

Irritable bowel syndrome (IBS) is considered to be the most common of all gastrointestinal dysfunctions<sup>[1-4]</sup>. Patients with IBS are suffering from a variety of gastrointestinal complaints, as well as associated symptoms like headache and dysuria. Furthermore, there is also a strong connection to fibromyalgia, chronic fatigue syndrome, anxiety, and depression<sup>[5,6]</sup>.

The understanding of IBS and especially the interaction between the central and enteric nervous systems has grown considerably over the last years<sup>[7]</sup>. There are several studies demonstrating abnormalities in the autonomic nervous system<sup>[8,9]</sup>, whereas the expression of different biochemical parameters has been studied with somewhat diverging results<sup>[10-14]</sup>.

The division of IBS into different subgroups is based on the fact that these patients behave in dissimilar ways. According to the Rome II criteria, building on stool and defecation patterns, IBS can be divided into diarrhoea

predominant (D-IBS), constipation predominant (C-IBS) and alternating (A-IBS) subtypes<sup>[15,16]</sup>. Although, lately questioned, the Rome II criteria are widely used in clinical practice<sup>[17]</sup>.

When comparing the various subtypes of IBS, Whitehead *et al*<sup>[18]</sup> did not find any disparity in colonic motility and psychological testing. In contrast, other authors have found differences in gender, abdominal discomfort/pain and psychological comorbidity between the IBS subtypes<sup>[19-22]</sup>. Disparities in endocrine factors between the subtypes of IBS have not been extensively studied. However, Elsenbruch *et al*<sup>[23]</sup> found a significant increase in postprandial saliva cortisol in D-IBS patients, not evident in C-IBS patients and controls. Jonsson and Theorell<sup>[24]</sup> found that plasma cortisol correlated negatively with diarrhoea symptoms and lower prolactin values were seen in patients with functional dyspepsia.

In earlier studies, we have shown that IBS could be associated with deviated tension in the body<sup>[13,25]</sup>. A physiotherapeutic approach to adjust and reduce pathological tension in the body is by the use of Body Awareness Therapy (BAT). This method is devoted to take care of, and improve, the patient's ability to become aware of his or her own capability by the use of self recruited resources to recapture a normal balance in the body<sup>[26-30]</sup>. We found that 12 wk of treatment with BAT gave symptom relief of both gastrointestinal and psychological symptoms<sup>[25]</sup>. However, patients with C-IBS were more relieved than the D-IBS and alternating types of IBS. In a second trial we treated the patients for 24 wk and satisfying effects were obtained for the entire group of IBS patients<sup>[13]</sup>.

The aim of the present study was to elucidate the differences in somatic and psychological symptoms, as well as biochemical stress markers in the IBS subtypes. The hypothesis was that these subtypes present with dissimilar symptomatic expressions.

## MATERIALS AND METHODS

### Study population

All patients with IBS as diagnosed by gastroenterologists, GI surgeons or GPs referred for Body Awareness Therapy at the Unit for Functional Gastroenterology, participated in the study. Patients with an acute psychiatric disease and patients not understanding the Swedish language were excluded from the study.

IBS patients, 73 women and 7 men (age, 21-65 years), with a BMI  $23.3 \pm 3.7$  participated in the study. According to the Rome II criteria patients were divided into 3 groups: D-IBS ( $n = 30$ , 24 women and 6 men), C-IBS ( $n = 16$ , 15 women and 1 man) and A-IBS suffering from combined symptoms ( $n = 34$ , all women). Fifty-six IBS patients had suffered from their gastrointestinal symptoms for more than 5 years (for D-IBS 67%, for C-IBS 88% and for A-IBS 65%). There were no differences in BMI between the subtypes.

A healthy control group consisting of 18 women and 3 men (age, 21-61 years) had a BMI of  $22.3 \pm 2.2$ . They

were free of gastrointestinal symptoms and without ongoing pharmacological treatment.

### Study design

The groups of IBS test patients and the AHC group underwent complete physiotherapeutic examinations in accordance to the Body Awareness Scale (BAS). They also filled in the questionnaires GIS, SCL90, SOC, PRS, and pain drawing. Blood samples were taken from an antecubital vein. The ethics committee of the University of Göteborg approved the study. All subjects gave their written consent before acceptance of inclusion into the study.

### Body examinations

BAS test is based on one hand observations by the physiotherapist of dysfunctions in defined items of basic movements (BASobs) and was carried out during video recording. In addition, standardized questions in order to measure the patients' own opinion concerning their body awareness (BASself) was performed. The variables were ranging from 0-6 where a higher score represented more symptoms<sup>[31,32]</sup>.

### Questionnaires

A modified form of Gastro Intestinal Symptom questionnaire (GIS) was used<sup>[33]</sup>. This survey evaluates 35 general gastrointestinal symptoms. A total score and scoring of specified symptoms were used. The test utilizes a seven-graded scale (0-6). A higher score means increased gastrointestinal complaints.

The Symptom Checking List questionnaire (SCL90) is a self-rating scale evaluating symptomatic behaviour of psychological state using questions related to everyday life<sup>[34]</sup>. The questionnaire includes 90 questions. The answers score in a five-graded scale (0-4) and allow subdivision into different items. A higher score reflected more symptoms.

The Sense of Coherence Scale (SOC) measures the degree to which individuals find the world around them comprehensible and manageable and thus represents a measurement of coping skills<sup>[35]</sup>. The questionnaire includes 29 questions. The answers score in a seven-graded scale (0-6) and allow subdivision into different items. A high SOC score is linked to successful coping with factors that induce stress and is consequently reflecting a higher health level/quality of life.

Psychosocial rating scale (PRS) (slightly modified from Headley Court psychosocial rating scale) has 33 items and score from 0-4 ranging from 'very severe problems' to 'no problems'<sup>[36]</sup>.

The distribution of pain was visualized on a pain-map, figuring the human body with a front and backside. When calculating the results, the body was divided into 45 sections<sup>[37]</sup>. Points were given for every section where pain was marked. The points were summed up to a score.

### Biochemistry in blood

Venous blood samples were taken under fasting condi-

**Table 1** Body awareness scale (BAS-H)

Category	AHC ( <i>n</i> = 21)		D-IBS ( <i>n</i> = 30)		C-IBS ( <i>n</i> = 16)		A-IBS ( <i>n</i> = 34)	
	M	Md (IQR)	M	Md (IQR)	M	Md (IQR)	M	Md (IQR)
BASobs								
Total	1.5	2 (2)	2.6	3 (3) <sup>c</sup>	2.7	3 (3) <sup>f</sup>	2.2	2 (4) <sup>c,f,i</sup>
Grounding	1.5	2 (2)	2.6	2 (2) <sup>c</sup>	2.9	3 (2) <sup>f</sup>	1.9	2 (2) <sup>b,f,i</sup>
Mid-line	2.1	2 (2)	3.6	4 (3) <sup>c</sup>	3.4	3 (2) <sup>f</sup>	3.0	3 (2) <sup>c,d</sup>
Centring	2.1	2 (1)	3.3	3 (2) <sup>c</sup>	3.4	4 (2) <sup>c</sup>	2.8	3 (2) <sup>c,e,g</sup>
Flow	1.5	1 (2)	2.5	3 (4) <sup>c</sup>	2.7	3 (3) <sup>f</sup>	2.4	2 (4) <sup>c</sup>
Respiration	1.4	1 (2)	3.9	4 (2) <sup>c</sup>	3.9	4 (2) <sup>c</sup>	3.6	4 (1) <sup>c</sup>
Boundaries	0.5	0 (0)	1.1	0 (2) <sup>c</sup>	1.1	0 (2) <sup>b</sup>	1.0	0 (2) <sup>b</sup>
BASself								
Total	1.6	2 (2)	1.8	2 (3) <sup>a</sup>	2.7	2 (4) <sup>c,f</sup>	2.3	2 (4) <sup>c,f,h</sup>
Grounding	0.8	0 (2)	1.0	0 (2)	1.8	2 (3) <sup>c,e</sup>	1.8	2 (4) <sup>c,f</sup>
Mid-line	1.6	2 (2)	1.8	2 (4)	3.2	4 (5) <sup>c,f</sup>	2.4	2 (4) <sup>b,e,g</sup>
Centring	1.3	0 (2)	1.3	1 (2)	2.2	2 (4) <sup>a,d</sup>	1.4	1 (2)
Flow	2.1	2 (4)	2.8	3 (5) <sup>a</sup>	3.8	4 (4) <sup>c,e</sup>	3.5	4 (4) <sup>c,d</sup>
Respiration	1.0	0 (2)	1.6	2 (2)	2.4	2 (3) <sup>c,d</sup>	2.9	4 (3) <sup>c,f</sup>
Boundaries	1.7	2 (3)	2.0	2 (3)	2.6	2 (4) <sup>c,e</sup>	2.0	2 (4) <sup>e</sup>

Results from BASobs and BASself examination score from AHC group and D-IBS, C-IBS and A-IBS patients. The results are shown in total and as items categorised. The higher score, the more symptoms. <sup>a</sup>P < 0.05, <sup>b</sup>P < 0.01, <sup>c</sup>P < 0.001 vs AHC; <sup>d</sup>P < 0.05, <sup>e</sup>P < 0.01, <sup>f</sup>P < 0.001 vs D-IBS; <sup>g</sup>P < 0.05, <sup>h</sup>P < 0.01, C-IBS vs A-IBS. M: Mean; Md: Median; IQR: Inter quartile range.

tions from an antecubital vein in the morning for analysis of C-peptide, triglycerides, prolactin and cortisol (at 8:00 and 13:00).

### Statistical analysis

This study consisted of ordinal data (BAS and questionnaires) and quantitative data (biochemical parameters). A high value for BAS, GIS, SCL90, PRS, and pain drawing meant more symptoms. A high SOC value reflected a higher degree of sense of coherence. Median (Md), inter-quartile range (IQR), mean (M), standard deviation (SD) and percentage were used for presentation of data. Although median and IQR were optimal for ordinal data, means were presented as well for better visualization. Mann Whitney *U* test was used for ordinal and quantitative data<sup>[38]</sup>.

## RESULTS

### Study population

There was an obvious gender difference between the IBS subtypes with 20% men in the D-IBS, 6.25% in the C-IBS and 0% men in the A-IBS subgroup.

### Physiotherapeutic data

**BAS:** All subtypes of IBS patients scored higher in the BASobs than the controls. Comparing the subtypes, the A-IBS group showed less body disturbances compared to the other two groups (Table 1). In BASself the D-IBS group expressed lower score compared to the other two groups, with levels similar to the AHC group.

### Questionnaires

**Gastrointestinal symptoms:** All subtypes scored higher than the control group. There was no difference between the subtypes in total score. The A-IBS group

scored higher for constipation and flatulence and less for diarrhoea and motility compared to the D-IBS group. The C-IBS group scored more constipation, less motility and less diarrhoea compared to the D-IBS group (Table 2). Thus, the patients scored in accordance with their own subtype.

**Psychological symptoms:** The C-IBS and the A-IBS group scored higher psychological symptoms compared to the D-IBS group and all groups scored higher than the controls. Also, the C-IBS group scored more symptoms compared to the A-IBS group, especially for sensitivity, phobic anxiety, psychoticism and somatisation (Table 3).

**SOC:** The SOC questionnaire revealed that there were significant differences between the subtypes compared to the controls. The A-IBS group and the C-IBS group scored lower sense of coherence than the D-IBS group, which differed from healthy controls only in the total score and present time and external conditions (Table 4).

**PRS:** All subtypes showed lower psychosocial rating compared to the control group (Table 5). Besides, both C-IBS and A-IBS showed lower psychosocial rating/quality of life compared to the D-IBS group.

**Pain:** The subgroups of IBS showed higher scores of pain presented on the body drawings compared to the healthy controls. However, there were no differences in pain score for the different locations between the subtypes (Table 6).

**Biochemical analysis:** The D-IBS group differed from the other two subtypes and the AHC with significantly higher C-peptide values (Table 7). The C-IBS patients

**Table 2 Gastrointestinal scale (GIS)**

Category	AHC ( <i>n</i> = 21)		D-IBS ( <i>n</i> = 30)		C-IBS ( <i>n</i> = 16)		A-IBS ( <i>n</i> = 34)	
	M	Md (IQR)	M	Md (IQR)	M	Md (IQR)	M	Md (IQR)
Total	0.4	0 (1)	1.9	1 (3) <sup>c</sup>	1.7	1 (3) <sup>c</sup>	2.0	1 (3) <sup>c</sup>
Pain	0.5	0 (1)	1.9	2 (2) <sup>c</sup>	2.0	2 (2) <sup>c</sup>	1.9	2 (2) <sup>c</sup>
Flatulence	0.5	0 (0)	2.4	2 (3) <sup>c</sup>	2.5	3 (3) <sup>c</sup>	2.9	3 (4) <sup>c,e</sup>
Nausea	0.5	0 (1)	1.5	0 (3) <sup>a</sup>	1.3	1 (2)	1.4	0 (2) <sup>a</sup>
Constipation	0.4	0 (1)	0.8	1 (1) <sup>a</sup>	3.8	4 (2) <sup>c,f</sup>	2.9	2 (3) <sup>c,f</sup>
Diarrhoea	0.5	0 (1)	3.2	3 (3) <sup>c</sup>	0.5	0 (1) <sup>f</sup>	2.2	2 (2) <sup>c,f</sup>
Motility	0.7	0 (1)	3.8	4 (3) <sup>c</sup>	2.6	2 (3) <sup>c,e</sup>	3.0	3 (3) <sup>c,d</sup>
Miscellaneous	0.3	0 (0)	1.2	1 (1) <sup>c</sup>	1.1	0 (1) <sup>c</sup>	1.2	1 (2) <sup>c</sup>

GIS score from AHC group, D-IBS, C-IBS, and A-IBS patients. The results are shown in total and as items categorised. The higher score, the more symptoms. <sup>a</sup>*P* < 0.05, <sup>c</sup>*P* < 0.001 vs AHC; <sup>d</sup>*P* < 0.05, <sup>e</sup>*P* < 0.01, <sup>f</sup>*P* < 0.001 vs D-IBS. M: Mean; Md: Median; IQR: Inter quartile range.

**Table 3 Psychological symptoms (SCL-90)**

Category	AHC ( <i>n</i> = 21)		D-IBS ( <i>n</i> = 30)		C-IBS ( <i>n</i> = 16)		A-IBS ( <i>n</i> = 34)	
	M	Md (IQR)	M	Md (IQR)	M	Md (IQR)	M	Md (IQR)
Total	0.3	0 (0)	0.8	0 (1) <sup>c</sup>	1.3	1 (2) <sup>c,f</sup>	1.1	1 (2) <sup>c,f,i</sup>
Obsessive-comp	0.4	0 (1)	1.0	1 (2) <sup>c</sup>	1.5	1 (2) <sup>c,f</sup>	1.3	1 (3) <sup>c,e</sup>
Sensitivity	0.3	0 (0)	0.6	0 (1) <sup>c</sup>	1.3	1 (2) <sup>c,f</sup>	0.9	0 (2) <sup>c,f,h</sup>
Depression	0.4	0 (1)	1.1	1 (2) <sup>c</sup>	1.6	2 (2) <sup>c,f</sup>	1.5	1 (3) <sup>c,f</sup>
Anxiety	0.4	0 (0)	1.0	0 (2) <sup>c</sup>	1.3	1 (2) <sup>c,e</sup>	1.2	1 (2) <sup>c,d</sup>
Hostility	0.2	0 (0)	0.3	0 (0) <sup>b</sup>	0.8	0 (1) <sup>c,f</sup>	0.7	0 (1) <sup>c,f</sup>
Phobic anxiety	0.4	0 (0)	0.4	0 (0)	0.7	0 (1) <sup>c,f</sup>	0.5	0 (0) <sup>c,g</sup>
Paranoid ideation	0.2	0 (0)	0.4	0 (1) <sup>b</sup>	1.2	1 (2) <sup>c,f</sup>	0.7	0 (1) <sup>c,d,i</sup>
Psychoticism	0.1	0 (0)	0.3	0 (0) <sup>c</sup>	0.7	0 (1) <sup>c,f</sup>	0.4	0 (1) <sup>c,d,i</sup>
Somatisation	0.2	0 (0)	1.3	1 (2) <sup>c</sup>	1.9	2 (2) <sup>c,f</sup>	1.5	1 (3) <sup>c,d,i</sup>

SCL 90 score from AHC group, D-IBS, C-IBS and A-IBS patients. The results are shown in total and as items categorised. The higher score, the more symptoms. <sup>b</sup>*P* < 0.01, <sup>c</sup>*P* < 0.001 vs AHC; <sup>d</sup>*P* < 0.05, <sup>e</sup>*P* < 0.01, <sup>f</sup>*P* < 0.001 vs D-IBS; <sup>g</sup>*P* < 0.05, <sup>h</sup>*P* < 0.01, <sup>i</sup>*P* < 0.001, C-IBS vs A-IBS. M: Mean; Md: Median; IQR: Inter quartile range.

**Table 4 Sense of coherence (SOC)**

Category	AHC ( <i>n</i> = 21)		D-IBS ( <i>n</i> = 30)		C-IBS ( <i>n</i> = 16)		A-IBS ( <i>n</i> = 34)	
	M	Md (IQR)	M	Md (IQR)	M	Md (IQR)	M	Md (IQR)
Total	4.2	4 (2)	3.8	4 (3) <sup>b</sup>	3.1	3 (3) <sup>c,f</sup>	3.4	4 (3) <sup>c,f,h</sup>
Comprehensibility	3.7	4 (2)	3.3	3 (3)	2.5	3 (3) <sup>c,f</sup>	3.1	3 (3) <sup>c,i</sup>
Manageability	4.4	5 (3)	4.0	4 (3)	3.3	3.5 (3) <sup>c,f</sup>	3.6	4 (3) <sup>c,h</sup>
Meaningfulness	4.6	5 (1)	4.2	5 (3)	3.8	4 (2) <sup>c,d</sup>	3.7	4 (2) <sup>c,f</sup>
Present time	4.5	5 (1)	4.0	5 (3) <sup>a</sup>	3.3	3 (3) <sup>c,f</sup>	3.5	4 (3) <sup>c,f</sup>
External conditions	4.1	4 (2)	3.7	4 (3) <sup>a</sup>	2.9	3 (4) <sup>c,f</sup>	3.4	3 (3) <sup>c,h</sup>

Sense of coherence score from the AHC group and C-IBS, D-IBS and A-IBS patients. The results are shown in total and as items categorised. The higher score, the better SOC. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01, <sup>c</sup>*P* < 0.001 vs AHC; <sup>d</sup>*P* < 0.05, <sup>e</sup>*P* < 0.001 vs D-IBS; <sup>g</sup>*P* < 0.01, <sup>h</sup>*P* < 0.001, C-IBS vs A-IBS. M: Mean; Md: Median; IQR: Inter quartile range.

**Table 5 Psychosocial rating scale (PRS)**

Category	AHC ( <i>n</i> = 21)		D-IBS ( <i>n</i> = 30)		C-IBS ( <i>n</i> = 16)		A-IBS ( <i>n</i> = 34)	
	M	Md (IQR)	M	Md (IQR)	M	Md (IQR)	M	Md (IQR)
PRS	3.75	4 (0)	3.33	4 (1) <sup>c</sup>	3.17	4 (1) <sup>c,e</sup>	3.12	4 (2) <sup>c,f</sup>

Psychosocial rating scale (PRS) presented as M, Md (IQR) from the AHC group and D-IBS, C-IBS and A-IBS patients. A higher score indicates a better psychosocial rating/quality of life. <sup>a</sup>*P* < 0.001 vs AHC; <sup>b</sup>*P* < 0.01, <sup>c</sup>*P* < 0.001 vs D-IBS. M: Mean; Md: Median; IQR: Inter quartile range.

expressed higher prolactin values both compared to the controls and the D-IBS subtype. Concerning the morning cortisol measurement the subtypes showed

higher values compared to the controls, while the mid day cortisol levels were only slightly raised. However, the differences in cortisol values did not attain statistical

**Table 6** Pain

Category	AHC ( <i>n</i> = 21)		D-IBS ( <i>n</i> = 30)		C-IBS ( <i>n</i> = 16)		A-IBS ( <i>n</i> = 34)	
	M	Md (IQR)	M	Md (IQR)	M	Md (IQR)	M	Md (IQR)
Total	4.2	4 (4)	12.4	11 (12) <sup>b</sup>	14.1	11 (13) <sup>b</sup>	13.3	11 (12) <sup>c</sup>
Abdominal	0.3	0 (0)	1.5	2 (1) <sup>b</sup>	2.4	2 (1) <sup>c</sup>	1.9	2 (1) <sup>c</sup>
Rest of body	3.9	4 (6)	10.1	9 (10) <sup>a</sup>	13.1	14 (14) <sup>a</sup>	11.4	9 (11) <sup>b</sup>

Pain score presented as M, Md (IQR) experienced as drawings from the AHC group and D-IBS, C-IBS, and A-IBS patients. The higher score the more symptoms. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01, <sup>c</sup>*P* < 0.001, vs AHC. M: Mean; Md: Median; IQR: Inter quartile range.

**Table 7** Biochemical levels (mean ± SD, %)

Category	AHC ( <i>n</i> = 21)	D-IBS ( <i>n</i> = 30)	C-IBS ( <i>n</i> = 16)	A-IBS ( <i>n</i> = 34)
C-peptide	0.46 ± 0.14	10/10	0.62 ± 0.28 <sup>a</sup>	40/3
Triglyceride	0.7 ± 0.3	10/0	1.3 ± 0.6 <sup>c</sup>	57/3
Prolactin	264 ± 118	5/10	232 ± 94	7/23
Cortisol 8	491 ± 144	10/14	539 ± 239	10/6
Cortisol 13	316 ± 65	10/14	334 ± 157	27/30

Biochemical status presented as mean ± SD and percent (%) above/below ± 1SD for the AHC group and D-IBS, C-IBS, and A-IBS patients. Cortisol 8 and 13 equals cortisol level at 8 am, and at 1 pm, respectively. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01, <sup>c</sup>*P* < 0.001, vs AHC; <sup>a</sup>*P* < 0.01, vs D-IBS.

significance. All subgroups showed higher triglyceride levels than controls.

Looking at the variation above or below one standard deviation all subtypes had a larger variation compared to the controls. There was also a difference in the patterns of standard deviation, e.g. the prolactin values were, for the D-IBS group mostly below one standard deviation while for the C-IBS and A-IBS group the values were mostly above one standard deviation.

## DISCUSSION

In the present study our IBS population compared to healthy controls, showed overall a higher degree of dysfunctions in basic movements and awareness, as well as more psychological and gastrointestinal symptoms. They also scored a lower sense of coherence and increased pain. In addition the IBS patients had higher and more edged values of biochemical parameters.

When dividing the patients into subgroups according to their stool and defecatory patterns, i.e. D-IBS, C-IBS and alternating type of IBS the following characteristics were identified.

The D-IBS group showed a disturbed body movement pattern on BASobs in the same magnitude as the other two groups. However, on self-estimation (BASself) they rated themselves as having less dysfunction reflecting a lower sense of body awareness compared to the other two groups. They had the same amount of gastrointestinal, but less psychological symptoms compared to C-IBS and A-IBS. The D-IBS patients scored a nearly normal degree of sense of coherence and thus a good quality of life, also reflected in a slightly less distorted psychosocial rating scale compared to the other subgroups. However, they expressed a high pain score similar to the other subtypes. They also had a higher C-peptide value, not being so prominent in C-IBS and

A-IBS. All subgroups also showed higher triglyceride values compared to controls.

The C-IBS and A-IBS groups exhibited to some extent similar patterns. However, the A-IBS patients revealed less body disturbance than the C-IBS. On self-estimation (BASself) both groups rated themselves at the same level as the physiotherapist. Both subtypes suffered from more gastrointestinal and psychological symptoms, than the AHC group. However, the C-IBS patients had more psychological symptoms than the D-IBS and A-IBS groups. Both groups displayed a lowered sense of coherence, and thus a lower quality of life, also outlined in the psychosocial rating scale. Furthermore, they were afflicted with high pain scores compared to the controls. When looking at the biochemistry the C-IBS patients had elevated prolactin values compared to the other groups.

The outcome of the gastrointestinal scale (GIS) reflecting the same symptom patterns as the subtype is supportive of an initially correct subtyping of the patients prior to referral. Actually, GIS could be used as a tool to subtype the IBS patients.

From the present study it seems as the D-IBS patients differed from the other two groups. They were not aware of their dysfunctional body awareness not realising their depreciated state of health, thus coping with preserved quality of life. These patients had less psychological symptoms, and higher C-peptide values. Thus, the increased C-peptide value could be secondary to hyperinsulinemia, reflecting an altered adrenergic drive. Although, sympathetic activation normally inhibits bowel motility, one could speculate whether this tentative adrenergic abnormality may be one component of the enteric neuropathy seen in D-IBS. Overall, they revealed themselves as ambitious persons, with a higher proportion of men compared to the other subtypes and many of them in the midst of their professional careers.

The higher C-peptide and triglyceride levels may be part of a metabolic syndrome, which is known to correlate with psychosocial stress<sup>[39]</sup>. Prolactin may be important in the process of coping with stress and traumatic experience<sup>[40]</sup> and Sivik *et al*<sup>[41]</sup> reported active soldiers to have lower prolactin values. Sondergaard *et al*<sup>[42]</sup> have shown a strong correlation between prolactin and alexithymia specially the item 'difficulty to identifying feelings'. The D-IBS group in our study had both lowered prolactin values and lower body awareness.

These results are in accordance with the outcome of a study by Aggarwal *et al*<sup>[8]</sup>. When studying predominant symptoms in IBS and the correlation with autonomic nervous system abnormalities they found that the D-IBS subgroup was associated with adrenergic nervous system malfunctions. They also found that C-IBS patients were more psychologically distressed, with higher degree of depression and anxiety. Also, the C-IBS patients were found to have vagal cholinergic dysfunction in that study. This may also be in line with our results of higher prolactin values for the C-IBS group, which may correspond to increased vagal tone<sup>[43]</sup>, as well as higher SCL90 scores. The C-IBS and A-IBS patients are characterised by their psychological symptoms of anxiety and depression. Emotional strain is correlated to increased levels of prolactin and this could be one of the reasons for the prolactin increase in the C-IBS group<sup>[44]</sup>.

Also in agreement with our results, Elsenbruch *et al*<sup>[12]</sup> found in a study on postprandial autonomic and cortisol responses that D-IBS patients elicited an enhanced sympathetic drive as measured by heart rate variability compared to the C-IBS patients and controls. The D-IBS had significantly higher postprandial saliva cortisol levels, but the cortisol values at baseline were equal for these groups, which is in conformity with our results on cortisol levels. Although morning fasting cortisol levels were increased equally for all subtypes, the differences compared to controls did not turn out as statistically significant. There was also a considerable spread of the values above and below one standard deviation compared to controls. These findings were also partly true for the mid day cortisol levels.

Although the sample size of the present study is fairly modest in this context, the recruitment of subjects was from patients presenting with fairly advanced disease, with several years history of symptoms. However, since they are referred from different types of care providers they can be regarded as representing a general population of IBS patients. Thus, our results can probably be generalised for a larger IBS population.

IBS is described as a gastrointestinal functional disorder, which onset and course is affected by psychological factors. Asahina *et al*<sup>[45]</sup> suggest that treatment of psychological factors should also be considered when dealing with IBS. Moser<sup>[46]</sup> points out that functional gastrointestinal disorders are the most frequent clinical conditions seen in practice and suggests that integrated psychosomatic care should be provided i.e. the patient's psychosocial status and the demand for additional psychological care should be assessed and offered<sup>[47]</sup>. This

is supported by the results of randomised controlled studies having shown that psychotherapy is superior to conventional therapy<sup>[46]</sup>. This is also in line with the results from our studies with physical, psychological and biochemical examinations and treatment of the 'whole person' with body awareness therapy. Jones *et al*<sup>[48]</sup> showed that IBS patients had lower quality of life and less interpersonal support and greater reliance on passive coping strategies. IBS patients show in our study lower quality of life and lower body awareness which could be connected to passive coping strategies. The disparities seen in our study of the subtypes of IBS are in agreement with these studies mentioned and may be different expressions of the functional gastrointestinal disorder. With the knowledge gained from the present study body awareness therapy could be adjusted to the different manifestations encountered in the subtypes of IBS.

In conclusion, this study has shown that the D-IBS patients, with a higher proportion of men, scored less body awareness, less psychological symptoms, better sense of coherence and showed higher C-peptide values, possibly indicating an adrenergic drive representing unconscious mental stress. The C-IBS and A-IBS patients expressed higher body awareness, more depression and anxiety with impaired sense of coherence. The raised prolactin levels in C-IBS patients may reflect an increased vagal tone and emotional strain. The importance of the differences seen between the IBS subtypes in the present study and its implications for future treatment of IBS will have to be elucidated in further investigations.

## COMMENTS

### Background

Irritable Bowel Syndrome (IBS) is the most common of all gastrointestinal disorders, affecting around 15% of the population at least in the Western societies. The division of IBS into subgroups is based on the fact that these patients behave in dissimilar ways. IBS subgroups building on stool and defecation patterns can be divided into diarrhoea predominant (D-IBS), constipation predominant (C-IBS) and alternating (A-IBS) subtypes.

### Research frontiers

The understanding of IBS and especially the interaction between the central and enteric nervous systems has grown considerably over the last years. Therefore, in recent year's research has focused more and more on the psychosomatic (body and mind) aspect of the disease. IBS patients are therefore examined more comprehensible with gastrointestinal symptoms, psychological symptoms, biochemical stress markers, quality of life and body awareness. Thus, psychosomatic remedies like hypnotherapy, psychotherapy and body awareness therapy have been applied.

### Innovations and breakthroughs

Subgroups of IBS as shown in this study differ in body awareness, quality of life, psychological symptoms and biochemical stress markers.

### Applications

Treatment like Body Awareness Therapy (BAT) which is a physiotherapeutic approach, to adjust and reduce pathological tension in the body may in the future also be applied and streamlined for these subgroups in order to get a more optimal treatment.

### Peer review

This manuscript reports results of a study of differences in body awareness, pain scores, psychological symptoms and blood levels for prolactine, triglycerides and morning cortisol between healthy controls and patient diagnosed by Rome criteria with either D-IBS, C-IBS and A-IBS. The manuscript is well constructed and written.

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# Ultrastructural changes in hepatocytes after taurine treatment in CCl<sub>4</sub> induced liver injury

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## INTRODUCTION

Liver cirrhosis is the terminal stage of various chronic liver diseases. Even mild but continuous injury in the liver soon results in excessive production of extracellular matrix components<sup>[1]</sup>. Deposition in the space of Disse causes capillarization of sinusoids and alterations in liver functions. This fibrotic stage ultimately progresses to cirrhosis, which is characterized by nodule formation and corruption of liver architecture.

Overproduction and accumulation of extracellular matrix proteins in the liver start usually after chronic hepatocyte injury that initiates a series of complicated cell-to-cell and cell-to-matrix interactions, eventually leading to activation of hepatic stellate cells, which are the main producers of excessive collagen during cirrhosis process<sup>[2]</sup>. Since hepatocyte injury seems to be the first and the main fibrogenic stimulus in the liver, healing of these cells could be a desirable goal in preventing progression of fibrosis.

Taurine, 2-aminoethanesulphonic acid, is an essential  $\beta$ -amino acid. It is present at high concentrations in many tissues. It plays important roles in numerous physiological functions including conjugation with bile acids, modulation of calcium levels, and maintenance of osmolarity, antioxidation and stabilization of membranes<sup>[3]</sup>. It was reported to have beneficial effects in various physiological and pathological conditions<sup>[4-7]</sup> by mainly diminishing production of reactive oxygen species (ROS). It also can prevent DNA damage at physiological concentrations<sup>[8]</sup>. Taurine has also hepatoprotective effects such as inhibition of extracellular matrix accumulation in experimental liver fibrosis<sup>[9,10]</sup> and improvement of liver function tests in fatty liver disease of children<sup>[11]</sup>. Hepatoprotective feature of taurine is attributed to its inhibitory activity on generation of ROS, which are

## Abstract

**AIM:** To search the organelle based changes in hepatocytes after taurine treatment in experimental liver fibrosis induced by CCl<sub>4</sub> administration.

**METHODS:** Thirty rats were divided into two groups. Group 1 ( $n = 15$ ) was injected with CCl<sub>4</sub> plus taurine and Group 2 ( $n = 15$ ) with CCl<sub>4</sub> plus saline for 12 wk. At the end of 12th wk, mitochondria, rough and smooth endoplasmic reticulum, and nuclei of hepatocytes were evaluated using a scoring system. The results were compared with histopathological findings, as well.

**RESULTS:** Taurine treatment reduced fibrosis scores significantly as compared to placebo. Organelle injury scores decreased significantly with taurine treatment. Ultrastructural and histopathological scores in both groups were in strong correlation ( $r = 0.931$  for CCl<sub>4</sub> plus taurine and  $r = 0.899$  for CCl<sub>4</sub> plus saline group).

**CONCLUSION:** Organelle based transmission electron microscopy findings can reflect successfully histological results as well as tissue healing in hepatocytes from hepatotoxin-induced liver fibrosis.

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**Key words:** Taurine; Liver fibrosis; Hepatocyte; Ultrastructure

known to play an important role in hepatic injury both *in vitro* and *in vivo*<sup>[12,13]</sup>.

The effects of acute oxidative stress on the ultrastructure of sinusoidal endothelium, space of Disse, hepatocytes and Kuppfer cells in perfused rat liver have been studied previously by Cogger *et al*<sup>[14]</sup>. They successfully demonstrated the alterations in the mitochondria of injured hepatocytes. Recently, we have shown beneficial effects of taurine on histopathology and oxidative stress parameters in a rat model of CCl<sub>4</sub>-induced liver fibrosis<sup>[15,16]</sup> where remarkable histopathological improvement in taurine treated animals subjected to hepatotoxin was observed, and this was associated with oxidative stress reduction and hepatocellular apoptosis. In this work we report on the changes in the chronic setting, with more focusing on the multiple organelle based alterations after administration of CCl<sub>4</sub> that causes hepatic injury primarily *via* increasing ROS production in the liver<sup>[17]</sup>. We also studied the correlation of ultrastructural changes with histopathological findings.

## MATERIALS AND METHODS

The study was approved by the Institutional Animal Use and Care Committee of the Gulhane School of Medicine, Ankara, Turkey, and was performed in accordance with the National Institute of Health guidelines for the care and handling of animals. The animals were fed with free access to standard rat food and water, and housed in metabolic cages one in one at controlled temperature and 12-h light/dark cycles before and during the experiment. Electron microscopic examinations were performed at the Department of Anatomy, Hacettepe University Medical Faculty, Ankara.

### Animals and treatment strategies

Thirty male Sprague Dawley rats weighing 250-400 g were randomly divided into two groups. Group 1 (*n* = 15) was injected with CCl<sub>4</sub> (0.2 mL/100 g twice weekly; S.C) plus taurine (1000 mg/kg per day; I.P), and Group 2 (*n* = 15) with CCl<sub>4</sub> plus saline for 12 wk. At the end of 12th wk all rats were killed under anesthesia and the livers were excised. Adequate numbers of specimens from right and left lobes of each liver were collected for transmission electron microscopy and histopathological examination.

### Light microscopy analysis

For light microscopy, tissue sections were fixed in 10% neutral buffered formalin and embedded in paraffin. Paraffin sections were stained with hematoxylin-eosin, examined and scored by two pathologists who were blinded to the treatment protocol. Degree of necrosis, inflammation, fat accumulation, and fibrosis was scored as 0: Absent; 1: Slight; 2: Moderate; and 3: Severe as described elsewhere, with small modifications<sup>[18]</sup>. Histopathological evaluation was performed twice in four sections per slide from all animals in each group.

**Table 1 Ultrastructural scoring system used in experimental liver fibrosis<sup>[19-21]</sup>**

Ultrastructure Assessment	Score
Mitochondria	Normal
	Prominent cristae
	Edematous mitochondrion
	Collection of amorphous material
rER	Normal
	Dilatation
	Irregular lamellar organization
sER	Presence of focal breaks
	Normal
	Dilatation
Nucleus	Vacuolization
	Presence of large degenerated areas, myelin figures
	Normal
	Irregular chromatin distribution (margination, clumping)
	Increased heterochromatin
	Degenerated nucleus

rER: Rough endoplasmic reticulum; sER: Smooth endoplasmic reticulum.

### Ultrastructural analysis

The specimens were fixed in 2.5% glutaraldehyde for 24 h and subsequently washed in phosphate buffer (pH 7.4), post-fixed in 1% osmium tetroxide in phosphate buffer (pH 7.4) and dehydrated in increasing concentrations of alcohol. Afterwards, the tissues were washed with propylene oxide and embedded in epoxy-resin embedding media. Semi-thin sections about 3 mm in thickness were cut with a glass knife on a LKB Nova ultramicrotome (Sweden). These sections were stained with methylene blue and examined by a Nikon Optiphot (Japan) light microscope. Ultrathin sections were collected on copper grids, stained with uranyl acetate and lead citrate, and finally examined under a Jeol JEM 1200 Ex (Japan) transmission electron microscope. Twenty cells from each specimen were examined. Mitochondria, nuclei, rough endoplasmic reticulum (rER) and smooth endoplasmic reticulum (sER) of hepatocytes were evaluated by using a previously described scoring system (Table 1)<sup>[19-21]</sup>. Twenty nuclei, 50 mitochondria, 20 rERs and 20 sERs were examined for each animal.

### Statistical analysis

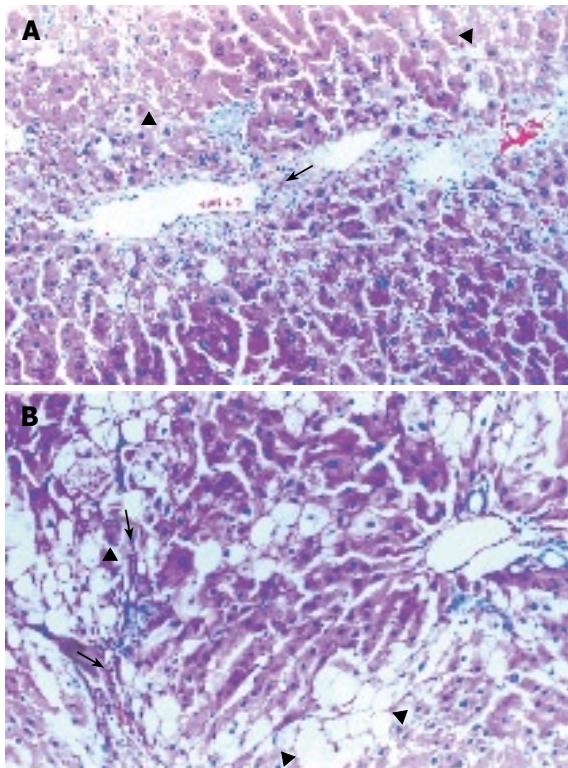
Results are expressed as mean ± SEM. Mann-Whitney U test for histopathologic scores and Student's *t*-test for ultrastructure scores were used to analyze significance of differences between groups. Correlation of histopathological and ultrastructural scores was assessed with Pearson correlation procedure. The differences were accepted as statistically significant when *P* < 0.05.

## RESULTS

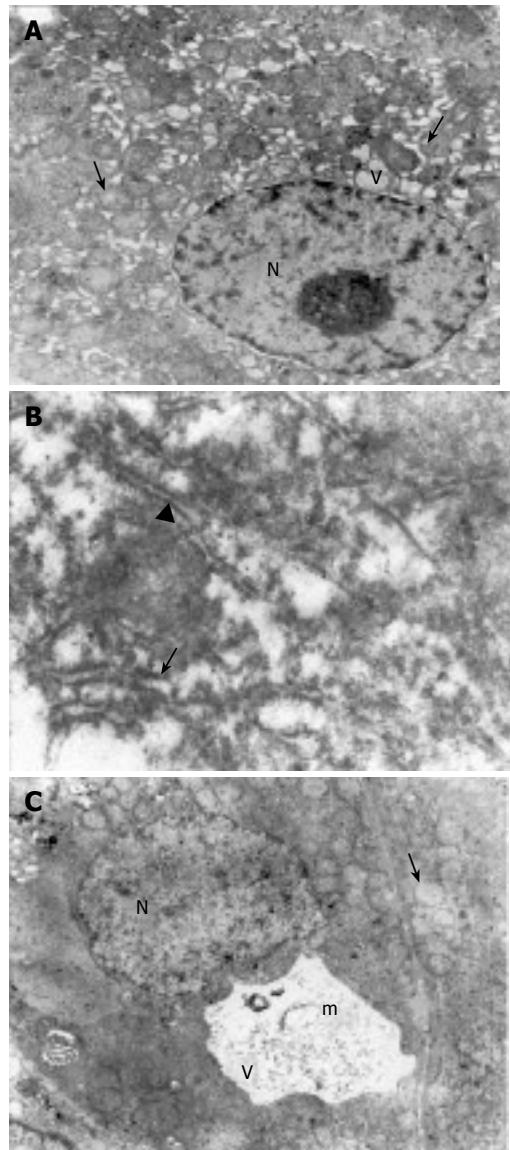
Two animals in Group 1 and four in Group 2 died before the end of experiment.

### Light microscopy

CCl<sub>4</sub> treatment produced hepatic necrosis, inflammation, fatty accumulation, and fibrosis by the 12th wk. Light



**Figure 1** **A:** Mild bile duct proliferation (arrow) and microvesicular changes (arrowheads) in Taurine treated animals (HE, x 200); **B:** Severe macro and microvesicular fatty accumulation (arrowheads) and fibrosis (arrows) in Taurine untreated group (HE, x 50).



**Figure 2** Electron micrographs. **A:** The dilatations in the rER (arrow) and sER (arrowhead), large vacuoles in the hepatocytes (V), a normal nucleus (N) and mitochondria with a prominent edema (small arrow) in  $\text{CCl}_4$  plus taurine treated group (x 4000); **B:** Large dilatations (arrow) and focal breaks (arrowhead) in the rER in  $\text{CCl}_4$  plus saline treated group (x 30 000); **C:** The vacuolizations (V) and myelin figures (m) in the sER, nucleus (N) and mitochondria with prominent edema (arrow) in  $\text{CCl}_4$  plus saline treated group (x 4000).

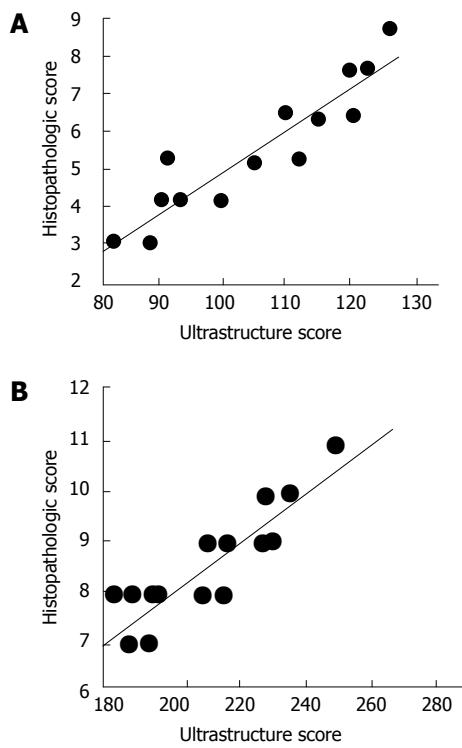
<sup>1</sup>Mann-Whitney U test; <sup>2</sup>Student's *t*-test. ER: Endoplasmic reticulum.

microscopy evaluation of liver sections from animals treated with  $\text{CCl}_4$  and taurine are shown in Figure 1, and Table 2. Necrosis, inflammation, fatty accumulation and fibrosis were significantly lower in Group 1 when compared with Group 2 (Figure 1B).

#### Transmission electron microscopy

Ultrastructural analysis of the liver sections revealed significantly lower organelle injury scores in  $\text{CCl}_4$  plus taurine treated group when compared with  $\text{CCl}_4$  plus saline treatment (Table 2). Mitochondrial edema was seen in both groups but it was more extensive in saline treated animals. Additionally, mitochondrial cristae were

much more visible in taurine treated animals (Figure 2A). While there were irregular lamellar organization and large dilatations with focal breaks in rERs of hepatocytes in  $\text{CCl}_4$  plus saline treated group in many areas (Figure 2B), only some focal slight dilatations were observed in taurine treated animals. Dilatations in taurine treated animals (Figure 2A) but vacuolization and myelin figures in saline treated group were sER findings (Figure 2C). Although there was irregular chromatin distribution in some areas, nuclei were almost normal in appearance with taurine treatment (Figure 2A). However, chromatin distribution was irregular and nuclei showed extensive margination and clumping in saline treated group. Other prominent findings were large vacuolization of hepatocyte cytoplasm in taurine treated animals and presence of active fibroblasts in some focal



**Figure 3** Correlation of histopathologic and ultrastructure scores in  $\text{CCl}_4$ . **A:** Plus Taurine treated group ( $r = 0.931$ ); **B:** Plus Saline treated group ( $r = 0.899$ ).

areas in saline treated group. Though the model was hepatotoxin-based, disruption of membranes was rarely seen; therefore, we did not modify the scoring table regarding the changes in membranes.

Ultrastructural scores in both groups highly correlated with the histopathologic scores ( $r = 0.931$  for Group 1,  $r = 0.899$  for Group 2) (Figure 3).

## DISCUSSION

The present study addresses the ultrastructural findings in hepatocytes and their correlation with histopathological scores after taurine treatment in  $\text{CCl}_4$  induced experimental liver fibrosis. As was shown previously in several studies, taurine treatment inhibits production of excess extracellular matrix in experimental liver fibrosis<sup>[8,10]</sup>. Although we did not measure tissue content of collagen in liver tissues, we found visible evidence under light microscopy that taurine decreased the degree of necrosis, inflammation, fatty accumulation and fibrosis. Antifibrotic activity of this endogenous amino acid in the liver was confirmed once more in the present study. Since  $\text{CCl}_4$  results in hepatotoxicity through its enzymatic conversion to reactive trichloromethyl free radicals<sup>[17]</sup>, antioxidant treatment is essential to be effective in reducing the degree of injury.

We analyzed mitochondria, rER, sER and nuclei according to a previously described ultrastructural scoring table<sup>[19-21]</sup> that was successfully used in experimental pancreatitis to demonstrate the level and characteristics of injury in parenchymal cells. Such a scoring system may be useful for the researchers in following the changes in ultrastructure of hepatocytes

under pathogenic stress or medications.

The most significant ultrastructural recovery with taurine treatment occurred in mitochondria and sER. Mitochondria are the energy source of the cell, as was specifically demonstrated in hepatocytes, with two membranes, one of which limits the organelle and the other of which is inside the organelle and is thrown into folds that project inward in a tubular nature called cristae mitochondria<sup>[22]</sup>. The molecules in the electron transport chain, which play the central role in ATP synthesis, are found in the cristae. It is also well known that mitochondria are both a major source of endogenous production of ROS<sup>[23]</sup>. The major mitochondrial pathology in our work was edema, the major ultrastructural sign of cellular injury, extensively invading the matrix resulting in swelling of the organelle and structural damage to cristae. Though edema was seen in both groups, it was much less in taurine treated animals. In addition, there was minimal disruption in cristae with taurine treatment and cristae integrity was preserved quite well. Swelling and loss of regular cristae structure with rupture of outer membrane, which are characteristics of deteriorated function of mitochondria<sup>[22,24,25]</sup>, decreased with taurine in our study.

Endoplasmic reticulum is normally composed of tubules, vesicles and sacs forming lamel-like shape<sup>[26]</sup>. Crucial pathways that are related to carbohydrate metabolism, biotransformation, steroid metabolism and protein processing function in the ER with integration to other organelles<sup>[27]</sup>. rER carries ribosomes on its surface different from sER, which is the place of action and storage of important cellular enzymes<sup>[28]</sup>. The proteins synthesized in rER are stored in cristae. Glycogen synthesis and detoxification of drugs are performed in sER in the liver. Severely dilated ER is representative of severely damaged hepatocytes<sup>[26]</sup>. When we examined ERs of the rats treated with saline, we found serious damage as irregular lamellar organization, large dilatations and focal breaks in rERs, and vacuolizations and myelin figures in sERs in many areas. However, only some focal and slight dilatations were observed in rERs and sERs of taurine treated animals. Besides absence of dilation that is known to be reversible when the toxic state is properly opposed or ceases<sup>[29]</sup>, intact lamellar organization and absence of vacuolization seem to be required for normal functioning of ER.

Nuclei, the hallmark of eukaryotic cells, contain DNA with its associated protein called chromatin<sup>[30]</sup>. The density of chromatin varies throughout the nucleus. Dense regions inside the nucleus are called heterochromatin and are found in parts of the chromosome where there are few or no genes. It lies next to inner side of the nuclear envelope. The genes in heterochromatin are generally inactive, that is, not transcribed. Euchromatin is found in parts of the chromosome that contain many active genes. We found alterations in chromatin distribution in the nuclei of hepatocytes from saline treated animals. Although we could not detect abundant heterochromatin, disorganization of nuclear content as margination

and clumping of chromatin in saline treated animals may be morphological evidence of injury in the nucleus. However, severely degenerated nuclei were rarely detected. Nuclear content was almost normal in appearance and organization with taurine treatment, which was previously reported to prevent DNA damage<sup>[8]</sup>. These results confirm the basic knowledge that nucleoplasmic constituents represent the structural counterpart of transcription and processing of messenger and ribosomal RNAs, and therefore constitute fine and highly sensitive indicators of cellular activity.

Electron microscope findings in hepatocytes after hepatotoxin have not been defined systematically to date. Moreover, which changes in each organelle are reversible or not is not clear. Dincer *et al*, previously reported partly the ultrastructural changes in hepatocytes after taurine treatment<sup>[31]</sup>. However, the present study not only defines the organelle changes in more detail; it also provides a better assessment and measurement of ultrastructural injury.

The change in ultrastructural scores between the two groups was nearly the same when compared with histopathological scores. This indicates that the current histopathological scoring system used to describe tissue injury in the present study successfully reflects organelle based ultrastructural changes in hepatocytes. On the other hand, the results obtained in this study should not be overwhelmed, because taurine's most significant action is directly counteracting the effect of CCl<sub>4</sub>. Protective effects of this antioxidant in clinical conditions related to other injury mechanisms in the liver might not be so strongly evident.

In conclusion, this study brought us direct view evidence of changes in morphology of hepatocyte organelles after induction of a certain hepatotoxin. Taurine preserves morphology of major organelles of hepatocytes and delays the development of fibrosis. Structural changes in hepatocyte organelles we observed in this study are likely the cause of significant histological improvements. Since transmission electron microscopy is the highest magnification tool at present, modeling new ultrastructural scoring systems including more organelles and parameters can be useful in estimating the degree of injury and outcome of alternative treatment strategies in management of chronic liver diseases.

## COMMENTS

### Background

Liver fibrosis and cirrhosis are untreatable conditions at present. Antioxidant medications including taurine have been reported to possess antifibrotic efficacy in experimental liver fibrosis. Taurine is one of the main components of energy drinks, which are widely consumed by healthy people around the world. The present study addresses organelle based changes in hepatocytes after taurine treatment in rat liver fibrosis.

### Research frontiers

Preventive effect of taurine in continuing liver injury was tested. The study design does not include treatment after establishment of liver fibrosis. Antioxidants may be taken into consideration not only for their efficacy on established liver fibrosis but also for their hepatoprotective efficacy in the long term.

### Innovations and breakthroughs

Organelle based effects of taurine in hepatocytes is to be shown for the first time on animals.

### Applications

Taurine administration was previously shown to be effective in delaying the development of fibrosis in experimental conditions. The present findings support the idea that conduct of large scale clinical studies on the efficacy of taurine in human liver fibrosis or cirrhosis should be encouraged.

### Terminology

Liver fibrosis refers to invasion of normal liver by collagen deposits in chronic liver injury leading to destruction of normal tissue architecture and functional insufficiency. Taurine is a potent antioxidant not present in the market as a drug but is included in the majority of energy drinks.

### Peer review

This paper is an experimental study in rats demonstrating that taurine protects the liver at the ultrastructural level after the administration of carbon tetrachloride. The paper is novel, well written and well organized, particularly the discussion which is comprehensive and easy to read.

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RAPID COMMUNICATION

## Up-regulation of $\alpha$ -catenin is associated with increased lymph node involvement in colorectal cancer

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SWM, but no such effect on disease free survival (DFS) or disease specific survival (DSS). As to co-expression with another member of the adhesion complex ( $\beta$ -catenin), high  $\alpha$ -catenin/ $\beta$ -catenin MI index was of marginal significance in predicting longer DSS ( $P = 0.063$ , log-rank).

**CONCLUSION:** The results implicate that high  $\alpha$ -catenin expression is intimately involved in the key regulatory mechanisms leading to invasive phenotype, lymph node metastases, and progressive disease in CRC.

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**Key words:** Colorectal carcinoma; Alpha-catenin; Membrane staining; Cytoplasmic staining; Prognosis

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### Abstract

**AIM:** To investigate the changing pattern of  $\alpha$ -catenin expression and its relationship to clinical and pathological features of colorectal cancer (CRC) patients.

**METHODS:** Archival tumor samples were analyzed using immunohistochemistry (IHC) for  $\alpha$ -catenin in 91 patients with advanced CRC.

**RESULTS:** The values of  $\alpha$ -catenin membrane index (MI) and cytoplasmic index (CI) were significantly related to the depth of tumor invasion ( $P = 0.027$ ,  $P = 0.020$ , respectively), high indices being associated with increased depth of the primary tumor invasion (T3 and T4). Similarly, patients with high  $\alpha$ -catenin expression had a significantly increased risk of lymph node metastasis (32/39 vs 37/52 for MI and 37/45 vs 32/46 for CI) ( $P = 0.001$ ,  $P = 0.0001$ , respectively, for LNN status). An altered expression (i.e., cytoplasmic pattern) was also related ( $P = 0.047$ ) to the response to chemotherapy; patients with low CI were more responsive (CR: 7/46) than patients with high CI values (CR: 0/45). There was a marginal effect on survival in patients time with metastases (SWM) ( $P = 0.087$ ); patients with low CI showing slightly longer

### INTRODUCTION

Under normal conditions, cell-cell adhesion molecules maintain epithelial cell integrity and cellular architecture. The process of tumor invasion and metastasis is associated with alterations in the functions of several adhesion molecules. In general, tumor cells lose their capacity for normal adherence, which facilitates their detachment from their site of origin<sup>[1,2]</sup>.

Homotypic cell-cell adhesion is regulated mostly by the cadherin-catenin complex. Alterations in E-cadherin and catenins have been linked with more aggressive behavior of several human tumors<sup>[3-6]</sup>. Catenins are divided according to their molecular weight as  $\alpha$ -catenin (102 kDa),  $\beta$ -catenin (88 kDa) and  $\gamma$ -catenin (80 kDa)<sup>[7-9]</sup>.

$\alpha$ -catenin links E-cadherin to the actin cytoskeleton via its association with either  $\beta$ - or  $\gamma$ -catenin<sup>[10,11]</sup>.

Abnormal  $\alpha$ -catenin expression has been reported in many human cancers<sup>[12-15]</sup>. Reduced  $\alpha$ -catenin expression was associated with tumor invasion and metastases in colorectal cancer (CRC)<sup>[6]</sup>.

In this study, we examined  $\alpha$ -catenin expression in a series of CRC, and analyzed the relationship with clinical and pathological features of CRC patients.

## MATERIAL AND METHODS

### Study material

The material of the present study consists of a series of 91 patients with advanced CRC, enrolled among the consecutive CRC patients attending our clinic for therapeutic procedures. Of these 91 patients, 58 had metastases at diagnosis (Stage IV disease), while the remaining 33 patients (with stage II and III disease at baseline) subsequently developed a metastatic disease during the mean follow-up (FU) time of 25.1 ± 27.8 mo. All patients were treated for advanced and metastatic disease at the Department of Oncology and Radiotherapy, Turku University Hospital, according to the protocols used for CRC patients with stage II, III or IV disease at that time. These 91 patients included in the present study were enrolled into this prospective cohort between October 1998 and August 2003. All patients have been prospectively followed-up until death or when last seen alive at their clinical visit (March 2007), with the median FU-time of 27.6 mo (range 3-150 mo). The study was approved by the TUH Ethics Committee and was conducted in accordance with the Declaration of Helsinki. Samples were collected with the endorsement of the National Authority for Medico-legal Affairs.

The key clinical data of the patients are shown in Table 1. Of these 91 cases, 34 were women and 57 were men. The mean age was 61.5 years (range 24-78 years). The majority ( $n = 38$ ) of the tumors were localized in the left colon, followed in order of frequency by the right colon ( $n = 23$ ), rectum ( $n = 22$ ), and colon transversum ( $n = 7$ ). At the time of diagnosis, 14 patients had Stage II disease, 19 Stage III and 58 tumors were at Stage IV. The majority ( $n = 59$ , 64.8%) were T3 tumors, and almost half ( $n = 46$ ) had known lymph node involvement at the time of diagnosis, including the cases with Nx status. The patients were selected into the cohort on the basis of both the diagnosis and treatment received, and were assigned to one of the two treatment arms: (1) 20 were treated with irinotecan alone; and (2) 71 received a combination of irinotecan and 5-fluorouracil (5-FU) as the first line treatment.

### $\alpha$ -catenin immunostaining

Formalin-fixed, paraffin-embedded primary colorectal tumor tissue was obtained from 91 patients. Sections were cut serially at 5 µm for routine haematoxylin and eosin staining and for immunohistochemical (IHC) analysis. An experienced pathologist confirmed all histological diagnoses. IHC analysis was done using the automatic system (BenchMark XT, Ventana Medical

**Table 1 Key characteristics of the patients and their tumors**

Variable	n or value	% <sup>1</sup>
Patients	91	
Male	57	63.0
Female	34	37.0
Age (yr)		
Median (range)	60.7 (24-80)	
Primary tumour status <sup>2</sup>	91	
T1	1	1.0
T2	6	6.5
T3	59	64.8
T4	16	17.6
Tx	9	9.8
Primary nodal status <sup>2</sup>	91	
N0	22	24.0
N+	49	54.0
Nx	20	22.0
Metastases at diagnosis	91	
M0	34	37.0
M1	57	63.0
Histological grade	91	
Gr I	11	12.1
Gr II	62	68.1
Gr III	18	19.8
Stage	91	
Stage II	14	15.0
Stage III	19	21.0
Stage IV	58	64.0
Survival (mo)		
From primary diagnosis		
Median (range)	27.3 (3-150)	
From metastasis		
Median (range)	21.5 (3-80)	

<sup>1</sup>When applicable; <sup>2</sup>TNM classification. Tx: Unknown; Nx: Unknown.

Systems, Inc. Tucson, Arizona, USA). This fully automated processing of bar code labeled slides included baking of the slides, solvent free deparaffinization, antigen retrieval in a cell conditioning buffer CC2 (Mild: 36 min conditioning, and standard: 60 min conditioning), incubation with the monoclonal mouse  $\alpha$ -catenin antibody (clone  $\alpha$  CAT-7A4, isotype IgG1- $\kappa$ , Zymed Laboratories, San Francisco, CA), at a dilution 1:100 (32 min, 37°C). The dilution of the primary antibody was based on dilution experiments. Application of ultraViewTM Universal DAB (a biotin-free, Multimer-based detection system for the specific and sensitive detection of mouse IgG, mouse IgM, and rabbit IgG primary antibodies). UltraView DAB includes: ultraView Universal HRP, ultraView Universal DAB Inhibitor, ultraView Universal DAB Chromogen, ultraView Universal DAB H<sub>2</sub>O<sub>2</sub>, and ultraView Universal DAB Copper. Counterstaining with haematoxylin (2021) took 4 min, and post-counterstaining with bluing reagent (2037) took 4 min as well. After staining, the sections were dehydrated in ethanol, cleared in xylene, and covered with Mountex and cover slips.

### Evaluation of $\alpha$ -catenin staining

The  $\alpha$ -catenin staining was evaluated by observers blinded to the clinical data using regular light microscopy. Membranous and cytoplasmic expression was evaluated separately. For the cell membrane staining,

four categories were used, (+++, ++, +, -), starting from equivalent to normal to entirely negative<sup>[16]</sup>. The cytoplasmic staining was also graded into four categories: (0) Negative, no detectable staining; (1) Weak, but still detectable staining; (2) Moderate, clearly positive but still weak; (3) Heavy staining, intense<sup>[17]</sup>. In calculating the staining indexes: membrane index (MI), and cytoplasmic index (CI), both the intensity of staining and the fraction of positively-stained cells were taken into account, using the following formula:  $I = 0 * f_0 + 1 * f_1 + 2 * f_2 + 3 * f_3$ , where I is the staining index,  $f_0-f_3$  are the fractions of the cells showing a defined level of staining intensity (from 0 to 3). Theoretically, the index could vary between 0 and 3n<sup>[18]</sup>. The  $\alpha$ -catenin expression was evaluated independently by two observers (AE, AB). The agreement of the evaluation of  $\alpha$ -catenin staining indices was tested between two observers, and the agreement was good as suggested by the correlation coefficient (Pearson's  $r$ : MI, CI, and NI were 0.77, 0.91, and 0.90, respectively,  $P < 0.001$ ).

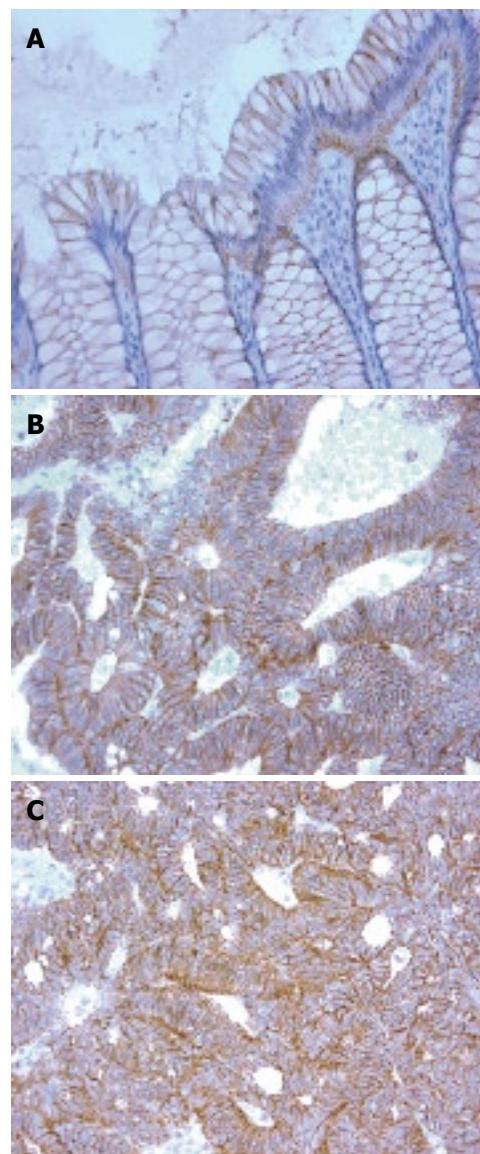
### Statistical analysis

Statistical analyses were performed using the SPSS® (SPSS, Inc., Chicago, USA) and STATA (Stata Corp., Texas, USA) software packages (SPSS for Windows, version 14.0.1 and STATA/SE 9.2). Frequency tables were analyzed using the  $\chi^2$  test, which evaluation included likelihood ratio (LR), or Fischer's exact test to assess the significance of the correlation between the categorical variables. Odds ratios and their 95% confidence intervals (95%CI) were calculated where appropriate, using the exact method. Differences in the means of continuous variables were analyzed using non-parametric tests (Mann-Whitney or Kruskal-Wallis) for 2- and K-independent samples, respectively. ANOVA (analysis of variance) was only used for deriving the mean values (and their SD) of each individual category. Univariate survival (life-table) analysis for the outcome measure (disease specific survival, DSS; disease free survival, DFS) was based on Cox's method (indices treated as continuous variables), and/or using Kaplan-Meier analysis (indices with Median as cut-off). In all tests,  $P < 0.05$  was regarded statistically significant.

## RESULTS

In normal colonic mucosa,  $\alpha$ -catenin expression was predominantly membranous but this pattern was disturbed (diffuse cytoplasmic and membranous) in the tumor tissues (Figure 1). The mean values of MI and CI were 1.3 and 1.1, respectively.

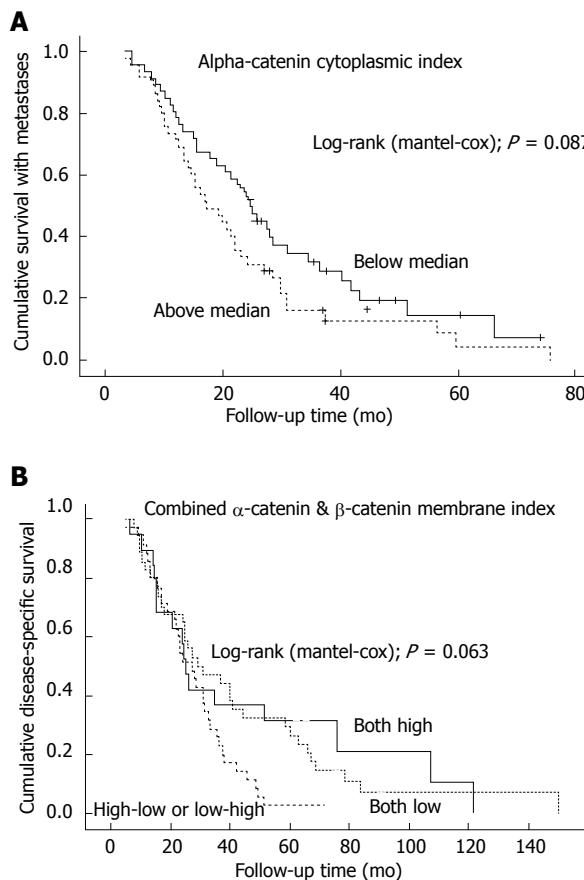
The two expression patterns of  $\alpha$ -catenin were related to all clinical and tumor variables recorded in this series. MI was significantly ( $P = 0.03$ ) related to the localization of the primary tumor, being more intense in carcinomas of the descending colon ( $n = 38$ ) and rectum ( $n = 22$ ) than in those of the ascending ( $n = 23$ ) and transverse colon ( $n = 7$ ). Both MI and CI were also correlated with the depth of the primary tumor (ANOVA;  $P = 0.027$ ,  $P = 0.020$ , respectively), higher



**Figure 1** Different immunohistochemical staining patterns of  $\alpha$ -catenin in colorectal carcinomas. **A:** In normal colonic epithelium,  $\alpha$ -catenin is predominantly expressed in the cell membrane. **B:** A medium-powered view of a colonic adenocarcinoma showing membranous expression of  $\alpha$ -catenin. **C:** This case shows both membranous and cytoplasmic expression of  $\alpha$ -catenin.

mean index values being associated with increased depth of the primary tumor invasion (i.e., T3 and T4). Interestingly, patients with high  $\alpha$ -catenin expression (MI, CI) had a significantly increased lymph node metastasis ( $32/39$  vs  $37/52$  for MI and  $37/45$  vs  $32/46$  for CI) ( $P = 0.001$ ,  $P = 0.0001$ , respectively). On the other hand, there was no correlation between  $\alpha$ -catenin expression and most of the clinical variables (e.g. age, sex, and stage). However, there was a marginal relation between MI and CI ( $P = 0.08$ ,  $P = 0.07$ , respectively) and the grade of the primary tumor.

Cytoplasmic  $\alpha$ -catenin expression was also significantly ( $P = 0.04$ ) related to the response to treatment in that the patients with low CI were more responsive (CR: 7/46) than patients with high CI values (CR: 0/45). No such association was established for the MI.



**Figure 2** A: Cytoplasmic  $\alpha$ -catenin expression and survival with metastasis in univariate (Kaplan-Meier) analysis; B: Combined  $\alpha$ -catenin and  $\beta$ -catenin (MI) expression and disease-specific overall survival in Kaplan-Meier analysis.

In univariate survival analysis, neither MI nor CI was a significant predictor of disease outcome. As to the survival with metastases, there was a marginal difference in the survival curves, patients with low CI showing slightly longer survival with metastases (SWM) (Figure 2A). Finally, when  $\alpha$ -catenin expression was analyzed jointly with  $\beta$ -catenin expression (with the combined MI and CI indices built up using the same criteria as described for  $\alpha$ -catenin), only the combined MI index was of marginal prognostic value in predicting DSS, those with high MI surviving longer (Figure 2B).

## DISCUSSION

As compared with the sub-cellular distribution of  $\alpha$ -catenin in normal colonic mucosa, neoplastic cells demonstrated a distinct shift from the membranous localization to more widespread distribution (membranous, cytoplasmic) in cancer cells. This is in line with the previous reports describing this type of altered pattern of  $\alpha$ -catenin expression in cancer cells<sup>[19,20]</sup>.

Interestingly,  $\alpha$ -catenin expression (MI) was more intense in carcinomas of the descending colon and rectum as compared with the lesions localized in the ascending and transverse colon. Similar observation was reported for  $\beta$ -catenin<sup>[21,22]</sup>, but not for  $\alpha$ -catenin. There is increasing evidence to suggest that molecular mechanisms and molecular phenotypes differ in

carcinomas arising in the proximal and distal segments of the large bowel<sup>[23]</sup>. The involvement of different molecular pathways in colorectal carcinogenesis is exemplified by the fact that cancers of “mutator” phenotypes preferentially occur in the proximal colon, whereas the adenoma-to-carcinoma sequence phenotype is characteristic of carcinomas in the distal colon and rectum<sup>[24,25]</sup>. Corresponding differences have also been demonstrated with other potential prognosticators<sup>[26]</sup>.

The correlation between  $\alpha$ -catenin expression pattern and the TNM categories is a controversial issue. Some studies report that there is no correlation between  $\alpha$ -catenin and these clinicopathological variables<sup>[3]</sup>. On the other hand, Gofuku *et al* 1999 demonstrated that reduced expression of  $\alpha$ -catenin was significantly correlated with the depth of invasion. Moreover, the frequency of lymph node metastases was significantly higher in those tumors with reduced  $\alpha$ -catenin expression<sup>[6]</sup>. Our data show that higher indices in both (MI, CI) have a correlation with increased depth of tumor invasion and to increased lymph node involvement. There are multiple reasons that could explain these inconsistent and in part discrepant results reported in these different studies<sup>[3,6]</sup>. Such potential confounding factors might include the size of tissue sample, intrinsic tumor heterogeneity, lack of standardization of the positive and negative results, and different immunohistochemical staining and grading methods, with varying degree of sensitivity. In addition, our patients represent advanced CRC, the majority of patients with stage IV disease, as compared e.g. with Gofuku’s material, in which only 24/100 patients had stage IV CRC<sup>[6]</sup>.

To our knowledge, this is the first study to investigate the relation between  $\alpha$ -catenin expression and response to treatment. Interestingly, a significant relation was observed between CI and the response to treatment. Accordingly, the patients with low CI were more responsive to treatment than patients with high CI values. The significance of these observations remains to be elucidated in a larger study, however.

As to the relation between  $\alpha$ -catenin expression pattern and clinical outcome, a marginally significant association was observed to survival with metastases. When we combined both MI and CI of  $\alpha$ -catenin and MI and CI of  $\beta$ -catenin (using 3 categories; high/high; high/low or low/high; low/low), only the combined MI index was of marginal prognostic value, high MI predicting longer DSS. Such information has not been reported previously. As shown by the data (Figure 2B), it seems feasible to speculate that patients with high MI of both  $\alpha$ - and  $\beta$ -catenin survive longer, because this is the normal expression pattern of these two adhesion molecules, and retaining this pattern could indicate a less pronounced dedifferentiation of the cancer cells.

Taken together, the present study examined the predictive and prognostic value of  $\alpha$ -catenin expression in advanced CRC. Our results substantiate the emerging evidence on different molecular events operating in CRC at different localizations, while demonstrating a

significant difference in  $\alpha$ -catenin expression between tumors of the proximal and distal sites. High  $\alpha$ -catenin expression was typical in advanced invasion of the primary tumour (i.e., T3 and T4) as well as in appearance of LNN metastases. An altered expression (i.e., cytoplasmic) pattern was also related to the response to treatment, and there was some marginal association with the SWM, but not with DFS or DSS. The latter was marginally predicted by the combined ( $\alpha$ -catenin/ $\beta$ -catenin) MI, however, patients with high MI showing longer DSS. The results implicate that  $\alpha$ -catenin expression is associated with the regulatory mechanisms leading to invasive phenotype and progressive disease in CRC. The full prognostic value of this adhesion molecule as a single marker remains to be established, however, and there is some circumstantial evidence (Figure 2B) that probably more valuable prognostic information could be obtained when  $\alpha$ -catenin is combined with the other constituents of the cell adhesion complex, e.g.  $\beta$ -catenin and the cadherins.

## COMMENTS

### Background

Process of tumor invasion and metastasis is associated with alterations in the functions of several adhesion molecules. In general, tumor cells lose their capacity for normal adherence, which facilitates their detachment from their site of origin.  $\alpha$ -catenin links E-cadherin to the actin cytoskeleton via its association with either  $\beta$ - or  $\gamma$ -catenin. Reduced  $\alpha$ -catenin expression was associated with tumor invasion and metastases in colorectal cancer (CRC). We examined  $\alpha$ -catenin expression in a series of 91 patients with advanced colorectal carcinoma, and analyzed the relationship with clinical and pathological features of CRC patients.

### Research frontiers

CRC is one of the commonest malignant tumours and has a relatively poor prognosis, where the outcome depends on the extent of local and particularly metastatic tumour spread. For example, in locally advanced disease (Dukes C), the 5-year relative survival rate is 65% but goes down to 8% in metastatic disease (Dukes D). Metastatic disease causes the majority of cancer-related deaths, either as a result of tumour involvement of critical organs or due to complications of therapy to control tumour growth and spread. This dramatic difference emphasizes the importance of delineating predictive factors capable of reliably distinguishing CRC patients at risk for developing a metastatic phenotype.

### Innovations and breakthroughs

Patients with high  $\alpha$ -catenin expression (MI, CI) had a significantly increased lymph node metastasis. There is also significant difference in  $\alpha$ -catenin expression between tumors of the proximal and distal sites. Interestingly,  $\alpha$ -catenin expression (MI) was more intense in carcinomas of the descending colon and rectum as compared with the lesions localized in the ascending and transverse colon. Similar observation was reported for  $\beta$ -catenin, but not for  $\alpha$ -catenin. There is increasing evidence to suggest that molecular mechanisms and molecular phenotypes differ in carcinomas arising in the proximal and distal segments of the large bowel. The involvement of different molecular pathways in colorectal carcinogenesis is exemplified by the fact that cancers of "mutator" phenotypes preferentially occur in the proximal colon, whereas the adenoma-to-carcinoma sequence phenotype is characteristic of carcinomas in the distal colon and rectum. Corresponding differences have also been demonstrated with other potential prognosticators. To our knowledge, this is the first study to investigate the relation between  $\alpha$ -catenin expression and response to treatment. Interestingly, a significant relation was observed between CI and the response to treatment. Accordingly, the patients with low CI were more responsive to treatment than patients with high CI values. The significance of these observations remains to be elucidated in a larger study, however.

### Applications

Valuable prognostic information could be obtained when  $\alpha$ -catenin is combined

with the other constituents of the cell adhesion complex, e.g.  $\beta$ -catenin and the cadherins.

### Peer review

The manuscript is well written and the results implicate that high  $\alpha$ -catenin expression is associated with invasive phenotype, lymph node metastases, and progressive disease in CRC. It includes interesting new data of  $\alpha$ -catenin expression in colon carcinomas of different locations. This is also the first study to investigate the relation between  $\alpha$ -catenin expression and the response to treatment.

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RAPID COMMUNICATION

## Laparoscopic *versus* open appendectomy: Which way to go?

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## INTRODUCTION

The introduction of laparoscopic surgery has dramatically changed the field of surgery. With improvements in the equipment and increasing clinical experience it is now possible to perform almost any kind of procedure under laparoscopic visualization.

Although more than a century has elapsed since McBurney first performed open appendectomy<sup>[1]</sup>, this procedure remains the treatment of choice for acute appendicitis for most surgeons.

In 1983, Semm performed the first laparoscopic appendectomy<sup>[2]</sup>. Ever since then, the efficiency and superiority of laparoscopic approach compared to the open technique has been the subject of much debate<sup>[3-23]</sup>. The idea of minimal surgical trauma, resulting in significantly shorter hospital stay, less postoperative pain, faster return to daily activities, and better cosmetic outcome has made laparoscopic surgery for acute appendicitis very attractive. However, several retrospective studies<sup>[3-12]</sup>, several randomized trials<sup>[13-19]</sup> and meta-analyses<sup>[20-24]</sup> comparing laparoscopic with open appendectomy have provided conflicting results. Some of these studies have demonstrated better clinical outcomes with the laparoscopic approach<sup>[13-17]</sup>, while other studies have shown marginal or no clinical benefit<sup>[18-22]</sup> and higher surgical costs<sup>[19,23]</sup>.

At present, although there is no consensus regarding the superiority of the laparoscopic approach over the conventional technique, there is trend towards greater utilization of laparoscopic appendectomy<sup>[24,25]</sup>.

In the present study, we aim to compare the laparoscopic approach and the conventional technique in the treatment of acute appendicitis, using prospectively collected data from patients subjected to appendectomy between January 2006 and January 2008.

## Abstract

**AIM:** To compare the outcome of laparoscopic *versus* open appendectomy.

**METHODS:** Prospectively collected data from 293 consecutive patients with acute appendicitis were studied. These comprised of 165 patients who underwent conventional appendectomy and 128 patients treated laparoscopically. The two groups were compared with respect to operative time, length of hospital stay, postoperative pain, complication rate and cost.

**RESULTS:** There were no statistical differences regarding patient characteristics between the two groups. Conversion to laparotomy was necessary in 2 patients (1.5%). Laparoscopic appendectomy was associated with a shorter hospital stay (2.2 d *vs* 3.1 d,  $P = 0.04$ ), and lower incidence of wound infection (5.3% *vs* 12.8%,  $P = 0.03$ ). However, in patients with complicated disease, intra-abdominal abscess formation was more common after laparoscopic appendectomy (5.3% *vs* 2.1%,  $P = 0.002$ ). The operative time and analgesia requirements were similar in the two groups. The cost of treatment was higher by 370 € in the laparoscopic group.

**CONCLUSION:** Laparoscopic appendectomy is as safe and efficient as open appendectomy, provided surgical experience and equipment are available.

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**Key words:** Laparoscopy; Appendicitis; Appendectomy; Conventional appendectomy

## MATERIALS AND METHODS

Data was collected prospectively on patients with acute appendicitis who underwent open or laparoscopic appendectomy from January 2006 to January 2008 in the surgery department of the University Teaching Hospital at Patras. The clinical data base contained information such as patient characteristics, postoperative course, length of hospital stay, postoperative morbidity and mortality, 30-d readmission and hospital charges.

All human studies were performed according to the principles of the declaration of Helsinki. The study was approved by the research and ethics committee at the University Hospital of Patras.

The diagnosis of appendicitis was made in the emergency department and was based on the presence of right lower quadrant pain, nausea or vomiting, and abdominal guarding on physical examination. In patients where a clinical diagnosis could not be established, imaging studies such as abdominal ultrasound or CT were performed. Exclusion criteria included pregnancy, hemodynamic instability, chronic medical or psychiatric illness, cirrhosis, coagulation disorders, previous laparotomy for small bowel obstruction, and ascites. In order to increase the homogeneity of the group, a total of 37 patients (11.2%), who underwent elective interval appendectomy or had incidental appendectomy in the presence of other intra-abdominal pathology were excluded from the study. The decision about the type of the operation was made according to the preference and experience of the surgical team on duty.

Prior to the surgery, all the patients received a standard regimen of intravenous antibiotics (1.5 g of Cefuroxime and 500 mg of Metronidazole). Provided purulent appendicitis was not observed at surgery, two additional doses were given. In patients with complicated appendicitis, antibiotics were not discontinued but were modified according to the culture results.

Open appendectomy was typically performed through a 3 cm McBurney muscle splitting incision in the right lower quadrant. Following appendectomy the stump was double ligated with an absorbable suture. In the presence of complicated appendicitis the abdomen was irrigated with warm saline solution and the skin incision was closed loosely.

In the laparoscopic group, pneumoperitoneum was produced by continuous pressure of 10–12 mmHg of carbon dioxide via a Verres canula, positioned in the left subcostal area. Following gas insufflation, a 12 mm trocar for the 30 degree angled laparoscope was placed in the left periumbilical area and two additional trocars, a 12 mm trocar in the suprapubic area to accommodate the stapling device and to facilitate specimen extraction, and a third 5 mm trocar in the left lower abdominal quadrant were introduced under direct visualization. The patient was placed in a Trendelenburg position, with a slight rotation to the left. The abdominal cavity was thoroughly inspected in order to exclude other intra-abdominal or pelvic pathology. After the mesoappendix was divided with bipolar forceps, the base of the

**Table 1 Patient demographics**

	Open appendectomy	Laparoscopic appendectomy	P
Number of patients	165	128	
Male (%)	55.1	44.5	0.33
Female (%)	44.9	55.5	0.38
Mean age	33.4 ± 18	33.8 ± 17.8	0.44
WBC count	15497 ± 3000/mm <sup>3</sup>	15728 ± 2793/mm <sup>3</sup>	0.80
Co-morbidities (%)			
CAD	6 (3.6)	5 (3.9)	0.63
Hypertension	13 (7.8)	9 (7)	0.71
COPD	5 (3)	4 (3.1)	0.27
DM	6 (3.6)	3 (2.3)	0.14

WBC: White blood cell; CAD: Coronary artery disease; COPD: Chronic obstructive pulmonary disease; DM: Diabetes mellitus.

appendix was secured with two ligating loops, followed by dissection distal to the second loop using a curved dissector. In patients with severe inflammation, a stapling device was used for the dissection of the appendix. The specimen was placed in an endobag and was extracted through the suprapubic trocar. All specimens were sent for histopathology.

The parameters examined in this study included patient's characteristics (age, sex), operation time (from skin incision to wound closure), conversion to open procedure, and intraoperative findings (normal, gangrenous or perforated appendix). Furthermore, during the post-operative follow up, pain was assessed both by the patient's requirements for analgesia, and with a visual analog score. The length of hospital stay, complications and cost were also added to the plot. The discharge criteria were met once the patients' were afebrile, with audible bowel sounds and were able to tolerate a liquid diet.

Statistical analysis was performed using SPSS statistical software, version 12.0 (SPSS Inc., Chicago, IL). The data were expressed as mean and standard deviation. Parameters such as length of hospitalization, mortality and morbidity, and hospital cost are given as mean variable. Bivariate analyses were performed to determine the differences between laparoscopic versus open appendectomy in patient characteristics, length of hospital stay and costs using independent sample *t* tests for continuous variables and chi-square analysis for categorical variables. A *P* value of less than 0.05 was considered statistically significant.

## RESULTS

A total of 293 patients with acute appendicitis were admitted during the study period. 165 patients were subjected to open appendectomy and 128 patients to laparoscopic appendectomy. The patient characteristics are shown in Table 1. There were no significant differences with respect to gender, age, white blood cell count at presentation, and associated co-morbidities.

Out of the total 165 open procedures, 118 (71.5%) were performed for uncomplicated appendicitis and 47 (28.5%) for complicated disease including appendiceal perforation with local or widespread peritonitis.

**Table 2 Intraoperative variables**

	Open appendectomy	Laparoscopic appendectomy	P
Intraoperative findings (%)			
Normal appendix	16 (9.6)	8 (6.2)	0.20
Acute appendicitis	102 (61.8)	82 (64)	0.72
Gangrenous appendicitis	19 (11.5)	20 (15.6)	0.17
Appendiceal abscess	19 (11.5)	12 (9.3)	0.32
Peritonitis	9 (5.4)	6 (4.6)	0.14
Mean operative time (min)	47 ± 19.7	44.3 ± 24	0.31

**Table 3 Postoperative complications n (%)**

	Open appendectomy	Laparoscopic appendectomy	P
Uncomplicated disease	118	90	
Wound infection	1 (0.8)	0 (0)	0.01
Bowel injury	0 (0)	1 (1.1)	< 0.001
Morbidity (%)	0.8	1.1	0.5
Complicated disease	47	38	
Wound infection	6 (12.8)	2 (5.3)	0.03
Intra-abdominal abscess	1 (2.1)	2 (5.3)	0.002
Bowel obstruction	5 (10.6)	3 (7.9)	0.37
Respiratory infection	4 (8.5)	2 (5.3)	0.18
Morbidity	34	23.7	0.12
Total morbidity (%)	10.3	7.8	0.43

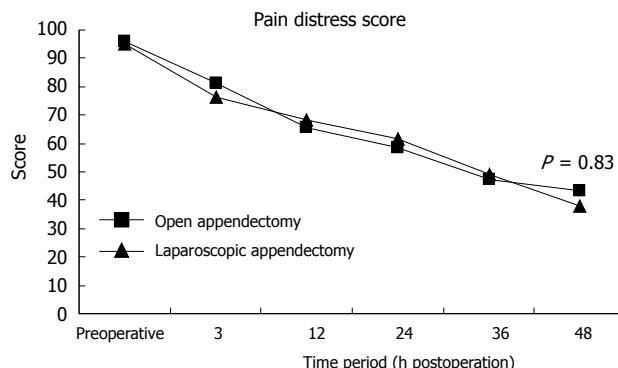
In the laparoscopic group, 90 (70.3%) procedures involved uncomplicated disease and 38 (29.7%) complicated appendicitis (Table 2). Additionally, in 16 (9.6%) open and 8 (6.2%) laparoscopic procedures, no pathology was observed in the appendix and other intra-abdominal structures (Table 2).

The actual operating room time was similar between the two groups (47 ± 19.7 min in the open group vs 44.3 ± 24 min in the laparoscopic group; P = 0.31, Table 2). Conversion to an open procedure was required in two patients (1.5%) with extensive cecal adhesions secondary to severe inflammation rendering appendiceal mobilization and visualization difficult and dangerous.

There was no mortality in either group and the overall morbidity was not significantly different (10.3% in the open group vs 7.8% in the laparoscopic group; P = 0.43, Table 3).

In patients with uncomplicated disease, the morbidity rates were low (0.8% in open appendectomy and 1.1% in laparoscopic appendectomy; P = 0.5, Table 3). One patient subjected to open appendectomy developed wound infection. The culture of pus revealed *E. coli* and the patient was successfully treated with antibiotics and wound debridement. Similarly, in one patient in the laparoscopic group, intestinal injury occurred during insertion of the visiport. The lesion was recognized intraoperatively and was successfully managed with endoscopic sutures. The end result was favorable and no further manipulations were required.

In contrast to uncomplicated disease, patients with complicated appendicitis were prone to postoperative complications (34% after open appendectomy and 23.7% after laparoscopic approach; P = 0.12, Table 3). Postop-

**Figure 1** Visual analogue score (VAS) for pain assessment.

erative bowel obstruction was observed in patients with complicated disease in both study groups (10.6% after conventional appendectomy and 7.8% after laparoscopic appendectomy; P = 0.37, Table 3). In addition, complicated appendicitis was associated postoperatively with respiratory infection in 4 patients subjected to open appendectomy, and 2 patients treated laparoscopically (P = 0.18, Table 3).

Infectious complications were seen in both study groups in patients with complicated disease. Open appendectomy was associated with a significantly higher incidence of wound infection compared with the laparoscopic group (12.8% vs 5.3%; P = 0.03, Table 3).

On the other hand, the incidence of intra-abdominal abscess formation was higher in patients with severe peritonitis who were treated laparoscopically (5.3% vs 2.1%; P = 0.002, Table 3). All patients who developed intra-abdominal abscess were treated successfully with antibiotics and CT-guided drainage of the collection, and had an uneventful recovery.

Bowel movements in the first postoperative day were observed in 92% patients subjected to laparoscopic appendectomy and 67% in the open group (P < 0.001). As a result, 78% patients in the laparoscopic group and 51% in the open group were able to tolerate a liquid diet within the first 24 postoperative hours (P < 0.001). The mean postoperative hospital stay was 2.2 d (range, 1-17 d) after laparoscopic appendectomy and 3.1 d (range, 1-18 d) after open appendectomy (P = 0.04).

Visual analogue pain scores were similar in the two groups for the first two postoperative days (Figure 1). There was a significant decline after the first 3 postoperative hours to 48 h in both groups. There was no difference between open and laparoscopic groups with respect to either overall pain level (P = 0.93) or degree of pain remission (P = 0.82). Eventually, the need for analgesic medication usage for the control of postoperative pain was similar in the two groups.

Finally, the operative costs were higher by 370 € in the laparoscopic group. In the present study, the costs were calculated based on the most cost effective materials used such as laparoscopic equipment, versatile laparoscopic instruments, endoloops and collection bags. Hospital charges regarding anesthesia were not added to the plot since there was no difference in the operative times.

## DISCUSSION

Acute appendicitis is the most common intra-abdominal condition requiring emergency surgery<sup>[26]</sup>. Although more than 20 years have elapsed since the introduction of laparoscopic appendectomy, there is no consensus on its advantages and disadvantages compared to the conventional technique.

Recent studies have shown significant advantages of laparoscopic appendectomy with respect to the length of hospital stay, postoperative pain and infectious complications<sup>[5,8,12,14,18]</sup>. These findings have been challenged by other authors who observed no significant difference in the outcome between the two procedures, and moreover noted higher costs with laparoscopic appendectomy<sup>[3,17,19,25,27]</sup>.

Bearing in mind that laparoscopic appendectomy, unlike other laparoscopic procedures, has not been found superior to open surgery for acute appendicitis, we designed the present study to determine any possible benefits of the laparoscopic approach.

Operation time remains a topic of much debate among experts. Preliminary studies<sup>[28-30]</sup> have shown significantly longer operative times for laparoscopic appendectomy. The inexperience of the surgeons with the new technique may contribute to the longer duration of the operation in the early studies. However, recent studies<sup>[16-18]</sup> have supported the initial findings. Because in these studies, most of the operations were performed by residents, the longer operation times can be attributed to the learning curve. By contrast, in the present study, the operation times were nearly similar in the two techniques, and the learning curve effect was minimal as the surgeons performing the procedures were highly experienced with a wide spectrum of laparoscopic procedures, including laparoscopic bariatric surgery and laparoscopic colectomy. This experience is reflected in our study by the relatively narrow range of operative times in the laparoscopic group ( $44.3 \pm 24$ ).

Previous studies have given conflicting results with respect to the length of hospital stay after laparoscopic appendectomy. Guller *et al*<sup>[12]</sup> in a population-based analysis using a national administrative data base showed that laparoscopic appendectomy is associated with significantly shorter hospital stay. These findings were supported by the Cochrane Collaboration large scale meta-analysis<sup>[24]</sup>. In agreement with these studies, we found that hospital stay was significantly shorter in patients subjected to laparoscopic appendectomy ( $P = 0.004$ ). In the present study, bowel movements were observed significantly earlier in patients managed laparoscopically, leading to earlier feeding and discharge from hospital.

In the present study, pain was assessed both subjectively *via* a visual analogue scale and objectively by the tabulation of analgesic use. Although some studies have reported less pain in the first 48 h after laparoscopic appendectomy<sup>[20,21,24,25,31]</sup>, in our series there was no difference between the two groups with respect to either the visual analogue scores or the use of analgesics. Our study suffered from the drawback that it

was not blinded. As a result, the perception of pain may have been influenced by the patient's enthusiasm for a novel technique.

There was no mortality in our study. This is consistent with the majority of previous publications. It has been reported that the mortality rate is 0.05% and 0.3% in laparoscopic and open appendectomy respectively<sup>[12]</sup>. The low mortality rates indicate that appendectomy, especially in the absence of complicated disease, is a safe procedure regardless of the technique used.

In the present study, the overall complication rates were 10.6% and 8.1% for open and laparoscopic appendectomy respectively. These results are in agreement with previous reports, which vary from 5.7% to 25.8% for open appendectomy and 3% to 19% for laparoscopic appendectomy<sup>[13-15,20-23]</sup>.

Complicated appendicitis was initially considered as a contraindication to laparoscopic appendectomy<sup>[32,33]</sup>. However, recent studies have shown that laparoscopic approach in complicated disease is feasible and may even be superior to the conventional approach<sup>[6,7,10]</sup>.

In our series, 28.5% patients in the open group and 29.7% in the laparoscopic group had complicated disease. These patients are considered to be at increased risk of postoperative infections such as wound infection and intra-abdominal abscess formation<sup>[34,35]</sup>. According to the Cochrane systemic review of the literature<sup>[24]</sup>, wound infection is about one-half after laparoscopic appendectomy, while intra-abdominal abscess formation is 3 times higher after laparoscopic appendectomy.

In the present study, the rate of wound infection in patients with complicated disease was significantly lower after laparoscopic appendectomy (5.3% vs 12.8%,  $P = 0.03$ ). Placement of the detached appendix into an endobag before its removal from the abdominal cavity reduces contact with the fascial surfaces and minimizes contamination.

Intra-abdominal abscess formation was more common after laparoscopic appendectomy in complicated disease (5.3% vs 2.1%,  $P = 0.002$ ). It has been suggested that carbon dioxide insufflation may promote mechanical spread of bacteria in the peritoneal cavity, especially in cases of ruptured appendix<sup>[21,36-38]</sup>. In order to decrease the bacterial load and hence the risk of abscess formation, we advocate extensive irrigation of the abdominal cavity. However, in our practice, we observed that meticulous irrigation was unnecessary and even more dangerous as it leads to contamination of the entire abdominal cavity, which is difficult to aspirate latter. That was the case in two patients with severe peritonitis where intra-abdominal abscess formation occurred. Ever since we have changed our practice to simple suctioning of the infected area, we have not observed any postoperative abscess formation, even in patients with severe peritonitis.

The higher cost of laparoscopic appendectomy compared to the conventional technique is considered as an obstacle to its greater use. However, hospital charges for laparoscopic appendectomy have reduced

dramatically over the past several years<sup>[39]</sup>. Surgical expertise and the abundance of laparoscopic equipment have significantly reduced the economical mismatch in favor of the conventional technique. In addition, Moore and coworkers, using a decision analysis model, have demonstrated an economic benefit of laparoscopic appendectomy from a social perspective, since shorter hospital stay and earlier return to daily activities is very important, especially for patients who are young and lead a productive life<sup>[40]</sup>.

In the present study, the operative costs for laparoscopic appendectomy were only 370 € higher. The greater cost of laparoscopic appendectomy observed in various studies<sup>[3,14,25]</sup> can be attributed to the use of disposable laparoscopic instruments and the longer operative time. In our series, we were able to minimize the operative costs, mainly by employing reusable laparoscopic instruments.

Although there is no consensus with regard to the advantages of the laparoscopic approach compared to the conventional technique, the use of laparoscopic appendectomy has increased significantly in the last several years. In the present study, we were able to demonstrate the superiority of the laparoscopic approach in terms of hospital stay and wound infection, with only marginally higher hospital costs. Although the incidence of intra-abdominal abscess formation was higher after laparoscopic appendectomy, all complications occurred early in our practice. Greater experience and improvements in our technique has made it possible to eradicate this catastrophic complication.

Provided that surgical experience and equipment are available, laparoscopic appendectomy is safe and equally efficient compared to the conventional technique. However, as long as there is no consensus to the best approach for appendicitis, the choice of the procedure will be based on the preference of the surgeons and patients.

## COMMENTS

### Background

Laparoscopic surgery has been available for a long time. Today, even the most complicated procedures can be performed laparoscopically. However, laparoscopic appendectomy, a relatively easy procedure, has not gained wide acceptance among surgeons, and the conventional technique remains the procedure of choice in many centres worldwide.

### Research frontiers

Intra-abdominal abscess formation is the most catastrophic complication of laparoscopic appendectomy. By simple suctioning of the infected area, rather than using widespread irrigation we were able to decrease the incidence of postoperative abscess formation.

### Innovations and breakthroughs

In the present study, we were able to demonstrate that laparoscopic appendectomy is superior to the conventional technique in terms of hospital stay and wound infection. Additionally, in expert hands, even the most serious complications such as an intra-abdominal abscess formation can be minimized. Furthermore, in the present study, we were able to decrease medical costs by employing reusable laparoscopic equipment.

### Applications

The present study has shown that laparoscopic surgery should be considered in every patient with appendicitis.

### Peer review

The authors demonstrated a prospective study of laparoscopic versus open appendectomy and concluded "Provided that surgical experience and equipment are available, laparoscopic appendectomy is safe and equally efficient alternative to conventional technique." This present study is an interesting and novel.

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RAPID COMMUNICATION

# Relationship between microvessel count and post-hepatectomy survival in patients with hepatocellular carcinoma

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## Abstract

**AIM:** To elucidate the relationship between the microvessel count (MVC) by CD34 analyzed by immunohistochemical method and prognosis in hepatocellular carcinoma (HCC) patients who underwent hepatectomy based on our preliminary study.

**METHODS:** We examined relationships between MVC and clinicopathological factors in 128 HCC patients. The modified Japan Integrated Staging score (mJIS) was applied to examine subsets of HCC patients.

**RESULTS:** Median MVC was 178/mm<sup>2</sup>, which was used as a cut-off value. MVC was not significantly associated with any clinicopathologic factors or postoperative recurrent rate. Lower MVC was associated with poor disease-free and overall survivals by univariate analysis ( $P = 0.039$  and  $P = 0.087$ , respectively) and lower MVC represented an independent poor prognostic factor in disease-free survival by Cox's multivariate

analysis (risk ratio, 1.64;  $P = 0.024$ ), in addition to tumor size, vascular invasion, macroscopic finding and hepatic dysfunction. Significant differences in disease-free and overall survivals by MVC were observed in HCC patients with mJIS 2 ( $P = 0.046$  and  $P = 0.0014$ , respectively), but not in those with other scores.

**CONCLUSION:** Tumor MVC appears to offer a useful prognostic marker of HCC patient survival, particularly in HCC patients with mJIS 2.

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**Key words:** Hepatocellular carcinoma; Hepatic resection; Microvessel count; CD34; Modified Japan integrated staging score

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## INTRODUCTION

Hepatic resection is a useful option for radical treatment of hepatocellular carcinoma (HCC). However, recurrence rate after resection remains high<sup>[1,2]</sup>. Patient survival thus remains unsatisfactory due to high recurrence rate even at this stage. Clinicopathological factors in HCC are related to tumor recurrence<sup>[3]</sup> and tumor biological characteristics provide useful information regarding the activity of HCC. According to previous reports, candidates for tumor biological factors and molecular markers include abnormal expression of p53<sup>[4]</sup>, nm23 (a tumor suppressor gene)<sup>[5]</sup>, tumor angiogenesis<sup>[6]</sup>, proliferative activity<sup>[7]</sup>, growth factors<sup>[8]</sup>, DNA ploidy<sup>[9]</sup> and other molecular markers<sup>[10]</sup>. Some of

these markers are related to prognosis in HCC patients. Combination of conventional clinicopathological factors and prognostic factors of tumor biology may improve prediction of prognosis after hepatectomy for HCC and may contribute to a new staging classification.

Tumor angiogenesis may be important to support tumor growth<sup>[11]</sup>, and HCC is a hypervascular tumor expressing several angiogenic factors<sup>[12]</sup>. Levels of angiogenic factors such as vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (b-FGF) are increased in HCC and might affect patient survival<sup>[12,13]</sup>. Recent studies have also shown that microvessel density (MVD) in HCC or non-cancerous liver surrounding tumor correlates with tumor aggressiveness and prognosis<sup>[6,14-16]</sup>. We have previously provided a preliminary demonstration of the comprehensive analysis of various biological factors, revealing that microvessel count (MVC) using CD34 antibody was independently associated with poor prognosis in HCC patients undergoing hepatic resection by multivariate analysis<sup>[17]</sup>. However, contrary to other reports, poor patient prognosis was related to lower MVCs. We hypothesize that hypovascularity in HCC represents a factor associated with treatment-resistance such as chemoembolization, which causes poor prognosis.

The present study examined the relationship between MVC in HCC using immunohistochemical stains and conventional clinicopathological factors and prognosis in a larger number of HCC patients with longer follow-up period to clarify our hypothesis. Furthermore, we examined this relationship in subsets of patients after applying the modified Japan Integrated Staging score (mJIS).

## MATERIALS AND METHODS

### **Patients**

HCC specimens from 128 patients (104 men, 24 women) were obtained during surgery on patients admitted to the Division of Surgical Oncology at Nagasaki University Graduate School of Biomedical Sciences (NUGSBS) between 1990 and 2005. Mean age for patients at the time of surgery was  $62.9 \pm 8.2$  years (range, 28-78 yr). Prior to surgery for HCC, 36 patients (28.1%) were treated using either chemoembolization ( $n = 30$ ) or local ablation ( $n = 6$ ), including alcohol injection in 2 patients and radiofrequency ablation (RFA) in 4 patients. After surgery, 3 patients (2.3%) received adjuvant 5-fluorouracil chemotherapy by intra-arterial injection through a subcutaneously implanted reservoir. Child-Pugh classification was B in 11 patients (8.6%) and A in 117 patients. The liver damage grade by the Liver Cancer Study Group (LCSG) of Japan in 2000 was B in 26 patients and A in 102 (Table 1)<sup>[18]</sup>. The operative procedures included lobectomy or extended lobectomy ( $n = 54$ ), segmentectomy or subsegmentectomy ( $n = 43$ ) and partial resection ( $n = 31$ ). Radical hepatectomy was performed to remove hepatic tumor without leaving any residual tumor. All hepatic tumors were completely

**Table 1 Definition and criteria of Child-Pugh classification and liver damage grade**

	<b>A</b>	<b>B</b>	<b>C</b>
Child-Pugh classification			
Encephalopathy	None	Mild	Coma
Ascites	None	Responsive	Unresponsive
Serum bilirubin (mg/dL)	< 2.0	2.0-3.0	> 3.0
Serum albumin (g/dL)	> 3.5	2.8-3.5	< 2.8
Prothrombin activity (%)	> 70	40-70	< 40
Liver damage grade <sup>[18]</sup>			
Ascites	None	Responsive	Unresponsive
Serum bilirubin (mg/dL)	< 2.0	2.0-3.0	> 3.0
Serum albumin (g/dL)	> 3.5	3.0-3.5	< 3.0
ICG R15 (%)	< 15	15-40	> 40
Prothrombin activity (%)	> 80	50-80	< 50

ICG R15: Indocyanine green retention rate at 15 min.

resected without macroscopic exposure of the amputated section to the remaining liver. The present series included no in-hospital deaths and the only causes of death were cancer-related. Minimum follow-up period after hepatic resection of HCC was 24 mo.

We used the classification system of the General Rules for the Clinical and Pathological Study of Primary Liver Cancer<sup>[19]</sup>. This system provides a clinicopathological evaluation of HCC. Macroscopic classification as described by Classification of Primary Liver Cancer<sup>[19]</sup> was also applied in the study. All study protocols were approved by the Human Ethics Review Board of our institution. Informed consent for data collection was obtained from each patient during this period. Anesthetic and patient data were retrieved from the NUGSBS database.

### **Immunohistochemical staining**

Resected specimens were fixed in 10% formalin and embedded in paraffin. Thin sections (4  $\mu$ m) were deparaffinized twice using xylene and rehydrated in a series of ethanol solutions (100%, 90% and 80%). Sections were placed in 0.01 mol/L trisodium citrate dehydrate buffer (pH 6.0) and treated in a microwave oven for 10 min at 500 W. For CD34 staining<sup>[17,20]</sup>, tissue sections were digested with 0.2% trypsin in 0.01 mol/L phosphate-buffered saline (PBS) for 20 min at 37°C. In the next step, tissues were immersed in 3% H<sub>2</sub>O<sub>2</sub> with distilled water for 10 min to inactivate endogenous peroxidases. After blocking non-specific binding by normal goat serum, sections were incubated overnight at 4°C with mouse anti-monoclonal CD34 antibody (1:25; QB-END/10, Novocastra Laboratories, Newcastle, United Kingdom) as the primary antibody. This was followed by reaction with biotinylated anti-immunoglobulin and reagent using labeled streptavidin-biotin (LSAB) kit peroxidase (Dako, Carpinteria, CA). The peroxidase reaction was visualized with 0.01% H<sub>2</sub>O<sub>2</sub> and 3,3'-diaminobenzidine under light microscopy ( $\times 200$ ). For MVCs using CD34 staining, average count was determined in the 5 most-vascular areas in the HCC examined at 200  $\times$  magnification<sup>[17,20]</sup>. Two pathologists blindly assessed each slide.

**Table 2 Definition and criteria of TNM stage for HCC according to the Liver Cancer Study Group of Japan<sup>[18]</sup>**

Criteria for TNM categories	
(1) Number of tumors:	1
(2) Tumor size:	≤ 2 cm
(3) No vascular or bile duct invasion	
T category	T1: Fulfilling all three criteria T2: Fulfilling two criteria T3: Fulfilling one criterion T4: Fulfilling none of the criteria
N category	N0: Absence of lymph node metastasis N1: Presence of lymph node metastasis
M category	M0: Absence of distant metastasis M1: Presence of distant metastasis
Stage I	T1 N0 M0
Stage II	T2 N0 M0
Stage III	T3 N0 M0
Stage IV-A	T4 N0 M0 or T1-T4, N1M0
Stage IV-B	T1-4, N0 or N1, M1

**Table 3 Definition and criteria for JIS and mJIS**

	Score			
	0	1	2	3
Original JIS score <sup>[21]</sup>				
Japanese TNM stage	I	II	III	IV
Child-Pugh Classification	A	B	C	
Modified JIS score <sup>[22]</sup>				
Japanese TNM stage	I	II	III	IV
Liver damage grade	A	B	C	

TNM: tumor-node-metastasis.

### Staging criteria for the mJIS

We used the pathological tumor-node-metastasis (pTNM) classification system as defined by the Liver Cancer Study Group (LCSG) of Japan in 2000<sup>[18]</sup>. T category was determined based on 3 factors: number, size, and vascular or bile duct invasion. N category was determined as the presence of lymph node metastasis, while M category represented the presence of distant metastases. TNM staging comprises 4 stages based on the combination of T, N, and M categories (Table 2). The original Japan Integrated Staging score proposed by Kudo *et al*<sup>[21]</sup> comprised the sum of scores for the two variables of Japanese TNM classification and Child-Pugh classification. In the mJIS proposed by our institute<sup>[18,22]</sup>, Child-Pugh classification was replaced by the score for liver damage grade as defined by the LCSG of Japan (Table 3).

### Statistical analysis

Continuous data are expressed as mean ± standard deviation. Data from different groups were compared using one-way analysis of variance (ANOVA) and examined by Student's *t*-test or Dunnett's multiple comparison test. For univariate analysis, categorical data were analyzed using Fisher's exact test. Disease-free and overall survival rates were calculated according to the Kaplan-Meier method, and differences between groups were tested for significance using the log-rank test.

Multivariate analysis was performed using proportional hazards regression modeling. A two-tailed value of *P* < 0.05 was considered statistically significant. All statistical analyses were performed using SAS software (Statistical Analysis System, Cary, NC).

## RESULTS

Among the 128 patients in the present study, disease-free 1-, 3-and 5-year survival rates were 63%, 39% and 29%, respectively, and median disease-free survival was 3.5 years. Overall 1-, 3-and 5-year survival rates were 89%, 65% and 48%, respectively, and median overall survival was 5.9 years. Of 94 patients (75.0%) who displayed tumor recurrence after hepatectomy, 85 (90.4%) received chemoembolization (*n* = 81) or alcohol injection (*n* = 4).

Median MVC within the tumor area was 178/mm<sup>2</sup>, and this value was applied as a cut-off value. Table 4 shows the relationship between MVC and clinicopathological features in 128 HCC patients. However, MVC was not significantly associated with any clinicopathological factors, TNM stage or postoperative recurrence.

Figure 1 shows disease-free and overall survival after hepatectomy compared to MVC. Disease-free survival rate was significantly lower in patients with lower MVC than in patients with higher MVC (*P* = 0.039). Overall survival rates tended to be lower in patients with lower MVC than in patients with higher MVC, but this difference was not significant. Table 5 shows the results of multivariate analysis for disease-free and overall survival after hepatectomy for various factors identified as displaying significant associations on univariate analysis. Multiple tumors, vascular involvement of the tumor, liver damage grade B and lower MVC represented independent risk factors for poor disease-free survival after hepatectomy (*P* = 0.0004, 0.003, 0.007 and 0.024, respectively). Multiple tumors, vascular involvement of the tumor and macroscopic findings were identified as independent risk factors for poor overall survival after hepatectomy (*P* = 0.025, 0.014 and 0.017, respectively), whereas MVC was not associated with overall survival (*P* = 0.266).

Figure 2 shows disease-free survival by comparing MVC for each mJIS score. In HCC with mJIS 0 or 1, MVC was not associated with disease-free survival. Lower MVC was significantly associated with poor disease-free survival in HCC with mJIS 2. Lower MVC tended to be associated with poor disease-free survival in HCC with mJIS ≥ 3, but this result was not significant. Figure 3 shows overall survival by comparing MVC at each mJIS score. In HCC with mJIS 0, 1 or ≥ 3, MVC was not associated with overall survival. Lower MVC was significantly associated with poor disease-free survival in HCC with mJIS 2.

## DISCUSSION

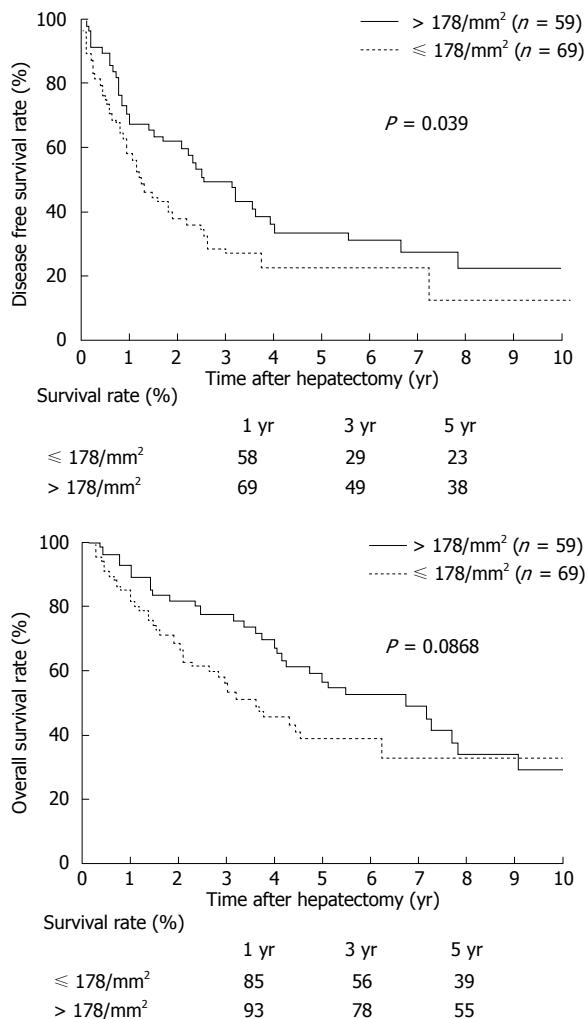
Previous studies have investigated prognostic factors in HCC for patients who underwent radical hepatectomy

**Table 4 Relationship between microvessel density and clinicopathological factors in HCC**

	<b>Microvessel count &gt; 178/mm<sup>2</sup>/≤ 178/mm<sup>2</sup></b>	<b>P</b>
Pretreatment		
No (n = 98)	53/45	1.0
Yes (n = 30)	16/14	
Liver damage		
A (n = 102)	54/48	0.969
B (n = 26)	15/11	
Background liver		
Normal liver (n = 7)	3/4	0.459
Chronic hepatitis (n = 75)	38/37	
Cirrhosis (n = 46)	28/18	
Viral status		
Hepatitis virus B (n = 50)	32/18	0.152
Hepatitis virus C (n = 61)	31/30	
Both hepatitis virus B and C (n = 4)	2/2	
Non-B, non-C (n = 13)	4/9	
Number of tumors		
Solitary (n = 92)	50/42	1.0
Multiple (n = 36)	19/17	
Tumor size		
< 3 cm (n = 31)	15/16	0.371
3-5 cm (n = 49)	24/25	
> 5 cm (n = 48)	30/18	
Macroscopic finding <sup>1</sup>		
SN (n = 37)	16/21	0.207
SNEG (n = 39)	25/14	
CMN (n = 52)	28/24	
Vascular involvement		
No (n = 83)	42/41	0.476
Yes (n = 45)	27/18	
Histopathological differentiation		
Well (n = 19)	12/7	0.437
Moderately (n = 97)	55/42	
Poorly (n = 6)	2/4	
Japan tumor-node-metastasis stage <sup>2</sup>		
I (n = 14)	5/9	0.542
II (n = 51)	27/25	
III (n = 44)	25/19	
VI-A (n = 19)	12/7	
Recurrence		
No (n = 32)	16/16	0.386
Yes (n = 96)	53/43	

<sup>1</sup>Macroscopic classification by Liver Cancer Study Group of Japan<sup>[19]</sup>; <sup>2</sup>The General Rules for the Clinical and Pathological Study of Primary Liver Cancer<sup>[18]</sup>. SN: Single nodular; SNEG: Single nodular with extranodular growth; CMN: Confluent multinodular group.

and in whom tumor stage had been determined<sup>[23,24]</sup>. The International Union against Cancer (UICC) and Japanese TNM classification systems for predicting patient prognosis in HCC did not provide good reflection of patient survival in HCC patients<sup>[25,26]</sup>. Combined staging systems with hepatic function have recently been applied<sup>[21,22,27,28]</sup>, and among these, the JIS score system was applied in Japan<sup>[21]</sup>. Child-Pugh classification was used in this system, but liver damage grade by the LCGS of Japan offers a better reflection of patient survival<sup>[18,29]</sup>. The mJIS score system thus includes liver damage grade and has been applied in a few reports<sup>[22]</sup>. The present study also applied mJIS score. In the next step, a staging system for HCC may need additional useful factors comprising tumor biological or molecular markers. The present study identified angiogenic factors of MVC



**Figure 1** Relationship between microvessel count (MVC) and disease-free and overall survival in patients with hepatocellular carcinoma (HCC) who underwent hepatic resection.

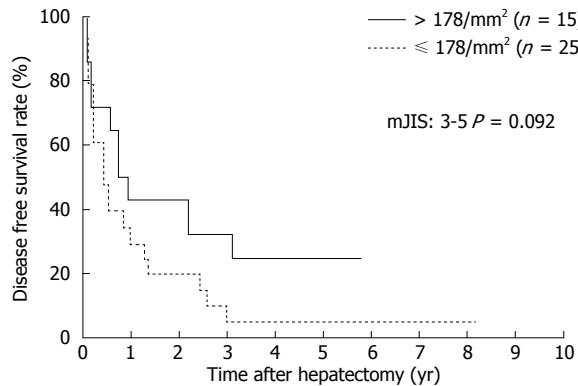
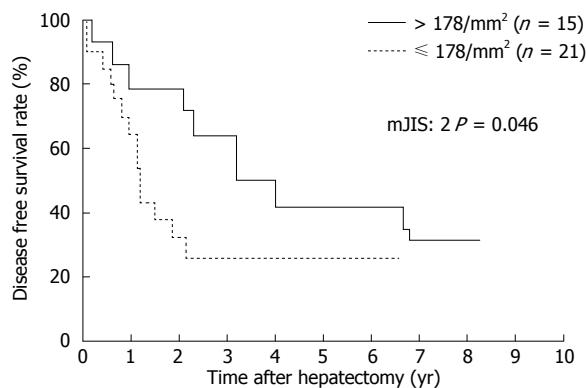
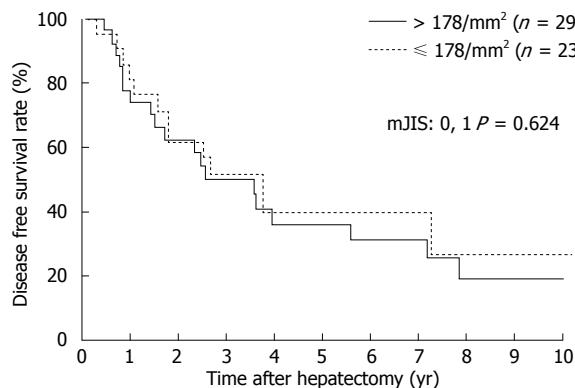
as potentially useful. In the last decade, microvessel density using CD34 as a prognostic parameter in HCC patients has been reported and appears consistently very useful<sup>[6,16,30,31]</sup>. We also performed a preliminary study of the significance of MVC for survival in HCC patients and, unlike other reports, revealed hypovascularity (i.e., lower MVC) as a poor prognostic parameter<sup>[17]</sup>.

MVC was not associated with any clinicopathological factors or recurrence rate after hepatectomy in either the present study or our pilot study<sup>[17]</sup>. In contrast to our results, El-Assal *et al*<sup>[30]</sup> and other investigators<sup>[6,14,15,30-32]</sup> have reported that microvessel density in HCC is increased in larger tumors, tumors with poor differentiation and cirrhotic patients, while higher microvessel density is associated with intra-hepatic recurrence. In the present study, however, MVC did not correlate with co-existing cirrhosis or with various etiological factors related to chronic hepatitis such as viral status. Conversely, Sun *et al*<sup>[33]</sup> found no relationship between MVC and either clinicopathological factors or patient prognosis. The relationship between tumor vascularity and clinicopathological features thus remains controversial. Increased micro-angiogenesis is definitely

**Table 5** Multivariate analysis by Cox's proportional hazard test of prognostic factors influencing disease-free survival and overall survival in HCC after hepatectomy

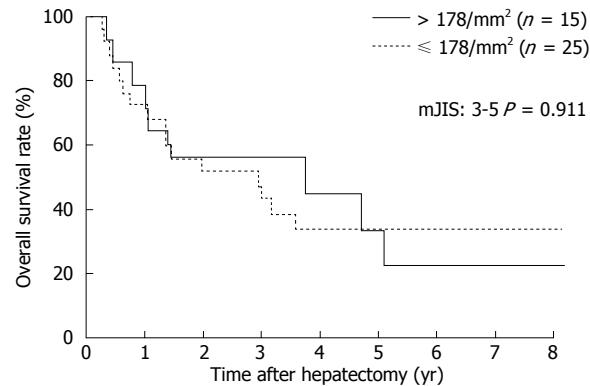
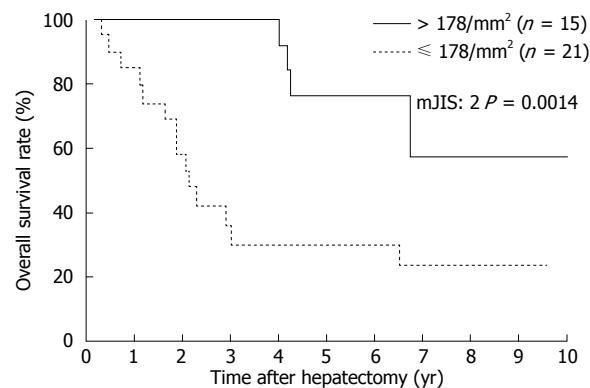
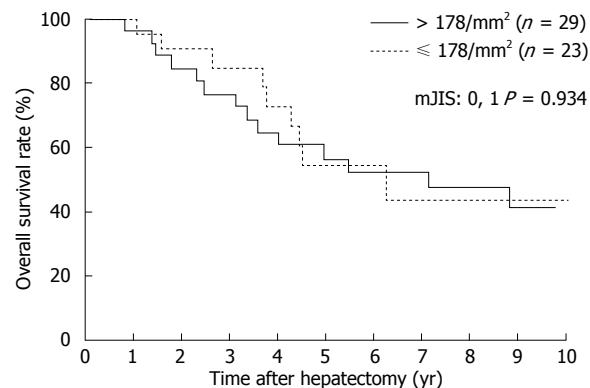
	Disease-free survival			Overall survival		
	Risk ratio	95% CI	P	Risk ratio	95% CI	P
Number ≥ 2 lesions	2.28	1.44-3.60	0.0004	1.87	1.08-3.24	0.025
Vessel involvement positive	2.04	1.28-3.24	0.003	2.07	1.16-3.68	0.014
Macroscopic finding SNEG or CMN	1.43	0.89-2.29	0.135	2.33	1.17-4.66	0.017
Surgical margin positive	1.75	0.93-3.33	0.086	1.59	0.65-3.91	0.310
Blood loss > 1500 mL	1.36	0.81-2.29	0.135	1.50	0.82-2.74	0.189
Liver damage grade B	1.99	1.20-3.31	0.007	1.44	0.77-2.69	0.255
PIVKA-II > 400 mAU/mL	1.30	0.81-2.13	0.274	1.15	0.65-2.04	0.636
Microvessel count ≤ 178/mm <sup>2</sup>	1.64	1.06-2.50	0.024	1.35	0.71-2.04	0.266

Macroscopic classification by Liver Cancer Study Group of Japan<sup>[19]</sup>. SN: Single nodular; SNEG: Single nodular with extranodular growth; CMN: Confluent multinodular group; CI: Confidence interval.



**Figure 2** Relationship between MVC and disease-free survival using the modified Japan Integrated Staging score (mJIS) in HCC patients who underwent hepatic resection.

associated with carcinogenesis of HCC and development



**Figure 3** Relationship between MVC and overall survival using the mJIS in HCC patients who underwent hepatic resection.

to advanced tumor<sup>[12,32]</sup>. At the stage of smaller-sized HCC, tumor vascularity is already rich on computed

tomography or enhanced ultrasonography<sup>[34,35]</sup>. Considering the mechanisms of HCC characterized above, increased microvessel density of the tumor is a logical result. When tumor demonstrates a large tumor size or poor histologic differentiation, tumor vascularity may be decreased. Previous reports also showed that HCC tumor vascularity decreased with the progression of histopathological grade<sup>[36,37]</sup>. Our results showed that lower MVC tended to be higher in poorly differentiated HCC, but this finding was not significant. A larger sample of poorly differentiated HCCs is needed to clarify this issue, as only 6 cases were included in the present series.

With respect to disease-free and overall survival after hepatectomy<sup>[1,2,6,24]</sup>, the results from our series were favorable. Counts  $\leq 178/\text{mm}^2$  for HCC were associated with worsened prognosis in patients undergoing hepatectomy, particularly in disease-free survival by univariate and multivariate analysis in the present study. Compared to other independent risk factors, the odds ratio was lower for MVC than for other factors such as a number of tumors, vascular involvement, macroscopic findings or liver damage grade. The role of MVC for tumor relapse or progression might not be particularly strong. This result contradicts findings in other reports described above<sup>[6,14,16,30,31]</sup>. As explained above, HCC naturally acquires rich tumor vascularity in the early stages and most clinically treated HCCs represent tumor with radiological enhancement<sup>[34,35,38]</sup>. Hypervascularity is observed in the majority of HCC<sup>[36]</sup>. As tumor vascularity decreases in HCC with deterioration of histological differentiation and/or aggressive invasiveness<sup>[36,37]</sup>, the malignant potential of hypovascular HCC could increase relative to that of hypervasculary HCC, thus leading to poorer prognosis. In cases of hypervasculary HCC, initial or recurrent tumor can be observed in earlier stages under various imaging modalities, and treatments combined with chemoembolization or chemotherapy via tumor microvessels may well prove effective<sup>[39]</sup>. We speculate that hypovascular HCC is difficult to detect by conventional imaging and to treat by chemoembolization or other therapy, and treatment selection is therefore limited. Previous studies showed that tumor vascularity correlates with the response to chemotherapy, which in turn correlates with survival<sup>[40-42]</sup>. Furthermore, tumor hypervascularity, which is observed in most HCC, correlated with good response to chemoembolization, whereas hypovascular HCC did not correlate with good response to chemoembolization<sup>[40,41]</sup>. Thus, a better response to arterial chemoembolization is associated with prolonged survival<sup>[40,41]</sup>. In our series, most patients with postoperative recurrence of HCC received arterial chemoembolization and, therefore, the prognosis of these patients could have been influenced by tumor vascularity and response to chemoembolization. Although we could not provide such evidence in recurrent tumors in our follow-up study, it is possible that the vascularity of primary HCC could influence the biological characteristics of recurrent HCC. For these

reasons, the survival of patients with hypovascular HCC logically would be worse after recurrence. Our results concerning MVC are thus biologically quite feasible. Anti-angiogenic therapy is a promising treatment for HCC<sup>[43]</sup>; however, the response of hypovascular HCC to such treatment remains problematic.

In addition to tumor-associated factors, hepatic functional reserve and liver function after surgery influence postoperative prognosis<sup>[3,6,44]</sup>. In the present series, we applied mJIS score to examine subsets of HCC patients. Poon *et al*<sup>[6]</sup> also reported that the significance of MVC differed between subsets of HCC patients. Our results showed no marked differences in disease-free and overall survival according to MVC in the early stage of mJIS 0 or the advanced stage of mJIS 3-5. A significant difference in MVC was only observed for mJIS 2. Poon *et al*<sup>[6]</sup> found microvessel density by CD34 as the only significant factor predictive of disease-free survival in patients with HCC (5 cm, but no significant prognostic influence was seen for larger HCC). In early-stage HCC, tumor can be sufficiently cured by hepatic resection even in the presence of malignant potential. Conversely, in severely advanced HCC, other significant prognostic factors might exert greater influence on tumor aggressiveness and survival, such as number of tumors, vascular involvement, macroscopic findings or liver damage grade, as indicated in the present study. For mJIS 2, patient survival with hepatic resection or ablation therapy was not particularly satisfactory compared to mJIS 0 or 1 according to our recent report<sup>[45]</sup>. In this stage of mJIS 2, treatment indications need to be defined according to the appropriate prognostic factors. In cases where lower vessel count was observed at initial resection, liver transplantation following recurrence may be a good option. Our results showed that MVC did not correlate with other clinicopathological parameters and is an independent risk factor for prognosis. Thus, according to the present results, MVC would represent a useful parameter to decide treatment modality.

In conclusion, we have demonstrated that lower MVC by CD34 in HCC offers an independent predictor of disease-free and overall survival in patients with HCC, particularly in HCC with mJIS 2. As a tumor biological factor, MVC representing tumor angiogenesis offer a new candidate prognostic factor in HCC to predict tumor recurrence and patient survival in combination with traditional pathological factors. Furthermore, this marker can be applied as a predictive marker to select molecular targeting treatments in future.

## COMMENTS

### Background

Tumor biological characteristics provide useful information on the activity of hepatocellular carcinoma (HCC). The combination of conventional clinicopathological factors and prognostic factors related to tumor biology may improve prediction of prognosis of patients with HCC. HCC is a hypervasculary tumor that expresses various tumor angiogenic factors. Microvessel density (MVD) in HCC may correlate with tumor aggressiveness and prognosis.

### Research frontiers

Tumor angiogenesis may be important for tumor growth. High levels of

angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (b-FGF) have been described in HCC, and they correlate with patient survival. Microvessel counts (MVC) in HCC or non-tumorous liver tissue that surrounds HCC correlate with tumor aggressiveness and prognosis. On the other hand, it should be difficult to chemoembolize hypovascular HCC. Therefore, we hypothesized that low MVCs correlates with poor prognosis.

### Innovations and breakthroughs

Our results presented new findings that were different than studies published previously by other investigators. However, we found that a low MVC is an independent prognostic factor for tumor relapse. This was particularly significant in HCC patients with mJIS 2.

### Applications

MVC could be a potentially useful marker of prognosis in HCC by predicting tumor recurrence and patient survival, in addition to traditional pathological factors. Furthermore, MVC could help in clinical decision making with respect to the selection of treatment modality.

### Peer review

Our study identified a novel prognostic factor that can be used to predict tumor recurrence and survival of patients with HCC. This marker can be potentially used to select the most appropriate surgical treatment modality, such as a liver transplantation particularly in patients with mJIS 2.

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RAPID COMMUNICATION

# OCTN and CARD15 gene polymorphism in Chinese patients with inflammatory bowel disease

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## INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC), the two common forms of idiopathic inflammatory bowel disease (IBD), are chronic, relapsing inflammatory disorders of the gastrointestinal tract. CD and UC are very common in developed countries with a prevalence of 0.7-11.6 per 100 000 and 2.0-14.3 per 100 000, while it is relatively uncommon in Asian countries with a prevalence of 0.08 per 100 000 and 0.5 per 100 000 in Japan<sup>[1]</sup>. However, the incidence of IBD has been increasing in some Asian countries in recent years, and China is one of the notable countries<sup>[2]</sup>.

The precise etiology of the disease is unknown, but interplay of environmental risk factors and immunologic changes will trigger the onset of the disease in a genetically susceptible host. Epidemiological studies in the past suggested a genetic susceptibility that has been confirmed by total genome scans and candidate gene studies. After the IBD1 locus in the chromosome 16 was identified as a CD locus by Hugot *et al*<sup>[3]</sup>, fine mapping of the IBD1 locus and following candidate gene approach led people to identify the *CARD15* (previously *NOD2*) as a susceptibility gene of CD. The *NOD2/CARD15* gene product is expressed in monocytes. It is involved in the binding of bacteria lipopolysaccharides and peptidoglycans so that it played an important role in activation of nuclear transcription factor kappa-B (NF-κB) in inflammatory response. Two missense mutations Arg702Trp (2104C→T), Gly908Arg (2722G→C) and one frame-shift mutation (3020insC) of the *NOD2/CARD15* gene affecting the function of binding microbial pathogens are independently associated with the development of CD<sup>[4]</sup>.

Numerous genome-wide scans and replication studies have identified IBD susceptibility loci since the initial

## Abstract

**AIM:** To investigate the single nucleotide polymorphism (SNPs) distribution of NOD2/CARD15 (R702W, G908R), OCTN1 1672C/T and OCTN2-207G/C in Chinese patients with inflammatory bowel disease (IBD).

**METHODS:** A total of 61 patients with Crohn's disease (CD), 151 patients with ulcerative colitis (UC), and 200 unrelated healthy controls were genotyped. Genotyping was performed by sequence specific primer polymerase chain reaction (PCR-SSP) or by restriction fragment length polymorphism (PCR-RFLP) analysis.

**RESULTS:** Among the subjects in our study groups, including patients with CD, UC and healthy controls, none had OCTN and CARD15 variants and very rare IBD family history was found in our patients with the percentage of 0 (0/61 with CD) and 1.3% (2/151 with UC).

**CONCLUSION:** Our results indicate that although OCTN or CARD15 variation is associated with susceptibility to IBD in Western populations, these might be rare and may not be associated with susceptibility to IBD in Chinese patients.

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**Key words:** Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; CARD15; Carnitine/organic cation transporter gene

study was published in 1996 by Hugot *et al*<sup>[3]</sup>. Recent studies suggested that *OCTN* (Carnitine/organic cation transporter gene) 1 and 2 in the IBD5 locus on chromosome 5 both encoded organic cation transporters and revealed significant associations with CD. The *OCTN* family is a family of transporter proteins for organic cations, and may also transport carnitine, an essential cofactor of the metabolism of lipids. *OCTNs* are therefore important in the maintenance of intracellular homeostasis and play an important role in the energy production of the cell. A C1672T substitution in exon 9 of the *OCTN1* gene and a G-207C in the *OCTN2* promoter region were indicated as functional and causative mutations to increase susceptibility to CD<sup>[5]</sup>.

The aim of the present study was to investigate the single nucleotide polymorphism (SNPs) distribution of *NOD2/CARD15* (R702W, G908R), *OCTN1* 1672C/T, *OCTN2*-207G/C and its association with IBD in Chinese patients.

## MATERIALS AND METHODS

### Study population

Blood samples from 61 patients with CD and 151 patients with UC were prospectively collected at the IBD Outpatient Clinic of the first affiliated hospital of Zhongshan University (Guangzhou, Guangdong Province, China) and Xijing Hospital of the Fourth Military Medical University (Xi'an, Shaanxi Province, China) between March 2005 and June 2006. All patients were followed up at least for one year and registered with an integrated clinical and epidemiological registry. A total of 212 healthy controls matched for age, sex and geography were healthy physical examinees in the two hospitals. All patients and healthy controls were of unrelated Chinese Han nationality. The diagnosis of either CD or UC was in accordance with previously established international criteria<sup>[6]</sup> based upon clinical, endoscopic, radiological and histopathological findings. All patients gave informed consent to participate in the study that was approved by the Ethics Committee of Zhongshan University and the Fourth Military Medical University.

### Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using the spin column technique (TIANamp Blood DNA kit, Tiangen Biotech, China).

Gene polymorphisms (R702W, G908R, *OCTN1* 1672C/T, *OCTN2*-207G/C) were determined using the sequence specific primers by polymerase chain reaction (SSP-PCR). Primer sequences and methods are depicted in Table 1. The PCR cycling parameters were a denaturing step at 94°C (3 min); 5 cycles of 94°C (30 s), 70°C (45 s), 72°C (30 s); 18 cycles of 94°C (30 s), 65°C (50 s), 72°C (30 s); 10 cycles of 94°C (30 s), 55°C (1 min), 72°C (1 min); and a final elongation step of 72°C (10 min). The PCR products were electrophoresed on 2% agarose gels containing ethidium bromide and viewed under ultraviolet light.

Restriction fragment length polymorphism (RFLP)

**Table 1** Primer sequences for SSP-PCR genotyping

SNP	Primers	PCR product (bp)
<i>OCTN1</i> 1672C/T (rs1050152)	W: TCTGACTGTCTGATTGGA ATCC	Allel C: 518 bp
	S: TAGTCTGACTGCTCTGATT CGAACACT C: TTTGAGACGGAGTT TGCTTGT	Allel T: 520 bp
<i>OCTN2</i> -207G/C (rs2631367)	W: GCACGACCAGGGAAGGTTC	Allel G: 493 bp
	S: GCACGACCAGGGAAGGTTC C: TCCCAGCCTCTCTAC TAGGGTAGTT	Allel C: 493 bp
R702W 2104C/T (rs2066844)	W: CTGAGAAGGCCCTGCTCC	Allel C: 396 bp
	S: CATCTGAGAAGGCCCTGCTCT C: CAATGCCAGTAAC ACTCACTACAG	Allel T: 399 bp
G908R 2722G/C (rs2066845)	W: TGGCCTTTCAGATTCTGGG	Allel G: 308 bp
	S: TGGCCTTTCAGATTCTGGC C: TGTATCAAAACCCCTG AGAGGACAA	Allel C: 308 bp

and sequencing were performed as means of verifying the PCR results. Five samples were chosen from each allele (including homozygous wild-type, heterozygous SNP and homozygous SNP) to perform RFLP and sequencing. Sequencing was performed by AuGCT Corporation of Beijing. The primers of sequencing were the same with RFLP. RFLP was performed as follows: (1) PCR amplification: initially a denaturing step at 96°C (1 min); 25 cycles of 96°C (30 s), 70°C (40 s), 72°C (30 s); 10 cycles of 96°C (30 s), 65°C (30 s), 72°C (30 s); and a final elongation step of 72°C (10 min). (2) RFLP: 10 μL of the PCR products mixed with 2 μL 10 × buffer and 2 μL restriction enzyme, then adding water to 30 μL, incubated for 12 h at 37°C and electrophoresed on 20% non-denaturing polyacrylamide gels, finally viewed under ultraviolet light after stained with ethidium bromide solution for 30 min. Primer sequences and restriction enzymes are depicted in Table 2.

The results of gene sequence and RFLP are identical. The result of SSP-PCR about G908R and *OCTN2*-207G/C was consistent with sequence and RFLP, so we chose 30 samples to perform RFLP and obtained the same result. But the result of SSP-PCR about R702W and *OCTN1* 1672C/T was not consistent with sequence or RFLP, so we changed all samples to perform RFLP.

### Statistics analysis

Comparison between cases and controls was made using the Chi-square test for categorical data with the SPSS software ver.13.0.

## RESULTS

In this study, we first performed genotyping by SSP-PCR with less cost and time than RFLP or sequencing. But the shortage of SSP-PCR is easy to result in false positivity. So after we completed the SSP-PCR, we used

**Table 2** Primer sequences and restriction enzymes used for RFLP genotyping

SNP	Primers	Restriction enzyme	Length of restriction fragments
OCTN1 1672C/T (rs1050152)	F: CGTCATGGTAGTCTGACTGTCCGTATTGGGATC R: TCCTACTTACCATTTCACTTCTGCATCTGCTTAAGG	BamH I	Allel C: 30 + 88 bp Allel T: 118 bp
OCTN2 -207G/C (rs2631367)	F: GCGCCGCTCTGCCAG R: AGGGTAGGCTCGCGAGCTGACACC	Msp I	Allel G: 44 + 83 bp Allel C: 127 bp
R702W 2104C/T (rs2066844)	F: TGGGGCCTGCTGGCTGAGTG R: GTGCAGCTGGCGGGATGGAG	Msp I	Allel C: 76 + 45 bp Allel T: 121 bp
G908R 2722G/C (rs2066845)	F: TCTGGCTGGGACTGCAGAGG R: CCCCTCGTCACCCACTCTGTCGC	BstU I	Allel G: 131 bp Allel C: 109 + 22 bp

**Table 3** Demographics and phenotype of IBD patients

	CD patients (n = 61)	UC patients (n = 151)
Sex (M:F)	40:21	91:60
Median age (yr, mean ± SD)	36.9 ± 13.7	43.8 ± 13.4
Patients with relative(s) who have IBD	0	2 (1.3%)
Location of CD	Location of ulcerative colitis	
Small bowel only (%)	25 (41.0)	Rectum sigmoid colon 86 (57.0)
Colon only (%)	7 (11.5)	Left hemicolon 18 (11.9)
Small bowel & colon (%)	29 (47.5)	Extensive 47 (31.1)
Behaviour of Crohn's disease (%)		Severe criteria of ulcerative colitis
Non-stricturing, non-penetrating (%)	26 (42.6)	Mild 74 (49.0)
Penetrating (%)	15 (24.6)	Moderate 57 (37.7)
Stricturing (%)	19 (31.1)	Severe 20 (13.2)
Stricturing & penetrating (%)	1 (1.6)	

RFLP and sequencing to verify the result. The SSP-PCR results of G908R and *OCTN2*-207G/C were consistent with RFLP or sequencing so that the SSP-PCR is successful in genotyping these alleles. On the contrary, we failed to genotype alleles of R702W and *OCTN1* 1672C/T by SSP-PCR, we therefore changed to use RFLP which had been used to detect polymorphism for a long time with reliable result.

We found very rare IBD family history in our patients with the percentage of 0 (0/61 with CD) and 1.3% (2/151 with UC). The demographics and phenotype of the IBD patients are described in Table 3.

As shown in Table 4, we found that the four SNPs, *OCTN1* 1672C/T, *OCTN2*-207G/C, R702W and G908R were completely absent in the Chinese Han nation population, in either the IBD patients or the control group. These results demonstrated that *OCTN* and *CARD15* variations might be rare and may not be associated with susceptibility to IBD in Chinese patients of Han nation.

## DISCUSSION

In the present study, we found that the polymorphism of C1672T in exon 9 of *OCTN1*, G-207C in the

**Table 4** Genotype and allele results

SNP	Group	Cases	Genotype			Allele	
			C/C	C/T	T/T	C	T
<i>OCTN1</i> 1672C/T (rs1050152)	CD	61	61	0	0	122	0
	UC	151	151	0	0	302	0
	Control	200	200	0	0	400	0
<i>OCTN2</i> -207G/C (rs2631367)	CD	61	61	0	0	122	0
	UC	151	151	0	0	302	0
	Control	200	200	0	0	400	0
<i>R702W</i> 2104C/T (rs2066844)	CD	61	61	0	0	122	0
	UC	151	151	0	0	302	0
	Control	200	200	0	0	400	0
<i>G908R</i> 2722G/C (rs2066845)	CD	61	61	0	0	122	0
	UC	151	151	0	0	302	0
	Control	200	200	0	0	400	0

*OCTN2* promoter region, and 2104C/T(R702W), 2722G/C(G908R) in *CARD15* were completely absent in Chinese patients with IBD and healthy controls. The study suggested that the four SNPs might not play a role in susceptibility of IBD in Chinese patients, thereby differing from the case of Western populations, but consistent with the results in Asian population. In our study, there were 212 IBD patients (61 CD, 151 UC) and 200 healthy controls. Compared with the studies in the Chinese population before, we had the largest number of cases. In Asia, it was the first study to detect the polymorphism of *OCTN* in UC patients.

In the last ten years, there have been tremendous researches on genetic susceptibility of inflammatory bowel diseases (IBD) and over 10 chromosomal regions have been identified by genome-wide scanning. The regions on chromosomes 16, 12, 6, 14, 5, 19 and 1 have been renamed IBD 1-7, respectively<sup>[7]</sup>. Further fine mapping as well as candidate gene studies have already led to the identification of a number of susceptibility genes including *CARD15*, *DLG5*, *OCTN1* and 2, *NOD1*, *HLA*, *TLR4*, *TNF-α*, *IL-1RA*, and *ICAM-1*<sup>[8]</sup>. The *CARD15* gene is undoubtedly replicated most widely at present. The three *NOD2/CARD15* variant alleles, Arg702Trp, Gly908Arg and 3020insC were found to increase the risk of CD in Caucasians, including those from Germany, England<sup>[9]</sup>, Australia<sup>[10]</sup> and America<sup>[11]</sup>. When *OCTN1* and 2 were reported to be associated to IBD, the west-

ern countries such as Canada<sup>[5]</sup>, England<sup>[12]</sup>, German<sup>[13]</sup>, Greek<sup>[14]</sup>, Spain<sup>[15]</sup> and New Zealand<sup>[16]</sup> carried out experiments and proved that the SNPs of *OCTN1* and 2 independently or the haplotype OCTN-TC (SNPs of *OCTN1* and 2 create a two-allele risk haplotype, TC) were positively associated with IBD (with CD only in most studies). On the contrary, studies performed in Asian population differed from the case in Caucasians. The three NOD2 mutations were proved to be totally absent in the studies of Yamazaki *et al*<sup>[17]</sup> in Japan with 483 CD patients, Lee *et al*<sup>[18]</sup> in Korea with 128 CD and 47 UC by sequencing, and Leong *et al*<sup>[19]</sup> in Hong Kong with 65 CD and 63 UC, Gao *et al*<sup>[20]</sup> in Zhejiang University of China with 32 CD and 110 UC by SSP-PCR. Guo QS in Wuhan University of China found Two heterozygotes of the 3020insC mutation in 74 UC patients and one in 15 CD, and only one in healthy controls by SSP-PCR. So they concluded that the NOD2 3020insC mutation was not associated with CD or UC in Hubei Han population<sup>[21]</sup>. Similar to the *CARD* gene, Yamazaki *et al*<sup>[22]</sup> in Japan found the SNPs of *OCTN1* and 2 were completely absent with 484 CD patients and 345 healthy control by means of sequencing. The studies above showed that there was apparent genetic heterogeneity among Caucasians and Asians, so there should be a presence of ethnic differences in susceptibility to IBD in Chinese population.

In our study, familial clustering was rare in IBD patients. Although Chinese, Korean and Japanese races differ, the familial aggregation was similarly rare in IBD patients from the three countries. Does low prevalence of familial clustering and absence of NOD2/CARD15 and OCTN gene variants suggest that genetic factors may play a less important role in the development of IBD in the Asian population? The study of Kim *et al*<sup>[23]</sup> in Korea did not support this point. He found that although a positive family history [21 of 1043 (2.01%) with UC and 6 of 397 (1.51%) with CD] is much lower than that with Western patients, the population relative risk was 13.8 in first-degree relatives, indicating that a positive family history is an important risk factor for IBD in Koreans. Montgomery *et al*<sup>[24]</sup> found that young Asians who were born in Britain are at a significantly higher risk of developing IBD than the indigenous European population with relative odds of 6.1. This may reflect a greater genetic predisposition to IBD when uncovered by exposure to environmental factors. Undoubtedly, genetic susceptibility plays a most important role in the etiology of IBD. Recently, IL23R has been regarded as a milestone in unraveling etiology of CD and SNPs of IL23R was reported to be associated with CD<sup>[25]</sup>. We are recruiting more cases and controls to investigate whether SNPs of IL23R would also play a protective role in Chinese CD patients. Since there is great genetic heterogeneity between Chinese and the Caucasians, further studies including total genome-wide scans among Chinese patients are warranted to identify genes susceptible to IBD which would shed more light on the etiology of this disease in our own country.

## COMMENTS

### Background

Inflammatory bowel disease (IBD), including two clinical subtypes: Crohn's disease (CD) and ulcerative colitis (UC), has been increasing in some Asian countries in recent years, and China is one of the notable countries. Previous epidemiological studies suggested a genetic susceptibility in IBD which has been confirmed by molecular biology techniques. CARD15 (previously NOD2) was first confirmed to be a susceptible gene of CD. Two missense mutations Arg702Trp (2104C→T), Gly908Arg (2722G→C) and one frame-shift mutation (3020insC) of the NOD2/CARD15 gene affecting the function of binding microbial pathogens are independently associated with the development of CD. In recent years, A C1672T substitution in exon 9 of the *OCTN1* gene and a G-207C in the *OCTN2* promoter region were indicated as functional and causative mutations to enhance the susceptibility to IBD.

### Research frontiers

In the last ten years, there have been tremendous researches on genetic susceptibility of IBD and over 10 chromosomal regions have been identified by genome-wide scanning. Further fine mapping as well as candidate gene studies have already led to the identification of a number of susceptible genes. When CARD15 was first confirmed to be a susceptible gene of CD, its three variant alleles, Arg702Trp, Gly908Arg and 3020insC were found to increase the risk of CD in many western countries but completely different in Asian countries. Similar to the *CARD* gene, many western countries have proved that the SNPs of *OCTN1* and 2 were positively associated with IBD but absent in Japanese. These studies showed that there was apparent genetic heterogeneity between Caucasian and Asian as well as Chinese population.

### Innovations and breakthroughs

The study suggested that the four SNPs, C1672T, G-207C, 2104C/T and 2722G/C, might not play a role in susceptibility of IBD in Chinese patients, thereby differing from the case of Western populations, but consistent with the results in Asian population. Compared with the studies in the Chinese population before, we had the largest number of cases. In Asia, it was the first study to detect the polymorphism of OCTN in UC patients.

### Applications

With great genetic heterogeneity between Chinese and the Caucasian, further studies including total genome-wide scans among Chinese patients are warranted to identify genes susceptible to IBD which would shed more light on the etiology of this disease in China.

### Peer review

In this study, the authors found that of the Chinese patients with IBD as well as the healthy controls none had OCTN and CARD15 variants. These results suggest that OCTN and CARD15 are rare in the Chinese population and may not be associated with susceptibility to IBD in Chinese patients. This was an interesting study with an aim that was well justified.

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RAPID COMMUNICATION

## Permeabilities of rebamipide *via* rat intestinal membranes and its colon specific delivery using chitosan capsule as a carrier

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totally within 6 h. The area under concentration-time profile of drug in the colon mucosa using chitosan capsules (AUCLI, 16011.2 ng·h/g) was 2.5 times and 4.4 times greater than using gelatin capsules and CMC suspension, respectively. Meanwhile, the area under concentration-time profile of drug in the plasma (AUCPL) was 1016.0 ng·h/mL for chitosan capsule, 1887.9 ng·h/mL for CMC suspension p and 2163.5 ng·h/mL for gelatin capsule. Overall, both AUCLI and AUCPL were increased when C12 was co-administrated, but the increase of AUCLI was much greater; the drug delivery index (DDI) was more than 1 compared with simple chitosan capsule group.

**CONCLUSION:** There was a regional difference in the permeability of Rebamipide across the jejunum, ileum and the colon, and passive diffusion seems to be one of the major transport mechanisms of rebamipide. Absorption enhancers can increase the permeability of rebamipide across the colon tissue significantly. In addition, chitosan capsule may be a useful carrier to deliver rebamipide to the colon specifically and the co-administration of C12 with rebamipide may also be very useful in local treatment.

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**Key words:** Rebamipide; Diffusion chamber; Permeability; Sodium laurate; Chitosan capsule; Colon-specific delivery

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Huang BB, Li GF, Luo JH, Duan L, Nobuaki K, Akira Y. Permeabilities of rebamipide *via* the rat intestinal membranes and its colon specific delivery using chitosan capsule as a carrier. *World J Gastroenterol* 2008; 14(31): 4928-4937 Available from: URL: <http://www.wjnet.com/1007-9327/14/4928.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4928>

## INTRODUCTION

Rebamipide (2-(4-chlorobenzoylamino)-3-[2(1H)-quinolinon-4-yl] propionic acid), a novel anti-ulcer drug, has been reported to prevent various acute

experimental gastric lesions and accelerate healing of chronic gastric ulcers<sup>[1]</sup>. This drug has been marketed in Japan since 1990 as a therapeutic agent treating gastric ulcer and acute and/or chronic gastritis. Although the characteristics of rebamipide in preclinical and clinical area<sup>[2,3]</sup>, which is one of key factors to develop its reasonable oral dosage form at the initial stage, has been investigated fully, little was known about the permeability of this drug in different gastrointestinal tissues. On the other hand, recent studies also have shown the beneficial effect of this drug on experimental colitis. It has been demonstrated that the attenuation of colitis indices induced by rebamipide was associated with its inhibition of inflammatory cytokine-mediated granulocyte (neutrophil) infiltration into the colon<sup>[4]</sup>. Other groups demonstrated that rebamipide can suppress chemically induced colitis in rodents, which appeared to be largely related with the inhibition of the production of reactive oxygen species. In clinical practice, rebamipide has been used to treat patients with proctitis<sup>[5]</sup>. Therefore, we hope to find some absorption enhancers to augment permeability of rebamipide across the colonic tissues, and in this case, this drug should be specifically localized in the large intestine by its colon-specific delivery to improve its therapeutic effect on colitis and to decrease its side-effects, as well. There are many investigations on absorption enhancers; meanwhile, it has been demonstrated previously that chitosan capsule could act as useful carriers for colon-specific delivery of peptide and anti-inflammatory drugs including insulin, calcitonin, 5-amino salicylic acid and ridogrel<sup>[6-9]</sup>. However, the effects of absorption enhancers increasing the distribution on colon and chitosan capsule on the colon-specific delivery of rebamipide ought to be established.

Therefore, we evaluated the permeability of rebamipide across different gastrointestinal membranes in the present study, analyzed potential transport mode, and investigated the effect of some absorption enhancers, such as sodium laurate (C12) and labrasol, on the permeability of rebamipide across colonic tissues. In addition, the effectiveness of chitosan capsule to the colon-specific delivery of rebamipide and the influence of rebamipide chitosan capsules with absorption enhancers on colon specific delivery were evaluated.

## MATERIALS AND METHODS

### Materials

Rebamipide was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid) and HPMCP (Hydroxypropyl methylcellulose phthalate) were purchased from Wako Pure Chemical Industries Co. C12 was from Tokyo Kasei Kogyo Co. Ltd, while labrasol was from Saint-Priest, France. Chitosan capsules and gelatin capsules were obtained from Aicello Chemical Company Ltd. (Toyohashi, Japan), and the mean diameters and weight of these capsules were 3.5 mm × 1.6 mm and about 1.0 mg, respectively. All other chemicals were of analytical grade.

### Instruments

Diffusion chamber apparatus was purchased from Harvard, US. The Shimadzu LC-10A system (Japan) as high performance liquid chromatography (HPLC) instrument was used in this study. The pH measuring instrument was from DKK.TOA Corporation in Japan. NTR-6000 Dissolution Tester made in Japan was chosen to test the dissolution rate of drug dosage forms.

### Animals

Male Wistar rats (280 ± 20 g) were purchased from Laboratory Animal Center, Southern Medical University. All of the animal experiments were performed according to the guideline of Experimental Animal Ethics Committee of Southern Medical University.

### Preparation of drug solutions for the *in vitro* permeability studies

For *in vitro* permeability studies, rebamipide was dissolved in oxygenated (O<sub>2</sub>/CO<sub>2</sub>, 95/5) HEPES buffer adjusted to pH 7.4, which was prepared daily, to yield final concentration of 80 μmol/L. In certain experiments, the dosing solutions were added to labrasol (0.4, 1, 2 g/L) or C12 (0.5, 1, 2 mmol/L).

### Permeability studies

The *in vitro* transport of rebamipide across different intestinal membranes was evaluated by a diffusion chamber method using stripped rat intestine for 2 h<sup>[10,11]</sup>. Male Wistar rats, weighing 280 ± 20 g, were fasted overnight and anesthetized with sodium pentobarbital (32 mg/kg, IP). The intestine of each rat was excised and rinsed in PBS of pH 7.4 and, avoiding Peyer's patches, experimental segments were obtained. The first 5 cm of the top of small intestine was cut away, the next 10 cm was used as the jejunum and the final 10 cm was considered to be the ileum. The first 2 cm of the large intestine was removed and the next 6 cm was used as the colon. The underlying muscularis from the serosal side of the tissue was removed and the final intestinal segments were mounted in the diffusion chamber in which a surface area of 1.78 cm<sup>2</sup> was exposed and preheated to 37°C. Immediately following tissue mounting, 7 mL of HEPES buffer at pH 7.4 was added to serosal side or mucosal side, while an equal volume of the rebamipide solution with or without an absorption enhancer was added to the opposite side. Each side of the chamber was bubbled with a mixture of 95 mL/L O<sub>2</sub> and 5 mL/L CO<sub>2</sub> to maintain the viability of the membrane. The heating unit was capable of holding six cells and the temperature was kept at 37°C during the whole procedure using a circulating water bath. At predetermined time intervals, 0.4 mL of solution at receiver side was sampled and it was immediately added to an equal volume of the HEPES buffer kept at the same temperature. The concentration of rebamipide in the samples was determined by HPLC. The apparent permeability coefficient (Papp) was calculated by the equation Papp = dC/dt × (1/A C<sub>0</sub>), where Papp is expressed in cm/s, dC/dt is the slope of the linear portion of the permeation curves, A is

the diffusion area, and  $C_0$  is the initial concentration of rebamipide in the donor side.

#### **Preparation of various rebamipide dosage forms**

One mg of rebamipide or a mixture of C12 (0.05, 0.15 and 0.25 mg) with rebamipide (1 mg) was filled in one chitosan capsule. Then, the surface of the chitosan capsule was coated with hydroxypropylmethylcellulose phthalate (HPMCP) as an enteric coating material. 150 g/L HPMCP dissolved in acetone/ethanol (1/1) solvent was used in the whole procedure. In addition, 1 mg of rebamipide was filled in one gelatin capsule as one control dosage form. The procedure of preparation of this dosage form was the same as that of chitosan capsule. For another control dosage form, 4 mg/mL of rebamipide solution in 5 g/L CMC (Carboxymethylcellulose) containing 10 mmol/L NaOH was also prepared.

#### **In vitro dissolution test**

The dissolution tests of rebamipide from chitosan capsules and gelatin capsules were carried out using the Japanese Pharmacopoeia (JP) rotating basket method with some slight modifications. Liquid 1 (a model medium of an artificial gastric juice for the Japanese Pharmacopoeia disintegration test) and liquid 2 (a model medium of an artificial intestinal juice for the Japanese Pharmacopoeia disintegration test) were used as media in these experiments. The rotation speed of the baskets was 100 r/min. Samples (0.2 mL) were taken every 60 min and the amount of rebamipide released from the capsules was determined by HPLC.

#### **Establishment of rebamipide determination in rat plasma and colonic tissue using HPLC**

**HPLC conditions:** Rebamipide in colonic tissue and plasma was assayed by reversed phase HPLC system containing 5-μm Cosmosil (4.6 mm × 150 mm) particles in an analytical column from Nacalai Tesque, a Shimadzu LC-10 pump system, a Shimadzu SIL-10A autoinjector and a Shimadzu CR-6A integrator. The mobile phase was mixture of 15 mL/L HAc solution (mobile phase A) and acetonitrile containing 100 mL/L tetrahydrofuran (mobile phase B). The gradient system was programmed by linearly increasing the proportion of mobile phase B from 18% to 40% within 55 min. The ultraviolet detector was set at 240 nm.

**Preparation of colon tissue sample:** Male Wistar rats weighing 280 ± 20 g were fasted for 16 h prior to experiments but allowed water *ad libitum*. They were anesthetized with sodium pentobarbital (32 mg/kg, ip). The abdomen was opened through a midline incision and the whole colon was removed from the body. After being washed with PBS, the colon tissue was cut into small pieces. The specimens were weighed. Methanol (5 mL) was added and the specimens were homogenated at ice-water bath using a POLYTRON homogenizer. The homogenate was centrifuged at 12 000 r/min for 15 min. The supernatant was evaporated at 60°C under nitrogen flow. The residue was re-dissolved in 0.25 mL

of 5 mmol/L NaOH solution by ultrasound for 15 min. The suspension was centrifuged at 12 000 r/min for 15 min. The resulting supernatant was taken as colon tissue sample to be analyzed by HPLC.

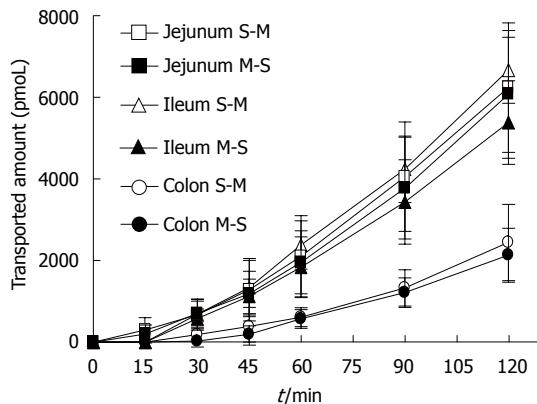
**Preparation of plasma sample:** Male Wistar rats, 280 ± 20 g, were fasted for 16 h prior to experiments but allowed water *ad libitum*. They were anesthetized with sodium pentobarbital (32 mg/kg, IP). The abdomen was opened through a midline incision and 5 mL blood was collected into heparinized syringes *via* the abdominal vein. Samples were immediately centrifuged at 10 000 r/min for 5 min to obtain plasma fraction. 5 mL methanol was added in 1 mL plasma, and the mixture was vortexed for 1 min and then centrifuged at 3600 r/min for 15 min. 4.5 mL of supernatant was evaporated at 60°C under nitrogen flow. The residue was re-dissolved in 0.25 mL 5 mmol/L NaOH solution by ultrasound for 15 min. The suspension was centrifuged at 12 000 r/min for 15 min. The resulting supernatant was taken as plasma sample to be analyzed by HPLC.

#### **In vivo absorption experiments**

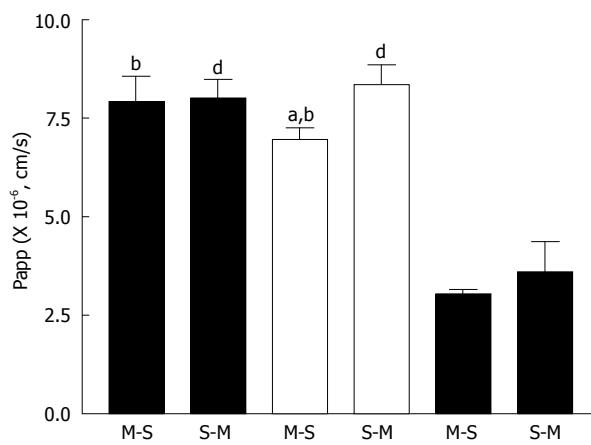
Male Wistar rats, 280 ± 20 g, were fasted for 16 h before the experiments, and then four chitosan or gelatin capsules (4 mg rebamipide) were administered orally to the stomach *via* polyethylene tubing under light ether anesthesia. One mL of distilled water was administered after that. For the CMC group, 1 mL of rebamipide CMC solution (4 mg rebamipide) was taken orally under the same conditions. At the predetermined time interval, blood samples and colonic tissue samples were prepared, and then the rebamipide contents in the samples were determined by HPLC. Drug delivery index (DDI) was calculated from the following equation: DDI = (AUC<sub>1LI</sub>/AUC<sub>2LI</sub>)/(AUC<sub>1PL</sub>/AUC<sub>2PL</sub>), where AUC<sub>1LI</sub> and AUC<sub>1PL</sub> represent the area under concentration-time profiles of rebamipide in the large intestinal mucosa and the area under the plasma concentration-time profiles of rebamipide after the oral administration of its chitosan capsules, respectively, while AUC<sub>2LI</sub> and AUC<sub>2PL</sub> represent the area under concentration-time profiles of rebamipide in the large intestinal mucosa and the area under the plasma concentration-time profiles of rebamipide, respectively, after the oral administration of its gelatin capsules or its CMC solution. The *in vivo* absorption experiments were run with C12, and then four chitosan with C12 were orally administered to the stomach, other procedures same as above, DDI = (AUC<sub>3LI</sub>/AUC<sub>1LI</sub>)/(AUC<sub>3PL</sub>/AUC<sub>1PL</sub>). AUC<sub>3LI</sub> and AUC<sub>3PL</sub> represent the area under concentration-time profiles of rebamipide in the large intestinal mucosa and the area under the plasma concentration-time profiles of rebamipide, respectively, after the oral administration of chitosan capsules with different dosage of C12.

#### **Statistical analyses**

Results were expressed as the mean ± SE and statistical significance was performed by the Student's *t*-test



**Figure 1** Time course of M-S and S-M transport of rebamipide across the different intestinal tissues. Each value represents mean  $\pm$  SE ( $n = 10$ ).



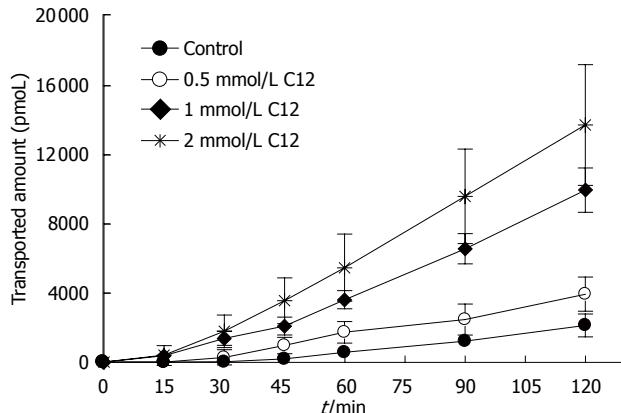
**Figure 2** The Papp of rebamipide across the different intestinal regions. Each value represents mean  $\pm$  SE ( $n = 10$ ). <sup>b</sup> $P < 0.01$  vs colon M-S, <sup>d</sup> $P < 0.01$  vs colon S-M, <sup>a</sup> $P < 0.05$  vs ileum S-M.

or Dunnett's test for multiple comparisons with the minimum levels of significance,  $P < 0.05$ .

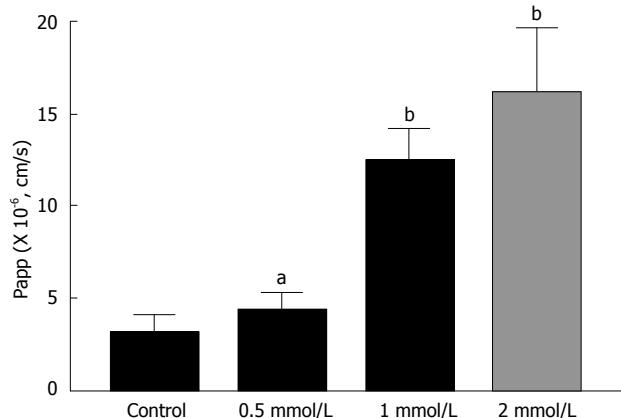
## RESULTS

### Regional differences in the permeability of rebamipide across different intestinal membranes

Figure 1 shows the time course of absorptive (mucosal to serosal, M-S) and secretory (serosal to mucosal, S-M) transport of rebamipide across the rat various intestinal membranes. As shown, there were regional differences in the *in vitro* permeability of rebamipide. The permeability of rebamipide across the jejunal or ileal membranes was higher than that across the colonic membrane. The S-M transport of rebamipide in the jejunum and colon was almost as same as its M-S transport, while the S-M transport of rebamipide across ileal tissues was slightly greater than that from M-S transport. Figure 2 summarizes the Papp of rebamipide across the different intestinal regions. As shown in Figure 2, Papp in jejunum and ileum was higher than that in colon, but no significant difference of drug permeability was observed between jejunal and ileal region. Based on the regionally different absorption studies, we selected colon as a model region to estimate the permeability of rebamipide in the presence of some



**Figure 3** Time course of transport of rebamipide across the colonic tissues in the presence of various concentrations of C12. Each value represents mean  $\pm$  SE ( $n = 10$ ).



**Figure 4** Effect of various concentrations of C12 on the Papp of rebamipide in the colonic tissues. Each value represents mean  $\pm$  SE ( $n = 10$ ). <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs control.

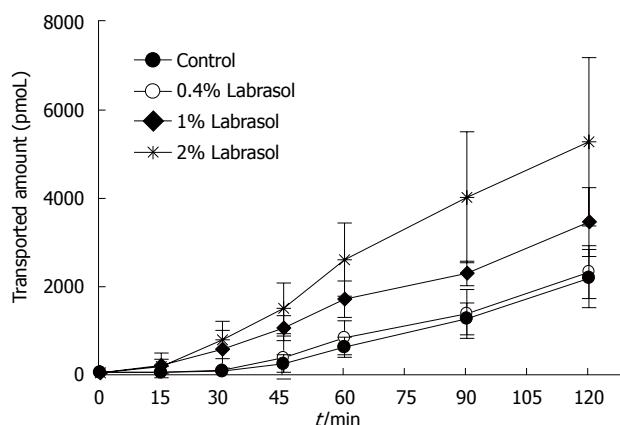
absorption enhancers in the following, because the colon is the pharmacodynamic position of rebamipide.

### Effect of C12 on the permeability of rebamipide across the colonic membranes

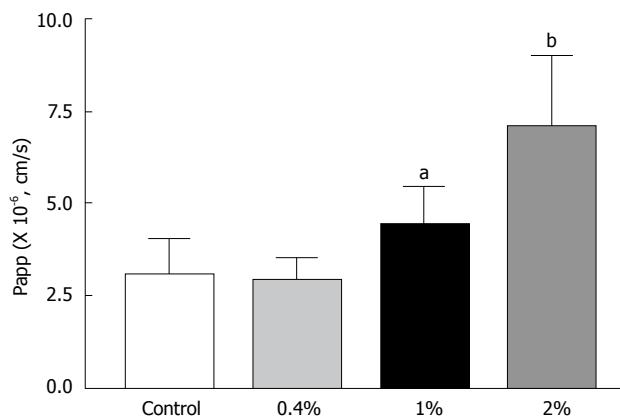
Figures 3 and 4 show the effects of different concentrations of C12 on the cumulative amount and Papp of rebamipide across the colonic region. As showed in Figures 3 and 4, the permeability of rebamipide from the colonic region was remarkably enhanced by the addition of C12. Also, there exists a concentration-dependent effect of C12 on the absorptive transport of rebamipide over the range of 0.5 to 2 mmol/L. In general, the higher concentrations of C12 gave the greater enhancement of rebamipide transport. In this experiment, we also have used 0.4% labrasol to dissolve C12, since C12 itself was not dissolved easily in the HEPES buffer. Therefore, we also investigated the influence of labrasol on the permeability of rebamipide across the colonic membranes.

### Effect of labrasol on the permeability of rebamipide across the colonic membranes

The transport of rebamipide with labrasol across



**Figure 5** Time course of transport of rebamipide across the colonic tissues in the presence of various concentrations of labrasol. Each value represents mean  $\pm$  SE ( $n = 10$ ).



**Figure 6** Effect of various concentrations of labrasol on the Papp of rebamipide in the colonic tissues. Each value represents mean  $\pm$  SE ( $n = 10$ ). <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs control.

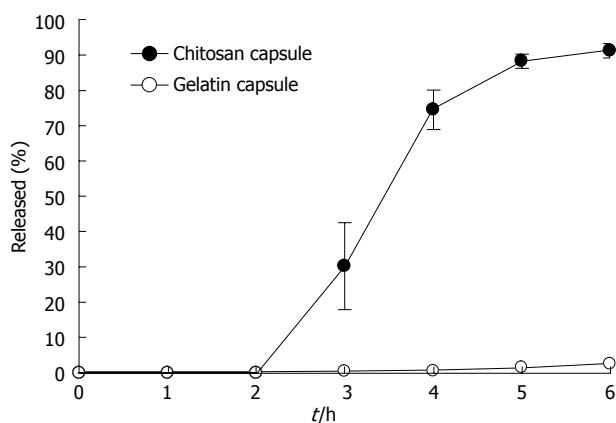
the colonic membranes was examined. We observed a concentration-dependent effect of labrasol on the cumulative amount and Papp of rebamipide across the colonic region, as indicated in Figures 5 and 6. However, there exists no effect of labrasol at lower concentration (0.4 g/L) on the transport of rebamipide. In addition, the enhancement effect of labrasol for the permeability of rebamipide was not as strong as that with C12.

#### In vitro releasing pattern of rebamipide from chitosan and gelatin capsules

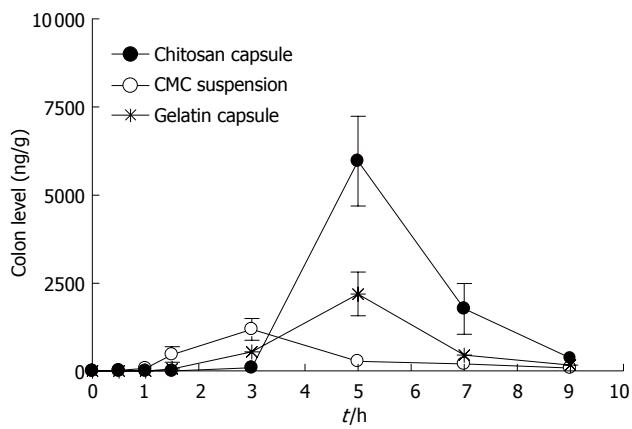
Figure 7 shows the release-time profiles of rebamipide from chitosan capsules and gelatin capsules. We studied drug release in liquid 1, an artificial gastric juice (pH 1), during the period 0–2 h after dosing, and in liquid 2, an artificial intestinal juice (pH 7) during the period 2–6 h after dosing. The release of rebamipide from the chitosan capsules in the protection of HPMCP was less than 10% totally within 6 h, while its release from the gelatin capsules reached about 100% in the same conditions.

#### Plasma concentration and colonic tissue distribution after oral administration of rebamipide in different dosage forms

Figure 8 shows the time course of rebamipide content

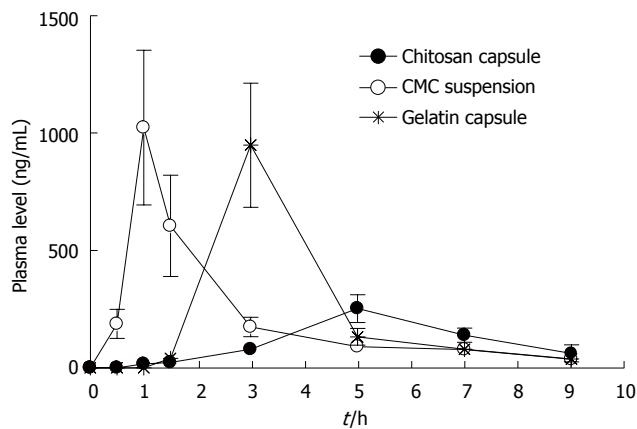


**Figure 7** Release of rebamipide from chitosan capsules or gelatin capsules by Japanese pharmacopoeia rotating basket method ( $n = 6$ ). 0–2 h: In the artificial gastric juice; 2–6 h: In the artificial small intestinal juice.

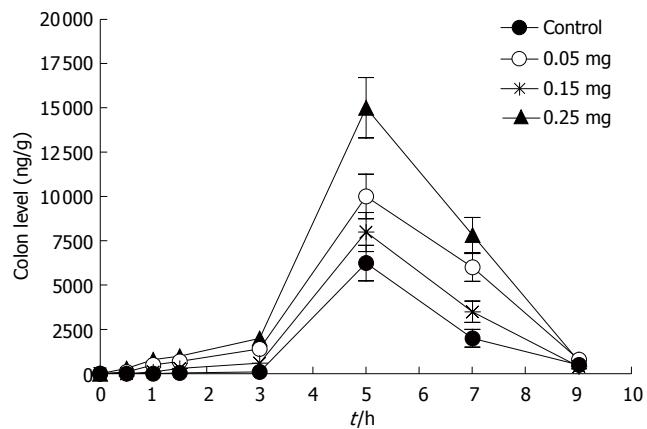


**Figure 8** Concentration-time profiles of rebamipide in the colon tissue after oral administration to rat with various dosage forms. Each value represents mean  $\pm$  SE ( $n = 3$ ).

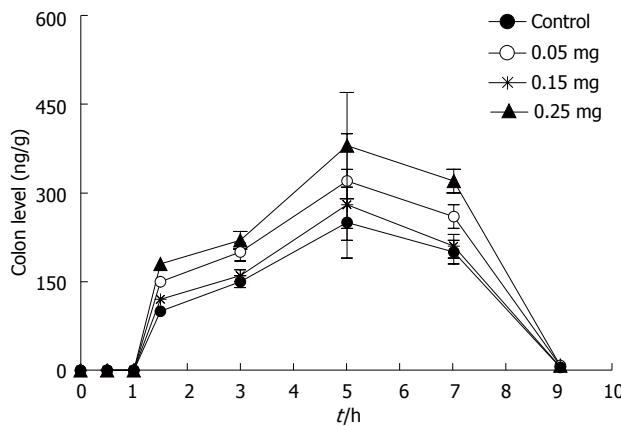
in the large intestine after the oral administration of rebamipide in different dosage forms. The area under concentration-time profile of drug in the large intestinal mucosa (AUCLLI, 16011.22 ng · h/g) after the oral administration of rebamipide using chitosan capsules was 2.5 times and 4.4 times greater than that of rebamipide using gelatin capsules and CMC solution, respectively. The target site of rebamipide is the large intestine, while the transfer amount of rebamipide to the systemic circulation after the oral administration is an index of the drug level in non-targeted sites and is related to the manifestation of adverse effects. We therefore determined the plasma concentrations of rebamipide after its oral administration with chitosan capsules and gelatin capsules as well as CMC solution. Figure 9 shows the plasma concentration-time profiles of rebamipide after the oral administration of rebamipide in different dosage forms. The area under the curve in the plasma (AUCPL) in CMC solution group was 1887.92 ng · h/mL, and AUCPL in gelatin capsule group was 2163.52 ng · h/mL. On the other hand, we observed an at least 1 h lag time in plasma concentration after the oral administration of chitosan capsules containing rebamipide. The AUCPL was 1016.02 ng · h/mL. Overall, the AUCPL value of rebamipide with chitosan capsule was lower than that seen with gelatin capsule or CMC



**Figure 9** Plasma concentration-time profiles of rebamipide after oral administration to rat with various dosage forms. Each value represents mean  $\pm$  SE ( $n = 3$ ).



**Figure 10** Concentration-time profiles of rebamipide in the colon tissue after oral administration to rat with or without C12. Each value represents mean  $\pm$  SE ( $n = 3$ ).



**Figure 11** Plasma concentration-time profiles of rebamipide after oral administration to rat with or without C12. Each value represents mean  $\pm$  SE ( $n = 3$ ).

solution. Thus the absorption of rebamipide from the gastrointestinal tract to the systemic circulation after the oral administration was inhibited by the use of chitosan capsules. In addition, the DDI, which shows the ratio of drug amount in targeted and non-targeted sites in different dosage forms, constitutes a good index for drug efficacy and safety. By using the equation indicated in Materials and Methods, the DDI of rebamipide in chitosan capsules was calculated to be 8.3 and 5.4, compared with CMC solution and gelatin capsules, respectively, and thus confirmed the effectiveness of chitosan capsules in ensuring the colon-specific delivery of rebamipide.

#### Plasma concentration and colonic tissue distribution after oral administration of rebamipide with different dosage of C12

The time course of rebamipide content in the large intestine and plasma of rebamipide after the oral administration of rebamipide with different dosage of C12 is shown in Figures 10 and 11. The area under concentration-time profile of drug in the large intestinal mucosa (AUCL) increased remarkably after the oral administration of rebamipide with C12, especially

the dosage of 0.25 mg; AUCL of chitosan capsules containing rebamipide was 38 458.2 ng · h/g. Though AUCPL (1536.1 ng · h/mL) also enhanced, the increment of AUCL was larger than AUCPL statistically. By using the three dosage of C12, the DDI of rebamipide in chitosan capsules was calculated to be 1.8, 1.4 and 1.2, respectively, compared with chitosan capsules. These indicated that absorption enhancer C12 can promote plasma concentration and colonic tissue distribution of rebamipide, but is much more profitable to colonic tissue distribution. In addition, there existed a concentration-dependent effect of C12 on the plasma concentration and colonic tissue distribution over the range of 0.05 to 0.25 mg. In general, the higher dosage of C12 gave the larger enhancement of distribution.

## DISCUSSION

Most drugs (approximately 60%) treat diseases by oral administration, which is the easiest and most useful method for drug delivery, and prediction of drug absorption is therefore very important for the design of an oral preparation. Only a few experimental *in vitro* methods have so far been established for prediction of drug absorption capability *in vivo*. Caco-2 monolayers are generally accepted to be a suitable *in vitro* model for drug transport studies as these cells have been shown to express most of the enzymatic, functional and morphological characteristics of the intestinal and morphological characteristics of the intestinal mucosa<sup>[12,13]</sup>. However, Caco-2 monolayers cannot be used to predict the regional differences in the permeability of drugs in the gastrointestinal tract. Also, the expression levels of transporters in Caco-2 cells were usually variable and were dependent on the culture condition, which is one of the major disadvantages to estimate the actual permeability of drugs, especially drugs mediated by these transporters. On the other hand, diffusion chamber technique<sup>[14-16]</sup> is one of the other effective means to predict the absorbability of drugs in humans based on rat intestinal permeability.

Recently, Watanabe *et al* investigated the correlation between the apparent permeability coefficients (Papp) of 15 water-soluble and poorly water-soluble drugs based on the diffusion chamber experiment and the fractions absorbed (Fa) in humans. A good correlation was found between Papp and Fa of the test drugs<sup>[17]</sup>. Therefore, in this study, we also investigated the permeability of rebamipide across different gastrointestinal membranes using diffusion chamber experiment.

The low solubility and low permeability of rebamipide indicate that it should be classified into Class IV in Biopharmaceutics Classification System (BCS)<sup>[18]</sup>. It has been known that many drugs categorized into BCS Class IV are P-gp substrates<sup>[19]</sup>. Also, it has also been demonstrated that the intestinal P-gp, an ATP-dependent multidrug efflux pump, can be an active secretion system or an absorption barrier by transporting some drugs from cells into intestinal lumen. Meanwhile, our results revealed that the permeability of rebamipide across the different gastrointestinal regions in M-S or S-M direction is almost equal, although S-M transport of rebamipide across the ileal tissue was slightly greater than that from M-S transport. Based on these results, it may be inferred that carrier-mediated intestinal transport was not involved in the transport of rebamipide and passive diffusion seems to be one of the major mechanisms for the intestinal transport of rebamipide. However, we have used 80 μmol/L of rebamipide in this study to avoid the limitation of detectable amount by the present HPLC method. For most of carrier-mediated intestinal transports, it has been said that there exists a saturated effect of drug concentration on the transport of drug<sup>[20]</sup>. Therefore, it is necessary to investigate the characteristics of the permeability of rebamipide across M-S or S-M direction at much lower concentration in order to elucidate the exact mechanism of its transport across the intestinal membrane in the future, as more sensitive determination of rebamipide will have to be set up. In addition, we found in this study that the Papp of rebamipide across the colonic membranes in the absorptive direction was about  $(3.06 \pm 0.07) \times 10^{-6}$  cm/s ( $n = 15$ ), while Miyake *et al*<sup>[21]</sup> reported the Papp across the same membrane was about  $(2.93 \pm 0.35) \times 10^{-6}$  cm/s. The slight difference may be result from the different experimental conditions.

As far as the discordance of the absorbability or bioavailability of rebamipide in human and its *in vitro* permeability is concerned, it can be inferred that some rebamipide metabolism or any others could be involved in human intestinal tract, as the bioavailability of rebamipide was only about 10%, while the permeabilities of rebamipide across the absorptive jejunal and ileal membranes in our study reached  $8.0 \times 10^{-6}$  cm/s and  $6.9 \times 10^{-6}$  cm/s, respectively. Consistent with our results, Walle *et al* also showed that the Papp of taxol was  $4.4 \times 10^{-6}$  cm/s in Caco-2 cells, and in general a Papp value in Caco-2 cells of  $> 1 \times 10^{-6}$  cm/s is associated with efficient intestinal absorption in humans. Therefore, it was hypothesized that the low oral bioavailability of taxol may be more dependent on presystemic metabolism in

the liver than on lack of absorption. Also, the authors pointed out that a novel development involving increased expression of CYP3A4 should be helpful to examine the potential contribution of CYP3A4 to the transport of drugs such as taxol using the Caco-2 cell system<sup>[22]</sup>. Hence, these factors also should be involved to evaluate the absorption characteristics of rebamipide in human, based on the obtained permeability parameters of rebamipide using diffusion chamber in our present study.

As the improvement of drug permeability by using an absorption enhancer has been very attractive from the aspects of biopharmaceutics, pharmacology, and economics, many researchers investigated the absorption enhancement using various adjuvants<sup>[23-25]</sup>. However, it has been very difficult to use those adjuvants for practical formulation, because they possibly cause local toxicity. Although many compounds have been reported to have absorption enhancing ability, medium-chain fatty acids and medium-chain glycerides are thought to be relatively safe because they are used as nutritional dietary supplements. Currently, only sodium caprate (C10) has been used and marketed as an absorption enhancer in ampicillin suppository marketed in Japan, Denmark, and Sweden, and in ceftizoxime suppository in Japan<sup>[26,27]</sup>. Furthermore, Miyake *et al* demonstrated that C12 was a more effective and safer adjuvant than C10 at the same concentration. Additionally, rebamipide is used to treat the proctitis in colon; therefore, we examined the effect of C12 on the permeability of rebamipide across colonic membranes in the absorptive direction, and we found that the permeability of rebamipide from the colonic region was remarkably enhanced by the addition of C12. Generally, the effects of the absorption enhancing agents are also often intestinal site-dependent. Hence, we also determined the effect of C12 on the permeability of rebamipide across the absorptive ileum membranes. We found the increased Papp ratio of rebamipide with 1 mmol/L of C12, compared with no C12 in this region was 1.92 (data was not shown in the results section), while it was 4.04 in the colon region, where Papp ratio = Papp (with C12)/Papp (without C12). It seems that using C12 as absorption enhancer to increase the absorption of rebamipide is feasible. On the other hand, C12 is not easily dissolved in the experimental HEPES buffer solution; therefore, labrasol was used to increase the solubility of C12 in this test medium. Labrasol is a surfactant that contains saturated polyglycolized C6-C14 glycerides, and its NMR characterization indicated that it is a mixture consisting of 30% mono-, di- and triglycerides of C8 and C10 fatty acids, 50% of mono- and diesters of poly (ethylene glycol) (PEG) and 20% of free PEG 400<sup>[28]</sup>. It shows high tolerance and low toxicity, and has a LD50 of 22 g/kg in rats. Labrasol has been included as a pharmaceutical excipient in European Pharmacopoeia in 1998. As a result, 0.4 g/L labrasol can make 2 mmol/L C12 dissolve in the HEPES buffer. Also, it was found that there was no influence on the permeability of rebamipide at this low concentration, though increased transport of rebamipide with 1 g/L or 2 g/L of labrasol across the colon membrane was found

and also showed concentration-dependent effect of labrasol on the permeability of rebamipide.

As the action and some mechanisms of rebamipide on treating colitis in animals and in human beings have been demonstrated, we investigated the colon specific delivery of rebamipide using chitosan capsule. Chitosan is a high molecular weight cationic polysaccharide, which can be prepared by alkaline N-deacetylation of chitin, the second most abundant natural polymer<sup>[29]</sup>. It has been known that chitosan possesses many advantages including low toxicity, with an oral LD<sub>50</sub> in mice of > 16 g/kg, moderate immunostimulating effect, and inert and biodegradable characteristics. In addition, chitosan has been widely applied as a potential formulation excipient in conventional pharmaceutical devices. This polymer also has been investigated as a potential adjuvant for orally controlled release systems<sup>[30]</sup> and colon targeting<sup>[4-7]</sup>. In addition, it was reported that chitosan had mucoadhesive properties, which probably was mediated through ionic interactions with negative charges in mucus or on cell surfaces. In this study, the chitosan material with average molecular weight of 43000 and average deacetyl degree of 83% was chosen to prepare the capsules. As results, we also have demonstrated that this kind of chitosan capsule coated with HPMCP could be a useful carrier to the colon specific delivery of rebamipide, as has been shown in that the DDI of rebamipide chitosan capsule was calculated to be 8.3 or 5.4, compared with CMC solution group or gelatin capsule group, respectively. Based on our findings, we proposed mechanisms for the colon-specific delivery of rebamipide using chitosan capsule. In the case of rebamipide CMC solution, rebamipide has been absorbed from the small intestine region. Additionally, rebamipide in the gelatin capsule also is absorbed from the small intestinal region, though HPMCP coating the surface of gelatin capsule can protect this kind of capsule from the attack of strongly acidic surroundings in the stomach. However, both HPMCP and gelatin material are easily dissolved in the small medium. Therefore, rebamipide is released and absorbed from the small region when HPMCP-coated gelatin capsule reaches this region. On the other hand, when rebamipide was orally administered using HPMCP-coated chitosan capsules, HPMCP first protected the dissolution of chitosan capsule in stomach. Then, the HPMCP is rapidly dissolved and there is little influence of the intestinal medium on the chitosan capsule when HPMCP-coated chitosan capsule enters into the small region. After that, this dosage form reaches the cecum and then the colon. The capsule was disintegrated by microorganisms richly distributed in these regions, and hence rebamipide was released and exerted its local action on colitis. Alternatively, it was reported recently that the pH may actually fall in the colon, when compared with the pH of the small intestine. This low pH, which is caused by acidification of the colonic contents by the products of bacterial fermentation, may be also related to the degradation of chitosan capsule in rat cecal contents and in colon, since chitosan is

unstable and easily degraded under acidic conditions. For one thing, rebamipide has weak acidity and can be dissolved in basic solution. Therefore, it can be dissolved in the colonic medium due to the basic property of this medium, which makes rebamipide much easier to distribute into the colon tissue and exert its local action.

Based on these mechanisms, it is also necessary to make the model drug released from the chitosan capsule easily dissolve in the colon medium by some means to insure that a large amount of drug is distributed in the tissue or absorbed into the circulation system, if we carry out the colon-specific delivery of some other model drug using chitosan capsule in the future. Since there were many experiments done to demonstrate the possibility of chitosan capsule as a specific colon delivery carrier in animals, we should pay close attention to the large scale production of drug-filled chitosan capsules and its prospects in clinical practice from now on. Further investigation should involve: (1) the difference of colon-specific delivery from other chitosan capsules with different molecular weights and degrees of deacetylation; (2) besides HPMCP, the possibility of some other enteric compounds as the coating material and the effect of the coating thickness on colon specific delivery of drugs; (3) the toxicity of cyanoacrylate adhesive used to seal the body and cap in the preparation of drug-filled chitosan capsules; (4) the evaluation of the advantages and disadvantages of chitosan capsules, compared with other colon specific delivery carriers.

Moreover, it is an important method to co-fill with some absorption enhancer in chitosan capsule to increase the drug absorption when systemic action is expected, while it is doubtful that some absorption enhancer is co-administered if the goal is to exert drug's local treatment and reduce the systemic side-effects. In our experiment, rebamipide content in the large intestine increasing notably after the oral administration of rebamipide with C12, which may indicate that absorption enhancer can enhance effect of therapeutic on colitis. On the other hand, DDI got raised, but absolutely speaking, the increasing of plasma concentration may produce adverse effects. Therefore, considering such factors, we should choose a reasonable dosage of C12, which can improve the distribution in colon of rebamipide and control the level of blood concentration; thus, rebamipide with chitosan capsule and C12 produces the best treatment outcome. In future, we will study the effects on colon absorption of rebamipide with various kinds of absorption enhancers, and the safety and toxicity of combinations of absorption enhancer and chitosan capsule.

In addition, in the case of ulcerative colitis, it is reported<sup>[31,32]</sup> that rebamipide enema treatment is useful; however, the treatment is mainly effective in local inflammation of rectum and descending colon due to the limitation of enema technique. Using chitosan capsule, rebamipide could be first distributed in the ascending colon, and then in the transverse colon and other colon. Therefore, rebamipide chitosan capsule may be more helpful in treating the extensive colitis or pancolitis.

In conclusion, we demonstrated a regional difference in the *in vitro* permeability of rebamipide across the small intestine and the colon in rats. Also, absorption enhancers such as C12 and labrasol can increase the permeability of rebamipide across the colon tissue significantly. We demonstrated that rebamipide can be specifically delivered to the colon using HPMCP-coated chitosan capsule. Meanwhile, we also confirm that rebamipide chitosan capsule, when added with C12, is more useful in treating colitis.

## COMMENTS

### Background

Rebamipide has been used to treat patients with proctitis using enema, but this treatment is inconvenient and circumscribed. It has been demonstrated previously that chitosan capsules could act as useful carriers for colon-specific delivery of peptide and anti-inflammatory drugs. However, the effects of absorption enhancement of the distribution on colon and of chitosan capsule on the colon-specific delivery of rebamipide ought to be established.

### Research frontiers

It is very important to improve therapeutic effects of rebamipide on colitis and decrease its side-effects, so it is suggested to find some absorption enhancers to augment permeability of rebamipide across the colonic tissues, and to localize specifically the drug in the large intestine by colon-specific delivery.

### Innovations and breakthroughs

Rebamipide chitosan capsule is more convenient and helpful than enema in treating colitis, especially extensive colitis or pancolitis.

### Applications

Since there were many experiments done that have demonstrated the possibility of chitosan capsule as a specific colon delivery carrier in animal, the large scale production of drug-filled chitosan capsules and its prospects in clinical practice from now on should be paid much attention. Rebamipide chitosan capsule, added with absorption enhancer, can be using in treating colitis in future.

### Terminology

The apparent permeability coefficient ( $P_{app}$ ) is the apparent parameter of permeability ( $\text{cm/s}$ ), was calculated by the equation  $P_{app} = dC/dT \times (1/A \cdot C_0)$ , where  $dC/dT$  is the slope of the linear portion of the permeation curves,  $A$  is the diffusion area, and  $C_0$  is the initial concentration of rebamipide in the donor side.

### Peer review

The authors explained rebamipide delivery using chitosan capsule. The study seems to be interesting and promising.

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RAPID COMMUNICATION

## Compression anastomosis clip for gastrointestinal anastomosis

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### Abstract

**AIM:** To investigate the feasibility of compression anastomosis clip (CAC) for gastrointestinal anastomosis proximal to the ileocecal junction.

**METHODS:** Sixty-six patients undergoing gastrointestinal anastomosis proximal to the ileocecal junction were randomized into two groups according to the anastomotic method, CAC or stapler.

**RESULTS:** The postoperative recovery of patients in CAC and stapled anastomosis groups was similar. No postoperative complication related to the anastomotic method was found in either group. Both upper gastrointestinal contrast radiography at the early postoperative course and endoscopic examination after a 6-mo follow-up showed a better healing at the compression anastomosis.

**CONCLUSION:** CAC can be used not only in colonic surgery but also in gastrointestinal anastomosis. Our result strongly suggests that CAC anastomosis is safe in various complication circumstances. However, it should be further confirmed with a larger patient sample.

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**Key words:** Gastrointestinal anastomosis; Compression anastomosis clip; Stapler

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### INTRODUCTION

Compression anastomosis was first reported in 1826 by Denan, who conceived a sutureless bowel anastomosis that encompassed the inverting technique proposed by Lembert<sup>[1]</sup>. The idea is to compress two bowel walls together and induce a simultaneous necrosis and healing process leading to the joining of the two lumens. In 1892, Murphy introduced a mechanical device known as "Murphy's Button", which has been used for years<sup>[2-4]</sup>.

Additional compression devices, such as magnetic ring, AKA-2 device, and biofragmentable anastomosis ring (BAR) were developed after almost 100 years<sup>[5-7]</sup>. Although animal experiments and clinical trials have reported the high efficacy and safety of these anastomotic devices, they are not widely accepted because of their narrow inner caliber and high expenses. Finally, these compression anastomotic devices were substituted by staplers which are used routinely at present<sup>[3,8]</sup>.

However, compression anastomosis with no foreign bodies left at the anastomotic site permanently, is considered an ideal anastomotic method. Many surgeons still make effort to improve this compression device. Recently, a new device, compression anastomosis clip (CAC) emerged in 2000, was approved for its use by the FDA<sup>[9]</sup>. Clinical trials performed in Israeli Medical Center have confirmed its safety and efficacy for colonic anastomosis in laparoscopic procedures<sup>[10-12]</sup>. However, its application in gastrointestinal anastomosis proximal to the ileocecal junction is limited at an experimental level<sup>[9]</sup>. This prompts us to study its clinical effects on gastrointestinal anastomosis proximal to the ileocecal junction.

### MATERIALS AND METHODS

#### Sample size and randomization

This study was approved by the institutional committee on ethics in clinical trials. Sixty-six patients at the age of 35-83 years, who underwent gastroenterostomy or enteroenterostomy in July 2005 - December 2006,

**Table 1** Clinical data obtained from the patients (mean  $\pm$  SD)

	Study group (n = 33)	Control group (n = 33)
Sex (M:F)	24:9	21:12
Age (yr)	57.0 $\pm$ 11.85	58.6 $\pm$ 13.23
Height (cm)	167.7 $\pm$ 7.54	166.4 $\pm$ 8.59
Body weight (kg)	61.7 $\pm$ 10.92	62.7 $\pm$ 7.49
BMI (kg/m <sup>2</sup> )	21.9 $\pm$ 3.13	22.7 $\pm$ 3.54
Albumin (g/L)	41.6 $\pm$ 4.44	39.9 $\pm$ 4.77
Hemoglobin (g/L)	125.8 $\pm$ 17.48	118.6 $\pm$ 16.34
Prealbumin (mg/L)	276.1 $\pm$ 105.74	301.9 $\pm$ 100.18

**Table 2** Primary disease and distribution of patients

Dagnosis	Study group	Control group	Total
Malignant			
Gastric cancer	21	24	45
Gastric stump cancer	1	0	1
Reflux esophagitis	1	0	1
Anastomotic stenosis	1	1	2
Intestinal tumor	2	0	2
Total	26	25	51
Benign			
Intestinal adhesion	3	2	5
Intestinal tumor	1	1	2
Peptic ulcer disease	1	3	4
Annular pancreas	1	0	1
Inflammatory bowel disease	1	2	3
Total	7	8	15

were recruited into the study. Patients with an emergency surgical history or a nickel allergy history were excluded. All participants signed an informed consent form and were randomly divided into a study group or a control group according to the anastomotic method. Randomization was based on computer-generated codes that were maintained in sequentially numbered opaque envelopes. Compression anastomosis clip (CAC) (NiTi Medical Technologies, Netanya, Israel), 30 mm in diameter, was used for anastomosis in the study group ( $n = 33$ ). In the control group, an anastomosis was performed with a stapler. Bowel preparation, consisting of oral magnesium sulphate, gentamycin, and metronidazole, was performed on the day prior to surgery in all patients except for seven patients with complete intestinal obstruction. There was no statistical difference in preoperative conditions between the two groups (Table 1). The main indication for underlying disease was gastric cancer in both groups (Table 2). One patient in the study group was complicated by postgastrectomy reflux esophagitis due to a previous radical gastrectomy and Brown's reconstruction. She complained of serious nausea and vomiting after surgery and was readmitted to our hospital for reconstruction. The afferent loop was resected and a side-to-side anastomosis to distal jejunum was performed with CAC. The distribution of anastomotic sites and operative procedures are listed in Table 3. Two anastomoses were performed for the patient who underwent total gastrectomy and Roux-en-Y reconstruction during esophagojejunostomy and jejunoojejunostomy. Only the difference in the latter was compared in both

**Table 3** Anastomotic site and operative procedure

Site	Study group	Control group	Total
Stomach-small bowel			
Subtotal gastrectomy with a Billroth II reconstruction	12	11	23
Gastrojejunostomy	6	3	9
Total	18	14	32
Small bowel-small bowel			
Total gastrectomy with a Roux-en-Y reconstruction	9	14	23
Enterenterostomy	6	5	11
Total	15	19	34

groups based on the different anastomotic methods.

The compression anastomosis was completed with CAC which is a shape memory nitinol double ring clip. The hollow viscera to be anastomosed were parallel to each other and two 5-mm incisions were made at both sides. The CAC mounted on a deployment device was cooled in ice-cold water for 2 min before use. After the clip was opened at an angle of approximately 30°, it was inserted into the hollow viscera through the two 5-mm incisions. The device returned to its original closed shape when it was warmed by body temperature and compressed the two visceral walls firmly. Then, the scalpel incorporated in the applier created a slit through the entrapped walls to allow free passage of air and feces through the anastomosis until the healing process ended. Finally, the applier was withdrawn and the two small incisions through which the clip was inserted were sutured. The compression anastomotic procedure was completed. The postoperative parameters were followed in three aspects.

### Clinical evaluation

Vital sign, recovery of bowel function, antibiotics application, duration of nasogastric drainage, time to start oral feeding and duration of postoperative hospitalization were recorded. We also recorded the time to complete the anastomosis, intraoperative and postoperative complications in all patients, and the time to expel the ring in the study group.

### Radiological examination

Four days after surgery, patients in the CAC group received abdomen plain X-ray examination to locate the ring in body. In addition, the integrity of anastomosis was examined by upper gastrointestinal contrast radiography with dilute gastrografin 7 d after operation.

### Endoscopy

After a six-month follow-up period, endoscopy was performed to evaluate the healing of anastomosis.

### Statistical analysis

Results were expressed as mean  $\pm$  SD. Differences in means between the two groups were calculated for significance with independent-samples *t* test using SPSS 10.0.  $P < 0.05$  was considered statistically significant.

**Table 4 Recovery-related variables after operation (mean  $\pm$  SD)**

Variable	Study group (n = 33)	Control group (n = 33)	P
Gases started (d)	4.3 $\pm$ 1.24	4.7 $\pm$ 1.28	0.230
Bowel movement (d)	5.7 $\pm$ 2.36	6.2 $\pm$ 2.53	0.407
Antibiotics stopped (d)	3.8 $\pm$ 0.74	4.0 $\pm$ 0.83	0.258
Duration of nasogastric drainage (d)	2.6 $\pm$ 0.98	2.8 $\pm$ 0.64	0.314
Start of oral intake (d)	4.8 $\pm$ 1.82	5.4 $\pm$ 1.69	0.174
Postoperative hospital duration (d)	14.9 $\pm$ 6.01	15.1 $\pm$ 8.35	0.946

## RESULTS

All patients recovered from operation uneventfully. The parameters collected during their early postoperative course were similar in both groups (Table 4). No difference was observed in the duration of operation between the two groups ( $139.4 \pm 41.39$  min vs  $143.2 \pm 40.53$  min). No intraoperative and postoperative complications were found, including anastomotic leakage, bleeding, and stenosis, which were related to the anastomotic methods. However, postoperative complications unrelated to the anastomosis occurred in two patients with compression anastomosis. One patient who underwent subtotal gastrectomy and Billroth II reconstruction showed delayed emptying of the stomach. The intestinal adherence distant from the compression anastomosis was observed at the second operation. The patient recovered finally. Another gastric cancer patient with pyloric obstruction underwent a palliative gastrojejunostomy and was refed with normal diet gradually. However, the patient's condition deteriorated suddenly and the patient died on day 35 after operation because of cardiopulmonary decompensation.

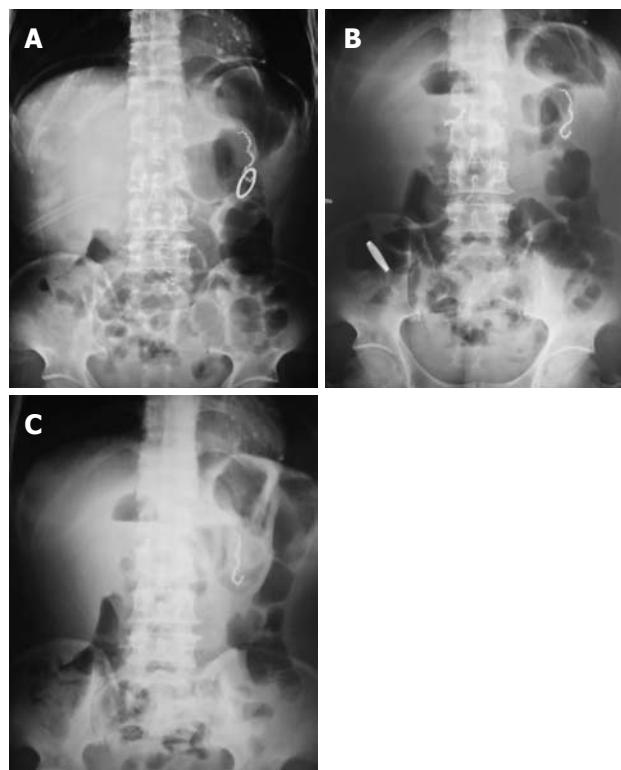
In the CAC group, all of the anastomosis clips were expelled with stool except two, and none complained of discomfort or tenesmus. The mean time of ring expulsion was  $15.1 \pm 6.04$  d (5-29 d). One patient who died 35 d after surgery did not expel the ring. The anastomotic ring in another patient with adhesive ileus was taken out by gastroscopy on the 16th postoperative day.

Using the radiological examination, we were able to locate the anastomosis ring in the patient's body dynamically (Figure 1). The upper gastrointestinal contrast radiography showed that the anastomoses in both groups were intact. No stenosis was observed in all patients, even with the CAC left at the anastomotic site (Figure 2).

After a 6-mo follow-up period, the anastomotic healing was evaluated in all patients by gastroscopy or enteroscopy. No relevant stenosis was detected in either group. All anastomoses showed good healing without scar. The mucosa of compression anastomosis was smooth (Figure 3). However, slight edema and congestion occurred in three of the stapled mucosa.

## DISCUSSION

Compression anastomosis is very close to the sutureless



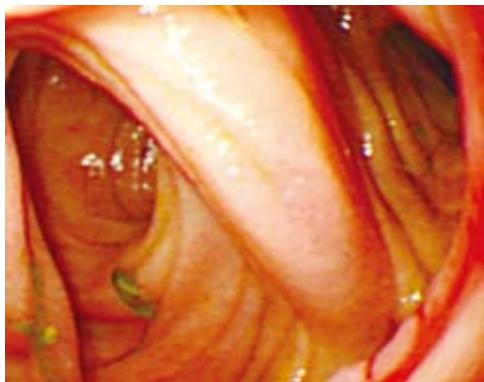
**Figure 1** Plain X-ray film showing the CAC *in situ* in abdomen of patients who underwent radical resection of gastric cancer and Billroth II reconstruction 5 d after operation (A), the ring moving to the right lower abdomen 11 d after operation (B), and expelling of the ring with stools 15 d after operation (C).



**Figure 2** Upper gastrointestinal contrast radiography showing no stenosis with CAC *in situ* in the patient who underwent alleviative gastrojejunostomy 7 d after operation.

anastomosis in the absence of permanent foreign bodies at the anastomotic site<sup>[2]</sup>. Although the technique of compression anastomosis was introduced more than 180 years ago and the theoretical advantages were appreciated, the technique has not been accepted as a standard for gastrointestinal anastomosis, which could be explained by its narrow inner caliber, difficult application and assemblage, and high cost<sup>[3,8]</sup>. It seems that these barriers can be tackled well with the new device, compression anastomosis clip.

The mechanism of CAC is similar to that of Murphy's button. The walls of hollow organs are placed between coils of the ring, pressed and clamped together. Its contin-



**Figure 3** Gastroscopic examination revealing a good healing at the compression anastomosis without scar, edema or congestion six months after subtotal gastrectomy with a Billroth II reconstruction.

uous pressure creates gradual and controlled necrosis of the tissue in the area limited by the CAC coil's perimeter while the external edges of this area become sealed, forming a smooth tight homogeneous anastomosis. The device is biofragmented or detached from the anastomotic site and is excreted from the body. The innovation of CAC is the shape memory nitinol double ring that becomes flexible in cool water and resumes its programmed shape when warmed by body temperature.

Technical failure rates of a new technique must be reduced by eliminating the learning curve on a suitable animal model<sup>[13]</sup>. In our study, no intraoperative problems were observed and no additional supportive measures were needed, suggesting that the increasing experience with our previous animal experiments may significantly shorten the duration of technique learning. The learning process of this new technique is short, indicating that this technique is easy to learn and handle. We also confirmed that CAC could be used not only for colonic anastomosis but also for gastrointestinal anastomosis and enterostomy. In addition, we also performed gastrojejunostomy and enterostomy with CAC for patients with intestinal adhesion, peptic ulcer, or inflammatory bowel disease. Almost all the patients in the CAC group passed the ring without discomfort only with two exceptions. No obstruction due to anastomotic device was observed, indicating that the air and feces are able to pass through the temporary passage of anastomosis during the healing process of anastomosis. The CAC, 30 mm in diameter and 8 mm in inner caliber, could pass through the ileocecal junction successfully.

Anastomotic leakage is a serious postoperative complication of gastrointestinal anastomosis. However, surgical procedure is one of the important factors that affect the healing of anastomosis<sup>[14]</sup>. Our data are consistent with a parallel study in Israel<sup>[10-12]</sup>. No anastomotic leakage was observed in our study, which may be partially explained by a constant stress plateau, which is exerted by coils at about 400 Mpa and up to 6% perfect recoverable strain<sup>[8]</sup>. The constant pressure makes the ring detached from the anastomotic site at an appropriate time. The unique physical properties of nitinol, mostly the constancy of stress, enable the device to exert

a relatively constant pressure irrespective of the bowel wall thickness.

Ischemia, inflammation, and fibrosis due to anastomosis leakage are the factors for anastomotic stenosis<sup>[15,16]</sup>. No sign of anastomotic stenosis at compression anastomotic site was observed either in upper gastrointestinal contrast radiography early postoperatively or in the endoscopy after a long-term follow-up period. The compression anastomosis showed a smooth and intact healing. In contrast, edema and congestion occurred in three of the stapled anastomoses. These discrepancies may be explained by the following. First, the absence of foreign bodies at CAC anastomotic site may greatly decrease inflammatory stimuli and formation of fibrous tissue. Second, the compression anastomotic diameter is determined by the outer diameter of the CAC device, as opposed to staplers, which is determined by its inner diameter. Thus, the CAC is capable of creating a larger anastomosis using a small device. Finally, the raw surface at the edge of the stapler line after firing may also increase the possibility of stricture.

The mean time of expulsion of anastomosis clip was  $15.1 \pm 6.04$  d after surgery. However, significant variations were found in different individuals. Half of the patients in the CAC group expelled the anastomotic clip in about 2 wk. The patients who passed the ring within about 1 wk were always younger and more likely to have benign diseases. They recovered from surgical stress faster and started activities earlier. In contrast, the elder patients with malignant disease often had complex conditions and malnutrition before surgery. They were bedridden for a longer time after surgery and took a longer time to expel the anastomotic ring, suggesting that many factors including anastomotic healing, enterokinesia, and distance of pathway, etc, can affect the duration of the ring expelling. Therefore, we hold that the duration for the ring to detach from the anastomotic site is more important. Further study is needed to confirm our findings.

The characteristics of postoperative recovery were similar in patients with CAC or stapled anastomosis. The anastomotic device did not influence the normal diet intake. None of the patients complained of abdominal pain due to dietary. However, Thiede and colleagues conducted a multicenter prospective trial of biofragmentable anastomosis ring and found that a low fiber diet until the evacuation of ring fragments from the bowel could prevent obstruction with no discomfort<sup>[16]</sup>.

The recovery course of 7 patients with a compression anastomosis who had complete intestinal obstruction before surgery, was uneventful and similar to those without complete intestinal obstruction before operation, which might be related to the characteristics of the CAC. When a CAC anastomosis was performed, the coils were inserted into the bowel lumens that were to be anastomosed through the small incisions and the compression anastomosis was completed after closure of the incisions. However, performing a stapled anastomosis needed a relatively larger surgical field and had more chances to contaminate the abdominal cavity when the applier was withdrawn. In addition, application of

the CAC may simplify the gastrointestinal bypass operation and shorten the operative time. Therefore, surgical injury to the patient could be minimized and the bowel function could recover earlier. Moreover, previous studies demonstrated that persistent foreign bodies may be responsible for anastomotic recurrence of cancer and inflammatory bowel disease and affect radiological examination<sup>[17,18]</sup>. Although no recurrence at anastomotic site was found in our study during the 6-mo follow-up period, further study is needed.

In conclusion, CAC is an alternative method for gastrointestinal anastomosis under different disease circumstances. Further study with a larger patient sample and a longer follow-up period is needed to confirm our findings before CAC application in emergency and laparoscopic surgery.

## COMMENTS

### Background

Gastrointestinal anastomosis is a common surgical procedure in abdominal surgery. Compression anastomosis, which was first reported in 1826 by Denan, is one of the methods. Because there is no foreign body left at the anastomotic site permanently, it is considered an ideal anastomotic method. However, the compression anastomotic devices have been substituted by staplers due to their narrow inner caliber and high cost.

### Research frontiers

Recently, a new device, compression anastomosis clip (CAC), which was emerged in 2000, was approved by the FDA. The mechanism of CAC is similar to that of Murphy's button. The innovation of CAC is the shape memory nitinol double ring that becomes flexible in cool water and resumes its programmed shape when warmed by body temperature. Clinical trials performed in Israeli Medical Center confirmed the safety and efficacy of CAC for colonic anastomosis in laparoscopic procedures.

### Innovations and breakthroughs

The application of CAC in gastrointestinal anastomosis proximal to the ileocecal junction was evaluated in this study. Besides, it can also be applied in selected patients who suffered from inflammatory bowel disease, peptic ulcer disease and intestinal obstruction, etc.

### Applications

CAC can be used for gastrointestinal anastomosis proximal to the ileocecal junction.

### Peer review

This study elucidated the safety of CAC for gastrointestinal anastomosis under different disease circumstances, which may be an alternative method for abdominal surgery and decrease the anastomotic recurrence of cancer and inflammatory bowel disease. The study was well designed and the findings were reliable and significant.

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RAPID COMMUNICATION

# Expression transformation of claudin-1 in the process of gastric adenocarcinoma invasion

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**Author contributions:** Wu YL and Zhang S contributed equally to this work; Wu YL and Zhang S designed the research; Wu YL, Zhang S, Wang GR and Chen YP performed the research; Chen YP contributed to new reagents; Wu YL and Zhang S analyzed the data and wrote the paper.

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lymph node metastasis than in those without lymph node metastasis.

**CONCLUSION:** Up-regulation of claudin-1 expression and its transformation in invasive and metastatic gastric carcinoma suggest that claudin-1 participates in the transformation of biological behaviors in neoplasms. Further study is needed to elucidate the precise mechanism and the relation of claudin-1 expression with the neoplasm progress.

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**Key words:** Gastric carcinoma; Claudin-1; Expression transformation; Invasiveness; Metastasis; Immunohistochemistry

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## Abstract

**AIM:** To investigate the relation of expression transformation of claudin-1 with invasiveness and metastasis of gastric carcinoma.

**METHODS:** By using immunohistochemistry, expression of claudin-1 in mucosa and invasive front of 136 gastric adenocarcinoma cases and proliferative index (Ki-67) were detected and analyzed.

**RESULTS:** In mucosa, the claudin-1 over-expression rate of mucinous adenocarcinomas (including signet-ring cell carcinomas) was the highest. It was negatively related with the differentiation but positively related with the invasiveness and metastasis of gastric cancer. In invasive front, the claudin-1 over-expression rate was positively related with the differentiation, invasiveness and metastasis of gastric carcinoma. The expression transformation of claudin-1 was found in gastric carcinoma. The expression of claudin-1 in invasive front was transformed in 28/136 gastric carcinoma cases. The transformation rate in highly differentiated tubular adenocarcinomas was the highest (51.5%, 17/33). The deeper was the invasiveness, the higher was the transformation rate. The claudin-1 expression transformation rate in serosa and omenta was significantly higher (92.9%) than in tunica muscularis of invasive gastric cancer cases, as well as in patients with

## INTRODUCTION

Gastric carcinoma is one of the most frequent malignant tumors in the world. Gastric carcinoma cells with a fibroblastic pattern in which intracellular adhesion is decreased show active mobility and invasiveness, indicating that epithelial mesenchymal transition (EMT) has occurred<sup>[1]</sup>. Tight junction (TJ) proteins participate in EMT of tumors<sup>[2]</sup>. The four-time transmembrane proteins of claudin family are essential components of TJ<sup>[3]</sup>, but the role of TJ proteins in the development of malignant tumor is not clear. By using immunohistochemistry, claudin-1 expression in gastric carcinoma was investigated and its relation with biological behaviors of gastric carcinoma was discussed in this study.

## MATERIALS AND METHODS

### **Patients**

A total of 136 patients (106 males and 30 females) with primary gastric carcinoma, who underwent surgery between January and December 2007 in the First Affiliated Hospital of Fujian Medical University, were enrolled in this study. Their median age was 64 years, ranging 28-80 years. All patients did not receive radiation therapy or chemotherapy prior to operation. The histological findings, lymph node metastasis and TNM stage were evaluated based on World Health Organization Classification of Tumors<sup>[4]</sup>.

### **Immunohistochemistry**

Specimens were fixed in formalin, embedded in paraffin wax, cut into 4 μm thick sections and stained with hematoxylin and eosin.

The sections were immunostained with anti-rabbit polyclonal antibodies for claudin-1 (1:100, ZYMED) and Ki-67 (MB67, Ready, NeoMarkers) with the EnVision method. The sections were deparaffinized and heated in a microwave oven for 10 min to retrieve the antigens. After immersed in 3% hydrogen peroxide of 100% methanol for 10 min to block the endogenous peroxidase activity, the sections were incubated with primary antibodies for 60 min at room temperature, with EnVision™ for 20 min, and then immersed into a DAB solution. The sections were counterstained with haematoxylin, dehydrated and mounted. Between steps, the sections were washed three times with phosphate-buffered saline (PBS). As a negative control, PBS was used instead of primary antibody. Two independent observers without knowledge of the clinical outcomes evaluated the immunohistochemical staining of sections till a complete agreement on the classification.

### **Immunohistochemical analysis of claudin-1 and Ki-67 labeling index**

Claudin-1 was expressed in the cell membrane and/or cytoplasm (Figure 1). The intensity of staining in cell membrane and cytoplasm and the percentage of immunoreactive cells over the total tumor cells were evaluated as previously described<sup>[5]</sup>. The intensity of staining was graded as 0 when staining was not greater than negative control, 1 as light staining, and 2 as heavy staining. Immunoreactivity was scored according to the percentage of immunoreactive cells over the total tumor cells counted as 0 if < 5% cells were stained, 1 if 5%-25% cells were immunoreactive, 2 if 26%-50% cells were immunoreactive, and 3 if > 50% cells were immunoreactive. The expression of claudin-1 was finally defined according to the score obtained from the grade of intensity multiplied by the score of cell immunoreactivity, i.e. negative (-, scored 0-1), positive (+, scored 2-3), and strongly positive (++, scored 4 or above). Positive expression of Ki-67 staining was found in nuclei of carcinoma cells. Ki-67 labeling index was defined as the ratio of immunoreactive cells over 1000 tumor cells.

### **Statistical analysis**

Chi square test was used for univariable categorical analysis. All statistical analyses were performed with SPSS 10.0.  $P < 0.05$  was considered statistically significant.

## RESULTS

### **Relation between claudin-1 expression and clinicopathological parameters of gastric carcinoma**

Claudin-1 was mainly expressed in the cell membrane and/or cytoplasm of gastric carcinoma cells. The expression of claudin-1 was related with the histological type, degree of invasiveness and lymph node metastasis of gastric cancer ( $P < 0.05$ ). However, the expression of claudin-1 was not significantly related with the sex and age of gastric cancer patients (Table 1).

The claudin-1 over-expression rate was the highest in mucinous adenocarcinomas, and lower in poorly differentiated carcinomas than in well-moderately differentiated carcinomas. It was significantly higher in mucosa of patients with their tumors invaded muscularis propria and visceral peritoneum, or with lymph node metastasis than in mucosa of patients with their tumors only invaded lamina propria or submucosa, or without lymph node metastasis.

The claudin-1 expression in invasive front was different from that in the mucosa. The claudin-1 over-expression was the highest in well-moderately differentiated carcinomas and the lowest in poorly differentiated carcinomas. The deeper was the invasive depth, the higher was the claudin-1 over-expression rate. The incidence of claudin-1 over-expression rate was 50% in invasive front with tumors invaded visceral peritoneum and significantly higher in patients with lymph node metastasis. The expression of claudin-1 was not related with the proliferation index of gastric carcinoma cells.

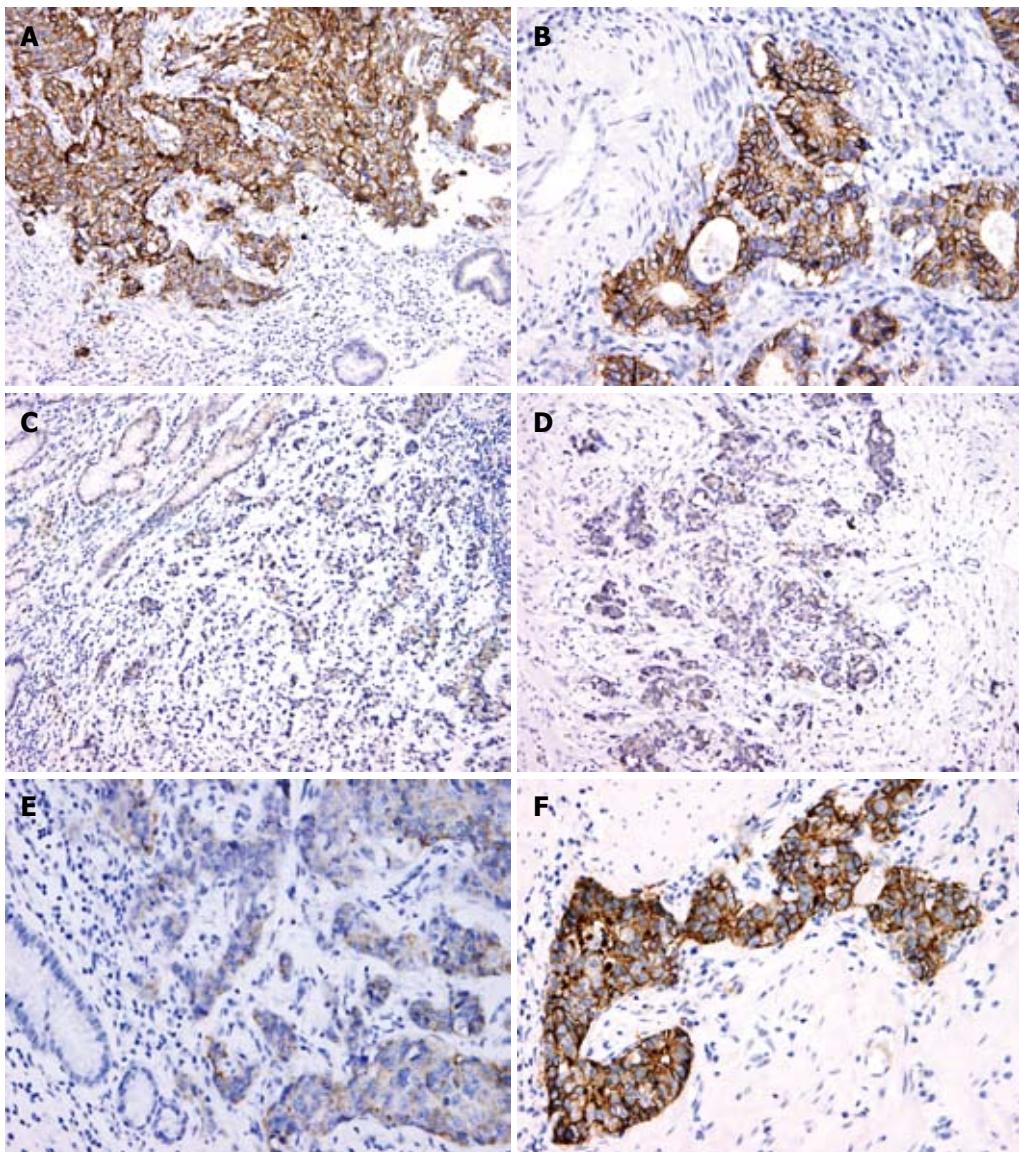
### **Relation between expression transformation of claudin-1 and biological behaviors of gastric carcinoma**

The expression of claudin-1 was transformed in mucosa and invasive front of gastric carcinoma patients, which was 26.2% (28/107) in mucosa and 49.1% (28/57) in invasive front (Table 2). The expression transformation rate of claudin-1 was 51.5% (17/33) in well-moderately differentiated carcinoma patients, 16.0% (9/57) in poorly differentiated carcinoma patients, and 11.8% (2/17) in mucinous carcinoma patients, respectively (Table 3).

The deeper was the invasiveness, the higher was the transformation rate of claudin-1 expression. The claudin-1 expression transformation was significantly higher in patients (26/28) with their tumors invaded visceral peritoneum than in those with their tumors only invaded muscularis propria ( $P < 0.05$ ), and in patients (20/61) with lymph node metastasis than in those (8/46) with no lymph node metastasis ( $P < 0.05$ ).

## DISCUSSION

TJs, adherent junctions and desmosomes form the apical



**Figure 1** Immunohistochemical staining ( $\times 200$ ) showing over-expression of claudin-1 in mucosa (A) and invasive front (B) of gastric carcinoma, low-expression in mucosa (C) and invasive front (D), and transformation of claudin-1 low-expression in mucosa (E) and over-expression in invasive front of gastric carcinoma (F).

junctional complex in epithelial cellular sheets. Adherent junctions and desmosomes are responsible for the mechanical adhesion between adjacent cells, while TJs play a mainly role in the tight sealing of cellular sheets, thus controlling the paracellular ion flux and maintaining tissue homeostasis<sup>[6]</sup>. By forming a fence that prevents lateral diffusion of membrane proteins and lipids, TJs also play a crucial role in the maintenance of cell polarity. TJs also participate in the regulation of cell proliferation and differentiation, or other cellular functions<sup>[7]</sup>.

TJs are consisted of three major integral membrane proteins: claudins, occludin and junctional adhesion molecules. The role of these proteins has not been completely elucidated. However, it is presumed that claudins form the principal chain of TJ strands. The claudin protein family is consisted of 24 members of closely correlated transmembrane proteins. A number of tissues express multiple claudin proteins and form TJ strands by interacting through homotypic and/or heterotypic fashion, though the expression pattern of claudins is tissue specific.

The histological grade of carcinomas is a significant

prognostic parameter and depends on the differentiated degree of glandular epithelium and cellular polarity. The invasiveness in high-grade and poorly-differentiated carcinoma is stronger than that in low-grade and well-differentiated carcinoma. One of the key determinants controlling cellular adhesion and polarity is the TJs<sup>[6]</sup>. Carcinoma cells frequently show deficiencies in structure and function of the TJs<sup>[8]</sup>. It was supposed that the TJs play a critical role in the progress of neoplasm through acting as a connector of extracellular environment affecting the intercellular signal pathway and cellular skeleton<sup>[9]</sup>. The changes of permeability in TJs may also permit the diffusion increase in nutrient substances and other factors for growth and survival of tumors. Otherwise, the loss of integration of TJs in the development of metastatic phenotype is also an important step.

The expression of claudin proteins may change in carcinoma cells. The expression of claudin-1 and claudin-4 in ovarian and prostatic carcinomas is increased<sup>[10-12]</sup>, claudin-4 is over-expressed in pancreatic carcinomas<sup>[13,14]</sup>, while claudin-1 expression is down-

**Table 1** Claudin-1 expression and clinicopathologic characteristics of gastric carcinoma *n* (%)

	<i>n</i>	Expression in mucosa			Expression in invasive front		
		Low	Over	<i>P</i>	Low	Over	<i>P</i>
<b>Sex</b>							
Male	106	80 (75.5)	26 (24.5)	0.144	58 (54.7)	48 (45.3)	0.198
Female	30	27 (90)	3 (10)		21 (70)	9 (30)	
<b>Age</b>							
≤ 50	20	17 (85)	3 (15)	0.491	13 (65)	7 (35)	0.665
> 51	116	90 (77.6)	26 (22.4)		66 (56.9)	50 (43.1)	
<b>Histological type</b>							
Well-moderately differentiated	46	33 (71.7)	13 (28.3)	0.046	16 (34.8)	30 (65.2)	0.000
Poorly differentiated	65	57 (87.7)	8 (12.3)		48 (73.8)	17 (26.2)	
Mucinous	25	17 (68)	8 (32)		15 (60)	10 (40)	
<b>Depth of invasion</b>							
Lamina propria or submucosa	18	17 (94.4)	1 (5.6)	0.099	16 (88.9)	2 (11.1)	0.007
Muscularis propria	28	19 (67.9)	9 (32.1)		18 (64.3)	10 (35.7)	
Visceral peritoneum	90	71 (78.9)	19 (21.1)		45 (50)	45 (50)	
<b>Lymph node metastasis</b>							
Negative	52	46 (88.5)	6 (11.5)	0.048	38 (82.6)	14 (17.4)	0.009
Positive	84	61 (72.6)	23 (27.4)		41 (48.8)	43 (51.2)	
<b>Ki-67 index</b>							
I	20	17 (85)	3 (15)	0.529	14 (70)	6 (30)	0.389
II	57	43 (75.4)	14 (24.6)		33 (57.9)	24 (42.1)	
III	54	42 (77.8)	12 (22.2)		28 (51.9)	26 (48.1)	
IV	5	5 (100)	0		4 (80)	1 (20)	

**Table 2** Expression transformation of claudin-1 in gastric carcinomas

Expression in mucosa	Expression in invasive front	<i>n</i>
Low-expression	Low-expression (expression invariably)	79
Low-expression	Over-expression (expression variably)	28
Over-expression	Low-expression (expression variably)	0
Over-expression	Over-expression (expression invariably)	29

regulated in breast and colon carcinomas<sup>[15-17]</sup>.

The three dimensional cultures of breast cancer cells showed that the reexpression of claudin-1 may increase apoptosis of cancer cells<sup>[18]</sup>. It was reported that the expression of claudin-1 in stage II colon carcinomas is related with a poor prognosis<sup>[17]</sup>. Another study showed that the expression of claudin-1 is up-regulated in colon carcinomas<sup>[19]</sup>, and the expression level of claudin-1 is negatively related with the histological grade of tumors<sup>[17]</sup>.

However, investigations on the role of claudin-1 expression in the progression of gastric carcinomas are relatively few and only two studies on the expression of claudin-1 in gastric carcinomas can be found in PubMed so far. Through tissue microarray, Resnick *et al*<sup>[20]</sup> found that claudin-1, -3, -4 and ZO-1 are expressed in non-tumor mucosa, tumor mucosa and invasive front of gastric carcinomas, the expression of caldudin-1 is higher in intestinal subtype than in diffuse subtype of adenocarcinomas. Soini *et al*<sup>[21]</sup> found that the expression of claudin-1 is significantly higher in intestinal-type gastric than in diffuse-type gastric carcinomas, indicating that claudin-1 expression is the determiner of diffuse phenotype of gastric carcinoma. Our results show that claudin-1 over-expression occurred in mucinous gastric adenocarcinomas, and was negatively related

**Table 3** Relation between the expression transformation of claudin-1 and biological behaviors of gastric carcinomas

	<i>n</i>	Low-expression in mucosa	Over-expression in invasive front
<b>Histological type</b>			
Well-moderately differentiated	17	33	30
Poorly differentiated	9	57	17
Mucinous	2	17	10
<b>Depth of invasion</b>			
Lamina propria or submucosa	1	17	2
Muscularis propria	1	19	10
Visceral peritoneum	26	71	45
<b>Lymph node metastasis</b>			
Negative	8	46	14
Positive	20	61	43

with the differentiation degree of adenocarcinomas, but positively related with the invasiveness and metastasis of adenocarcinomas in mucosa. However, the expression of claudin-1 in invasive front was different from that in mucosa of gastric carcinoma. The claudin-1 over-expression in invasive front was positively related with the differentiated degree and the invasiveness and metastasis of gastric adenocarcinomas.

The transformation of claudin-1 expression accompanied the progression of gastric carcinomas. The expression of claudin-1 in invasive front of gastric carcinomas was transformed. The claudin-1 expression transformation percentage of well-differentiated adenocarcinomas was the highest (51.5%, 17/33). The deeper the invasiveness of gastric carcinomas was, the higher the transformation rate was. The transformation was significantly higher in patients with tumors invaded visceral peritoneum than in those with tumors only

invaded muscularis propria and in patients with lymph node metastasis than in those with no lymph node metastasis. These results suggest that transformation of claudin-1 expression participates in the progression of gastric carcinomas.

The role of TJ proteins in the development of cancer is not clear. Carcinoma cells, especially those exhibiting a higher potentiality of metastasis, frequently show loss of functional TJs, such as ZO-1, -2 and occludin is decreased in tumor and its metastasis<sup>[22,23]</sup>. The exact action of claudin on cancers is not clear. It was reported that the expression of claudin-1 is decreased in invasive duct carcinoma of breast<sup>[24]</sup> while the expression of claudin-3 and -4 is increased in some other carcinomas<sup>[25,26]</sup>. The expression of claudin-1 may promote the activation of pro-MMP-9<sup>[26-28]</sup>, suggesting that the expression of claudin-1 may involve the invasiveness and metastasis of adenocarcinoma. Caludin-1 is regarded as a target site of  $\beta$ -catenin/Tcf signals, which supports that claudin-1 down-regulates the formation of colorectal carcinomas<sup>[29]</sup>. The expression of claudin-1 mRNA is decreased in breast carcinomas<sup>[15]</sup> while the expression of claudin-23 is down-regulated in intestinal subtype of gastric carcinomas<sup>[30]</sup>. It was reported that the claudin-1 expression is frequently up-regulated in tumor tissues and its expression level is equal to or higher than in consecutive normal colon mucous membrane, suggesting that the expression of claudin-1 is related with poorly-differentiated adenocarcinoma. The loss of claudin-1 expression is a strongly predictive parameter for tumor recurrence and survival of patients. It was reported that expression of claudin-1 and -4 is increased in ovarian carcinomas and prostatic carcinomas<sup>[26,31]</sup> and over-expression of claudin-4 in pancreatic carcinomas and precancerous lesion<sup>[32]</sup> are the causative action of claudin on cellular transformation and progression of invasiveness<sup>[33]</sup>.

Usually, a low expression level of claudin may result in functional damage to TJs. However, how the over-expression of claudin promotes tumor progression remains unclear<sup>[26,31,32]</sup>. One possible mechanism is that up-regulation or abnormal expression of some claudins may facilitate tumor formation by directly altering the function of TJ. Furuse *et al*<sup>[34]</sup> reported that up-regulation of claudin-2 expression in renal cells of Madin-Darby dogs decreases the function of TJ, and Tan *et al*<sup>[35]</sup> showed that the expression and distribution of claudin-1 are related with cell dissociation in pancreatic carcinoma by activating the mitogen-activated protein kinase-2. Up-regulation of claudins may also affect cell signaling pathways by binding domains to ZO-1<sup>[36]</sup>. ZO-1 interacts with several signaling proteins related with the neoplastic process, such as ras substrate AF-6<sup>[37]</sup>, G-protein and connexin 43<sup>[38]</sup>.

In conclusion, up-regulation and transformation of claudin expression in the invasive process of gastric carcinomas are involved in the biological behavior transformation of tumors. The exact role of claudin protein in the development of malignant tumors and their prognosis remains unclear and should be further studied.

## COMMENTS

### Background

Tight junction (TJ) protein participates in the processes of epithelial-mesenchymal transformation (EMT) of carcinomas. Claudin proteins are the major members of the TJ family. In this study, the relation between the expression transformation of claudin-1 and the invasiveness and metastasis of gastric carcinoma was evaluated.

### Research frontiers

The expression of claudin-1 was found to be significantly related with the biological behavior of gastric cancers, indicating that TJ plays a role in the development of neoplasms. The mechanism of TJ underlying the progress of cancer remains to be further studied.

### Innovations and breakthroughs

This study evaluated the relation between the expression of claudin-1 and the invasiveness and metastasis of gastric carcinoma.

### Applications

The importance of TJ in tumor development has not been extensively studied. The role of the claudin family in the invasiveness and metastasis of cancers is controversial. The relation between the expression transformation of TJ proteins and MET in cancer was clarified in the present study, which contributes to the exploitation of its mechanism underlying the development and progress of gastric carcinoma.

### Terminology

TJs, adherent junctions and desmosomes form the apical junctional complex in epithelial cellular sheets. The claudin protein family is consisted of 24 members of the transmembrane proteins, such claudins1-4.

### Peer review

This study investigated the relation between the expression transformation of claudin-1 and the invasiveness and metastasis of gastric carcinomas. The results suggest that claudin-1 participates in the transformation of the biological behaviors of gastric carcinomas. The study was well designed and the findings may be valuable for the further study on mechanism of claudin-1 underlying the development and progress of gastric carcinoma.

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RAPID COMMUNICATION

# Role of transforming growth factor-beta signaling pathway in pathogenesis of benign biliary stricture

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research; Zheng JB, Zhang XX contributed new analytic tools;  
Geng ZM, Tao J and Zheng JB analyzed data; and Geng ZM  
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expression ratio of Smad7 in cases with benign biliary stricture was 70.0%, higher than that in normal bile duct, but this difference is not statistically significant 70.0% vs 50%,  $P > 0.05$ ). There was a positive correlation between positive expression of TGF- $\beta_1$ , Smad4 and CTGF in cases with benign biliary stricture.  
**CONCLUSION:** The high expression of TGF- $\beta$ /Smad/CTGF signaling pathway plays an important role in the pathogenesis of benign biliary stricture.

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**Key words:** Biliary stricture; Transforming growth factor-beta 1; Smad; Connective tissue growth factor; T $\beta$ R

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## Abstract

**AIM:** To characterize the expression of members of the transforming growth factor-beta (TGF- $\beta$ )/Smad/connective tissue growth factor (CTGF) signaling pathway in the tissue of benign biliary stricture, and to investigate the effect of TGF- $\beta$  signaling pathway in the pathogenesis of benign biliary stricture.

**METHODS:** Paraffin embedded materials from 23 cases of benign biliary stricture were analyzed for members of the TGF- $\beta$ /Smad/CTGF signaling pathway. TGF- $\beta_1$ , T $\beta$ R I, T $\beta$ R II, Smad4, Smad7 and CTGF protein were detected by immunohistochemical strepto-avidin-biotin complex method, and CTGF mRNA was evaluated by hybridization *in situ*, while 6 cases of normal bile duct served as controls. The percentages of positive cells were counted. The correlation between TGF- $\beta_1$ , Smad4 and CTGF was analyzed.

**RESULTS:** The positive expression ratios of TGF- $\beta_1$ , T $\beta$ R I, T $\beta$ R II, Smad4, CTGF and CTGF mRNA in 23 cases with benign biliary stricture were 91.3%, 82.6%, 87.0%, 78.3%, 82.6% and 65.2%, respectively, significantly higher than that in 6 cases of normal bile duct respectively (vs 33.3%, 16.7%, 50.0%, 33.3%, 50.0%, 16.7%, respectively,  $P < 0.05$ ). The positive

## INTRODUCTION

Benign biliary strictures are caused by a heterogeneous group of benign conditions. They are usually iatrogenic, most frequently as a result of cholecystectomy<sup>[1]</sup>. The incidence of this complication has increased with the widespread use of laparoscopic cholecystectomy (LC)<sup>[2-4]</sup>. Other causes of benign biliary strictures include hepatolithiasis, recurrent cholangitis, infection with the fluke, Mirizzi syndrome, etc<sup>[5]</sup>. Anastomotic strictures are seen following bile duct reconstruction or orthotopic liver transplant (OLT)<sup>[6,7]</sup>.

The main manifestations of benign biliary stricture are scar contracture and stenosis of bile duct, especially at the hepatic hilum or above<sup>[8]</sup>. The diagnosis and treatment of benign biliary strictures remains a clinical challenge, requiring a multidisciplinary approach. The pathogenesis of benign biliary stricture is still unclear.

It is well known that the cytokine transforming growth factor beta 1 (TGF- $\beta_1$ ) has a key role either in

the wound healing process or induction of fibrosis. The biological effect of TGF- $\beta_1$  is regulated by a special signal transduction pathway<sup>[9,10]</sup>. Following activation of the TGF- $\beta$  receptor, intracellular signal transduction is mediated by a variety of Smad proteins<sup>[11-13]</sup>. Connective tissue growth factor (CTGF), which promotes cell proliferation and deposition of extracellular matrix (ECM), is a downstream medium in the process in which TGF- $\beta_1$  produces a marked effect on connective tissue cell. The aim of the present study was to determine the expressions of various cytokines in TGF- $\beta$ /Smad/CTGF signaling pathway in tissue of benign biliary stricture, and to further investigate the role of TGF- $\beta$  signaling pathway in the pathogenesis of benign biliary stricture.

## MATERIALS AND METHODS

### **Patients and pathologic specimens**

The study population included 23 patients (10 males and 13 females; mean age 50 years) who underwent benign biliary strictures (between June 2003 and November 2005, in Department of Hepatobiliary Surgery, First Affiliated Hospital, Medical College, Xi'an Jiaotong University, Xi'an). The average time interval between the first surgery and the second surgery was 16 mo, and the average admission time was 22 d. The causes of benign biliary stricture were bile duct injury or anastomotic stricture after biliary reconstruction ( $n = 17$ ), hepatolithiasis ( $n = 3$ ) and recurrent cholangitis ( $n = 3$ ). The specimens were obtained from the cicatrices of bile duct. Fibrosis was seen from HE staining. Six normal specimens of bile duct were obtained from donators in liver transplantation. The study protocol was approved by the Ethics Committee of the First Affiliated Hospital, Medical College, Xi'an Jiaotong University.

### **Antibodies**

Antibodies to TGF- $\beta_1$  (Rabbit anti-human monoclonal antibody, Santa-dilution 1:400), T $\beta$ R I (Rabbit anti-human monoclonal antibody, Santa-dilution 1:400), T $\beta$ R II (Rabbit anti-human monoclonal antibody, Santa-dilution 1:300), Smad4 (Rabbit anti-human monoclonal antibody, Santa-dilution 1:400), Smad7 (Rabbit anti-human monoclonal antibody, Santa-dilution 1:400) and CTGF (Rabbit anti-human monoclonal antibody, Santa-dilution 1:300) were purchased from Wuhan Boster Biological Technology Co. Ltd.

### **Immunohistochemical analysis**

The expression of TGF- $\beta_1$ , T $\beta$ R I, T $\beta$ R II, Smad4, Smad7 and CTGF was detected by SABC immunohistochemical method. The test kit of SABC was the product of Wuhan Boster Biological Technology Co. Ltd. In the control group, the primary antibody was replaced with PBS or normal rabbit serum. All paraffin embedded sections were deparaffinized and rehydrated, and pretreated for 20 min at 75°C in a microwave oven. After being treated with 1 mL/L H<sub>2</sub>O<sub>2</sub> for 30 min to block the

endogenous peroxidase, the sections were incubated with 20 mL/L fetal calf serum for 30 min to reduce nonspecific binding. Then the primary TGF- $\beta_1$ , T $\beta$ R I, T $\beta$ R II, Smad4, Smad7 and CTGF antibodies were applied to the sections and incubated at 4°C overnight. The sections were subsequently incubated with goat anti rabbit IgG at 37°C for 30 min, followed by incubation with SABC at 37°C for 30 min, and stained with DAB-H<sub>2</sub>O<sub>2</sub> for 5-10 min and counterstained with hematoxylin.

### **In situ hybridization for CTGF mRNA**

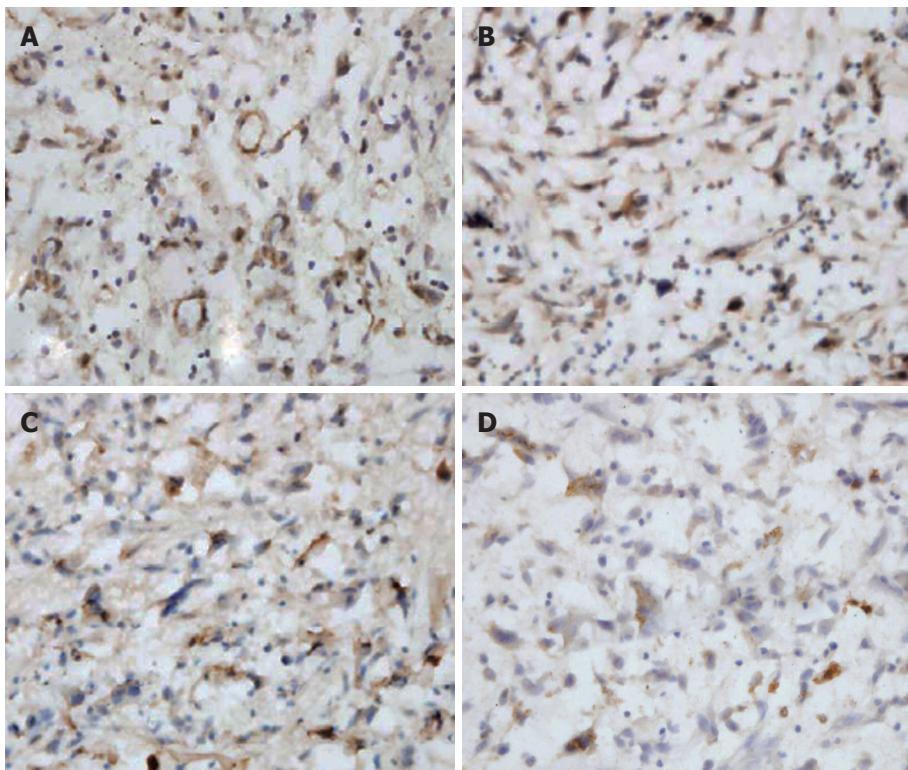
CTGF mRNA ISH detection kit and antisense oligonucleotide probe (digoxin-labeled) were purchased from Wuhan Boster Biological Technology Co. Ltd. A 30-mer sequence that is complementary to the region of CTGF mRNA was synthesized as follows: (1) 5'-CTG CTGCCCGTCTGCG CCAAGCAGCTGGG-3'; (2) 5'-CAACTGCCT GGTCAGACCACAGAGTGGAG-3'; (3) 5'-TGTACTACAGGAAGATGTACGGAGACATGG-3'. In brief, deparaffinized sections were incubated with 3% hydrogen peroxide for 30 min and then with 1 g/mL pepsin for 15 min. The prehybridization was performed at 37°C for 2 h, and the hybridization was conducted in a 42°C water bath for 18 h with each section covered with a soil coverslip. After thorough washing, tissue sections were preblocked for 20 min with blocking solution. Then, mouse anti-digoxin antibody was added for 60 min at 37°C. After washed in PBS, the sections were visualized according to the manufacturer's instructions. A negative control was prepared by using a hybridization solution without the probe.

### **Assessment of staining reactions**

A positive reaction was detected as plasmatic stain presenting in yellow or brown-yellow color. A modified Shimizu's method<sup>[14]</sup> was used to assess staining reactions. We selected 10 visual fields under HP microscope and counted 100 cells randomly. The following scoring system was used: (1) score 0, positively stained cytoplasm in less than 5% of cells; score 1, 5% - 35% positive; score 2, 36%-65% positive; and score 3, > 66% positive; (2) The staining intensity was estimated using a 4-grade scoring system (0, 1, 2, 3): very weak (1+ staining in some cells) (Score 0); weak (1+ staining in cells) (Score 1); moderate (2+ staining in cells) (Score 2); strong (3+ staining in cells) (Score 3). The examiners were blinded to patients' clinical and histological (HE staining) profile. Two investigators evaluated the staining levels independently, after which any discordant evaluations were adjusted by connected microscopes and scored jointly. The 1st and 2nd score were added together and divided 2, then the mean was made the final score; 0, 1, 1.5-2, 2.5-3 were recorded as (-), (+), (++) and (+++) respectively.

### **Statistical analysis**

The percentages of positive cells according to the final score of TGF- $\beta_1$ , T $\beta$ R I, T $\beta$ R II, Smad4,



**Figure 1** **A:** TGF- $\beta_1$  staining at stenotic bile duct (SABC,  $\times 400$ ); **B:** Smad4 staining at stenotic bile duct (SABC,  $\times 400$ ); **C:** CTGF staining at stenotic bile duct (SABC,  $\times 400$ ); **D:** CTGF mRNA staining at stenotic bile duct (INS,  $\times 400$ ).

**Table 1 Positive expression of various cytokines in TGF- $\beta_1$ /Smad/CTGF signaling pathway in benign biliary stricture group and normal control group**

Group	TGF- $\beta_1$	T $\beta$ R I	T $\beta$ R II	Smad4	Smad7	CTGF	CTGF mRNA
Benign biliary stricture (%)	91.3 <sup>a</sup>	82.6 <sup>a</sup>	87.0 <sup>a</sup>	78.3 <sup>a</sup>	70.0	82.6 <sup>a</sup>	65.2 <sup>a</sup>
Normal control (%)	33.3	16.7	50.0	33.3	50.0	50.0	16.7

<sup>a</sup> $P < 0.05$  vs control.

Smad7, CTGF and *CTGF* mRNA were calculated. Fisher's exact probability test was used to analyze the relationship between stricture group and normal control group. Spearman linear correlation analysis was used to analyze correlativity between TGF- $\beta_1$ , Smad4 and CTGF, respectively.  $P < 0.05$  was considered statistically significant in difference. Statistical analyses were performed with SPSS version 11.0 (SPSS Inc., Chicago, IL, USA).

## RESULTS

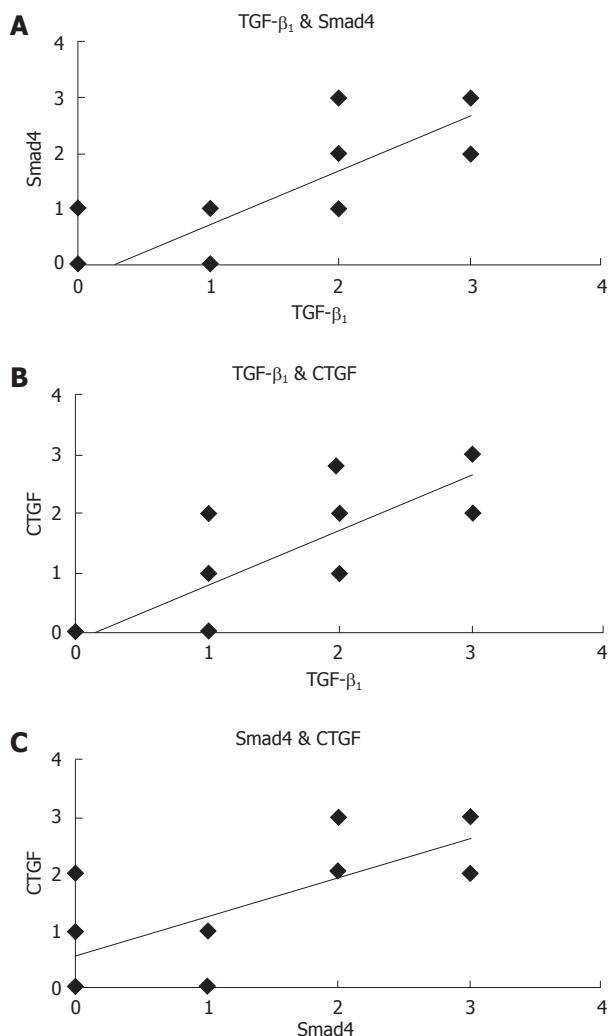
### Expression of TGF- $\beta_1$ , T $\beta$ R I, T $\beta$ R II, Smad4, Smad7, CTGF and CTGF mRNA in benign biliary stricture group and normal control group

TGF- $\beta_1$ /T $\beta$ R was expressed focally or diffusely in granulation tissue and cytoplasm of fibroblasts, macrophages, vascular endothelial cells, and inflammatory cells (Figure 1A). The expression of TGF- $\beta_1$  was weak in fibrous tissue of normal bile duct wall. The positive percentages of TGF- $\beta_1$ , T $\beta$ R I and T $\beta$ R II at benign biliary stricture group were 91.3% (21/23), 82.6% (19/23) and 87.0% (20/23), respectively. Smad4/Smad7 was mainly expressed in cytoplasm and nucleus of fibroblasts, and some was expressed

in fibrocytes (Figure 1B). The positive percentages of Smad4 and Smad7 at benign biliary stricture group were 78.3% (18/23) and 70.0% (16/23), respectively. Positive cells of CTGF were stained as yellow or brownish yellow granules in cytoplasm at immunohistochemical staining and *in situ* hybridization staining (Figure 1C and D). CTGF was expressed mainly in cytoplasm of fibroblasts in benign biliary stricture group, while it was scarcely expressed in fibrocytes in the wall of common bile duct of normal control group. The positive percentages of CTGF and *CTGF* mRNA of benign biliary stricture group were 82.6% (19/23) and 65.2% (15/23), respectively. The percentages of positive expression of various cytokines in TGF- $\beta_1$ /Smad/CTGF signaling pathway in benign biliary stricture group and normal control group are shown in Table 1.

### Linear correlation of TGF- $\beta$ /Smad/CTGF expression

By Spearman linear correlation analysis, expression of TGF- $\beta_1$  has positive correlation with expression of Smad4 ( $r = 0.848$ ,  $P = 0.000$ ) and CTGF ( $r = 0.848$ ,  $P = 0.000$ ); furthermore, Smad4 also has positive correlation with CTGF ( $r = 0.764$ ,  $P = 0.000$ ). Linear correlations of TGF- $\beta$ /Smad/CTGF expression are shown in Figure 2.



**Figure 2** A: Correlation between TGF- $\beta_1$  and Smad4; B: Correlation between TGF- $\beta_1$  and CTGF; C: Correlation between Smad4 and CTGF.

## DISCUSSION

In our previous study, we found that epithelial cells of the bile duct recovered poorly, chronic inflammation existed continuously, fibroblasts proliferated actively, collagens were over-deposited in submucosa, and reconstruction was poor after injury of bile duct. All these result in proliferation of cicatrix and high morbidity of stenosis of anastomosis<sup>[8]</sup>. Many studies have shown that TGF- $\beta_1$  is the most important cytokine in the pathogenesis of over proliferation of cicatrix<sup>[15-17]</sup>. High expression of TGF- $\beta_1$  and its receptors was found in keloid, and the expression of TGF- $\beta_1$  was significantly high in fibroblasts of hypertrophic scar tissues that were cultivated *in vitro*<sup>[18]</sup>. In the present study, we observed that the expression of TGF- $\beta_1$  in stenotic bile duct is significantly higher than that in normal bile duct, further confirming the outcome of our previous animal experiment<sup>[19]</sup>.

Biological effects of TGF- $\beta$  are regulated by specific signal transduction pathway. Smad family proteins have been identified as signal transducers for the TGF- $\beta$  superfamily<sup>[20-22]</sup>. Following activation of the TGF- $\beta$  receptor, intracellular signal transduction is mediated

by a variety of Smad proteins. TGF- $\beta_1$ /Smad signal transduction pathway can regulate itself by positive and negative feedback regulation loops<sup>[23,24]</sup>. Smad4/Smad7 are the important factors in the two circuit loops, respectively. In the T $\beta$ R I, T $\beta$ R II and Smad signaling pathway, TGF- $\beta_1$  can transfer and amplify signal, initiating its diverse cellular responses by binding to and activating specific cell surface receptors that have intrinsic serine/threonine kinase activity. These activated TGF- $\beta$  receptors stimulate the phosphorylation of receptor-regulated Smad proteins (PSmad), which in turn form complexes with Smad4 that accumulate in the nucleus, activate the promoter of TGF- $\beta_1$  and induce the expression of endogenous TGF- $\beta_1$  by itself; this forms the positive feedback regulation loop. Meanwhile, PSmad proteins also can activate the promoter of Smad7 that inhibits TGF- $\beta$ -induced transcriptional responses<sup>[25]</sup>. Psmad2 and Psmad3 may also affect its own promoter region, inhibiting transcription itself, and down-regulate the expression of R-Smad proteins, inhibiting signal transduction<sup>[26]</sup>. Accordingly, TGF- $\beta_1$  inhibits its signal transduction by negative feedback regulation loop made by activating Smad7 expression and down-regulating R-Smad expression.

This study indicates that T $\beta$ R I, T $\beta$ R II, Smad4 in tissue of stenotic bile duct also have high expression besides TGF- $\beta_1$ ; furthermore high expression of TGF- $\beta_1$  has positive correlation with Smad4 ( $r = 0.848$ ,  $P = 0.000$ ). We believe that this may be because high-expression of TGF- $\beta$  can promote the expression of itself and its receptor by a positive feedback regulation loop. This over-expression then could amplify bioactivity and constantly activate smads complexes, which accumulate in the nucleus and regulate the transcription of target genes, resulting in active proliferation of fibroblasts and excessive collagen deposition. So, we can presume that high-expression of R-Smad and Co-smad maybe an important mechanism in pathogenesis of benign biliary stricture. Lasting stimulus of TGF- $\beta_1$  that was constantly secreted in tissue of stenotic bile duct and high-sensitivity of the receptor to TGF- $\beta_1$  possibly result in the above consequences.

This study also has observed that the expression of Smad7 is higher in tissue of stenotic bile duct than that in normal bile duct, but there was no statistical significance between the two groups. We think that because of the tissue difference, Smad7, which is up-regulated in benign biliary cicatrix does not predominate in the competition with R-smad that was also activated and up-regulated, so Smad7 can't play a role in inhibitive function. R-smad, which predominates in the competition, is phosphorylated by T $\beta$ R I and further binds to Smad4; the complex transfers into nucleus and regulates the expression of target genes.

CTGF is considered a modulator and assisted mediated factor that mediates biologic function of other molecules, and promotes fission of fibroblasts and accumulation of collagen<sup>[27]</sup>. CTGF can promote the synthesis of ECM such as collagen I, collagen III and FN. Lasky *et al* observed that CTGF is over-expressed in

pathogenesis of fibrosis in skin, kidney, liver and heart<sup>[28]</sup>. Igarashi *et al*<sup>[29]</sup> thought that the expression of CTGF gene is directly regulated by TGF- $\beta_1$ . Grotendorst *et al*<sup>[30]</sup> also believed that CTGF, which promoted cell proliferation and deposition of ECM, was a downstream medium in the process in which TGF- $\beta_1$  produced a marked effect on connective tissue cell.

The results have shown that expression of CTGF in stenotic bile duct tissue is significantly higher than in normal bile duct specimens. This difference indicated that CTGF plays an important role in the pathogenesis of over proliferation of cicatrix and benign biliary stricture. The expression of TGF- $\beta_1$  has a positive correlation with that of CTGF ( $r = 0.848, P = 0.000$ ); this confirms that both of them play roles of superior and inferior grade in signal transduction pathway in the pathogenesis of benign biliary stricture, similar to other fibrotic disease, whereas the positive correlation between expression of Smad4 and CTGF ( $r = 0.764, P = 0.000$ ) indicates that the relationship between TGF- $\beta_1$  and CTGF is connected by Smads signaling pathway.

Taken together, the present study indicates that the mechanism of effect in TGF- $\beta_1$ /Smad/CTGF signaling pathway is as follows. The continuity of bile duct wall is destroyed after bile duct injury, which results in changes of adjacent intra-cellular metabolism. Then, TGF- $\beta$  is released and binded to the type II receptor at first; thus a binary complex is formed. Type I receptor then is recruited and phosphorylated in its GS domain by T $\beta$ R II, leading to activation of its kinase activity and subsequent formation of a signaling complex. R-Smads are phosphorylated by this signaling complex, and in turn can form heteromeric complexes with Smad4. These activated Smad complexes accumulate in the nucleus, where they directly or indirectly bind to specific promoter regions on target genes together with transcription factor (TF) and/or co-activators/repressors, and downstream mediator CTGF is activated. The activation of TGF- $\beta$ /Smad/CTGF signal transduction pathway can result in fibrosis in the interaction between cell and ECM, cause disorder of metabolism and regulation between inflammatory cell, repairing cell and collagen. Fibrocytes in submucosa are transformed into activated fibroblasts, which proliferate abundantly, and synthesize and secrete collagen fibers. Under the stimulus of bile and secondary infection, inflammation extends and the wound healing process disorders, chronic inflammation exists continuously, fibroblasts proliferate constantly, collagens are over-deposited in submucosa. All these result in a prolonged healing process of bile duct, proliferation of cicatrix and morbidity of benign biliary stricture.

## COMMENTS

### Background

Transforming growth factor beta 1 (TGF- $\beta_1$ ) has a key role either in the wound healing process or induction of fibrosis. The biological effects of TGF- $\beta_1$  are regulated by TGF- $\beta$ /Smad/connective tissue growth factor (CTGF) signaling pathway. The main manifestations of benign biliary stricture are scar contracture

and stenosis of bile duct. The exact role of TGF- $\beta$  signaling pathway in the pathogenesis of benign biliary stricture is still unknown.

### Research frontiers

In the last few years, many studies have shown that TGF- $\beta_1$  is the most important cytokine in the pathogenesis of over proliferation of cicatrix. Biological effects of TGF- $\beta$  are regulated by specific signal transduction pathway. Smad family proteins have been identified as signal transducers for the TGF- $\beta$  superfamily. The expression of CTGF gene is regulated directly by TGF- $\beta_1$ , CTGF, which promotes cell proliferation and deposition of extracellular matrix (ECM), is a downstream medium in the process in which TGF- $\beta_1$  produces a marked effect on connective tissue cell.

### Innovations and breakthroughs

The high expression of TGF- $\beta$ /Smad/CTGF signaling pathway plays an important role in the pathogenesis of benign biliary stricture. These results demonstrate a new view of TGF- $\beta$  signaling pathway involved in the benign biliary stricture.

### Applications

This study indicates that the mechanism of TGF- $\beta$ /Smad/CTGF signaling pathway in pathogenesis of benign biliary stricture. It may provide new targets for further understanding of the pathogenesis of benign biliary stricture and new therapeutic targets.

### Terminology

Smads are the only substrates for type I receptor kinases known to have a signalling function; they were first identified as the products of the Drosophila Mad and C. elegans Sma genes, which lie downstream of the BMP-analogous ligand-receptor systems in these organisms. Smad proteins transduce signals from TGF- $\beta$  superfamily ligands that regulate cell proliferation, differentiation and death through activation of receptor serine/threonine kinases.

### Peer review

The study is well designed and contains important information and novelties concerning TGF- $\beta_1$  signaling pathway in benign biliary stricture. The authors performed a great amount of experiments and a comprehensive statistical analysis of data.

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RAPID COMMUNICATION

# Inhibitory effects of genistein and resveratrol on guinea pig gallbladder contractility *in vitro*

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**Author contributions:** Wang LD, Li HF designed the research; Qiu XQ, Tian ZF and Zhang YF performed the research; Wang LD and Li HF analyzed the data, wrote the paper and edited the manuscript.

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solution containing 0.01 mmol/L egtazic acid (EGTA), genistein and resveratrol inhibited the first phasic contraction induced by acetylcholine (ACh), but did not affect the second contraction induced by CaCl<sub>2</sub>. In addition, genistein, resveratrol and 17β-estradiol also could reduce the contractile responses of ACh and KCl, and shift their cumulative concentration-response curves rightward.

**CONCLUSION:** Phytoestrogen genistein and resveratrol can directly inhibit the contractile activity of isolated gallbladder muscle both at rest and in response to stimulation. The mechanisms responsible for the inhibitory effects probably due mainly to inhibition of tyrosine kinase, Ca<sup>2+</sup> influx through potential-dependent calcium channels (PDCs) and Ca<sup>2+</sup> release from sarcoplasmic reticulum (SR), but were not related to the estrogen receptors.

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**Key words:** Phytoestrogen; Estradiol; Gallbladder; Smooth muscle; Ca<sup>2+</sup> channel

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## Abstract

**AIM:** To observe and compare the effects of phytoestrogen genistein, resveratrol and 17β-estradiol on the tonic contraction and the phasic contraction of isolated gallbladder muscle strips and to study the underlying mechanisms.

**METHODS:** Isolated strips of gallbladder muscle from guinea pigs were suspended in organ baths containing Kreb's solution, and the contractilities of strips were measured before and after incubation with genistein, resveratrol and 17β-estradiol respectively.

**RESULTS:** Similar to 17β-estradiol, genistein and resveratrol could dose-dependently inhibit the phasic contractile activities, they decreased the mean contractile amplitude and the contractile frequencies of gallbladder muscle strips, and also produced a marked reduction in resting tone. The blocker of estrogen receptor ICI 182780 failed to alter the inhibitory effects induced by genistein and resveratrol, but potassium bisperoxo (1, 10 phenanthroline) oxovanadate bpV (phen), a potent protein tyrosine phosphatase inhibitor, markedly attenuated the inhibitory effects induced by genistein and resveratrol. In calcium-free Kreb's

## INTRODUCTION

Female sex hormones are known to affect cholesterol metabolism, gallbladder and sphincter of Oddi motility<sup>[1-4]</sup>. Phytoestrogens represent a wide group of compounds which are naturally found in many plants, and they are defined as plant substances that are structurally or functionally similar to estradiol<sup>[5,6]</sup>. These natural products possess a wide spectrum of physiological and pharmacological effects such as estrogenic effects<sup>[7,8]</sup>, anti-atherosclerosis<sup>[9]</sup>, anti-osteoporosis<sup>[10]</sup>, relieving menopausal symptoms<sup>[11]</sup> and the inhibitory effect of tyrosine kinases<sup>[12,13]</sup>. Recently, many papers indicate that phytoestrogen genistein and

resveratrol can inhibit vasocontractile responses and relax vascular smooth muscles by a  $\text{Ca}^{2+}$  antagonistic property which is very similar to estradiol<sup>[14,15]</sup>, and data from electrophysiological studies suggest genistein reversibly inhibits L-type calcium current in isolated guinea-pig ventricular myocytes in a concentration-dependent manner<sup>[16]</sup>. Therefore, the biological actions of genistein and resveratrol are very useful for medicine and nutrition, and they are proposed to have potential as natural substitutes of estrogen therapy. Despite the increasing interest in the effects of phytoestrogen in the cardiovascular system, little is known about the effect of genistein and resveratrol on gallbladder smooth muscle. The present study was designed to observe and compare the effects of phytoestrogen genistein, resveratrol and 17 $\beta$ -estradiol on the tonic contraction and the phasic contraction of isolated gallbladder muscle strips both at rest and in response to acetylcholine (ACh) and KCl and to study its underlying mechanisms.

## MATERIALS AND METHODS

### **Animal and tissue preparation**

The present work was conducted in conformity with the procedures described in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health, and the procedures performed were in accordance with institutional guidelines. Adult male or non-pregnant female guinea pigs (weighing 387.8  $\pm$  7.5 g, provided by the experimental animal center of Lanzhou University) were utilized in this study. Preliminary studies indicated that no differences existed between the sexes with respect to either the contractile responsiveness to the agonists or to the sensitivities to genistein, resveratrol and 17 $\beta$ -estradiol. All animals were fasted overnight prior to being sacrificed by overdose injection of pentobarbitone and the whole gallbladders were quickly removed and placed in Kreb's solution containing the following compositions (mmol/L): NaCl 120, KCl 5.9,  $\text{NaH}_2\text{PO}_4$  1.2, MgCl<sub>2</sub> 1.2,  $\text{NaHCO}_3$  15.4, CaCl<sub>2</sub> 2.5, and glucose 11.5, buffered at pH 7.4. After removal of the mucosa by blunt dissection, muscle strips (5 mm  $\times$  10 mm) were prepared from the body of the gallbladder by cutting parallel to the long axis of the tissue, and then were mounted horizontally in separate 5-mL tissue chambers containing 37  $\pm$  0.5°C Kreb's solution, bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The muscle preparations were allowed to equilibrate for 2-3 h with a resting tension of 1.0 g and the solution was changed every 20 min. The isometric contractions were measured with force transducers and recorded with the BL-420E<sup>+</sup> experimental system of biological function (TME, China) by microcomputer.

### **Experimental protocols**

In order to observe the effects of genistein, resveratrol and 17 $\beta$ -estradiol on the basal contractile activities of gallbladder, the different concentration (1, 10, 20 or 40  $\mu\text{mol}/\text{L}$ ) of genistein, resveratrol and 17 $\beta$ -estradiol or the same dose of solvent (control) was added

respectively to the tissue chamber for 10 min. A specific antagonist of estrogen receptor ICI 182780 (10  $\mu\text{mol}/\text{L}$ ) or a potent protein tyrosine phosphatase inhibitor bpV (phen) (1  $\mu\text{mol}/\text{L}$ ) was added 10 min before administration of 10  $\mu\text{mol}/\text{L}$  genistein or resveratrol to investigate whether their actions were relevant with the estrogen receptors and tyrosine kinase inhibition in gallbladder smooth muscle.

To evaluate the possible effect of genistein, resveratrol and 17 $\beta$ -estradiol on ACh-induced calcium release and calcium influx through receptor-operated calcium channels (ROCs), gallbladder muscle strips were incubated in calcium-free Kreb's solution containing 0.01 mmol/L egtazic acid (EGTA) for 30 min, and then treated with ACh (10  $\mu\text{mol}/\text{L}$ ). When the contractile response had reached a plateau, CaCl<sub>2</sub> (10 mmol/L) was added into the organ chamber and a further contraction was obtained. Tissues were washed with  $\text{Ca}^{2+}$ -free Kreb's solution and left to return to baseline tone. The strips were then treated by ACh and CaCl<sub>2</sub> again after being incubated with genistein (20  $\mu\text{mol}/\text{L}$ ) and resveratrol (20  $\mu\text{mol}/\text{L}$ ) or the same dose of solvent for 6-8 min.

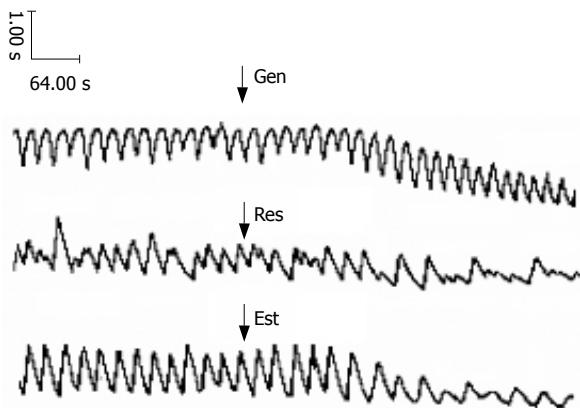
In some experiments, to determine the effect of genistein, resveratrol and 17 $\beta$ -estradiol on contractile response to ACh and potassium, the control contractile response curves to ACh ( $10^{-8}$ - $10^{-3}$  mol/L) and KCl (10-100 mmol/L) were obtained respectively, then the strips were washed repeatedly with Kreb's solution until the strips returned to the basal tension. The strips were then incubated with genistein (40  $\mu\text{mol}/\text{L}$ ), resveratrol (40  $\mu\text{mol}/\text{L}$ ) or 17 $\beta$ -estradiol (40  $\mu\text{mol}/\text{L}$ ) for 10 min respectively, and ACh or KCl concentration-dependent contraction curve was obtained again.

### **Drugs**

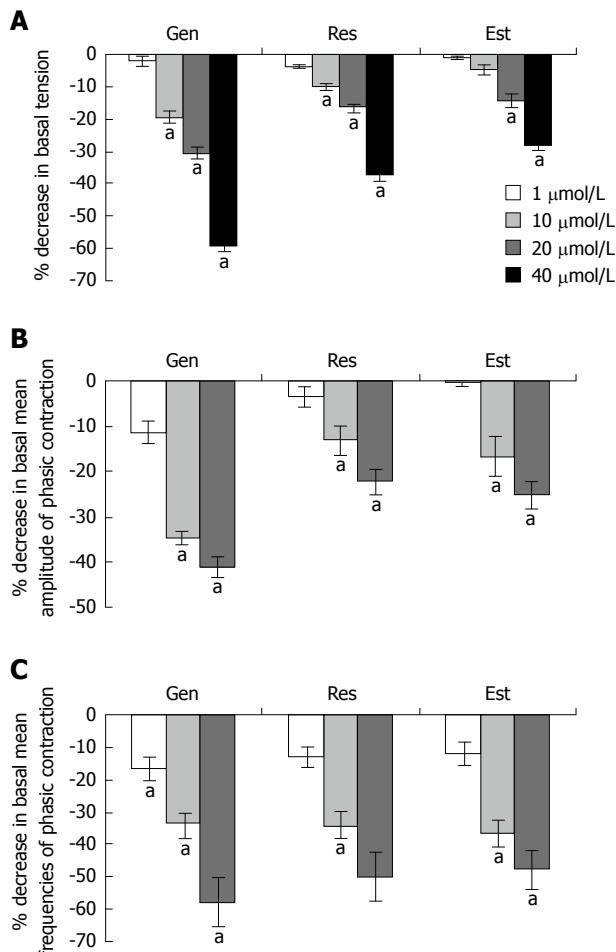
Genistein, resveratrol, 17 $\beta$ -estradiol (Sigma, Chemical Co, USA); ACh (the Second Pharmaceutical Factory of Beijing, China); ICI 182780 (Tocris Cookson Inc., Bristol, UK); potassium bisperoxo (1,10 phenanthroline)oxovanadate bpV (phen) (Alexis Biochemicals, San Diego, CA). ICI 182780, genistein, resveratrol and 17 $\beta$ -estradiol were dissolved with dimethyl sulphoxide (DMSO). The final concentration of DMSO in the bath in each case was always no more than 0.1% and had no effect on basal contraction.

### **Statistical analysis**

All results are expressed as mean  $\pm$  SE. "n" refers to the number of guinea pigs used in the study. Data were expressed as % decrease in the basal tension, mean amplitude and mean frequencies of phasic contraction. In experiments involving concentration-response curves, the results were expressed as percentage of control maximal contractile responses induced by  $10^{-3}$  mol/L ACh or 100 mmol/L KCl respectively. The EC<sub>50</sub> value of each strip was determined by the Scott Method, and was expressed as negative log molar (pD<sub>2</sub>) value. Statistical analysis was performed using the Student *t*-test and analysis of variance (ANOVA). Each group was compared with the solvent control. *P* < 0.05 was considered significant.



**Figure 1** Sample traces showing the basal contractile activity of the gallbladder before and after the administration of 20  $\mu\text{mol/L}$  genistein (Gen), resveratrol (Res) and 17 $\beta$ -estradiol (Est).

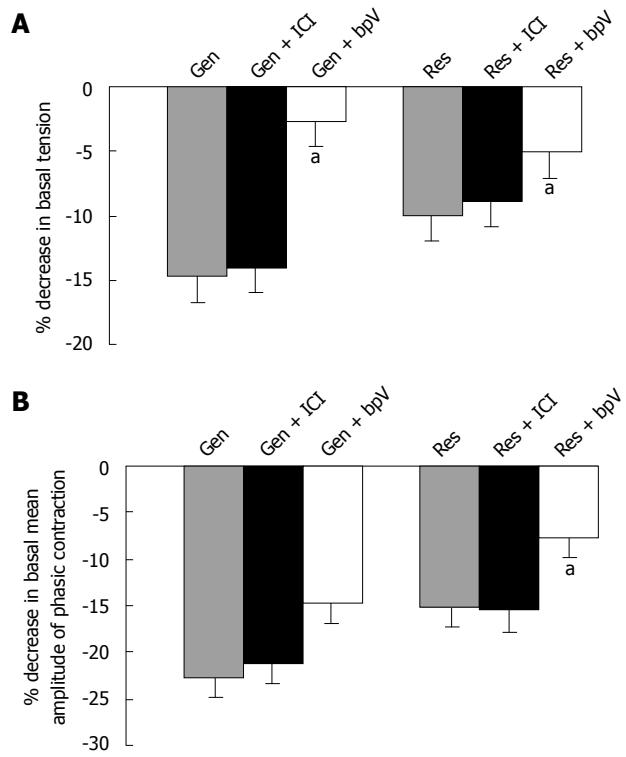


**Figure 2** Effects of genistein (Gen), resveratrol (Res) and 17 $\beta$ -estradiol (Est) on resting tension (A), mean contractile amplitude (B) and (C) mean frequencies of phasic contraction in isolated guinea pig gallbladder muscle strips ( $n = 10$ ).  $^aP < 0.05$  vs solvent control.

## RESULTS

### Effects of genistein, resveratrol and 17 $\beta$ -estradiol on basal activities of gallbladder muscle strips

The spontaneous contractile activities of isolated gallbladder smooth muscle were not very regular, and some strips had obvious spontaneous phasic



**Figure 3** Effects of genistein (Gen, 10  $\mu\text{mol/L}$ ) and resveratrol (Res, 10  $\mu\text{mol/L}$ ) on the basal tension (A) and mean amplitude (B) of phasic contraction in isolated guinea pig gallbladder muscle strips after preincubation with ICI 182780 (ICI) or bpV (phen) (bpV) ( $n = 5$ ).  $^aP < 0.05$  vs corresponding Gen or Res group.

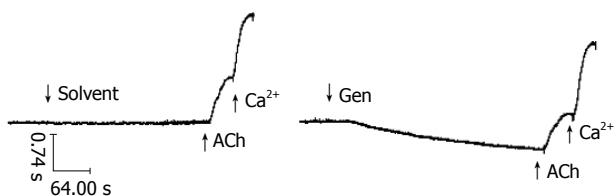
contractions with mean amplitude of  $0.49 \pm 0.06$  g and mean frequencies of  $2.80 \pm 0.25$  waves/min (Figure 1) while the others only possessed tonic contraction. In the strips with spontaneous phasic contractions, genistein, resveratrol and 17 $\beta$ -estradiol (1, 10, 20 or 40  $\mu\text{mol/L}$ ) could dose-dependently inhibit the phasic contractile activities, they decreased the mean contractile amplitude and the contractile frequencies and also produced a marked reduction in resting tone (Figures 1 and 2). Increasing the concentrations of the above three estrogens to 40  $\mu\text{mol/L}$ , the phasic contractile activities disappeared completely, the decreased percentages of the mean contractile amplitude and the contractile frequencies all reached 100%.

### Effects of genistein and resveratrol on basal activities of gallbladder in the presence of ICI 182780 and bpV (phen)

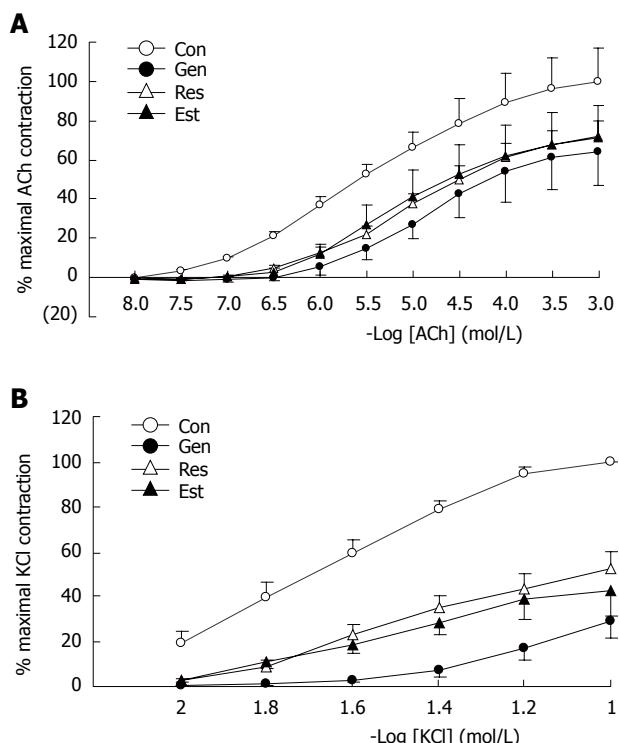
The inhibitory effects induced by genistein and resveratrol in gallbladder muscle strips had no obvious change in the presence of the specific estrogen receptor inhibitor ICI 182780 (10  $\mu\text{mol/L}$ ) (Figure 3), but after incubating the strips with the potent protein tyrosine phosphatase inhibitor bpV (phen) (1  $\mu\text{mol/L}$ ), the inhibitory effects induced by genistein and resveratrol markedly attenuated (Figure 3). ICI 182780 (10  $\mu\text{mol/L}$ ) and bpV (phen) (1  $\mu\text{mol/L}$ ) alone had no obvious effect on basal activity.

### Effects of genistein and resveratrol on biphasic contraction induced by ACh and CaCl<sub>2</sub>

In calcium-free (0.01 mmol/L EGTA) Kreb's solution,



**Figure 4** Traces of ACh and  $\text{CaCl}_2$ -induced contraction of gallbladder muscle strip in  $\text{Ca}^{2+}$ -free Kreb's solution in the absence and presence of genistein (Gen, 20  $\mu\text{mol/L}$ ).



**Figure 5** **A:** Line plots showing effects of genistein (Gen, 40  $\mu\text{mol/L}$ ), resveratrol (Res, 40  $\mu\text{mol/L}$ ) and 17 $\beta$ -estradiol (Est, 40  $\mu\text{mol/L}$ ) on ACh concentration-dependent contraction curves in isolated guinea pig gallbladder muscle strips ( $n = 6-7$ ). **B:** Effects of genistein (Gen, 40  $\mu\text{mol/L}$ ), resveratrol (Res, 40  $\mu\text{mol/L}$ ) and 17 $\beta$ -estradiol (Est, 40  $\mu\text{mol/L}$ ) on KCl concentration-dependent contraction curves in isolated guinea pig gallbladder muscle strips ( $n = 12$ ).

no spontaneous phasic contractions were observed, but ACh (10  $\mu\text{mol/L}$ ) could cause a transient contraction with the tensile increase of  $0.89 \pm 0.10$  g. As soon as such contraction reached a plateau,  $\text{CaCl}_2$  10 mmol/L was rapidly added into the bath and another higher contractile response occurred with the tensile increase of  $1.10 \pm 0.18$  g ( $n = 4$ ). Genistein (20  $\mu\text{mol/L}$ ; Figure 4) and resveratrol (20  $\mu\text{mol/L}$ ) reduced the first contraction induced by ACh from  $0.89 \pm 0.10$  g to  $0.50 \pm 0.18$  g and  $0.64 \pm 0.15$  g respectively (all  $P < 0.05$ ,  $n = 4$ ), but did not change the second contraction caused by  $\text{CaCl}_2$  ( $1.23 \pm 0.25$  in genistein groups and  $1.18 \pm 0.15$  in resveratrol groups  $\text{vs}$   $1.10 \pm 0.18$  g in control groups respectively, all  $P > 0.05$ ,  $n = 4$ ) in  $\text{Ca}^{2+}$ -free Kreb's solution.

#### Effects of genistein, resveratrol and 17 $\beta$ -estradiol on agonist-induced contractions

ACh ( $10^{-8}$ - $10^{-3}$  mol/L) and KCl (10-100 mmol/L)

elicited concentration-dependent contractile responses in isolated gallbladder muscle strips. However, genistein, resveratrol and 17 $\beta$ -estradiol significantly reduced the responses to ACh and KCl, and made their concentration-dependent contraction curves shift to the right (Figure 5). The pD<sub>2</sub> values of ACh in control and after incubation with 40  $\mu\text{mol/L}$  genistein, 40  $\mu\text{mol/L}$  resveratrol and 40  $\mu\text{mol/L}$  17 $\beta$ -estradiol were  $3.97 \pm 0.16$ ,  $3.38 \pm 0.17$  ( $P < 0.05$   $\text{vs}$  control,  $n = 6$ ),  $3.54 \pm 0.08$  ( $P < 0.05$   $\text{vs}$  control,  $n = 10$ ) and  $3.45 \pm 0.14$  ( $P < 0.05$   $\text{vs}$  control,  $n = 7$ ), respectively. The pD<sub>2</sub> values of KCl in control and after incubation with 40  $\mu\text{mol/L}$  genistein, 40  $\mu\text{mol/L}$  resveratrol and 40  $\mu\text{mol/L}$  17 $\beta$ -estradiol were  $1.61 \pm 0.30$ ,  $0.70 \pm 0.07$  ( $P < 0.05$   $\text{vs}$  control,  $n = 11$ ),  $1.12 \pm 0.03$  ( $P < 0.05$   $\text{vs}$  control,  $n = 14$ ) and  $1.10 \pm 0.05$  ( $P < 0.05$   $\text{vs}$  control,  $n = 7$ ).

## DISCUSSION

The gallbladder and gut should be viewed as hormonally responsive organs. The normal physiology of which may be altered by the sex hormones<sup>[1]</sup>. Also, it is well established that cholelithiasis is more frequent in women than in men. This difference is usually explained by the effects of estrogens and progesterone on the metabolism of bile acids, biliary cholesterol secretion and saturation, and gallbladder motility<sup>[3,4]</sup>. As we know, gallbladder motility has an important role in the regulation of bile flow, its function disturbances may prevent normal bile flow and thus enhance the probability of common bile duct stone formation. Sex steroid hormone have inhibitory effects on the contractility which may be mediated by the inhibition of the mobilization of intracellular calcium and calcium influx in gallbladder smooth muscles<sup>[4]</sup>.

Papers have shown that there are structural similarities between the steroid nucleus of 17 $\beta$ -estradiol and the rigid ring structure of phytoestrogen genistein, and because both of them are lipid-soluble compounds and their molecular weight are not large, they can easily enter cytoplasm through the cellular membrane to affect expression of some genes. The affinity of genistein to the classic estrogen  $\alpha$  receptor (ER $\alpha$ ) presented on reproductive organs is less than that of estrogen<sup>[17]</sup>, but it has a similar affinity as estrogen for the novel estrogen  $\beta$  receptor (ER $\beta$ ) in the vasculature<sup>[18]</sup>. As well as evidence that resveratrol exhibits variable degrees of estrogen receptor agonism in different test systems, and the similarity in structure between resveratrol and the synthetic estrogen diethylstilbestrol (DES; 4,4'-dihydroxy-trans- $\alpha$ ,  $\beta$ -diethylstilbene) prompted us to investigate whether resveratrol might exhibit estrogenic activity in gallbladder motility<sup>[19]</sup>. The present study has shown that the phytoestrogen genistein and resveratrol can induce significant inhibitory effects on isolated gallbladder contractility in a similar way as 17 $\beta$ -estradiol does, and the effects were dose-dependent. Gallbladder smooth muscle cells have been shown to express functional ERs<sup>[2]</sup>, so the effects of phytoestrogen genistein and resveratrol may be attributed to their combination with ERs, but our study demonstrates

that the inhibitory effects induced by genistein and resveratrol are unlikely to be mediated through the ER, as the actions of genistein and resveratrol had no obvious change in the presence of the pure and specific ER antagonist ICI 182780, although it can block not only the classical ER<sub>a</sub> but also the novel ER<sub>b</sub><sup>[20]</sup>. These results suggest that the acute inhibitory effects caused by genistein, resveratrol and 17-β estradiol are not mediated by the classical estrogen receptor and are independent of gene-mediated events.

It is well known that genistein and resveratrol are tyrosine kinase inhibitors<sup>[12,13]</sup> and tyrosine kinase activity has been demonstrated to play a role in smooth muscle contractility<sup>[4,21]</sup>. In the present experiment, a potent protein tyrosine phosphatase inhibitor bpV (phen), which can prevent the decrease of protein tyrosine phosphorylation, markedly attenuated the inhibitory effects of 10 μmol/L genistein and 10 μmol/L resveratrol on gallbladder smooth muscle contractile activities. Our results suggest that tyrosine kinase inhibition is probably responsible for the inhibitory effects induced by genistein and resveratrol in gallbladder smooth muscle contractility. These results are supported by the evidences that tyrosine kinase inhibition contributes to the decrease of Ca<sup>2+</sup> influx and Ca<sup>2+</sup> mobilization<sup>[21,22]</sup>.

The presence of cholinergic M receptors in guinea pig gallbladder smooth muscle cells has been reported<sup>[23]</sup>. ACh can activate receptor-operated calcium channels (ROCs) in the cellular membrane of gallbladder smooth muscle and increase calcium influx, while also activating G proteins and phospholipase C to produce inositol trisphosphate (IP<sub>3</sub>) which causes calcium release from endoplasmic reticulum<sup>[4,23]</sup>. As we know, the contractile response to ACh comprises two distinct components in Ca<sup>2+</sup>-free medium: an initial phasic component that results from IP<sub>3</sub>-mediated release of Ca<sup>2+</sup> from intracellular Ca<sup>2+</sup> stores followed by a tonic component that requires addition of Ca<sup>2+</sup> in the continuous presence of ACh, due to Ca<sup>2+</sup> influx. This is so-called biphasic contraction induced by ACh and Ca<sup>2+</sup>. In calcium-free Kreb's solution, genistein and resveratrol could significantly decrease ACh-induced contraction but they did not affect the latter CaCl<sub>2</sub>-induced contraction. In normal Kreb's solution, genistein, resveratrol and 17β-estradiol could also reduce the contractile responses of ACh and shift their cumulative concentration-response curves rightward in a parallel manner. Considering these observations, it seems reasonable to suggest that the inhibition of Ca<sup>2+</sup> release may involve in the inhibitory effects of genistein and resveratrol on gallbladder smooth muscle contractility.

Potential dependent calcium channels (PDCs) are activated by depolarization of the plasma membrane when the extracellular K<sup>+</sup> concentration is increased, and it has been reported that potassium-stimulated gallbladder contraction depends exclusively upon the influx of extracellular calcium<sup>[4]</sup>. In the present experiment, genistein, resveratrol or 17β-estradiol could shift the KCl concentration-dependent contraction

curves to the right in normal Kreb's solution and inhibited KCl concentration-dependent contractile responses in a noncompetitive manner. The results suggest that genistein and resveratrol may have Ca<sup>2+</sup> antagonistic properties which are consistent with the effect of 17β-estradiol, and inhibit extracellular Ca<sup>2+</sup> influx through PDC.

In summary, similar to 17β-estradiol, genistein and resveratrol have been shown to have a direct inhibitory effect on both the basal and agonist-stimulated contractile activity of guinea pig gallbladder *in vitro*.

## COMMENTS

### Background

Phytoestrogen genistein and resveratrol are structurally and functionally similar to estrogen and possess many physiological and pharmacological effects. Data indicate that genistein, resveratrol can inhibit vasocontractile responses and relax vascular smooth muscles by a Ca<sup>2+</sup> antagonistic property which is similar to estradiol, but little is known about the effect of genistein and resveratrol on gallbladder smooth muscle motility.

### Research frontiers

Gallbladder disease is more prevalent in women than men, and estrogen therapy has been associated with an increased incidence of gallbladder disease in both sexes, suggesting that hormones may play an important role in these conditions. Phytoestrogens such as genistein and resveratrol have estrogen agonistic/antagonistic effects, and are proposed to have potential as natural substitutes of estrogen therapy.

### Innovations and breakthroughs

This study aim is to compare the direct effects of genistein and resveratrol on isolated gallbladder smooth muscle motility with that of 17β-estradiol both at rest and in response to stimulation, and to elucidate the underlying mechanisms.

### Applications

The present results reflect the pharmacological actions of genistein and resveratrol and can provide the pharmacological guidance for the application of these compounds which are very valuable for medicine and nutrition.

### Peer review

This is an interesting report of effects of genistein and resveratrol on gallbladder contractility.

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CASE REPORT

# Laparoscopic resection for incidentally detected Meckel diverticulum

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## Abstract

The management of Meckel diverticulum found unexpectedly during an abdominal operation remains controversial. Most published reports have included only patients undergoing diverticulectomy or bowel resection through laparotomy. We report a case of a carcinoid tumor in a Meckel's diverticulum which was incidentally detected and removed during laparoscopic inguinal hernia repair. Although there is no compelling evidence in the literature to recommend prophylactic diverticulectomy, laparoscopic stapled resection represents a viable and safe approach in healthy individuals undergoing elective surgery for other purposes.

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**Key words:** Laparoscopy; Incidental findings; Meckel's diverticulum; Carcinoid tumor

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## INTRODUCTION

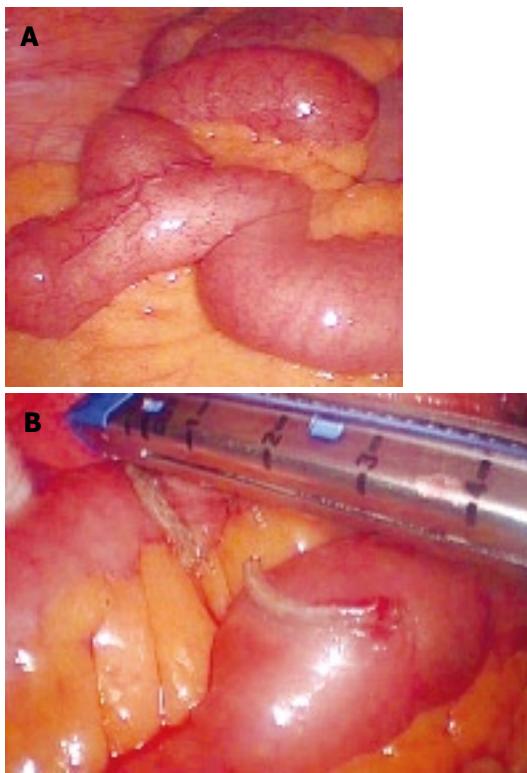
Meckel's diverticulum (MD) is one of the most common congenital abnormalities of the gastrointestinal tract, as it occurs in approximately 2% of the population. This true diverticulum results from an incomplete obliteration of the vitelline duct during the fifth week of gestation and arises from the antimesenteric border of the distal ileum, within 100 cm of the ileocecal valve in the adult. In the majority of cases, the MD is asymptomatic and the diagnosis is made during elective surgery for other intra-abdominal disorders. In some circumstances, the MD can be associated with heterogeneous clinical manifestations ranging from recurrent abdominal pain to life-threatening problems, such as gastrointestinal bleeding or acute bowel obstruction. The diverticulum may contain areas of ectopic mucosa, more commonly of gastric type, or malignant tumors such as carcinoids<sup>[1]</sup>.

The decision to perform diverticulectomy for MD incidentally detected during an abdominal operation is still controversial. Over the past two decades, laparoscopy has been extensively used in the diagnosis and treatment of various abdominal disorders. The opportunity provided by the laparoscopic approach to perform a complete abdominal exploration may increase the number of incidental findings, and this may again pose a dilemma to the surgeon who is more and more committed to the principles of evidence-based medicine for a better and more cost-effective patient care.

## CASE REPORT

A 66 year-old man was seen in the outpatient clinic because of the chronic complaint of pain on exertion and a bulge localized at his left groin. His medical history was unremarkable, except for a mild hypertension. The body mass index was 30. Physical examination showed a bilateral inguinal hernia. An abdominal ultrasonography was negative. Elective laparoscopic repair of the bilateral hernia was scheduled after the patient gave his informed consent.

Preoperative antibiotic prophylaxis (Cefazolin, 2 g i.v.) was administered. Under general anesthesia, pneumoperitoneum was established using a Veress needle. Three bladeless trocars were inserted along the transverse umbilical line, and a 30° angled scope was introduced through the umbilical port. Laparoscopic explora-

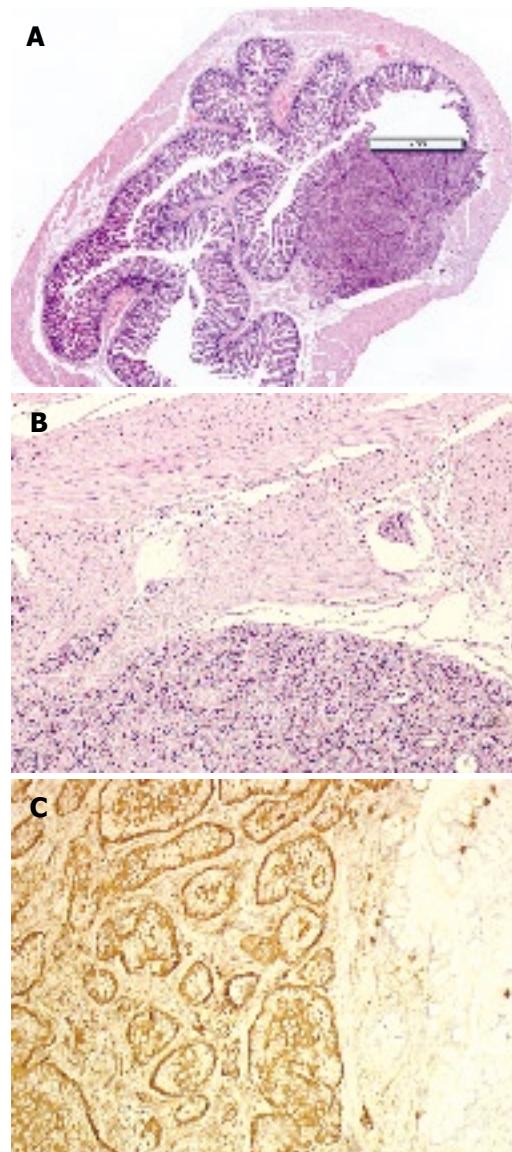


**Figure 1** Meckel diverticulum found unexpectedly during laparoscopic inguinal hernia repair (**A**) and resection of the diverticulum with an endoscopic linear stapler (**B**).

tion immediately revealed a 4 cm long MD arising from the distal ileum, about 70 cm proximal to the ileocecal valve (Figure 1). Priority was given to the inguinal hernia repair. Starting from the left groin, a curvilinear incision was made in the peritoneum beginning laterally and extending to the medial umbilical ligament. A peritoneal flap was created medially by blunt dissection to expose the Cooper's ligament. A preformed polypropylene mesh was placed within the pre-peritoneal pocket and secured with staples and fibrin glue. The peritoneal layer was closed with a running suture (PDS, Ethicon). The same procedure was repeated in the right groin. Upon completion of the hernia repair, the MD was held with an atraumatic grasper through the left port and an endoscopic linear stapler (EndoGIA II, 60 mm, Covidien) was tangentially applied across the base of the diverticulum and fired. The specimen was put into a plastic bag and removed through one of the ports. The patient had an uncomplicated recovery and was discharged home on postoperative day 2. Pathologic examination of the surgical specimen showed a normal ileal mucosa lining and a nodule, 1.5 cm in diameter, with a pattern indicative for a carcinoid tumor located in the proximity of the tip of the diverticulum (Figure 2).

## DISCUSSION

It has long been stated that the risk of developing complications following the incidental removal of MD can offset the potential benefits of this procedure<sup>[2]</sup>. Opponents to incidental diverticulectomy often cite Soltero



**Figure 2** Meckel diverticulum with a nodule 1.5 cm in size (HE, x 1.5) (**A**), superficial infiltration of the diverticulum wall (HE, x 10) (**B**), and immunohistochemical stain with chromogranin A showing a strongly positive reaction and normal intestinal mucosa with control stain of endocrine single cells on the right (x 10) (**C**).

and Bill<sup>[3]</sup> who, in 1976, estimated that the life-time risk of complications from an untreated MD was 4.2%, and that this risk decreased to zero with age. These authors recommended refraining from incidental diverticulectomy because there is only a small chance of MD-related complications in later life. In contrast, almost twenty years later, the results of a large population-based study in Olmsted County, Minnesota, provided data in support of prophylactic diverticulectomy<sup>[4]</sup>. This study reported a 6.4% cumulative rate of developing complications of MD that required surgery over a life-time, especially in male patients up to 80 years of age. Diverticulectomy for complicated MD carried an operative mortality and morbidity of 2% and 12%, and a cumulative risk of long-term complications of 7%. The corresponding rate for incidental diverticulectomy is 1%, 2% and 2%, respectively. Interestingly, in this latter subgroup of patients,

the mortality was related to the primary operation or the patient fitness but not to the diverticulectomy itself<sup>[4]</sup>. A subsequent report from the Mayo Clinic recommended MD resection only in male patients younger than 50 years of age, or when the diverticulum length is greater than 2 cm, or when abnormal features are detected within the diverticulum<sup>[1]</sup>. Of interest, a carcinoid tumor was found in 2.2% of the symptomatic patients and in 2.1% of the asymptomatic ones in this series<sup>[1]</sup>. More recently, however, a systematic review of the English literature has shown that there is no compelling evidence to support prophylactic resection<sup>[5]</sup>. In fact, resection of incidentally detected MD has a significantly higher early complication rate than leaving the diverticulum *in situ* (5.3% vs 1.3%,  $P < 0.0001$ )<sup>[4]</sup>.

It should be noted that most of the data upon which recommendations have been based so far originate from retrospective studies in patients who underwent incidental diverticulectomy or bowel resection through laparotomy<sup>[5]</sup>. The advent of laparoscopy may have changed this scenario. Laparoscopy allows a complete abdominal exploration in patients undergoing minimally invasive procedures and has the potential to reveal additional pathological findings. Moreover, laparoscopy has been used to diagnose and treat patients with MD complicated by small bowel obstruction or bleeding caused by occult heterotopic gastric mucosa<sup>[6,7]</sup>.

To our knowledge, this is the first reported case of carcinoid in MD which was incidentally found and removed during laparoscopy for inguinal hernia repair. Carcinoids are slow growing tumors with a malignant potential arising from the diffuse neuroendocrine system. The gastrointestinal tract is the largest neuroendocrine organ in the body and the site of origin for 90% of all carcinoids. The most common location of carcinoid is the appendix, where tumors are usually small and benign, followed by the ileum, where they are often multiple and characterized by a more aggressive biological behavior. More than 100 cases of carcinoids in MD have been reported in the literature, 72% of the tumors were located at the tip of the diverticulum, and distant metastases were

present in 24% of the patients at the time of diagnosis<sup>[8]</sup>. Immunohistochemical studies have shown that Meckel's carcinoids are closer to the ileal rather than to the appendiceal carcinoids<sup>[9]</sup>. Histopathological analysis in our patient demonstrated a R0 resection and the presence of limited superficial invasion of the diverticulum wall, confirming that diverticulectomy is adequate. Although there is no compelling evidence in the current literature to support prophylactic diverticulectomy for patients with MD, we believe that simple laparoscopic tangential resection with an endostapler is a viable and safe procedure during elective operations for healthy patients.

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CASE REPORT

## A case of long survival in poorly differentiated small cell carcinoma of the pancreas

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### INTRODUCTION

Small-cell carcinoma (SCC) is common malignancy of the lung and represents 20%-25% of all bronchogenic carcinomas<sup>[1]</sup>. It seldom originated from extrapulmonary sites (2.5%-4% of all SCC). The primary site of extrapulmonary SCC (EPSCC) can be in a variety of organs and the clinical course of these tumors has been found to be aggressive, with early dissemination and frequent recurrence. Primary SCC of the pancreas is rare, comprising about 1% of all pancreatic malignant tumors<sup>[2]</sup>. Because of its aggressiveness, most of patients with SCC of the pancreas are diagnosed at an advanced stage of disease. According to previous reports, survival of patients with SCC in the pancreas varies between 2 and 5 mo<sup>[3]</sup>. Here, we report a case of unusually long-term survival after curative surgery and combined chemotherapy for poorly differentiated SCC of the pancreas.

### CASE REPORT

A 62-year-old woman presented to our institute with a 3-wk history of anorexia, dyspepsia, epigastric pain. She had drunken extracts of Hovenia dulcis for 5 mo. There was no history of smoking and regular alcohol consumption. Her family history revealed no abnormalities. She had no relevant previous medical or surgical history. Physical examination was unremarkable except for epigastric tenderness.

Laboratory studies revealed increased total bilirubin (4.9 mg/dL) and direct bilirubin (3.5 mg/dL). Liver enzymes were raised, with serum alkaline phosphatase (1072 U/L), SGOT (233 U/L) and SGPT (101 U/L). The calcium was 9.9 mg/dL and phosphate was 4.0 mg/dL. Serum viral markers, including HIV, HBsAg, and HCV were nonreactive. Tumor markers included carbohydrate antigen 19-9 (CA 19.9, 291.6 U/mL), alpha-fetoprotein (AFP, 3.6 ng/mL), and carcino-embryonic antigen (CEA, 3.2 ng/mL). Serum neuron-specific enolase (NSE) was

### Abstract

Small cell carcinoma (SCC) of the pancreas is rare. It has similar histological features to pulmonary small cell carcinoma and is equally aggressive. Most patients with SCC in the pancreas reported in case studies died within 1 year after diagnosis. We present a case of unusually long-term survival after surgery and combined chemotherapy for SCC of the pancreas. A 62-year-old woman presented with epigastric pain and jaundice. Computed tomography revealed dilated common bile duct caused by external compression of the mass in the pancreatic head. Exploratory laparotomy and pancreaticoduodenectomy (PPPD) was performed with histopathological analysis confirming a primary small cell carcinoma of the pancreas. After an uneventful postoperative recovery, the patient was treated with 6 cycles of combined chemotherapy consisting of cisplatin and ectoposide. During the follow-up, there was no evidence of recurrence and the patient has remained in a good health condition for 36 mo since the diagnosis.

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**Key words:** Small cell carcinoma; Pancreas; Pancreatic carcinoma; Extrapulmonary

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**Figure 1** **A:** Abdominal computed tomography in the arterial phase shows poorly demarcated mass compressing CBD (black arrow); **B:** In delayed phase, mass (white arrows) at the pancreatic head revealed with relatively well delineated margin.

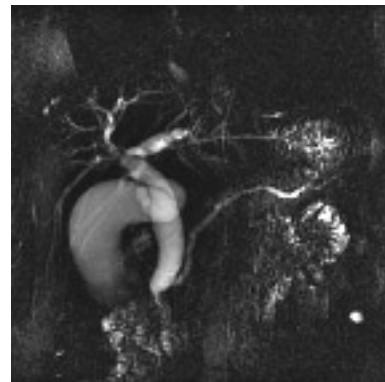
12.5 ng/mL (normal, 0.1-16.3 ng/mL).

A contrast-enhanced computed tomography (CT) of the abdomen showed dilatation of intrahepatic duct, common bile duct (CBD), and pancreatic duct. There was 1.3 cm sized mass at the head of pancreas compressing the distal CBD (Figure 1). There was no evidence of pancreatitis. Magnetic resonance cholangiopancreatography and endoscopic-retrograde cholangiopancreatography (ERCP) was performed and confirmed abrupt CBD narrowing due to extrinsic compression (Figure 2). During the ERCP, it was possible to obtain tissue from the CBD.

The histopathologic study revealed small uniform nuclei with inconspicuous nucleoli and scanty cytoplasm. The morphology of the cells was similar to that of small cell carcinoma of the lung. The immunohistochemical (IHC) stain results were positive for CD56 and thyroid transcription factor-1 (TTF-1) stain and negative for NSE. Typical microscopic features confirmed small cell carcinoma. Chest X-ray and bone scan were normal.

The tumor was localized in the head of the pancreas and no extension beyond the locoregional boundaries; we performed PPPD (Longmire III operation). During the operation, there was no peritoneal seeding or invasion to adjacent organs. Common hepatic artery (No 8) and para-aortic (No 16) lymph node were enlarged but they were found out to have no metastasis in frozen section biopsy. The patient recovered without any postoperative complications.

Examination of the surgical specimen showed an



**Figure 2** Magnetic resonance cholangiopancreatography (MRCP) shows abrupt narrowing of the distal common bile duct and mild dilatation of pancreatic duct.



**Figure 3** Gross appearance of the specimen shows a tumor nodule 2 cm in diameter at the head of the pancreas.

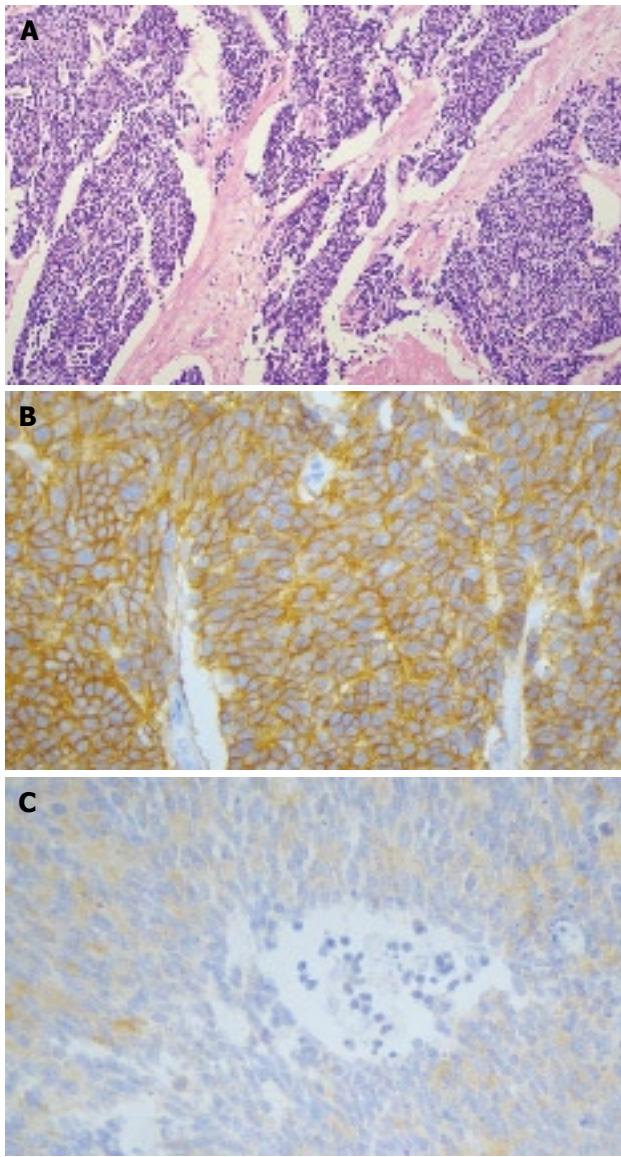
ill demarcated grayish white and firm mass measuring 2.0 cm × 1.2 cm in size in the head of the pancreas. 1 out of 16 lymph nodes showed tumor metastasis (Figure 3). There was lymphatic and perineural invasion. The tumor was composed of small monotonous and hyperchromatic poorly differentiated cells with higher nuclear to cytoplasmic ratio, and were positive for CD56, cytokeratin, chromogranin, TTF-1 and CEA, but negative for NSE, CD99 and equivocal for synaptophysin (Figure 4).

Four weeks after the operation, the patient received chemotherapy consisting of cisplatin (100 mg/m<sup>2</sup>) and ectoposide (60 mg/m<sup>2</sup>, day 1-3) for 4 wk intervals. After first cycle of chemotherapy CA 19-9 decreased to 19.0 U/mL. The chemotherapy was tolerated well and was continued for 6 cycles. During the follow-up there was no evidence of recurrence and the patient has remained in a good health condition for 36 mo since the diagnosis.

## DISCUSSION

Extrapulmonary small cell carcinomas (EPSCC) are rare with an incidence between 0.1%-0.4% of all cancers<sup>[1]</sup>. Approximately 2.5% of all SCC's arise in extrapulmonary sites such as head and neck region, esophagus, stomach, colon, rectum, gallbladder, uterine cervix, breast, urinary bladder, liver, and prostate<sup>[4-8]</sup>.

SCC of the pancreas is a rare entity. Since the earliest case report of pancreatic SCC with clinical



**Figure 4** A: The tumor consisted of small round or oval cells with hyperchromatic nuclei and scant cytoplasm; B and C: Immunohistochemical staining for CD56 (B) and synaptophysin (C) reveals a positive reaction.

and pathologic findings in 1973<sup>[9]</sup>, only a few reports have been published with fewer than 40 cases<sup>[3,10-12]</sup>. According to the previous studies, SCC of the pancreas is likely to occur in the head of pancreas in patients with average age of 60 and common clinical manifestations are abdominal pain, weight loss and jaundice. Many of the cases reported were diagnosed at autopsy, since most of patients with SCC of the pancreas are diagnosed at an advanced stage of disease and their survival varied between 2-5 mo<sup>[1,3]</sup>.

SCC is thought to originate from neuroendocrine cells, which are found in the epithelium of many mucosal surfaces. Despite evidence of neuroendocrine involvement, the origin of EPSCC is still unclear as development from undifferentiated airway epithelium has also been suggested along with the amine precursor uptake and decarboxylation (APUD) system hypothesis which proposes a common ancestral cell derived from the neural crest, migrating to various epithelial tissues

and sites within the body<sup>[13,14]</sup>. Since SCC cell has similar properties to the endocrine cells of APUD system, EPSCC may also secrete hormones. But in the case of SCC of the pancreas, there is only one report of ACTH-producing tumor by Corrin *et al* and one case associated with hypercalcemia<sup>[9,15]</sup>, which is different from frequent paraneoplastic syndromes in pulmonary SCC. There were no abnormal findings suggesting paraneoplastic hormone syndromes in the case of our patient. O'Connor *et al* reported NSE was clearly elevated in SCC of the pancreas. They suggested that serum NSE could be a tumor marker for SCC of the pancreas, but NSE was within the normal range in this case<sup>[16]</sup>.

EPSCC of the pancreas is histologically indistinguishable from metastatic pulmonary SCC. Therefore, exclusion of pulmonary small cell carcinoma is a prerequisite for the diagnosis of EPSCC. In our case, chest X-ray and PET-CT were negative for the lungs. Since the biopsy from the pancreas is difficult, most of the cases were diagnosed by biopsy from the liver metastasis or lymph node or autopsy and a few cases after surgical removal<sup>[3,11,17,18]</sup>. To evaluate dilated CBD, our patient received ERCP before surgery. During the procedure it was possible to obtain tissue from the narrowing portion of the CBD. We could confirm the tumor as EPSCC before the surgery.

On gross findings, SCC of the pancreas appears as a poorly demarcated white-gray mass with areas of necrosis and hemorrhage. It usually involves the head of the pancreas with a mean diameter of 4.2 cm<sup>[3]</sup>. The histopathologic appearance of the tumor consists of nest of small to medium sized round to oval shaped cells with a finely granular and hyperchromatic nucleus, inconspicuous nucleoli and scanty cytoplasm. Primary small cell carcinoma of the pancreas has a varied immunohistochemical profile.

Neuroendocrine markers such as CD56, chromogranin, TTF-1 and CEA were positive but NSE and synaptophysin was negative in current case.

Unfortunately clinical presentation of EPSCC is usually at an advanced stage due to the aggressive nature of the disease. Therapeutic modalities are determined by the location and extent of disease. Usually chemotherapy remains the treatment of choice and local modalities such as surgery and radiotherapy remain limited in localized disease<sup>[19]</sup>.

In our case, the tumor was localized in the pancreas with regional lymph node involvement. Since there was no extension beyond the locoregional boundaries (limited disease), we could perform curative surgery.

Because of the high incidence of metastasis, chemotherapy should be given after a successful resection of the tumor. Only one case was reported long survival after curative resection of SCC of the pancreas without adjuvant chemotherapy<sup>[18]</sup>. There are no definite chemotherapeutic regimens for SCC of the pancreas due to the small patient numbers. But the combination cisplatin and etoposide showed best result with response rates reaching 70% in an analysis of the

different patients of EPSCC with chemotherapeutic regimens. Doxorubicin-based regimens appear to be less effective<sup>[20]</sup>.

Complete response has been observed with cisplatin-etoposide based treatment in a patient with widespread metastatic disease<sup>[11]</sup>. The two patients reported by van der Gaast had extensive disease with a survival of 16 and 11 mo after combined chemotherapy with cyclophosphamide, doxorubicin and etoposide<sup>[21]</sup>. Another patient survived 14 mo with combined chemotherapy and the radiotherapy<sup>[22]</sup>. Our patient received chemotherapy consisting of cisplatin (100 mg/m<sup>2</sup>), etoposide (60 mg/m<sup>2</sup>, day 1-3) for 4 wk after the surgery. A CT scan of the abdomen after 6 cycles of chemotherapy showed no evidence of metastasis. The patient remains in good health 36 mo after the surgery. The reason for the good prognosis may be associated with an early detection of the tumor and the fact that the tumor was localized and showed no metastasis or dissemination.

Because of the unfavorable prognosis of EPSCC, multimodal therapy was used in most of reported cases with limited disease. In the case of resectable SCC of the pancreas, it is reasonable to perform the extensive surgery followed by chemotherapy. We report a case of primary SCC of the pancreas with unusually long-term survival after multimodal therapy.

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CASE REPORT

## Primary malignant melanoma of the liver: A case report

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### INTRODUCTION

Malignant melanoma occurs most frequently in skin but also in many organs and tissues of the body. However, primary hepatic malignant melanoma is exceedingly rare. Only 12 cases, including 8 cases from PubMed and 4 cases from Chinese literature<sup>[1-4]</sup>, have been reported. In PubMed, there are only 3 cases of definite primary melanoma<sup>[5-7]</sup>. Microscopically, it may be easily misdiagnosed because of morphological heterogeneity and hypomelanotic appearance. The present case represents the only case encountered in our department. In this report, we describe our pathological observations and review the literature in order to improve our understanding of the disease, avoid misdiagnosis and provide evidence for its clinical treatment and prognosis.

### Abstract

Primary malignant melanoma of the liver is an exceedingly rare tumor. Only 12 cases have been reported in the worldwide literature. We present a case of isolated malignant melanoma of the liver occurring in a 36-year-old Chinese male patient. Comprehensive dermatologic and ophthalmologic examinations revealed no evidence of a cutaneous or ocular primary lesion. Other lesions in brain, respiratory tract, lung, gastrointestinal tract and anus, were not demonstrated by serial positron emission tomography (PET). Microscopic examination of the resected specimen revealed a malignant melanoma, which was confirmed by immunohistochemical staining for HMB-45, S-100 protein, melanoma-pan and vimentin. Moreover, electron microscopy demonstrated melanosomes in tumor cell cytoplasm. Our case shows that primary malignant melanoma may occur in the liver and should be considered when the histopathological appearance is not typical for other hepatic neoplasm.

**Key words:** Primary malignant melanoma; Liver;  
Diagnosis; Histopathology; Immunohistochemistry

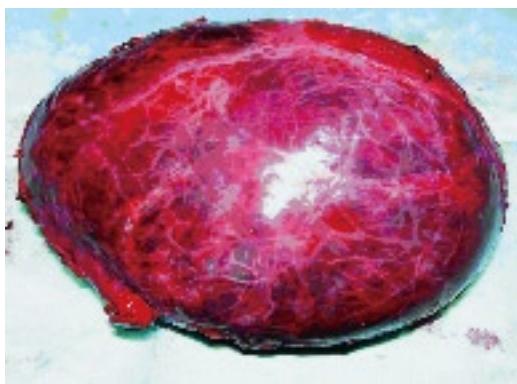
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### CASE REPORT

A 36-year-old man was admitted to the Department of General Surgery, Tangdu Hospital, Fourth Military Medical University (Xi'an, Shaanxi Province, China) because a mass in his liver was found 4 d ago at a routine health check. He had no history of alcohol abuse or hepatitis. No record of hepatocellular carcinoma (HCC) or any hereditary disease was found in his family members. Routine clinical biochemistry showed normal levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH),  $\gamma$ -glutamyltransferase ( $\gamma$ -GTP),  $\alpha$ -fetoprotein (AFP) and plasma proteins. Laboratory tests failed to show any positive hepatitis B surface antigen (HBsAg) or anti-hepatitis C virus (HCV) antibody. Contrast-enhanced abdominal computerized tomography (CT) displayed a 10.1 cm  $\times$  12.8 cm well-defined hepatic mass in the right-posterior lobe of the liver without evidence for spread to neighboring lymph nodes or abdominal dropsy



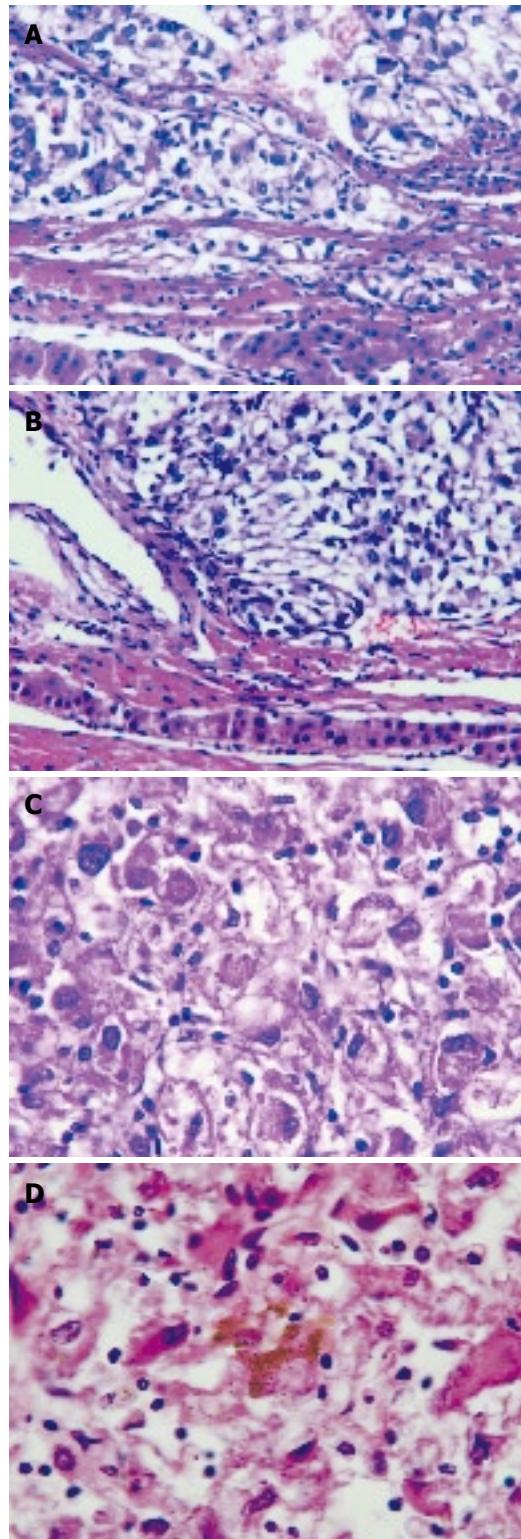
**Figure 1** Contrast-enhanced abdominal CT scan showing an oval, low-density, well-defined mass measuring 10.1 cm × 12.8 cm in the right posterior lobe of the liver.



**Figure 2** Gross appearance of the resected specimen showing the oval outline of tumor adjacent to normal liver tissue.

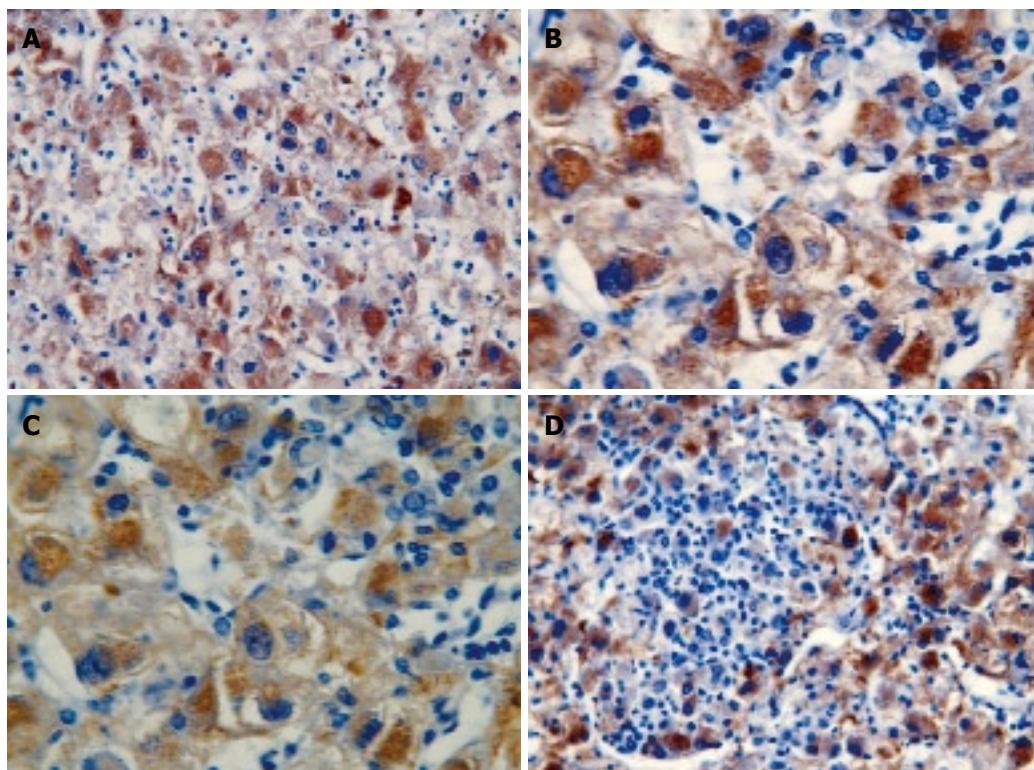
(Figure 1). The resected fresh tissue was fixed in 40 g/L formaldehyde solution, embedded in paraffin. Sections of 4 μm in thickness were prepared and stained with hematoxylin and eosin (HE). Immunostaining was carried out using a streptavidin-labeled peroxidase (S-P) kit (KIT9730) according to its manufacturer's instructions. Primary antibodies used in this study included those against HMB-45, melanoma-pan, epithelial membrane antigen (EMA), carcinoembryonic antigen (CEA), cytokeratins (18, 19, 7, 8), high-MW-CK, chromogranin A (CgA), synaptophysin (syn), sesmin, nerve specificity enolase (NSE), smooth muscle actin (SM-actin), vimentin, CD34, AFP, S-100 protein, CD117, human chorionic gonadotrophin (HCG), leucocyte common antigen (LCA), HBsAg, hepatitis B core antigen (HBcAg), as well as anti-HCV antibody. All of the reagents for immunostaining were supplied by Maxim Biotechnology Corporation Ltd, Fuzhou, China.

Grossly, the resected mass measured 12 cm × 11 cm × 6 cm (Figure 2). There was a clear differentiation between the mass and its surrounding normal tissue, and the cut surface of the tumor was grayish-yellow in color. Microscopically, the tumor cells showed a diffuse infiltration, and fibrous tissue could be observed between the lesion and normal hepatocytes. A few tumor cells breaking through the capsule infiltrated the adjacent liver tissue (Figure 3A and B). The tumor cells were

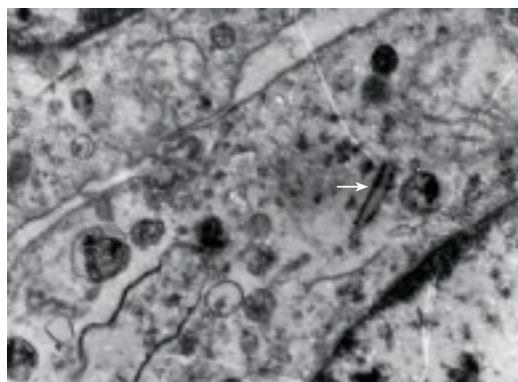


**Figure 3** Microscopy showing diffusely infiltrating tumor cells (A) and fibrous tissues (B) between the lesion and normal hepatocytes with a few broken tumor cells through the capsule infiltrating the adjacent liver tissue (x 200), pleomorphic tumor cells with round, spindle-shaped and irregular morphologies (x 400) (C), and some tumor cells containing melanin deposition (x 200) (D).

pleomorphic, with round, spindle-shaped and irregular morphologies. The tumor cell nuclei were round or oval, and the nucleoli were prominent, with both eosinophilic and basophilic varieties observed (Figure 3C). Some



**Figure 4** Immunohistochemistry revealing tumor cells positive for vimentin ( $\times 200$ ) (A), melanoma-pan ( $\times 400$ ) (B), HMB45 ( $\times 400$ ) (C) and S-100 protein ( $\times 200$ ) (D).



**Figure 5** Electron microscopy displaying occasional melanosomes in the cytoplasm (arrow).

tumor cells contained melanin deposition (Figure 3D). Immunohistochemically, the tumor cells were positive for vimentin (Figure 4A), melanoma-pan (Figure 4B), HMB-45 (Figure 4C) and S-100 protein (Figure 4D), but did not display AFP, high-MW-CK, CK18, CEA, CK19, CD34, CD117, NSE, LCA, CgA, HCG, EMA, desmin, SM-actin, SC-actin, myoglobin and immunoreactivity. Electron microscopy revealed occasional melanosomes in cytoplasm (Figure 5).

The complete absence of evidence for any cutaneous, ocular, mucosal lesions in all organs examined by serial position emission tomography (PET) supported the final diagnosis of primary melanoma.

After a wide local surgical resection, the patient received a 4-wk immunomodulatory therapy at a high dose of IV interferon alpha-2b, once a day. He then received a lower dose subcutaneous regimen, three times a week for a further 9 wk. This regimen was

well-tolerated. He was regularly followed up. Three months after operation, he had no recurrence of the disease. However, recurrent foci were found 5 mo after operation.

## DISCUSSION

Melanoma is a melanin-induced malignant tumor, most prevalent in patients over the age of 30 years. It mainly occurs in skin, but may be found in retina, anorectal canal, genital tract, gastrointestinal (GI) tract, accessory nasal cavity and parotid. Non-cutaneous primary melanomas carry a particularly high mortality because of their propensity for dissemination and invasion, often before they are clinically apparent.

Primary malignant melanoma of the liver is an extremely rare non-epithelial neoplasm, and few cases have been reported. We thus summarize, in this paper, the clinical characteristics and histopathologic manifestations of this unusual tumor.

Malignant melanomas of the skin originate from epidermal melanocytes or neural cells, both are derived from neural crest precursors<sup>[8]</sup>. Several factors, including race, heredity, tissue injury, stimulation, viral infection, sun-exposure and immunization, can lead to malignant transformation<sup>[9]</sup>. The origin of mucosal malignant melanoma is unclear. Most experts hold that malignant melanomas of non sun-exposed tissues are linked to stimulation by the blood-borne sunlight circulation factor, expressed in sun-exposed melanoblasts<sup>[10]</sup>. Based on the origin of primary melanomas of the esophagus and stomach, some authors believe that these alimentary tract neoplasms originate from migrating melanocytes invaginated by digestive tract epithelial cells during

embryogenesis<sup>[11,12]</sup>, which is supported by the distribution of melanocytes in other typically-occurring mucosal cells of the rectum<sup>[13]</sup>. However, the origin and pathogenesis of primary melanomas arising in parenchymal organs are still unclear.

It is difficult to show the clinical characteristics of primary hepatic malignant melanoma because case reports are available prior to the 1970s, except for 5 Chinese patients (2 males and 3 females, mean age 42.2 years, range 27-60 years). Previously reported patients had no readily identifiable risk factors when compared to patients with primary HCC.

Pathologically, hepatic malignant melanoma resembles that of the skin or mucosa, exhibiting morphologic variability within the tumor sample. Microscopically, the tumor mass is comprised of epithelioid cells arranged in nests, or spindle cells arranged in fascicles, with or without melanin pigment deposition. Mitotic figures are readily apparent. However, our case suggests that it may be difficult to identify malignant melanoma from a biopsy sample in some cases, because portions of the tumor may be amelanotic. In these cases, ancillary immunohistochemical staining may be extremely valuable<sup>[14]</sup>. In our case, when specimens of HCC, haemangioma, large-B cell lymphoma, smooth muscle tumor and rhabdomyosarcoma were all considered in the differential diagnosis, all of which were potentially consistent with the intact capsule, large size, and well-circumscribed boundaries. However, all the above tumors could be ultimately excluded based on their histopathologic characteristics and immunohistochemical staining. After reviewing a number of additional sections and detecting sporadic pigment granules in the tumor cytoplasm, we considered a diagnosis of malignant melanoma. The tumor cells expressed HMB45, S-100 protein, vimentin and melanoma-pan, all of which were consistent with hepatic melanoma. Our preliminary diagnosis was then confirmed by electron microscopy.

Once the pathologic diagnosis was established, whether the tumor was a primary or secondary lesion should be considered. An extensive investigation of potential primary sites demonstrated no evidence for hepatic melanoma, suggesting that the tumor is a primary melanoma of the liver.

Given the rarity of this tumor, the optimal therapeutic regimen is not known. In fact, the natural

history of these cases also remains unclear. Since surgical therapy is usually palliative, a more aggressive oncologic regimen consisting of chemotherapy, immunotherapy and radiotherapy may be required. With the natural history largely unknown, it is necessary to find treatment modalities for other deep primary melanomas,

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## Meetings

### Events Calendar 2008-2009

FALK SYMPOSIA 2008  
January 24-25, Frankfurt, Germany  
Falk Workshop: Perspectives in Liver Transplantation

International Gastroenterological Congresses 2008  
February 14-16, Paris, France  
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies  
[www.easl.ch/hepatitis-conference](http://www.easl.ch/hepatitis-conference)

February 14-17, Berlin, Germany  
8<sup>th</sup> International Conference on New Trends in Immunosuppression and Immunotherapy  
[www.kenes.com/immuno](http://www.kenes.com/immuno)

February 28, Lyon, France  
3<sup>rd</sup> Congress of ECCO - the European Crohn's and Colitis Organisation Inflammatory Bowel Diseases 2008  
[www.ecco-ibd.eu](http://www.ecco-ibd.eu)

February 29, Québec, Canada  
Canadian Association of Gastroenterology  
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK  
British Society of Gastroenterology Annual Meeting  
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China  
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea  
Asian Pacific Association for the Study of the Liver  
18<sup>th</sup> Conference of APASL: New Horizons in Hepatology  
[www.apaslseoul2008.org](http://www.apaslseoul2008.org)

March 29-April 1, Shanghai, China  
Shanghai-Hong Kong International Liver Congress  
[www.livercongress.org](http://www.livercongress.org)

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco  
OESO 9<sup>th</sup> World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas  
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA  
SAGES 2008 Annual Meeting - part of Surgical Spring Week  
[www.sages.org/08program/html/](http://www.sages.org/08program/html/)

April 18-22, Buenos Aires, Argentina  
9<sup>th</sup> World Congress of the International Hepato-Pancreato Biliary Association  
Association for the Study of the Liver  
[www.ca-ihpba.com.ar](http://www.ca-ihpba.com.ar)

April 23-27, Milan, Italy  
43<sup>rd</sup> Annual Meeting of the European Association for the Study of the Liver  
[www.easl.ch](http://www.easl.ch)

May 2-3, Budapest, Hungary  
Falk Symposium 164: Intestinal

### Disorders

May 18-21, San Diego, California, USA  
Digestive Disease Week 2008

May 21-22, California, USA  
ASGE Annual Postgraduate Course Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion  
E-mail: education@sgo.org

June 4-7, Helsinki, Finland  
The 39<sup>th</sup> Nordic Meeting of Gastroenterology  
[www.congrex.com/ngc2008](http://www.congrex.com/ngc2008)

June 5-8, Sitges (Barcelona), Spain  
Semana de las Enfermedades Digestivas  
E-mail: sepd@sepdi.es

June 6-8, Prague, Czech Republic  
3<sup>rd</sup> Annual European Meeting: Perspectives in Inflammatory Bowel Diseases  
E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey  
ESGAR 2008 19<sup>th</sup> Annual Meeting and Postgraduate Course  
E-mail: fca@netvisao.pt

June 11-13, Stockholm, Sweden  
16<sup>th</sup> International Congress of the European Association for Endoscopic Surgery  
E-mail: info@aes-eur.org

June 13-14, Amsterdam, Netherlands  
Falk Symposium 165: XX International Bile Acid Meeting, Bile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic  
Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management  
E-mail: idca2008@guantar.cz

June 25-28, Barcelona, Spain  
10<sup>th</sup> World Congress on Gastrointestinal Cancer  
Imedex and ESMO  
E-mail: meetings@imedex.com

June 25-28, Lodz, Poland  
Joint Meeting of the European Pancreatic Club (EPC) and the International Association of Pancreatology (IAP)  
E-mail: office@epc-iap2008.org  
[www.e-p-c.org](http://www.e-p-c.org)  
[www.pancreatology.org](http://www.pancreatology.org)

June 26-28, Bratislava, Slovakia  
5<sup>th</sup> Central European Gastroenterology Meeting  
[www.ceurgem2008.cz](http://www.ceurgem2008.cz)

July 9-12, Paris, France  
ILTS 14<sup>th</sup> Annual International Congress  
[www.ilts.org](http://www.ilts.org)

September 10-13, Budapest, Hungary  
11<sup>th</sup> World Congress of the International Society for Diseases of the Esophagus  
E-mail: isde@isde.net

September 13-16, New Delhi, India  
Asia Pacific Digestive Week  
E-mail: apdw@apdw2008.net

APDW 2008  
September 13-16, New Delhi, Indian Organized: Indian Society of Gastroenterology

III FALK GASTRO-CONFERENCE

September 17, Mainz, Germany  
Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany  
Falk Symposium 166: GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic  
Prague Hepatology Meeting 2008  
[www.czech-hepatology.cz/phm2008](http://www.czech-hepatology.cz/phm2008)

September 20-21, Mainz, Germany  
Falk Symposium 167: Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France  
Third Annual Meeting  
European Society of Coloproctology  
[www.escp.eu.com](http://www.escp.eu.com)



October 8-11, Istanbul, Turkey  
18<sup>th</sup> World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists  
E-mail: orkun.sahin@serenas.com.tr

October 18-22, Vienna, Austria  
16<sup>th</sup> United European Gastroenterology Week  
[www.negf.org](http://www.negf.org)  
[www.acv.at](http://www.acv.at)

October 22-25, Minnesota, USA  
Anstralian Gastroenterology Week 2008  
E-mail: gesa@gesa.org.au

October 22-25, Brisbane, Australia  
71<sup>st</sup> Annual Colon and Rectal Surgery Conference  
E-mail: info@colonrectalcourse.org

October 31-November 4, Moscone West Convention Center, San Francisco, CA  
59<sup>th</sup> AASLD Annual Meeting and Postgraduate Course  
The Liver Meeting Information: [www.aasld.org](http://www.aasld.org)

November 6-9, Lucerne, Switzerland  
Neurogastroenterology & Motility Joint International Meeting 2008  
E-mail: ngm2008@mci-group.com  
[www.ngm2008.com](http://www.ngm2008.com)

November 12, Santiago de Chile, Chile  
Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

November 28-29, Cairo, Egypt  
1<sup>st</sup> Hepatology and Gastroenterology Post Graduate Course  
[www.egyptgastrohep.com](http://www.egyptgastrohep.com)

December 7-9, Seoul, Korea  
6<sup>th</sup> International Meeting Hepatocellular Carcinoma: Eastern and Western Experiences  
E-mail: sglee@amc.seoul.kr

INFORMATION FOR ALL  
FALK FOUNDATION e.V.  
E-mail: [symposia@falkfoundation.de](mailto:symposia@falkfoundation.de)  
[www.falkfoundation.de](http://www.falkfoundation.de)

Advanced Courses - European

Institute of Telesurgery EITS - 2008 Strasbourg, France  
January 18-19, March 28-29, June 6-7, October 3-4

N.O.T.E.S  
April 3-5, November 27-29  
Laparoscopic Digestive Surgery

June 27-28, November 7-8  
Laparoscopic Colorectal Surgery

July 3-5  
Interventional GI Endoscopy Techniques  
Contact address for all courses:  
E-mail: [info@eits.fr](mailto:info@eits.fr)

International Gastroenterological Congresses 2009  
March 23-26, Glasgow, Scotland  
Meeting of the British Society of Gastroenterology (BSG)  
E-mail: [bsg@mailbox.ulcc.ac.uk](mailto:bsg@mailbox.ulcc.ac.uk)

May 17-20, Denver, Colorado, USA  
Digestive Disease Week 2009

November 21-25, London, UK  
Gastro 2009 UEGW/World Congress of Gastroenterology  
[www.gastro2009.org](http://www.gastro2009.org)



### Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London, The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.



## Instructions to authors

### GENERAL INFORMATION

*World Journal of Gastroenterology* (*World J Gastroenterol* ISSN 1007-9327 CN 14-1219/R) is a weekly open access peer-reviewed journal supported by an editorial board consisting of 1208 experts in gastroenterology and hepatology from 60 countries. The aim of the journal is to deliver the most clinically relevant original and commentary articles to readers, and to make the full text publicly available to all clinicians, scientists, patients and biomedical students on an unrestricted platform, so that they can access and learn about the most recent key advances in the field.

In addition to the open access nature, another key characteristic of *WJG* is its reading guidance for each article which includes background, research frontier, related reports, breakthroughs, applications, terminology, and comments of peer reviewers for the general readers.

*WJG* publishes articles on esophageal, gastrointestinal, hepatobiliary and pancreatic tumors, and other esophageal, gastrointestinal, hepatic-biliary and pancreatic diseases in relation to epidemiology, immunology, microbiology, motility & nerve-gut interaction, endocrinology, nutrition & obesity, endoscopy, imaging and advanced hi-technology.

The main goal of *WJG* is to publish high quality commentary articles contributed by leading experts in gastroenterology and hepatology and original articles that combine the clinical practice and advanced basic research, to provide an interactive platform for clinicians and researchers in internal medicine, surgery, infectious diseases, traditional Chinese medicine, oncology, integrated Chinese and Western medicine, imaging, endoscopy, interventional therapy, pathology and other basic medical specialties, and thus eventually improving the clinical practice and healthcare for patients.

#### Indexed and abstracted in

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#### Published by

The WJG Press

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Manuscripts should be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Number all pages consecutively, and start each of the following sections on a new page: Title Page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables, Figures, and Figure Legends. Neither the editors nor the publisher are responsible for the opinions expressed by contributors. Manuscripts formally accepted for publication become the permanent property of The WJG Press, and may not be reproduced by any means, in whole or in part, without the written permission of both the authors and the publisher. We reserve the right to copy-edit and put onto our website accepted manuscripts. Authors should follow the relevant guidelines for the care and use of laboratory animals of their institution or national animal welfare committee. For the sake of transparency in regard to the performance and reporting of clinical trials, we endorse the policy of the International Committee of Medical Journal Editors to refuse to publish papers on clinical trial results if the trial was not recorded in a publicly-accessible registry at its outset. The only register now available, to our knowledge, is <http://www.clinicaltrials.gov> sponsored by the United States National Library of Medicine and we encourage all potential contributors to register with it. However, in the case that other registers become available you will be duly notified. A letter of recommendation from each author's organization should be provided with the contributed article to ensure the privacy and secrecy of research is protected.

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Full manuscript title, running title, all author(s) name(s), affiliations, institution(s) and/or department(s) where the work was carried out; author contributions; disclosure of any financial support for the research; and the name, full address, telephone and fax numbers and email address of the corresponding author should be included. Titles should be concise and informative (remove all unnecessary words), emphasize what is new, and avoid abbreviations. A short running title of less than 40 letters should be provided. List the author(s)' name(s) as follows: initial and/or first name, middle name or initial(s), and full family name.

**Author contributions:** The format of this section should be like this: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed research; Wang CL, Zou CC, Hong F and Wu XM performed research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed data; and Wang CL, Liang L and Fu JF wrote the paper.

**Peer reviewers:** All articles received are subject to peer review. Normally, three experts are invited for each article. Decision for acceptance is made only when at least two experts recommend an article for publication. Reviewers for accepted manuscripts are acknowledged in each manuscript, and reviewers of articles which were not accepted will be acknowledged at the end of each issue. To ensure the quality of the articles published in *WJG*, reviewers of accepted manuscripts will be announced by publishing the name, title/position and institution of the reviewer in the footnote accompanying the printed article. For example, reviewers: Professor Jing-Yuan Fang, Shanghai Institute of Digestive Disease, Shanghai, Affiliated Renji Hospital, Medical Faculty, Shanghai Jiaotong University, Shanghai, China; Professor Xin-Wei Han, Department of Radiology, The First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan Province, China; and Professor Anren Kuang, Department of Nuclear Medicine, Huaxi Hospital, Sichuan University, Chengdu, Sichuan Province, China.

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An informative, structured abstract of no more than 350 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections: AIM: Only the purpose should be included. METHODS: The materials, techniques, instruments and equipment, and the experimental procedures should be included. RESULTS: The observed and experimental results, including data, effects, outcome, etc. should be included. Authors should present *P* value where necessary, and also include any significant data. CONCLUSION: Accurate view and the value of the results should be included.

The format for structured abstracts can be found at: <http://www.wjnet.com/wjg/help/11.doc>.

#### Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

#### Text

For articles of these sections, original articles, rapid communication

and case reports, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the body text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, should be found at: <http://www.wjgnet.com/wjg/help/instructions.jsp>.

### Illustrations

Figures should be numbered as 1, 2, 3, etc., and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: <http://www.wjgnet.com/1007-9327/13/4520.pdf>; <http://www.wjgnet.com/1007-9327/13/4554.pdf>; <http://www.wjgnet.com/1007-9327/13/4891.pdf>; <http://www.wjgnet.com/1007-9327/13/4986.pdf>; <http://www.wjgnet.com/1007-9327/13/4498.pdf>. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: ...etc. It is our principle to publish high resolution-figures for the printed and E-versions.

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### Notes in tables and illustrations

Data that are not statistically significant should not be noted. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 should be noted (*P* > 0.05 should not be noted). If there are other series of *P* values, <sup>c</sup>*P* < 0.05 and <sup>d</sup>*P* < 0.01 are used. A third series of *P* values can be expressed as <sup>e</sup>*P* < 0.05 and <sup>f</sup>*P* < 0.01. Other notes in tables or under illustrations should be expressed as <sup>1</sup>F, <sup>2</sup>F, <sup>3</sup>F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, □, ▲, △, etc., in a certain sequence.

### Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscripts and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

## REFERENCES

### Coding system

The author should number the references in Arabic numerals according to the citation order in the text. Put reference numbers in square brackets in superscript at the end of citation content or after the cited author's name. For citation content which is part of the narration, the coding number and square brackets should be typeset normally. For example, "Crohn's disease (CD) is associated with increased intestinal permeability<sup>[1,2]</sup>". If references are cited directly in the text, they should be put together within the text, for example, "From references<sup>[19,22-24]</sup>, we know that..."

When the authors write the references, please ensure that the order in text is the same as in the references section, and also ensure the spelling accuracy of the first author's name. Do not list the same citation twice.

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The accuracy of the information of journal citations is very important. We will interlink all references with DOI in ASP file so that readers can access the abstracts of cited articles online immediately.

### Style for journal references

Authors: the name of the first author should be typed in bold-faced letters. The family name of all authors should be typed with the initial letter capitalized, followed by their abbreviated first and middle initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR). The title of the cited article and italicized journal title (journal title should be in its abbreviated form as shown in PubMed), publication date, volume number (in black), start page, and end page [PMID: 11819634 DOI: 10.3748/wjg.13.5396].

### Style for book references

Authors: the name of the first author should be typed in bold-faced letters. The surname of all authors should be typed with the initial letter capitalized, followed by their abbreviated middle and first initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR) Book title. Publication number. Publication place: Publication press, Year: start page and end page.

### Format

#### Journals

English journal article (list all authors and include the PMID where applicable)

- 1 Jung EM, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

- 2 Lin GZ, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixudiarrhoea. *Shijie Huaren Xiaohua Zaishi* 1999; **7**: 285-287

In press

- 3 Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

- 5 Vallancien G, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

Volume with supplement

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EDITORIAL

# Update and actual trends on bacterial infections following liver transplantation

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## Abstract

Recent advances in effective antimicrobial prophylactic strategies have led to a decline in the incidence of opportunistic infections in liver transplant recipients. However, morbidity and mortality due to infectious diseases remain as major problems. Bacterial infections occurring early after transplant are mainly related to the technical aspects of the procedure. By contrast, after the first postoperative days and beyond, the nature and variety of infectious complications change. Opportunistic bacterial infections are uncommon after 6 mo in patients receiving stable and reduced maintenance doses of immunosuppression with good graft function and little is documented about these cases in the literature. Transplant recipients may be more susceptible to some pathogens, such as the *Nocardia* species, *Legionella* species, *Listeria monocytogenes*, *Mycoplasma* species, *Salmonella* species or *Rhodococcus equi*. Respiratory infections due to capsulated bacteria, such as *Streptococcus pneumoniae* and *Haemophilus influenzae*, can be life-threatening if not promptly treated in this population. These late bacterial infections may be very difficult to recognize and treat in this population. In this article, we review what has been described in the literature with regards to late bacterial infections following liver transplantation.

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**Key words:** Liver transplant; Bacterial infections; *Nocardia* species; *Listeria* species; *Legionella* species; *Mycoplasma* species; *Bartonella* species

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## INTRODUCTION

Since the first report in 1963, liver transplantation has become an established treatment for properly selected patients with end-stage liver disease, primary and some secondary hepatic malignancies and some rare metabolic liver-based disorders. During the past decade, survival rates after liver transplantation have steadily improved, with one-year survival exceeding 90% and approaching 70% at 8 years<sup>[1]</sup>. Increasingly, potent immunosuppressive agent use has dramatically reduced the incidence of rejection in the transplanted population, while increasing patient susceptibility to opportunistic infections and cancer<sup>[2]</sup>. However, recent advances in effective antimicrobial prophylactic strategies have led to a decline in the incidence of opportunistic infections in liver transplant recipients. Nevertheless, morbidity and mortality due to infectious complications remain as major problems in this selected population.

Infection and rejection remain major causes of morbidity after a liver transplant, accounting for up to 85% of deaths in some studies<sup>[3]</sup>. Infections were the most common cause of death after liver transplantation, and were present in 64% of a total of 321 cases in a study<sup>[4]</sup>. Two-thirds of all infections occurred during the first 100 d post transplantation in this study. Overall, the infections were bacterial in 48% of the cases, fungal in 22%, and viral in 12%<sup>[4]</sup>. The ratio of infectious to non-infectious causes of death did not change significantly during the 15-year study period, and the relative percentages of bacterial, fungal, and viral infections showed relatively little discrepancy on a year-to-year basis in this study<sup>[4]</sup>. In one other study, 83% of a liver transplant population had 1 or more episodes of infection and 67% had severe infections<sup>[5]</sup>. 70% of severe infections occurred within the first 2 mo after transplantation. The most frequent severe

infections occurred were abdominal abscesses, bacterial pneumonia, invasive candidiasis, *Pneumocystis jirovecii* pneumonia, and symptomatic cytomegalovirus infection<sup>[5]</sup>. In a 10-year Swiss single-centre study<sup>[6]</sup>, 80% of patients developed an infection following transplantation. Almost half of these were bacterial with an infection rate that peaked during the first month following transplantation. The temporal relation between graft rejection and the occurrence of infection showed that the incidences of both viral and bacterial infections were increased by immunosuppressive supplementation. The overall incidence of bacterial infections was much higher during the first month following transplantation than after 1 mo in this study; 188.2 vs 5.8 episodes per 1000 patient-days<sup>[6]</sup>.

Patterns of opportunistic bacterial infections after transplantation have been altered by routine antimicrobial prophylaxis for *Pneumocystis jirovecii* (trimethoprim-sulfamethoxazole prophylaxis for as little as 3 mo or for as long as a lifetime), and by infections due to organisms with antimicrobial resistance. Infections occur in a generally predictable pattern after solid-organ transplantation and most can be grouped into three major periods: the early period posttransplant (up to 6 wk), an intermediate period (1 to 6 mo); and a late period (more than 6 mo)<sup>[2]</sup>.

Infections occurring immediately post-transplantation are similar to those seen in post-operative immunocompetent hosts. Bacterial infections predominate; they usually have a nosocomial source, such as central vascular access sites, external drainage catheters or are related to foreign bodies, necrotic tissue, or prolonged endobronchial intubation. Abdominal abscesses and peritonitis, intrahepatic abscesses (often associated with hepatic artery thrombosis), cholangitis, wound infections, nosocomial pneumonias (in patients who require prolonged ventilation) are common during this time frame. Patients may become infected with nosocomial, antimicrobial-resistant bacteria, including methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus faecalis*, *Clostridium difficile*, and antimicrobial-resistant gram-negative bacteria.

During the intermediate period, the nature of common infections changes and patients are most at risk for the development of opportunistic infections due to the cumulative effect of relatively high-dose immunosuppression, although residual problems from the perioperative period can persist. Viral pathogens and allograft rejection are responsible for the majority of febrile episodes that occur during the period from 1 to 6 mo after transplantation<sup>[2]</sup>.

Opportunistic infections are uncommon after 6 mo in patients receiving stable and reduced maintenance doses of immunosuppression with good graft function, and little is documented about them in the literature. Potential etiologies of infection in these patients are diverse, including common, community-acquired bacterial infections seen in the general population, and uncommon opportunistic infections of clinical significance only in immunocompromised hosts. Respiratory infections due to capsulated pathogens,

such as *Streptococcus pneumoniae* and *Haemophilus influenzae*, can be life-threatening if not promptly treated in this population. In addition, transplant recipients may be more susceptible to certain bacterial pathogens, such as the *Nocardia* species, *Legionella* species, *Listeria monocytogenes*, *Mycoplasma* species, *Salmonella* species *Bartonella* species, or *Rhodococcus equi*. These late bacterial infections may be very difficult to recognize and treat in this population. Little information is available on the opportunistic late bacterial infections in liver transplant recipients. This review will focus on the risk, recognition and management of specific late bacterial infections, which are anticipated to occur more than 6 mo after liver transplantation.

## SPECIAL INFECTION CHARACTERISTICS OF THE LIVER TRANSPLANT PATIENT

In general, it is more difficult to recognize infection in transplant recipients than it is in patients with normal immune function. Inflammatory responses associated with infection may be impaired by immunosuppressive therapy, and signs and symptoms of infection may often be diminished. Furthermore, noninfectious causes of fever, such as allograft rejection, may develop in these patients. Because of the immunosuppressed state of this population, they are susceptible to a broad range of opportunistic infections, which frequently present with multi-organ system involvement and progress rapidly. In addition, altered anatomy following transplant surgery may alter the physical signs of infection.

Cytomegalovirus infection can be documented in more than half of patient populations after organ transplantation<sup>[7]</sup>; with viral replication persisting long term. It is known that cytomegalovirus can affect the capacity of the host to mount a defense against complicating infections. Cytomegalovirus infection was associated with a 2.45-fold higher incidence of major infections between day 30 and 180 after orthotopic liver transplantation in a study, with most of these infections caused by gram-positive cocci<sup>[8]</sup>.

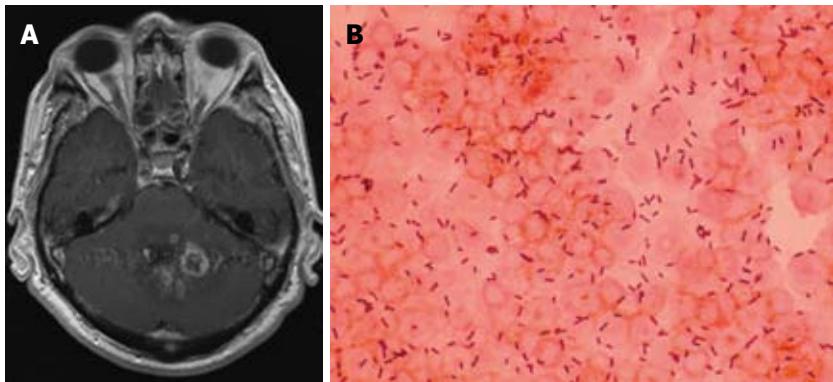
Serologic testing is not generally useful for diagnosis since seroconversion is often delayed in these patients (antigen-based tests or nucleic acid-based molecular assays are recommended for diagnosing in this population). In the same way, tissue biopsies with histopathology and microbiology are often needed to make an early and specific microbiologic diagnosis.

The choice of antimicrobial regimens is often more complex than in other patients due to increased antimicrobial resistance, urgency of therapy and the high frequency of drug related toxicities and interactions with immunosuppressant drugs.

## SPECIFIC LATE BACTERIAL INFECTIONS

### *Listeria monocytogenes* infection in the liver transplant patient

Listeriosis is a clinical condition occurring with an annual



**Figure 1** A: Gadolinium enhanced brain MRI demonstrating multiple ring-enhancing lesions, compatible with abscesses, located in the cerebellum; B: Gram stain of the spinal fluid of the same patient showing gram-positive rods. *Listeria monocytogenes* was isolated in spinal fluid culture.

incidence of 4.4/million individuals in the US and an associated mortality of approximately 20%<sup>[9]</sup>. *Listeria monocytogenes* has long been known as a pathogen of immunocompromised hosts (Figure 1). However, *Listeria* infections have only rarely been reported following orthotopic liver transplantation. The low incidence may be due, in part, to the prophylactic use of trimethoprim-sulfamethoxazole for *Pneumocystis jiroveci* pneumonia for up to 1 year following liver transplantation. Listeriosis occurs rarely after this 1 year period, so that further use of prophylactic antibiotics against this infection seems unjustified according to some authors<sup>[10]</sup>. The route of infection is usually through the intestinal tract following ingestion of contaminated food products, as well as from mother to child either transplacentally or during childbirth. The importance of dietary and other environmental exposures cannot be underestimated<sup>[11]</sup>. Most reported cases of listeriosis occurred months to years following liver transplantation, but it may also occur in the early postoperative period<sup>[12]</sup>. Its principal manifestations include bacteremia<sup>[10,13]</sup> and meningitis<sup>[14,15]</sup>, although endocarditis<sup>[16]</sup>, peritonitis<sup>[17]</sup>, hepatitis<sup>[18,19]</sup>, epididymitis or orchitis<sup>[20]</sup> have been reported. *Listeria* meningitis can be associated with profound changes in mental status or focal neurologic signs from involvement of the brain stem (rhombencephalitis) or even presence of a brain abscess<sup>[15]</sup>. Clinically, bacteremia is almost always present and is usually instrumental in getting the diagnosis. Treatment with antibiotics including ampicillin or trimethoprim-sulfamethoxazole with or without an aminoglycoside has proven successful in the majority of the cases.

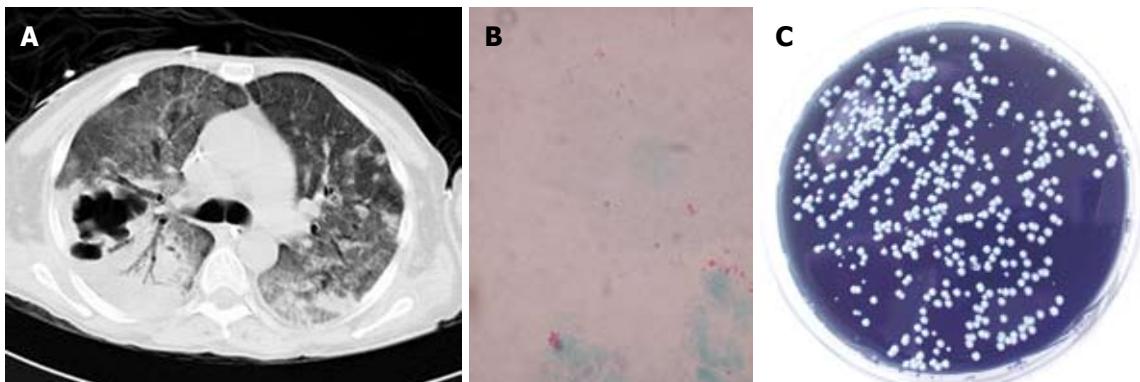
Five cases of listeriosis following liver transplantation have been reported<sup>[14]</sup>, four within 3 wk and one within 4 mo following transplantation. Rettally *et al*<sup>[10]</sup> reported a case of a patient presenting with *L. monocytogenes* bacteremia at 32 mo following orthotopic liver transplantation. The patient was successfully treated with 3 wk of intravenous treatment with ampicillin and vancomycin. In the case reported by Avery *et al*<sup>[16]</sup>, the patient was receiving trimethoprim-sulfamethoxazole prophylaxis for *P. jiroveci* pneumonia until 7 mo post-transplant, at which time aerosolized pentamidine was substituted because of the concern about possible drug hepatotoxicity. Three months later, the patient was diagnosed with a tricuspid valve *L. monocytogenes*

endocarditis. The patient was successfully treated with a total of 6 wk of ampicillin followed by penicillin therapy, with gentamicin administered for the first 4 wk of this course. Doses of her immunosuppressive medications were decreased. Bourgeois *et al*<sup>[18]</sup> reported the case of a liver transplant recipient who was diagnosed with *L. monocytogenes* acute hepatitis 8 mo after grafting. The patient made a full clinical recovery after therapy with ampicillin and gentamicin intravenously for 4 wk.

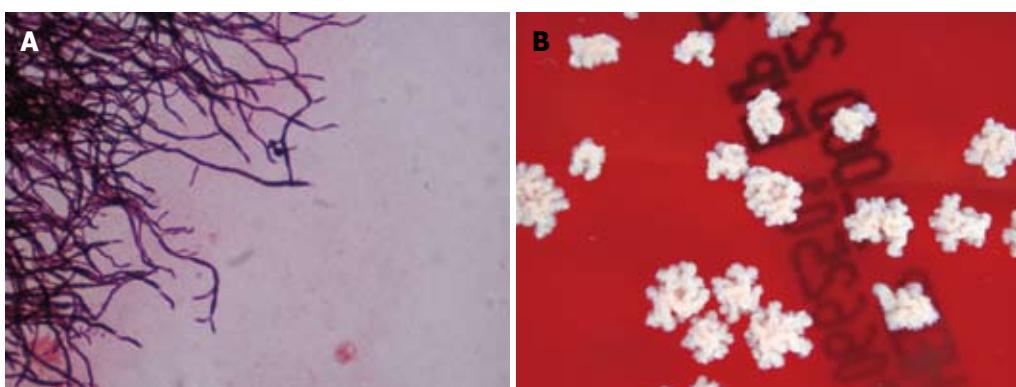
#### ***Legionella* species infection in the liver transplant patient**

*Legionella* species account for approximately 5% of cases of pneumonia in the general US population. Although more than 30 species have been described in the *Legionellae* family, 70% to 90% of infections are caused by *L. pneumophila*, particularly serogroups 1 and 6, followed by *L. micdadei*<sup>[21]</sup>. Aquatic habitats are considered the environmental reservoir for community and nosocomial acquired pneumonia<sup>[22]</sup>. Susceptible hosts include the elderly, cigarette smokers, individuals receiving immunosuppressive therapy, and organ transplant recipients<sup>[23]</sup>. Although community acquisition occurs, nosocomial *L. pneumonia* is more commonly reported in transplant patients (Figure 2). In solid organ recipients, infection often occurs early in the post-transplantation period or coincidentally with corticosteroid therapy for allograft rejection<sup>[22]</sup>. The radiographic findings of *L. pneumonia* in immunosuppressed patients can vary and include unilateral or bilateral dense, patchy, or nodular pulmonary infiltrates that can progress to cavitation.

The first case of infection with *Legionella* species in a liver transplant recipient was reported by Tokunaga *et al*<sup>[24]</sup> in 1992. *L. pneumophila*, serogroup 1, was identified by direct immunofluorescence in the lung and liver graft from a 2-mo-old infant who underwent orthotopic liver transplantation because of fulminant hepatic failure secondary to neonatal hepatitis. The patient died of respiratory failure due to this infection 22 d after transplantation despite treatment with erythromycin. Singh *et al*<sup>[25]</sup> described 3 adult liver transplant patients with evidence for Legionellosis acquired within 2 mo of surgical transplantation, including one case of *L. bozemanii* pneumonia, one case of *L. pneumophila* pericarditis, and one case of *L. pneumophila* pneumonia. In addition, the third case also had *L. micdadei* isolated from



**Figure 2** **A:** Chest CT scan showing multilobar pneumonic consolidation with cavitary lesions; **B:** Gimenez stain of a bronchoalveolar lavage specimen of the same patient showing small red coccobacilli; **C:** *Legionella pneumophila* was cultured on selective buffered charcoal yeast extract agar.



**Figure 3** **A:** Gram stained smear of a subcutaneous lesion aspirate demonstrating branching and beaded filamentous appearance gram-positive rods; **B:** *Nocardia asteroides* was isolated in culture.

pleural fluid. All three cases responded to a combination of erythromycin and ciprofloxacin. A *L. pneumonia* nosocomial outbreak after liver transplantation has been described<sup>[26]</sup>. Patients presented with fever, nonspecific constitutional symptoms, and either unilateral or bilateral infiltrates developing 3 wk to 12 wk after transplantation. Treatment with either erythromycin (four cases) or ciprofloxacin (one case) was successful in three of five patients. A hospital-acquired pneumonia due to *L. parisiensis* was reported by Presti *et al*<sup>[27]</sup> in 1997. The diagnosis was made on the basis of clinical and radiological signs of pneumopathy in a liver transplant patient and was confirmed by microbiological data. The patient was successfully treated with erythromycin for 3 wk, allowing resolution of the pneumonia. Ernst *et al*<sup>[28]</sup> reported a liver transplant recipient with community acquired *L. midiae* pneumonia 8 years after transplantation. The patient was successfully treated with a 3-wk course of ciprofloxacin. According to the authors, the predisposition in this case might in part be attributed to the need for a course of heightened immunosuppressive agents to treat an episode of acute organ rejection. Fraser *et al*<sup>[29]</sup> reported a liver transplant recipient who presented with cavitary pneumonia caused by *L. pneumophila* 7 years after transplantation. The patient required surgical resection and a 21-d course of therapy with levofloxacin for diagnosis and cure.

*Legionella* pneumonia is a rare complication of orthotopic liver transplantation in adults, and it remains a significant cause of morbidity and mortality in the susceptible host. Definitive diagnosis of *Legionella* pneumonia can be particularly elusive. As the urinary *Legionella* antigen is negative when other species or serogroups different than *L. pneumophila* serogroup 1 are implicated, examination of respiratory secretions in combination with immunofluorescent staining or specialized culture is required to establish the diagnosis. Erythromycin, rifampin, trimethoprim-sulfamethoxazole, doxycycline, clindamycin, and quinolones, alone or in combination, for 3 wk are effective treatments. Due to the risk of undesirable drug interaction of erythromycin with tacrolimus or cyclosporine, fluoroquinolone agents such as levofloxacin or ciprofloxacin may be the preferred treatment for legionellosis in transplant recipients.

#### ***Nocardia* species infection in the liver transplant patient**

*Nocardia* species are ubiquitous environmental saprophytes, living in soil, organic matter and water<sup>[30]</sup>. There are at least 12 species with *N. asteroides* complex (*N. asteroides* sensu strictu, *N. farcinica* and *N. nora*), with *N. brasiliensis*, *N. otitidiscavarium* and *N. transvaliensis*<sup>[30]</sup> being the most important ones. *Nocardia* species are Gram-positive, variably acid-fast, actinomycetes with branching rods (Figure 3). The frequency of nocardial

infections in solid organ transplant recipients varies between 0.7% and 3% and has mostly been reported in heart, kidney and liver transplant recipients, and less frequently in lung transplantation<sup>[31]</sup>. In a recent study involving 1840 liver transplant recipients, only 2 (0.1%) had a Nocardia infection and only 1 of them had a disseminated infection consisting of bacteremia<sup>[31]</sup>. Disseminated nocardiosis is more frequent in cellular immunocompromised patients, and it is often a potentially life-threatening infection. It is endogenous (i.e., secondary to bloodstream spread) from a primary pulmonary infection<sup>[30]</sup>. However, it may result very rarely from a primary nonpulmonary (e.g. cutaneous, gastrointestinal) infection. The brain is the most frequent nonpulmonary site involved in disseminated nocardiosis<sup>[32]</sup>. A *N. farrinica* disseminated (subcutaneous and neural) infection has been described in a liver transplant recipient<sup>[33]</sup>. The patient presented in postoperative mo 3 with fever and pain in the lower leg. Ten days after admission, the patient developed neurological symptoms and 3 brain abscesses were detected in an MRI.

A case of *N. asteroides* osteomyelitis of the tibia accompanied by brain abscesses in a liver transplant recipient was recently described<sup>[34]</sup>. The majority of bone cases are presumed to be due to direct extension from local soft tissue infection. Imaging of the brain should be performed in all cases of nocardiosis affecting a liver transplant recipient, since central nervous system involvement may be present even in the absence of neurological findings. Pulmonary involvement should also be excluded.

An optimal therapeutic approach has not been well established, especially in the disseminated forms. It is debated whether invasive resection or aspiration of the CNS lesions are necessary<sup>[2,35,36]</sup>. Some authors recommend an early biopsy of the lesions to achieve specific identification, even in cases where an extra-cranial focus of infection is found<sup>[37]</sup>. However, there are some reports that suggest that brain surgery is not always needed<sup>[35,36]</sup>. Sulfonamides are the drugs of choice for treatment of nocardiosis; however, there are an increasing number of reports of resistance to these agents<sup>[38]</sup>. The length of therapy in the treatment of nocardial infections in the transplant patient is debated. Some authors suggest extending it for 9 mo to 12 mo if there is CNS involvement<sup>[37]</sup>. The clinical use of other drugs should be supported by susceptibility testing. Linezolid is an oxazolidinone with activity against all of the clinically relevant species of Nocardia<sup>[39]</sup>, which has been shown to be an effective alternative for the treatment of nocardiosis, especially for the CNS forms<sup>[34]</sup>. Lewis *et al* described a case in which minocycline was used for 12 mo with a good response. Doxycycline and minocycline<sup>[34,40]</sup> may be attractive options as rescue therapy in those patients in which other treatments failed.

### Rhodococcus equi infection in the liver transplant patient

*Rhodococcus equi* is an intracellular gram-positive,

aerobic, nonmotile, non-spore-forming bacillus that is an increasingly important opportunistic respiratory pathogen in immunocompromised hosts. Impairment of cell-mediated immunity is the most important risk factor for infection with *R. equi* in humans<sup>[41]</sup>. Several cases have been described in patients with immunosuppression due to either human immunodeficiency infection, therapy for neoplastic disorders, or kidney and heart transplantation. 15 patients with a *R. equi* infection following solid-organ transplantation have been described in the literature<sup>[42-45]</sup>. In all patients, rhodococcal infection presented late after transplantation (median, 4 years). *R. equi* resides in the soil and humans acquire the infection through the respiratory tract. Exposure to farm animals was reported in only two cases; in four cases contact with contaminated soil or manure was considered a likely infectious source. Thirteen of 15 patients had pulmonary and/or pleural involvement. Soft-tissue infections were common in this population. Most patients received erythromycin and/or ciprofloxacin in combination therapy, which was generally associated with a successful outcome. Recurrent disease was observed after initial treatment with imipenem and tobramycin and in patients receiving monotherapy.

Sabater *et al*<sup>[43]</sup> reported the first case of *R. equi* infection in a liver transplant recipient. The patient was diagnosed with two subcutaneous abscesses on his left arm and an asymptomatic necrotizing bilateral pneumonia 28 mo following transplantation. The patient had been intensively immunosuppressed and working with calf manure. He was successfully treated with a combination of erythromycin, ciprofloxacin, and rifampicin. A case of vertebral osteomyelitis due to *R. equi* in a liver transplant recipient 7 mo after transplantation was reported by Fischer *et al*<sup>[42]</sup>. The patient was initially diagnosed with a recurrent pneumonia and a pleura-based lung abscess and subsequently developed osteomyelitis of the lower thoracic spine. Drainage of the paraspinal abscess and removal of the infected bone was performed while he was being treated with erythromycin and imipenem. Postoperatively, the patient received intravenous imipenem, vancomycin, and erythromycin for 14 d and then oral clarithromycin and rifabutin. Schilz *et al*<sup>[44]</sup> reported a case of a liver transplant patient with a pulmonary nodule caused by *R. equi* that followed a benign clinical course and resolved spontaneously.

Although solid-organ transplant recipients are rarely affected, *R. equi* must be included in the differential diagnosis of recurrent pneumonia or lung abscess in liver transplant recipients. Metastatic spread of infection is common, and diagnosis of pulmonary rhodococcal infection should prompt a search for additional involvement of extra-pulmonary sites. Monotherapy must be considered insufficient for transplant patients and surgery may be helpful in some cases.

### Other late bacterial infections in the liver transplant patient

*Bartonella benselae*, the etiologic agent of cat-scratch

disease, may cause a spectrum of illness in both immunocompetent and immunocompromised hosts. Disseminated infections caused by *Bartonella* species have been reported infrequently in solid organ transplant recipients. Humar *et al*<sup>[46]</sup> reported a case of a liver transplant recipient who presented 4 years later with fever of unknown origin, and was found to have a granulomatous hepatitis of the transplant allograft with a *Bartonella* species. Diagnosis was performed by serology (immunofluorescent antibody test). The patient completed a successful 4 wk course of azithromycin.

Apalsch *et al*<sup>[47]</sup> described a case of a disseminated cat-scratch disease in a 12-year-old liver transplant recipient with fever and lymphadenopathy. Granulomatous inflammation was shown in liver and lymph node biopsy specimens.

Because this organism may be difficult to grow in culture, serological and molecular tests are important for diagnosis. Antimicrobial therapy can result in prompt resolution of illness and usually consists of a macrolide or tetracycline derivative. However, the optimal antibiotic and duration of therapy for disseminated disease in immunocompromised patients is unknown.

Three cases of *Mycoplasma hominis* infections have been reported in the literature. Jacobs *et al*<sup>[48]</sup> reported the case of a recipient of liver transplantation with a postsurgical infection and Vogel *et al*<sup>[49]</sup> reported two patients with extragenital infections.

*Clostridium difficile* remains a significant complication in liver transplant recipients. In this patient population, *C. difficile* diarrhea may be more difficult to diagnose and may have a more complicated clinical course than in non-immunocompromised patients. Although the prevalence of *C. difficile* infection is usually the highest during the early post-transplant period, when immunosuppression is the highest, Albright *et al*<sup>[50]</sup> described 3 cases of late onset *C. difficile* infection (2, 3 and 5 years after transplantation). Nevertheless, the prognosis is good in most cases with timely diagnosis and treatment.

## CONCLUSION

In summary, late opportunistic bacterial infections following liver transplantation are rare. Trimethoprim-sulfamethoxazole taken daily or three times weekly in patients without an allergy to sulfonamides for the first 4 to 12 mo posttransplant primarily reduce the risk of *P. jiroveci* pneumonia and probably also helps to prevent infections with *L. monocytogenes*, *N. asteroides*, or *Toxoplasma gondii*. Early detection and treatment of these late bacterial infections is the key to obtaining a better prognosis in liver transplant patients.

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EDITORIAL

## Calcium signaling and T-type calcium channels in cancer cell cycling

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### Abstract

Regulation of intracellular calcium is an important signaling mechanism for cell proliferation in both normal and cancerous cells. In normal epithelial cells, free calcium concentration is essential for cells to enter and accomplish the S phase and the M phase of the cell cycle. In contrast, cancerous cells can pass these phases of the cell cycle with much lower cytoplasmic free calcium concentrations, indicating an alternative mechanism has developed for fulfilling the intracellular calcium requirement for an increased rate of DNA synthesis and mitosis of fast replicating cancerous cells. The detailed mechanism underlying the altered calcium loading pathway remains unclear; however, there is a growing body of evidence that suggests the T-type  $\text{Ca}^{2+}$  channel is abnormally expressed in cancerous cells and that blockade of these channels may reduce cell proliferation in addition to inducing apoptosis. Recent studies also show that the expression of T-type  $\text{Ca}^{2+}$  channels in breast cancer cells is proliferation state dependent, i.e. the channels are expressed at higher levels during the fast-replication period, and once the cells are in a non-proliferation state, expression of this channel is

minimal. Therefore, selectively blocking calcium entry into cancerous cells may be a valuable approach for preventing tumor growth. Since T-type  $\text{Ca}^{2+}$  channels are not expressed in epithelial cells, selective T-type  $\text{Ca}^{2+}$  channel blockers may be useful in the treatment of certain types of cancers.

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**Key words:** T-type calcium channels; Cancer; Cell cycle; Calcium

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### INTRODUCTION

Calcium is an essential signal transduction element involved in the regulation of many eukaryotic cellular functions including cell cycle progression<sup>[1]</sup>. Control of intracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]$ ) is crucial for the orderly progression of the cell cycle and plays a vital role in the regulation of cell proliferation and growth<sup>[2]</sup>; however, excessive calcium or loss of control in calcium signaling can lead to cell death<sup>[3]</sup>. Therefore, careful control of calcium signaling is required for cell survival. Upon stimulation, the intracellular calcium concentration can increase dramatically, often reaching micromolar amounts. This increase in cytoplasmic calcium can occur via release from intracellular stores or influx through a variety of plasma membrane ion channels. Voltage-gated and ligand-gated  $\text{Ca}^{2+}$  channels in the plasma membrane, along with ryanodine receptors (RyR) and inositol triphosphate receptors (IP3R) at the intracellular calcium stores, provide fluxes of  $\text{Ca}^{2+}$  to the cytoplasm.

The driving force for calcium entry is the result of an electrochemical gradient between the extracellular concentration ( $1.3 \times 10^{-3}$ - $2 \times 10^{-3}$  mol/L) of calcium and the intracellular concentration (<  $10^{-8}$  mol/L).

In general, non-excitable tissues, including the epithelium, do not express voltage gated  $\text{Ca}^{2+}$  channels. This is partly because the ranges of membrane potential changes in these cells are too small to activate these channels. However, recent studies show that T-type  $\text{Ca}^{2+}$  channels are expressed in cancerous cells, although their functional role has only begun to be investigated. Furthermore, there is a growing body of evidence suggesting that tumor cell proliferation can be halted by the use of ion channel blockers. T-type calcium channels are a class of calcium permeable low voltage activated (LVA) ion channels which open after small depolarizations of the membrane. Molecular biology has revealed the existence of three different T-type calcium channel subunits, the  $\alpha_{1G}$ ,  $\alpha_{1H}$  and  $\alpha_{1I}$ . The  $\alpha_1$  designation refers to the channels primary ion conducting protein, which consists of four domains each containing six transmembrane segments. There are other auxiliary calcium channel subunits; however, the LVA alpha1 subunits can function as stand alone complexes. The unique low voltage dependent activation/inactivation and slow deactivation of T-type  $\text{Ca}^{2+}$  channels indicate that these channels may play a physiological role in carrying depolarizing current at low membrane potentials. Therefore, these channels may play a direct role in regulating  $[\text{Ca}^{2+}]_i$ , especially in non-excitable tissues, including some cancerous cells. At low voltages, T-type  $\text{Ca}^{2+}$  channels are known to mediate a phenomenon known as "window current"<sup>[4-6]</sup>. The term "window" refers to the voltage overlap between the activation and steady state inactivation at low or resting membrane potentials. As a result, there is a sustained inward calcium current carried by a small portion of channels that are not completely inactivated. Window current allows T-type  $\text{Ca}^{2+}$  channels to regulate  $\text{Ca}^{2+}$  homeostasis under non-stimulated or resting membrane conditions<sup>[7]</sup>. The most direct evidence of T-type  $\text{Ca}^{2+}$  channel mediated  $\text{Ca}^{2+}$  window current is from a study conducted in HEK-293 cells expressing the T-type isoform  $\alpha_{1G}$ <sup>[8]</sup>, which demonstrated window current peaked at -48 mV. Membrane potentials around this voltage can occur in un-stimulated non-excitable cells.

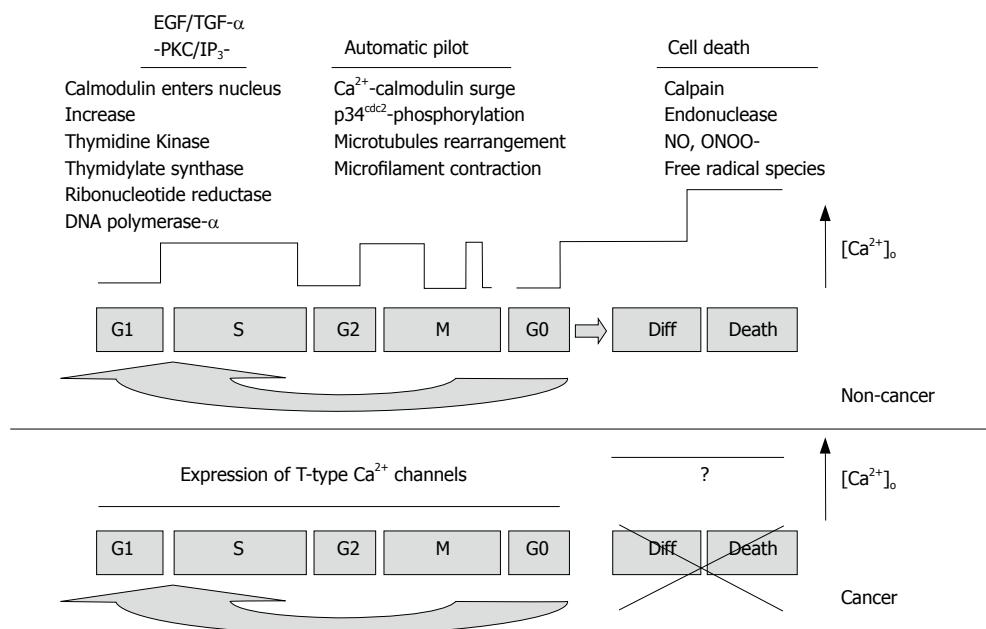
## CALCIUM SIGNALING AND CELL CYCLING

As shown in Figure 1, the cell cycle is divided into four stages: G1, S, G2 and M. DNA replication occurs in the S phase and mitosis occurs in the M phase. Cells must pass through a restriction point between the G1 and S phases before continuing proliferation; otherwise, they exit the cell cycle to G0 and differentiate or terminate. Another checkpoint in the cell cycle is between phases G2 and M. For cells to pass through these various points, one of the most prominent messengers is  $\text{Ca}^{2+}$ , as demonstrated by the induction of mitotic events by

injection of exogenous  $\text{Ca}^{2+}$  in a fertilized egg model<sup>[9]</sup>. Steinhardt *et al*<sup>[10]</sup> observed transient increases in cytosolic  $\text{Ca}^{2+}$  during late G1, prior to the initiation of the S phase and during G2 before entry into the M phase that were dependent upon external physiological  $\text{Ca}^{2+}$  concentration. In the transition from G1 to S phase, cells require external  $\text{Ca}^{2+}$  in addition to functional calcium channels in order to directly or indirectly trigger a myriad of critical downstream enzymes such as thymidine kinase, thymidylate synthase, ribonucleotide reductase and DNA polymerase and begin DNA replication. In the transition from G2 to M phase,  $\text{Ca}^{2+}$  flashes activate enzymes that are critical for microtubule rearrangement and microfilament contraction. In order to confirm the significant role that  $\text{Ca}^{2+}$  plays in the cell cycle, researchers have blocked the progression of the cell cycle *via* injection of  $\text{Ca}^{2+}$  chelators into the same fertilized eggs<sup>[11]</sup>. The influence of  $\text{Ca}^{2+}$  channels on cell growth is clearly demonstrated in pharmacological studies using  $\text{Ca}^{2+}$  channel antagonists. In a study by Zeitler *et al*<sup>[12]</sup>, various  $\text{Ca}^{2+}$  channel blockers, including verapamil, nifedipine, diltiazem and isradipine, caused G0/G1 cell-cycle arrest in growth factor induced human umbilical arterial endothelial cells (HUAEC) during proliferation. The  $\text{Ca}^{2+}$  signal has also been linked to activation of immediate early genes (e.g. *c-fos*) that are responsible for inducing resting cells in G0 to re-enter the cell cycle, an attribute most frequently up-regulated in rapidly proliferating cells<sup>[10]</sup>.

At the end of the cycle, cells can undergo suicide through a process known as apoptosis or active cell death, which is a genetic program specifically designed to shape organs during development and adjust cell population levels to appropriate values. The key players of apoptosis are a killer  $\text{Ca}^{2+}$  surge and the nuclear membrane  $\text{Ca}^{2+}$  activated endonuclease, which terminates the cell by cutting chromatin into fragments (Figure 1). Underlying mechanisms for  $\text{Ca}^{2+}$  mediated effects in cell proliferation may involve a wide variety of other intracellular signal transduction pathways such as G-proteins, protein kinase C (PKC), calmodulin, m-calpain, MAP kinase, phospholipase A2 and others<sup>[13,14]</sup>. Although the details of each pathway is beyond the scope of this discussion, there are several notable mechanisms that act to amplify  $[\text{Ca}^{2+}]_i$  for activation of gene transcription or cell migration. One mechanism is the hydrolysis of inositol lipids by the enzyme phospholipase C, the activation of which is itself dependent on an initial rise in  $[\text{Ca}^{2+}]_i$ , producing diacylglycerol (DAG) and InsP3. Resulting from the activation of G protein-linked or tyrosine-kinase linked receptors<sup>[15]</sup>, InsP3 thus causes a form of  $\text{Ca}^{2+}$  dependent  $\text{Ca}^{2+}$  release from intracellular stores. Described as "calcium puffs", which propagate into a local or global  $\text{Ca}^{2+}$  signal, this  $\text{Ca}^{2+}$  release is important for converting the cytoplasm into an excitable medium that can support repetitive  $\text{Ca}^{2+}$  oscillations<sup>[16]</sup>. The resulting amplification of  $[\text{Ca}^{2+}]_i$  contributes to the signal for mitosis and DNA synthesis.

In addition to InsP3, sensory proteins also play



**Figure 1**  $\text{Ca}^{2+}$  signaling pathways differ between cancerous and non-cancerous cells (adapted from <sup>[23]</sup>).

a role in maintaining the calcium signaling system. Calmodulin is a  $\text{Ca}^{2+}$  binding protein that acts as a  $\text{Ca}^{2+}$  sensor in the cell cycle. High expression of calmodulin has been observed during the S phase and mitosis, while inhibition of its activity by administration of calmodulin monoclonal antibodies is shown to block DNA synthesis<sup>[17]</sup>. Another sensory mechanism occurs through an extracellular calcium ion concentration sensing receptor (CaR) and calbindin, a high affinity  $\text{Ca}^{2+}$ -binding regulatory protein belonging to the same family as calmodulin. Parkash *et al* observed that CaR plays a role in sensing and responding to changes in extracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_o$ ). Upon activation by increased  $[\text{Ca}^{2+}]_o$ , CaR interacts with phospholipase C (PLC) via G proteins to produce DAG and InsP3<sup>[18]</sup>. The subsequent increase in  $[\text{Ca}^{2+}]_i$  is regulated by calbindin, which was previously found to bind to L-type HVA  $\text{Ca}^{2+}$  channels in pancreatic islets-cells<sup>[19]</sup>. It has also been shown that calbindin/CaR co-localization occurs in the estrogen receptor positive breast cancer cell line MCF-7<sup>[20]</sup>. Activated CaR also increases parathyroid hormone related protein (PTHrP), which appears to exacerbate cell metastasis in MCF-7 cells<sup>[21]</sup>. A study by Lewalle *et al*<sup>[22]</sup> shows that an increase in  $[\text{Ca}^{2+}]_i$  mediates tumor cell transendothelial migration *in vitro*. By associating the upregulation of these mechanisms in cancerous cells, increases in  $[\text{Ca}^{2+}]_i$  are shown to provide an important and pronounced signal for cell growth.

Calcium signaling in cancerous cells, however, uses an altered pathway during cell cycling<sup>[23]</sup>. Whitfield has shown that colon carcinomas undergoing carcinogenesis, which have lost their tumor-suppressing genes, have dramatically altered calcium signal mechanisms, and have ignored normal calcium-dependent restrictions by overproducing calcium-binding signal proteins (Figure 1)<sup>[18]</sup>. Attempts to terminate the mutant cells with  $\text{Ca}^{2+}$  signal

surges are futile, as the cells are no longer responsive to  $\text{Ca}^{2+}$  signals: instead, they produce and respond to their own renegade growth factors.  $\text{Ca}^{2+}$  and the signaling enzymes that are directly activated by  $\text{Ca}^{2+}$  or by  $\text{Ca}^{2+}$ -binding proteins play crucial roles in most cell signals and programs and must be understood and implemented in any future differentiation therapies. Since cancerous cells express T-type  $\text{Ca}^{2+}$  channels, it is possible that these channels provide an altered  $\text{Ca}^{2+}$  influx pathway in responding to the increasing demand of  $\text{Ca}^{2+}$  during rapid cell proliferation.

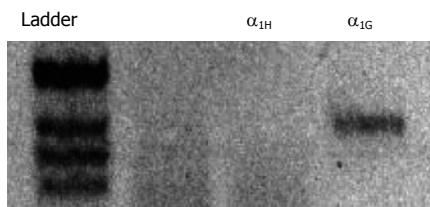
## T-TYPE CALCIUM CHANNELS IN CANCEROUS CELL PROLIFERATION

### *T-type $\text{Ca}^{2+}$ channels and non-cancerous cell cycling*

The function of regulating  $\text{Ca}^{2+}$  homeostasis may allow T-type  $\text{Ca}^{2+}$  channels to play an important role in controlling cell proliferation and differentiation in many tissues. In primary cultured rat aortic smooth muscle cells, a T-type  $\text{Ca}^{2+}$  current was found to be present in cells during the G1 and S phases but decreased or absent in all other phases of the cell cycle<sup>[24-26]</sup>. It was shown that cultured smooth muscle cells exhibited an increased T-type  $\text{Ca}^{2+}$  current during stages of proliferation and this current decreased as the cells became confluent or when they came into contact with one another<sup>[27]</sup>. T-type  $\text{Ca}^{2+}$  currents are also present in freshly dissociated or 1-2 d cultured neonatal rat ventricular myocytes when they are still able to proliferate, but are not observed in cells cultured greater than 3 d<sup>[28]</sup>. Likewise, older tissues under pathological conditions, such as cardiomyopathic hamster heart<sup>[29]</sup>, hypertrophied adult feline left ventricular myocytes<sup>[30]</sup>, and rat neointimal formation after vascular injury<sup>[31]</sup> have increased T-type  $\text{Ca}^{2+}$  current activity. These studies suggest that T-type calcium

**Table 1** Cancerous cells that expresses T-type  $\text{Ca}^{2+}$  channels

Cell type	Cell line	T-type isoform	Reference
Breast carcinoma	MCF-7, MDA-435	$\alpha_{1G}, \alpha_{1H}$	[33-36]
	MDA-231, MDA-361 MB-468, MB-474, BT-20, CAMA1, SKBR-3	$\alpha_{1G}$	[34-36]
Neuroblastoma	SK-N-SH, NG 108-15, SK-N-MC	$\alpha_{1G}$	[34,37-40]
	N1E-115	$\alpha_{1G}, \alpha_{1H}$	[37]
Retinoblastoma	Y-79, WERI-Rb1	$\alpha_{1G}, \alpha_{1H}, \alpha_{1I}$	[41,42]
Glioma	Primary (biopsy)	$\alpha_{1G}$	[36]
	U87-MG	$\alpha_{1G}, \alpha_{1H}$	[37]
Prostate carcinoma	TSU-PRL, DUPRO	$\alpha_{1G}$	[35,43]
	LNCaP	$\alpha_{1H}$	[1,34,35]
	PC-3, DU-145	$\alpha_{1G}, \alpha_{1H}$	[34,35]
Esophageal carcinoma	TE1, TE10, TE12, KYSE150, KYSE180, KYSE450	$\alpha_{1H}$	[44]
	SKGT4, TE3, TE7, KYSE70	$\alpha_{1G}, \alpha_{1H}$	[44]
	COLO-680N, SEG1, TE8, TE11, KYSE30, KYSE410, KYSE510	$\alpha_{1G}, \alpha_{1H}, \alpha_{1I}$	[44]
Fibrosarcoma	HT1080	$\alpha_{1G}$	[45]
Colorectal carcinoma	Caco2, DLD-1, Lovo, SW837	$\alpha_{1G}$	[35]
Pheochromocytoma	MPC 9/3L	$\alpha_{1G}$	[46]
	PC-12	$\alpha_{1H}$	[47]
Adenocarcinoma	H295R	$\alpha_{1H}$	[48]
Insulinoma	INS-1	$\alpha_{1G}$	[49]

**Figure 2** T-type  $\text{Ca}^{2+}$  channel expression in human malignant breast cancer tissue.

channels may play a vital role in regulating proliferation under specialized conditions.

#### **T-type $\text{Ca}^{2+}$ channels are broadly expressed in tumor cells**

If these channels do participate in proliferation under abnormal conditions, cells must first maintain control of the expression of  $\alpha_{1G}$  T-type  $\text{Ca}^{2+}$  channel messenger RNA in order to prevent functional expression of the protein. Otherwise, loss of this control may lead to aberrant cell growth and tumor progression. A recent study revealed the presence of T-type calcium channel mRNA expressed in breast tumor tissue that was removed from human biopsies (Figure 2)<sup>[32]</sup>. In this case, the tumor was later diagnosed as malignant and estrogen receptor positive by pathological examination. Expression of these channels in tumor cells has been reported broadly, as shown in Table 1<sup>[1,33-49]</sup>. For example, MCF-7 cells, a cell line derived from a human breast adenocarcinoma that has been shown to express  $\alpha_{1G}$  and  $\alpha_{1H}$  T-type  $\text{Ca}^{2+}$  channel mRNA and current transiently (Table 2)<sup>[33]</sup>. T-type  $\text{Ca}^{2+}$  channels have also been suggested as a potential therapeutic target for intracranial tumor and prostate cancer. Mibepradil was found to inhibit human astrocytoma (U87-MG) and neuroblastoma (N1E-115) proliferation and that over-expression of T-type  $\text{Ca}^{2+}$  channel protein doubled the proliferation rate while antisense treatment reduced

**Table 2** Q-RT-PCR detected T-type  $\text{Ca}^{2+}$  channels expression in non-confluent cultures of breast cancer cell lines

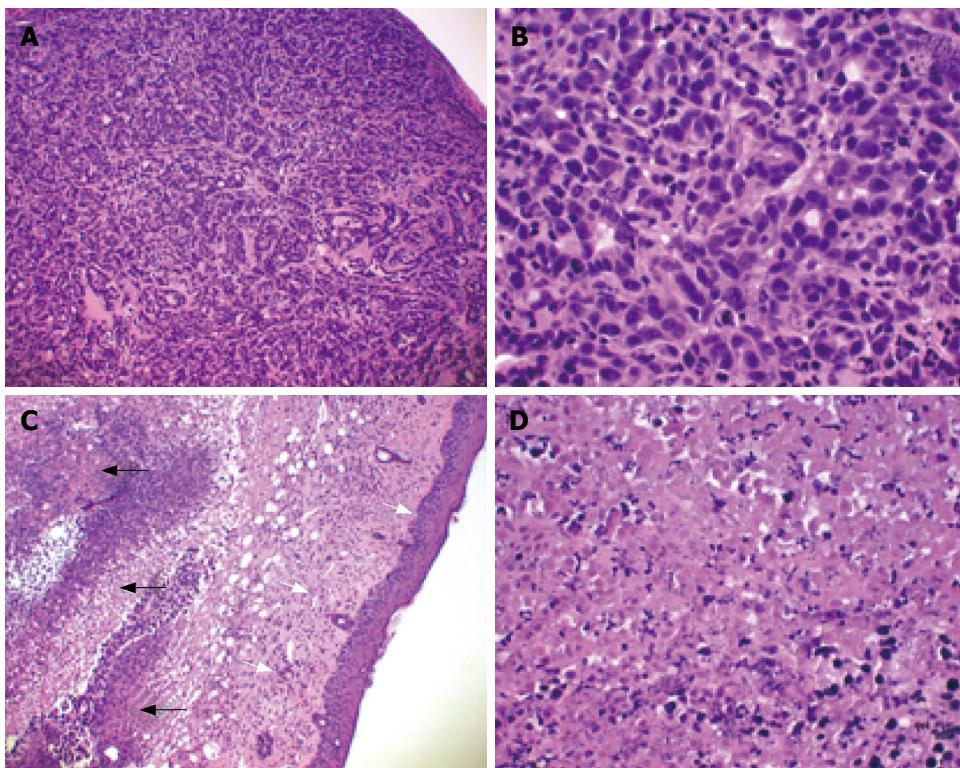
Cell types	T-channels	Non-confluent $\Delta\text{ct}$	Confluent $\Delta\text{ct}$
MDA-MB-231	$\alpha_{1H}$	14.28 ± 0.16	NA, ct > 40
MDA-MB-231	$\alpha_{1G}$	9.45 ± 0.87	NA, ct > 40
MCF-7	$\alpha_{1H}$	13.43 ± 0.24	NA, ct > 40
MCF-7	$\alpha_{1G}$	7.32 ± 0.3	NA, ct > 40

NA: Not applicable.

the proliferation rate of these cells<sup>[37]</sup>. Human prostate cancer epithelial cells (LNCap) have also been shown to express increased T-type  $\text{Ca}^{2+}$  channel ( $\alpha_{1H}$ ) current and mRNA. Similarly, increased T-type  $\text{Ca}^{2+}$  channel protein doubled proliferation while antisense treatment reduced the proliferation rate of these cells<sup>[1,43]</sup>. It was also shown that these channels were found to regulate intracellular calcium in LNCap cells. Another study examined the role of T-type  $\text{Ca}^{2+}$  channels in esophageal carcinoma cell proliferation; these data suggested that T-type  $\text{Ca}^{2+}$  channels may have a functional role in proliferation that can be reduced by inhibition of T-type  $\text{Ca}^{2+}$  channels<sup>[44]</sup>. Given the role that T-type calcium channels play in cell cycle progression and the relatively recent findings that show the functional expression of these channels in many different cancerous cell types, researchers have now been given the opportunity to investigate the potential of an entirely new target in the fight against cancer. Developing new compounds that target these proteins may hold the key to controlling certain types of cancer.

#### **EFFECT OF T-TYPE $\text{Ca}^{2+}$ CHANNEL BLOCKERS ON BREAST CANCER CELL PROLIFERATION**

The function of T-type  $\text{Ca}^{2+}$  channels with regards to



**Figure 3** Mibepradil destroys tumor cells implanted in a nude mouse. HE stain was applied to show the nuclei and cytoplasm of the cells (**A** and **C**,  $\times 100$ ; **B** and **D**,  $\times 400$ ).

tumor cell proliferation was also reviewed<sup>[50,51]</sup>. One study found the T-type  $\text{Ca}^{2+}$  channel to be particularly effective in controlling oscillations in intracellular  $\text{Ca}^{2+}$  as the result of the channels unique activation/inactivation properties. It was concluded that new selective antagonists may become helpful as a therapeutic approach against tumors in which proliferation depends on T-type  $\text{Ca}^{2+}$  channel expression<sup>[51]</sup>. A study performed in knockout animals found that selective inhibition of T-type  $\text{Ca}^{2+}$  channels may have impact upon the treatment of cancer<sup>[52]</sup>.

Studies have shown an inhibition in breast cancer proliferation by the channel blockers pimozide, thioridazine<sup>[53]</sup> and mibepradil<sup>[42]</sup>. The endogenous cannabinoid anandamide has also been shown to block T-type  $\text{Ca}^{2+}$  channels<sup>[54]</sup>, in addition to inhibition of breast cancer cell proliferation<sup>[55]</sup>, an effect that may be due to blockage of T-type  $\text{Ca}^{2+}$  channels.

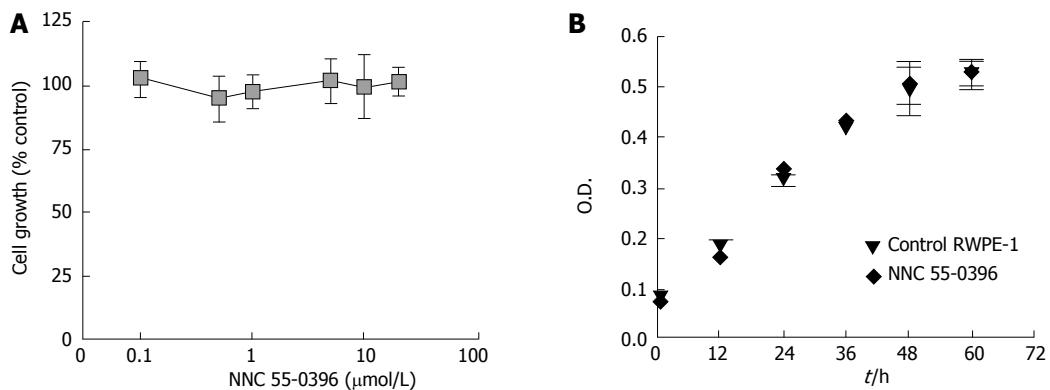
The anti-cancer effect of a T-type  $\text{Ca}^{2+}$  channel antagonists on tumor cells *in vivo* has been investigated<sup>[32]</sup>. MCF-7 cells were implanted into nude mice, athymic nude BSLB/c, and then either mibepradil (0.5 mg/100  $\mu\text{L}$ ) or saline (0.5 mg/100  $\mu\text{L}$ ) was injected locally at the tumor sites (s.c) twice a week. After 30 d of the treatment, mice were sacrificed and the tumors were removed for histochemistry examination.

As shown in Figure 3A and B, in the saline injected tissue the proliferation of the malignant tumor cells formed nodules in subcutis. The tumor cells were malignant as indicated by hyperchromatic nuclei with enlarged nuclei, irregular nuclear membrane, prominent nucleoli, and many mitotic features. No signs of degeneration and necrosis were detected. In contrast, the mibepradil injected tissue showed large areas of tumor degeneration and necrosis (Figure 3C and D). The

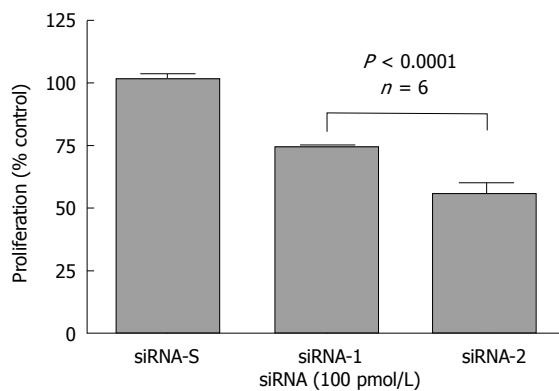
tumor necrosis was accompanied by prominent edema. Furthermore, as shown in Figure 3C, mibepradil more potently destroyed breast cancer cells (indicated by the black arrows) than non-cancerous cells at adjacent areas (indicated by the white arrows), including fibroblasts, endothelial cells and keratinocytes. These results indicate that a local injection of mibepradil induces necrosis of human breast carcinoma cells implanted into subcutaneous adipose tissue in mice.

More recently, the antiproliferative effect of the T-type calcium channel inhibitor NNC 55-0396<sup>[56]</sup> has been examined in cell lines derived from breast epithelial tissue, MCF-7, MDA-MB-231(ER- $\alpha$ ), and an adriamycin resistant cell line ADR. All three of these cell types express  $\alpha_{1G}$  and  $\alpha_{1H}$   $\text{Ca}^{2+}$  channel mRNA and their proliferation was suppressed by NNC 55-0396, with  $\text{IC}_{50}$  of about 1-2  $\mu\text{mol/L}$ <sup>[32,33]</sup>. The specificity of NNC 55-0396 antagonism on cancerous cell proliferation was investigated in a prostate epithelial cell line (RWPE-1) that does not express T-type  $\text{Ca}^{2+}$  channels<sup>[32]</sup>. As shown in Figure 4, NNC 55-0396 exhibited neither dose-dependent (up to 20  $\mu\text{mol/L}$  Figure 4A) nor time-dependent (up to 60 h, Figure 4B) inhibitory effects on RWPE-1 cell growth, suggesting that the anti-proliferation effect of NNC 55-0396 most likely resulted from blocking T-type  $\text{Ca}^{2+}$  channels of breast cancer cells. It also suggested that the general toxicity of NNC 55-0396 is minimal at the concentration that induces suppression of proliferation. T-type  $\text{Ca}^{2+}$  channel antisense treatment in these cells reduced the proliferation rate by 45% and antisense had no effect on proliferation on tumor cells not expressing T-type  $\text{Ca}^{2+}$  channels.

The role of T-type  $\text{Ca}^{2+}$  channels in cancerous cell proliferation has also been examined with specific siRNA



**Figure 4** Effect of NNC 55-0396 on RWPE-1 cell proliferation (Error bars represent SE;  $n = 3$ ).



**Figure 5** Effect of siRNAs on MCF-7 cell proliferation.

antagonism<sup>[33]</sup>. Specifically, MCF-7 cells were treated with siRNA targeting both  $\alpha_{1G}$  and  $\alpha_{1H}$  ( $\alpha_{1G/H}$ ). The cells were treated with scrambled (siRNA-S, 100 pmol/L),  $\alpha_{1G/H}$ -1 (siRNA-1) or  $\alpha_{1G/H}$ -2 (siRNA-2) for 48 h and subjected to MTT assay. The effects of siRNAs on cell proliferation were shown as percent (%) of vehicle control. As shown in Figure 5, scrambled siRNA was not significantly different than the control. However, both siRNA-1 and siRNA-2 treated cells had significantly lower proliferation rates compared to the scrambled and vehicle control siRNAs. These results strongly support the role of T-type  $\text{Ca}^{2+}$  channels in breast cancer cell proliferation and indicate that the effect of NNC 55-0396 on the breast cancer cell proliferation is due to the blockade of these channels.

## CONCERNs

Since T-type  $\text{Ca}^{2+}$  channels are normally expressed in the brain, heart and endocrine tissues of the human body, the potential side-effects of T-type  $\text{Ca}^{2+}$  channel blockers to these systems are of concern for therapeutic applications. Although T-type  $\text{Ca}^{2+}$  channel blockers have been used clinically for the treatment of neurological disorders (e.g. ethosuximide for absence seizures), the adverse effects of these drugs on the cardiovascular and central nervous systems are still unclear. Specifically, it is important to determine the possible arrhythmic and sedative effects of these drugs.

Human blood cells do not express T-type  $\text{Ca}^{2+}$

channels; therefore, it is advantageous to apply T-type  $\text{Ca}^{2+}$  channel blockers in the hemopoietic system, since current chemotherapeutic drugs have displayed severe side effects on this system. If we can locally deliver T-type  $\text{Ca}^{2+}$  channel blocker into the hemopoietic system, the compound should be very selective in eliminating the breast cancer cells in the blood stream. Thus, T-type  $\text{Ca}^{2+}$  channel blockers can be potential anti-metastasis drugs for adjuvant therapy of breast cancer.

## PERSPECTIVES

The function of T-type  $\text{Ca}^{2+}$  channels may not be restricted to cancerous cell proliferation. These channels may also play roles in cancerous cell colonization, invasion, secretion and angiogenesis. The growing number of proliferating cells need to attract blood vessels (angiogenesis) in order to receive nutrients,  $\text{O}_2$ , etc. to sustain themselves. The transformed cells are able to enter the blood stream and survive there, and colonize (metastasize) other tissues. Invasive growth or cell migration is a highly regulated process in which the migrating cells must secrete matrix proteases that disrupt the extracellular matrix (ECM) and permit easier transit through the surrounding environment. In addition, they must also profoundly reshape their structure, which involves massive cytoskeletal rearrangement. Precise regulation of intracellular calcium concentration is crucial for all of these processes. It is very possible that T-type  $\text{Ca}^{2+}$  channels also play significant roles in these processes<sup>[45]</sup>.

An expansion of the list of ion channels implicated in cancer development is expected, and the tools needed to investigate this issue are more readily available. As is the case with other protein families, it will be probably difficult to ascribe tumor development to the malfunction of a single ion channel. Rather, defects in T-type  $\text{Ca}^{2+}$  channels probably contribute to the neoplastic phenotype through complex interactions with other ion channels, most of which have not been properly identified. For instance, regulation of  $\text{K}^+$  channels can affect the membrane potential, which in turn regulates the window currents mediated by T-type  $\text{Ca}^{2+}$  channels. However, since in many cases there are already known pharmacological modulators (blockers

and activators) of ion channels, identification of a single defective ion channel in a particular cancer could provide a ready-to-go therapeutic approach.

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EDITORIAL

## Diagnostic criteria for autoimmune pancreatitis in Japan

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## INTRODUCTION

Autoimmune pancreatitis (AIP) is a particular type of pancreatitis of presumed autoimmune etiology. Currently, AIP should be diagnosed based on combination of clinical, serological, morphological, and histopathological features. When diagnosing AIP, it is most important to differentiate it from pancreatic cancer. Diagnostic criteria for AIP, proposed by the Japan Pancreas Society in 2002 first in the world, were revised in 2006. The criteria are based on the minimum consensus of AIP and aim to avoid misdiagnosing pancreatic cancer as far as possible, but not for screening AIP. The criteria consist of the following radiological, serological, and histopathological items: (1) radiological imaging showing narrowing of the main pancreatic duct and enlargement of the pancreas, which are characteristic of the disease; (2) laboratory data showing abnormally elevated levels of serum  $\gamma$ -globulin, IgG or IgG4, or the presence of autoantibodies; (3) histopathological examination of the pancreas demonstrating marked fibrosis and prominent infiltration of lymphocytes and plasma cells, which is called lymphoplasmacytic sclerosing pancreatitis (LPSP). For a diagnosis of AIP, criterion 1 must be present, together with criterion 2 and/or criterion 3. However, it is necessary to exclude malignant diseases such as pancreatic or biliary cancer.

## Abstract

Autoimmune pancreatitis (AIP) is a particular type of pancreatitis of presumed autoimmune etiology. Currently, AIP should be diagnosed based on combination of clinical, serological, morphological, and histopathological features. When diagnosing AIP, it is most important to differentiate it from pancreatic cancer. Diagnostic criteria for AIP, proposed by the Japan Pancreas Society in 2002 first in the world, were revised in 2006. The criteria are based on the minimum consensus of AIP and aim to avoid misdiagnosing pancreatic cancer as far as possible, but not for screening AIP. The criteria consist of the following radiological, serological, and histopathological items: (1) radiological imaging showing narrowing of the main pancreatic duct and enlargement of the pancreas, which are characteristic of the disease; (2) laboratory data showing abnormally elevated levels of serum  $\gamma$ -globulin, IgG or IgG4, or the presence of autoantibodies; (3) histopathological examination of the pancreas demonstrating marked fibrosis and prominent infiltration of lymphocytes and plasma cells, which is called lymphoplasmacytic sclerosing pancreatitis (LPSP). For a diagnosis of AIP, criterion 1 must be present, together with criterion 2 and/or criterion 3. However, it is necessary to exclude malignant diseases such as pancreatic or biliary cancer.

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**Key words:** Autoimmune pancreatitis; Diagnostic criteria; IgG4; Lymphoplasmacytic sclerosing pancreatitis

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## CLINICAL DIAGNOSTIC CRITERIA FOR AUTOIMMUNE PANCREATITIS 2006

The diagnostic criteria for AIP in Japan are based on the minimum consensus of AIP and aim to avoid

misdiagnosing pancreatic cancer as far as possible, but not for screening AIP. Therefore, the criteria emphasize the importance of imaging studies. In the 2002 criteria<sup>[3]</sup>, extent limitation on main pancreatic duct involvement (more than one-third the length of the entire pancreas) was required to diagnose only typical AIP cases and to avoid the possible inclusion of pancreatic cancer. However, with the accumulation of more AIP cases, it has become clear that, in several cases that are strongly suspected of having AIP, the degree of narrowing of the main pancreatic duct is less than one-third of the entire pancreas<sup>[5-7]</sup>. Furthermore, serum IgG4 levels are rather significantly and specifically elevated in AIP patients<sup>[8]</sup>. Thus, the 2006 criteria<sup>[4]</sup> delete the requirement that “more than one-third of the entire pancreas” is involved, allowing segmental AIP cases to be diagnosed. Furthermore, the 2006 criteria<sup>[4]</sup> include elevation of the serum IgG4 level as a diagnostic factor. Finally, the 2006 criteria<sup>[4]</sup> stress the need to exclude malignant diseases such as pancreatic or biliary cancer, before making the diagnosis of AIP (Table 1).

The preface of the criteria is described below<sup>[4]</sup>. It is suspected that the pathogenesis of autoimmune pancreatitis (AIP) involves autoimmune mechanisms. Currently, the main cases observed for characteristic findings of AIP are the diffuse enlargement of the pancreas and the narrowing of the pancreatic duct, which are associated with the findings that are suggestive of the involvement of autoimmune mechanisms such as increased levels of  $\gamma$ -globulin and IgG, the presence of autoantibodies, and the effective response to steroid therapy. In some cases, AIP shows extra-pancreatic manifestations such as sclerosing cholangitis, sclerosing sialadenitis, and retroperitoneal fibrosis, suggesting that AIP is a systemic disease. In Western countries, AIP is occasionally observed in association with ulcerative colitis and formation of tumors, suggesting that it is somewhat contrary to the definition and concept of the disease adopted in Japan.

Patients with AIP often show discomfort in the epigastrium, obstructive jaundice due to bile duct stricture, and diabetes mellitus. AIP is more common in middle-aged and elderly males. Although long-term prognosis of the disease is not clear, pancreatic stone formation has been found in some cases. When diagnosing AIP, it is important to differentiate it from neoplastic lesions such as pancreatic or biliary cancer, and to avoid facile therapeutic diagnosis by steroid administration. The present criteria, therefore, are based on the minimum consensus of AIP to avoid mis-diagnosing pancreas or biliary cancer as far as possible, but not for screening AIP.

Furthermore, description note of the criteria is reported as below<sup>[4]</sup>: (I) Imaging studies: (1) Diffuse or localized swelling of the pancreas. Abdominal ultrasonography (US), computed tomography (CT), and/or magnetic resonance imaging (MRI) show diffused or localized swelling of the pancreas. (A) The US feature of pancreatic swelling is usually hypoechoic, sometimes with scattered echogenic spots. (B) Contrast-enhanced CT generally shows delayed enhancement similar to normal pancreas with a sausage-like enlargement, and/or a capsular-like low density rim. (C) MRI shows diffuse or localized enlargement of the pancreas with a lower

**Table 1 Clinical diagnostic criteria for autoimmune pancreatitis 2006<sup>[4]</sup>**

**Clinical diagnostic criteria**

- 1 Diffuse or segmental narrowing of the main pancreatic duct with irregular wall and diffuse or localized enlargement of the pancreas by imaging studies, such as abdominal ultrasonography (US), computed tomography (CT) and magnetic resonance imaging (MRI)
- 2 High serum  $\gamma$ -globulin, IgG or IgG4, or the presence of autoantibodies, such as antinuclear antibodies and rheumatoid factor
- 3 Marked inter-lobular fibrosis and prominent infiltration of lymphocytes and plasma cells in the peri-ductal area, occasionally with lymphoid follicles in the pancreas

For diagnosis, criterion 1 must be present, together with criterion 2 and/or criterion 3. Diagnosis of autoimmune pancreatitis is established when criterion 1, together with criterion 2 and/or criterion 3, are fulfilled. However, it is necessary to exclude malignant diseases such as pancreatic or biliary cancers.

density in T1-weighted image and a higher density in T2-weighted image compared with each of the liver images. (2) Narrowing of the pancreatic duct. The main pancreatic duct shows diffuse or localized narrowing. (A) Unlike obstruction or stricture, narrowing of the pancreatic duct extends over a larger range where the duct is narrowed with irregular walls. In typical cases, more than one-third of the entire pancreatic duct is narrowed. Even in cases where the narrowing is segmental and extends to less than one-third, the upper stream of the main pancreatic duct rarely shows notable dilatation. (B) When the pancreatic images do show typical findings but laboratory data do not, there is a possibility of AIP. However, without histopathological examinations, it is difficult to distinguish AIP from pancreatic cancer. (C) To obtain the images of pancreatic duct, it is necessary to use endoscopic retrograde cholangiopancreatography (ERCP), and additionally the direct images taken during the operation or on specimens. Currently, the diagnosis is difficult to depend on magnetic resonance cholangiopancreatography (MRCP). (3) The pancreatic image findings described above may be observed retrospectively at the time of diagnosis. (II) Laboratory data: (1) In many cases, patients with AIP show increased levels of serum  $\gamma$ -globulin, IgG or IgG4. High serum IgG4, however, is not specific to AIP, since it is also observed in other disorders such as atopic dermatitis, pemphigus, or asthma. Currently, the significance of high serum IgG4 in the pathogenesis and the pathophysiology of AIP are unclear. (2) Although increased levels of serum  $\gamma$ -globulin ( $\geq 2.0$  g/dL), IgG ( $\geq 1800$  mg/dL), and IgG4 ( $\geq 135$  mg/dL) may be used as criteria for the diagnosis of AIP, further studies are necessary. Health insurance in Japan does not cover the cost of measuring serum IgG4 levels in AIP patients. (3) Autoantibodies, such as antinuclear antibody and rheumatoid factor, are often detected in patients with AIP. (III) Pathohistological findings of the pancreas: (1) Fibrotic changes associated with prominent infiltration of lymphocytes and plasma cells, occasionally with lymphoid follicles, are observed. In many cases, infiltration of IgG4-positive plasma cells is observed. (2) Lymphocytic infiltration is prominent

in the peri-ductal area, together with inter-lobular fibrosis, occasionally including intra-lobular fibrosis. (3) Inflammatory cell infiltration involving the ducts results in diffuse narrowing of the pancreatic duct with atrophy of acini. (4) Obliterative phlebitis is often observed. (5) Although fine needle biopsy under ultrasonic endoscope (EUS-FNA) is useful in differentiating AIP from malignant tumors, diagnosis may be difficult if the specimen is too small. (IV) Endocrine and exocrine function of the pancreas: Some patients with AIP show decline of exocrine pancreatic function and diabetes mellitus. In some cases, steroid therapy improves endocrine and exocrine pancreatic dysfunction.

AIP may be associated with sclerosing cholangitis and sialadenitis, or retroperitoneal fibrosis. Most of AIP patients with sclerosing sialadenitis are negative for both anti-SSA and anti-SSB antibodies, suggesting that AIP is different from Sjogren's syndrome. Sclerosing cholangitis-like lesions accompanying AIP and primary sclerosing cholangitis (PSC) respond differently to steroid therapy and follow different prognoses, suggesting that they are not the same disorder. Further studies are necessary to clarify the role of autoimmune mechanisms in AIP.

In the diagnostic criteria in Korea<sup>[5]</sup> and the United States (Mayo Clinic)<sup>[6]</sup>, "response to steroid" is included as one of the diagnostic items. When response to steroid therapy is added to the criteria, the diagnostic sensitivity is increased. Since relief of narrowing of the pancreatic duct can be seen as early as 2 wk after steroid therapy in AIP cases, it does not occur in pancreatic cancer cases. The Korean investigators advocate a short trial of steroid therapy to differentiate AIP from pancreatic cancer in cases that do not fulfill the Japanese criteria<sup>[5]</sup>. We also agree that a trial of steroid therapy can be used to assist in making the diagnosis when it is used appropriately. However, since general physicians who are not pancreatologists use the criteria, it is possible that the facile use of steroid trials will delay pancreatic cancer surgery, which could lead to cancer progression in some cases. Therefore, "response to steroid" is excluded in the diagnostic criteria in Japan<sup>[4,9,10]</sup>.

AIP with neutrophilic infiltration in the epithelium of the pancreatic duct (idiopathic duct-centric chronic pancreatitis: IDCP, or granulocyte epithelial lesion: GEL) has been reported by American<sup>[11]</sup> and Italian<sup>[12]</sup> pathologists. These patients showing different clinicopathological features from AIP are defined in Japan as follows: no predilection for elderly males, frequent association with inflammatory bowel disease, and weaker association with other sclerosing diseases. Histopathological finding of AIP in Japan is lymphoplasmacytic sclerosing pancreatitis (LPSP), and the above Western AIP cases have not been confirmed in Japan owing to the limited number of studies.

AIP patients usually have various extrapancreatic lesions such as sclerosing cholangitis and sialadenitis, and retroperitoneal fibrosis<sup>[13]</sup>. The histopathological findings of these extrapancreatic lesions are uniformly fibrosis with marked infiltration of IgG4-positive plasma cells and lymphocytes, which are similar to those in the

pancreas<sup>[14,15]</sup>. Therefore, the recent concept of AIP suggests that AIP is a pancreatic lesion of IgG4-related systemic disease<sup>[1,15,16]</sup>. In the future, criteria for AIP might develop into criteria for IgG4-related systemic disease.

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## Giant duodenal ulcers

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### INTRODUCTION

The history of giant duodenal ulcers (GDUs) dates back to 1931 when Brdiczka first described these exceptionally large ulcers<sup>[1]</sup>. Initially, GDUs were notable for being difficult to diagnose with barium roentgenogram<sup>[2,3]</sup> and their high morbidity and mortality<sup>[4-6]</sup>. Prompt and correct diagnosis was often delayed, and for decades the only curative intervention was invasive, surgical procedures fraught with technical difficulties. Until the early 1980's, few cases were ever described within medical literature in which patients with GDUs were successfully treated with medical therapy. Since the late 1970's and early 80's, technological and pharmacological advancements have markedly changed the manner in which physicians diagnose, treat and manage patients with GDUs. The widespread use of endoscopy, the introduction of H-2 receptor blockers and proton pump inhibitors, and the improvement in surgical techniques have all contributed to this evolution. Despite this, we suggest that GDUs remain an under recognized entity, and are often assumed to be identical to standard sized ulcers. A careful review of the literature highlights the important differences when comparing GDUs to classical peptic ulcers and why they must be thought of differently than their more common counterpart.

### Abstract

Giant duodenal ulcers (GDUs) are a subset of duodenal ulcers that have historically resulted in greater morbidity than usual duodenal ulcers. Until recently, few cases had been successfully treated with medical therapy. However, the widespread use of endoscopy, the introduction of H-2 receptor blockers and proton pump inhibitors, and the improvement in surgical techniques all have revolutionized the diagnosis, treatment and outcome of this condition. Nevertheless, GDUs are still associated with high rates of morbidity, mortality and complications. Thus, surgical evaluation of a patient with a GDU should remain an integral part of patient care. These giant variants, while usually benign, can frequently harbor malignancy. A careful review of the literature highlights the important differences when comparing GDUs to classical peptic ulcers and why they must be thought of differently than their more common counterpart.

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**Key words:** Giant duodenal ulcer; *Helicobacter pylori*; Nonsteroidal anti-inflammatory drugs; Malignancy; Endoscopy

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### HISTORY AND DEFINITION

Brdiczka is credited with being the first to describe GDUs and their radiographic appearance<sup>[1]</sup>. Most early case reports and small series emphasized the common problem of missed diagnosis on barium studies<sup>[7-10]</sup>. This was due to the fact that the ulcer crater was so large it would often be mistaken for a normal or slightly deformed duodenal cap. Kirsh and Brendel best illustrated this in their 1968 publication<sup>[4]</sup>. They examined 42 cases of GDU and found that only 24 were correctly diagnosed with a barium meal. They also established criteria for the diagnosis of GDUs. Their criteria included: an ulcerative crater greater than 2 cm, performance of the roentgen examination before surgical or pathological demonstration of ulcer, proof

that the lesion was benign and confirmation at surgery or post-mortem examination. Over the years, with the advent and technologic advancements of endoscopic evaluation, the criteria have unofficially evolved. Today, endoscopy has essentially replaced barium contrast studies for visualization of the upper gastrointestinal tract and there is little difficulty with the diagnosis of these lesions. GDUs are generally now defined simply as a benign, full thickness ulcer at least 2 cm in diameter, and usually involving a large portion of the duodenal bulb<sup>[11]</sup>.

## EPIDEMIOLOGY

Peptic ulcer disease remains a common medical problem worldwide, with the lifetime prevalence ranging from approximately 11% to 20% for men, and 8% to 11% for women<sup>[12]</sup>. Given this substantial lifetime prevalence, there are major economic loses and health care expenditures due to this problem. GDUs comprise approximately 1%-2% of all duodenal ulcers<sup>[13]</sup> and 5% of peptic ulcers requiring surgical intervention<sup>[14]</sup>. In most epidemiologic studies, the male to female ratio of standard sized ulcer disease is 2.3 to 1<sup>[15]</sup>, and the ratio in those with GDUs is approximately 3 to 1<sup>[11]</sup>.

The etiology of standard sized and GDUs has been associated with two major contributing causes: recent usage of nonsteroidal anti-inflammatory drugs (NSAIDs), and *Helicobacter pylori* (*H pylori*) infection. Worldwide, infection with *H pylori* continues to be widespread despite decreasing prevalence in select countries such as the United States. In addition, NSAID use has remained high, and the point prevalence of standard sized duodenal ulcers can reach 19% in chronic NSAID users<sup>[16]</sup>. Given these statistics, the clinician's awareness and familiarity with GDUs will be necessary, as patients will assuredly continue to develop these dangerous variants of peptic ulcer disease.

## PATHOPHYSIOLOGY

As stated above, GDUs have been most commonly associated with recent NSAID use and *H pylori* infection. The mechanism by which each of these factors causes duodenal ulcers is markedly different. Simply stated, *H pylori* infection has been shown to lead to a dysregulation of acid secretion, and an antral predominant gastritis. This leads to a high duodenal acid load and promotes gastric metaplasia within the duodenal bulb. This in turn favors *H pylori* colonization in the duodenum within these islands of gastric metaplasia. Through multiple different mechanisms, the bacteria cause inflammation within the duodenum, which is exacerbated by the increased acid load, and ultimately leads to the formation of an ulcer<sup>[17]</sup>. Meanwhile, NSAIDs lead to ulcer formation primarily through the inhibition of prostaglandins and direct mucosal injury<sup>[17]</sup>.

The true incidence of *H pylori* in standard sized duodenal ulcer formation is not known, but recent data suggests that it exceeds 85%<sup>[18,19]</sup>. However, two recent studies suggest that the percentage of GDUs caused by

*H pylori* is less than when compared to standard sized ulcers, and that NSAID use may play a more prominent role<sup>[20,21]</sup>. In 1999, Fischer *et al* reviewed 28 cases of GDU. In their study, only 39% of patients tested were found to have *H pylori* infection. In addition, Colleen *et al*<sup>[20]</sup> evaluated 184 patients with duodenal ulcers, and compared patients with standard sized ulcers to those with GDUs. They found that 53% of patients with GDUs had used daily NSAIDs in the month prior to presentation versus only 8% in the standard sized duodenal ulcer group. This same study also evaluated the basal acid output of patients with GDUs as compared to those with standard size ulcers; however, no significant difference existed<sup>[20]</sup>.

This collection of data suggests that daily NSAID use plays a more prominent role in the formation of GDUs than in standard sized duodenal ulcers. And while *H pylori* infection likely plays a role in the formation of GDUs, it is not as prevalent as it is with the formation of standard size duodenal ulcers.

Unfortunately, little data exists to explain the pathophysiologic differences that lead certain patients to develop these giant variants. Plausible explanations include genetic predisposition, dietary or environmental factors, microbial influence, variations in immunologic response or any combination of these factors<sup>[22,23]</sup>. Clearly, investigation and research are necessary to help provide further insight into these heretofore unexplained mechanisms.

## CLINICAL FEATURES

The presenting symptoms of most patients reflect the anatomical changes and the histopathology of the disease process. The most common of these symptoms is abdominal pain. Most patients describe the pain as involving the epigastric region, and some experience involvement of the right hypochondrium and/or radiation into the back. This requires the physician to include biliary and pancreatic pathology in their differential diagnosis. The pain of these ulcers has been described as more persistent than classically described with smaller duodenal ulcers. In addition, patients with GDUs are not provided relief from food or alkali<sup>[6,24]</sup>.

The majority of GDUs will present with hemorrhage<sup>[6,25]</sup>. This may manifest with melena, hematochezia, hematemesis, or any combination of the above. Anemia typically occurs in the setting of the bleeding ulcers. Surgical and endoscopic evaluation has often revealed the gastroduodenal artery within the ulcer bed. The size of the ulcer and the surrounding inflammation may cause gastric outlet obstruction. This often causes nausea and vomiting. Obstruction may be evident endoscopically by the presence of a large volume of gastric contents.

Additionally, the inflammatory mass can produce significant constitutional symptoms such as weight loss, cachexia, malnutrition and chronic abdominal pain. This constellation of symptoms can often mislead the clinician to suspect malignancy as the most likely

diagnosis.

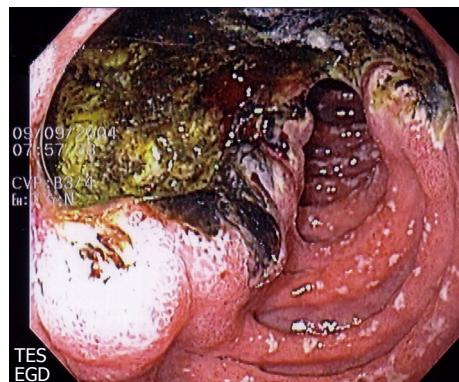
Other important historical features are the personal history of ulcer disease and the recent use of NSAIDs. Some case series note that over 40% of patients will have a prior history of a peptic ulcer<sup>[11]</sup>. In 1994, Colleen *et al*<sup>[20]</sup> revealed that patients with daily NSAID use greater than 1 mo prior to presentation had a markedly increased risk of forming GDUs.

It is well known that there is a high rate of complications with GDUs, and these complications directly lead to the high morbidity and mortality associated with this entity<sup>[5,6,25]</sup>. Common complications encountered with GDUs include bleeding and, on occasion, massive hemorrhage. One study showed that a GDU with adherent clot or a visible vessel on index EGD is a marker of an ulcer that is more likely to require surgical intervention<sup>[21]</sup>. Another feared complication is perforation. In previous case series, the perforation rate has varied between 0% and 7%<sup>[20,21,25,26]</sup>. Other complications include obstruction of the affected portion of the duodenum or proximal pylorus due to the massive inflammatory response, fistula formation, adhesions to or erosions into surrounding organs, and stricture formation in the biliary tree, pancreatic duct or the small bowel itself. These inflammatory changes are also one of the reasons that make a surgical approach fraught with technical difficulties<sup>[11,25,26]</sup>.

## DIAGNOSIS

As discussed above, the difficulty in diagnosis of GDUs has been one of the hallmarks of this disease entity since first described. Making the radiographic diagnosis by barium meal has been difficult<sup>[2,3]</sup>. The size of the ulcer often causes replacement of the duodenal bulb. As a result, GDUs may be altogether missed or misinterpreted as a deformed bulb, diverticulum or pseudodiverticulum during a barium study. The radiographic criteria to make the diagnosis of GDU were summarized by Klamer and Mahr in 1978<sup>[25]</sup>. Despite increased awareness of these criteria by clinicians, the successful diagnosis based solely on upper GI series remained unacceptably low. As a result, the entity of GDUs was likely under-diagnosed and frequently missed<sup>[4]</sup>.

The advent and widespread use of endoscopy has markedly improved clinician's ability to detect GDUs with greater accuracy<sup>[27]</sup>. Jaszewski *et al* highlighted this in 1983. In their series, seven cases of GDU were initially evaluated by upper GI barium meal. EGD then followed. GDUs were successfully diagnosed with roentgenography in only three of the seven cases. However, endoscopy confirmed the presence in all seven cases. This highlighted the importance of endoscopy in patients with symptoms suggestive of giant or regular duodenal ulcers. Subsequent case series have since used only endoscopy with measurements as diagnostic criteria<sup>[13,21]</sup>. Indeed, barium studies are now used far less frequently, typically when endoscopy is contraindicated or incapable of passing through a stricture. As a result, many clinicians may now encounter a GDU during an



**Figure 1** Endoscopic photograph of a Giant Duodenal Ulcer in a patient taking NSAIDs. The ulcer involves the proximal second portion of the duodenum. The ulcer seen is larger than 2 cm, and involves over fifty percent of the mucosal circumference.

endoscopy without the expectation of finding one, and may be inclined to misdiagnose a GDU as a simple peptic ulcer. Please see Figure 1 for an example of a GDU appearance during endoscopy.

The other obvious benefit of endoscopy is the ability to biopsy. This allows the clinician to exclude a neoplastic source as the cause of ulcer formation. While routine biopsy of standard sized duodenal ulcers is generally not recommended, this practice is worthwhile in the setting of GDUs, particularly those with nodularity at the edge. These giant variants, while usually benign, can frequently harbor malignancy. This was supported by a recent review of 52 cases of duodenal ulcers larger than 2 cm which were biopsied by Rathi *et al*. They found a malignancy rate of approximately 19% (primary duodenal carcinoma in 15%, lymphoma and tuberculosis in 2% each)<sup>[13]</sup>. Thus, we recommend multiple biopsies from the ulcer edges in all cases of GDUs.

## DIFFERENTIAL DIAGNOSIS

Prior to endoscopy, the differential diagnosis of benign GDUs had to include entities that mimicked its appearance on upper GI series. This included carcinoma of the duodenum, lymphosarcoma, duodenal diverticulum, pseudodiverticulum, regional enteritis, MALT lymphoma and tuberculosis. However, with the widespread use of endoscopy as detailed above, the differential diagnosis is somewhat narrowed. Frequently, the endoscopist cannot differentiate a benign giant ulcer from a malignant one macroscopically. As described above, patients may present with insidious symptoms such as weight loss, anorexia, and malnutrition raising concern for a malignant etiology. Therefore, again, biopsy and histopathologic examination is a necessity. Gastrinoma must also be included in the differential diagnosis, particularly in patients with evidence of acid hypersecretion, multiple ulcers extending beyond the second portion of the duodenum, diarrhea or a personal or family history of multiple endocrine neoplasia type 1 (MEN1).

## TREATMENT

Prior to the introduction of H<sub>2</sub> receptor blockers in the late 1970's, GDUs were managed primarily with surgery. This was reflected in the literature, as before 1982, there were very few cases published detailing successful medical management of GDUs<sup>[10-12]</sup>. Lumsden was able to document one patient who was a long-term (> 6 mo), asymptomatic survivor of a GDU after medical treatment only<sup>[6]</sup>.

Initially, the mortality rate associated with surgical management of GDUs was extremely high, reaching greater than 40% in early case series<sup>[24,25]</sup>. Those patients who had prompt and accurate pre-operative diagnosis had the lowest mortality. The inflammatory qualities of GDUs that make them distinct in size and nature from standard ulcers also make them more difficult to approach surgically<sup>[11]</sup>. Classically, the surgical technique most commonly recommended is truncal vagotomy and subtotal gastrectomy<sup>[21]</sup>. However, technical variations of the surgical approach have been debated. For example, management of the duodenal stump has been controversial<sup>[11,28]</sup>.

In subsequent decades, the mortality rate has fallen markedly due to numerous factors including improved radiographic technique, the advent of endoscopy and improved surgical and anesthetic techniques<sup>[11,21]</sup>. Most recently, since the 1970s, the advent of new acid suppression medication, the discovery of *H pylori* and its role in ulcer formation, and the importance of eradication therapy has led to the possibility of successful medical management of GDUs<sup>[21,27,29]</sup>.

In 1983, Jaszewski *et al*<sup>[27]</sup> reviewed 14 consecutive cases of GDUs between 1980 and 1982. Nine patients qualified for a trial of extended conservative medical treatment with close follow up. They were treated with cimetidine 1200 mg daily and antacids every 2 h while awake. Eight of the nine patients were successfully treated with the medical regimen and were asymptomatic for a mean of 14.9 mo. One patient failed medical treatment and had a perforation on day 23. This study suggested that medical treatment of uncomplicated GDUs could be accomplished with medical management, close observation and follow up with the assistance of endoscopic monitoring. In a Letter to the Editor responding to this article, Porro *et al* retrospectively reviewed 23 cases of GDUs, nine of which were eligible for similar treatment with cimetidine. Their letter agreed that H<sub>2</sub> receptor blockers could be used as short-term medical treatment of GDUs<sup>[30]</sup>.

Both of these reviews were performed before the release of proton pump inhibitors. In 1999, Fischer *et al*<sup>[21]</sup> published a prospective study of 28 patients with GDUs. One patient met criteria for immediate surgical referral, and the remaining 27 were placed on omeprazole 40 mg by mouth daily. Of these 27 patients, 7 required secondary surgical treatment for GDU complications or failure of medical therapy (4 emergent operations for re-bleeding, 3 elective operations for gastric outlet obstruction). Of these twenty remaining patients, fifteen of them had complete documented

healing on endoscopy. Of the eight patients requiring surgery, seven had a visible vessel or adherent clot on index EGD. Lastly, only 39% of patients had evidence of *H pylori* infection, and these patients were less likely to require surgery than those who were *H pylori* negative. The authors concluded that omeprazole appears to be a safe first-line treatment in stable patients, and should decrease the eventual need for operative intervention.

Several studies have confirmed the superiority of proton pump inhibitors (PPIs) versus H<sub>2</sub> receptor blockers in the treatment of standard sized gastric and duodenal ulcers<sup>[31-34]</sup>. Thus, attempts at medical treatment of GDUs should consist of proton pump inhibitors.

Despite the marked improvement delivered by the administration of proton pump inhibitors, GDUs are still associated with high rates of morbidity, mortality and complications. Thus, surgical evaluation of a patient with a GDU should remain an integral part of patient care. Indeed, there are indications for emergent and elective surgical intervention. The most common emergent indications include uncontrolled hemorrhage and perforation whereas unresolving obstruction, intractable or recurrent bleeding, and fistula formation are some of the elective indications<sup>[14]</sup>. Based on the data above, PPIs should be administered as a medical adjunct to the operation.

Lastly, discontinuation of NSAIDs and antimicrobial treatment of *H pylori* infection are recommended in the presence of these risk factors. Eradication of *H pylori* has been well documented to improve healing of peptic ulcers<sup>[29,35]</sup>.

## CONCLUSION

Historically, the two hallmark features of GDU disease were the difficulties in prompt diagnosis and the failures of medical management. A review of the literature suggests the changing nature of both of these features. The advent and widespread use of endoscopy has made a prompt diagnosis of GDU more accurate and easy to obtain. Once a diagnosis has been made, initiation of therapy can begin. Uncontrolled hemorrhage, perforation and unstable patients still should be cared for with immediate surgical evaluation and management. Recent data examining the use of H<sub>2</sub>-receptor blockers and proton pump inhibitors suggest that stable patients may be safely treated initially with medication, close observation and repeat endoscopic evaluation<sup>[21,27,30]</sup>. In addition, endoscopic biopsies allow a clinician to test for malignant etiologies of giant ulcers, which may be more common than suspected<sup>[13]</sup>. For this reason, we recommend that biopsies should be performed on all duodenal ulcers > 2 cm in diameter, particularly those with nodular appearing edges.

Due to evolving endoscopic and medical therapies, the management of GDUs has changed. What was once a disease that was difficult to diagnose, and managed solely with surgical intervention has become one easily diagnosed and potentially treated medically. It is of utmost importance that physicians recognize GDUs as

being different than their standard sized counterparts, and that we continue to further our understanding of this entity.

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## GASTRIC CANCER

# Mechanism and pathobiologic implications of CHFR promoter methylation in gastric carcinoma

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was significantly higher than that in well-differentiated GC samples ( $P = 0.014$ ). Moreover, the negative CHFR protein expression rate in paraffin-embedded GC samples was 55.07% (38/69), the positive rate in poorly differentiated GC samples was 36.73% (18/49), which was significantly lower than 65.00% (13/20) in well-differentiated GC samples ( $\chi^2 = 4.586, P = 0.032$ ).

**CONCLUSION:** Aberrant methylation of the *CHFR* gene may be involved in the carcinogenesis and development of GC, and is the predominant cause of down-regulation or loss of *CHFR* mRNA or protein expression. As aberrant methylation of CHFR promoter is correlated with tumor differentiation, it may help to predict the prognosis of GC and *CHFR* may become a novel target of gene therapy for GC in the future.

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**Key words:** *CHFR* gene; Gastric carcinoma; DNA methylation

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## Abstract

**AIM:** To investigate the aberrant methylation of CHFR promoter in human gastric cancer (GC) and its impact on the expression of *CHFR* mRNA and protein, as well as its correlation with clinical and histological features of human GC.

**METHODS:** Methylation-specific polymerase chain reaction (MSPCR) was used to detect the methylation status of CHFR promoter in 20 primary GC samples and paired normal gastric mucosa. The *CHFR* mRNA and protein expressions were investigated both by RT-PCR and by Western blotting. The CHFR protein expression in 69 GC samples was immunohistochemically examined.

**RESULTS:** The DNA methylation of the *CHFR* gene was found in 9 of the 20 GC samples (45%) and the down-regulation of *CHFR* mRNA and protein was significantly associated with the methylation status of the *CHFR* gene ( $P = 0.006$ ). In 20 samples of corresponding non-neoplastic mucosa, no DNA methylation of the *CHFR* gene was detected. The *CHFR* gene methylation in poorly differentiated GC samples

## INTRODUCTION

Gastric cancer (GC) is one of the most common malignancies worldwide<sup>[1]</sup>. As other malignant tumors, gastric carcinogenesis is a pathological process involving multiple genes and steps. The relevant genes are mainly oncogenes, tumor suppressor genes, and DNA mismatch repair genes. Tumor suppressor genes may lose their functions by gene mutation, loss of heterozygosity and methylation of promoters. Methylation is an epigenetic modification whereby the gene activity is controlled by adding methyl groups (CH<sub>3</sub>) to specific cytosines of the DNA. This control mechanism is important during mammalian embryonic development, and has received increasing attention in

carcinogenesis research<sup>[2]</sup>. Aberrant DNA methylation can change the chromosomal structure and DNA stability, cause abnormalities of gene expression, and affect proliferation and differentiation of tumors<sup>[3]</sup>. Hypermethylation in the promoter region, as a key factor for carcinogenesis, causes silencing of suppressor genes<sup>[4,5]</sup>. *CHFR* (checkpoint with FHA and RING finger) is a mitotic checkpoint gene that is localized at chromosome 12q24.33. *CHFR* encodes a protein with FHA and RING finger domains that governs transition from prophase to metaphase in the mitotic checkpoint pathway. In cellular response to mitotic stress by microtubule inhibitors, *CHFR* activation delays chromosome condensation during prophase and increases the cells' ability to survive the stress<sup>[6]</sup>. *CHFR* prevents errors in chromosome segregation that can lead to neoplasia. Recently, some studies showed that *CHFR* is an important tumor suppressor gene and its encoding product is a ubiquity ligase of Plk1<sup>[7-10]</sup>. Plk1 regulates both Wee1 kinase and Cdc25C phosphatase, which in turn control the Cdc2 kinase activity at the G2 to M transition. The *CHFR* gene can ubiquitinate and degenerate the Plk1, which prevents cells from entering prophase and metaphase. *CHFR* is ubiquitously expressed in normal human tissues while loss of *CHFR* expression has been observed in human tumors, in which it fails to prevent proliferation of abnormal cells from G2 to M phase, and abnormal differentiation and proliferation of cells occurs<sup>[11]</sup>. Moreover, CHFR protein, comprised of fork head-associated FHA and RING-finger (RF) domain, is frequently down-regulated in human colon cancer and GC (up to 50%). Loss of *CHFR* mRNA expression is a consequence of promoter methylation, suggesting that it plays a tumor suppressor role in gastrointestinal carcinogenesis. The checkpoint function of the FHA domain of *CHFR* is a core component of anti-proliferating properties against gastrointestinal carcinogenesis<sup>[12]</sup>. GC is the second most common cause of cancer-related death in Asia. Although surgery is the standard treatment for this disease, early detection and treatment are the only way to reduce its mortality<sup>[13]</sup>.

This study was performed to assess the methylation of CHFR promoter in Chinese GC tissue samples, its impact on gene expression, and its correlation with the clinical and pathobiological characteristics of GC.

## MATERIALS AND METHODS

### Tissue samples and DNA extraction

We studied GC samples and adjacent normal mucosa from 20 patients who underwent surgical resections at the Department of Surgery of Liaoning Tumor Hospital (Shenyang, China) from March to September 2007. None of these patients received chemotherapy or radiotherapy before surgery. Informed consent for use of the samples was obtained from each patient before surgery. All tissue samples were confirmed by histopathology. The samples were placed in liquid nitrogen immediately and then stored at -80°C until

analysis. For immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues from 69 GC patients and paired normal gastric mucosa, which were obtained from the First Affiliated Hospital of China Medical University during December 2003 to May 2004. Age and sex of the patients, tumor size, differentiation degree, Borrman type, depth of tumor invasion and status of lymph node metastasis were obtained from the histopathological reports of these patients. We attributed highly or moderately differentiated adenocarcinoma to "well differentiated", and attributed adenocarcinoma, mucinous carcinoma and signet cell carcinoma to "poorly differentiated". High molecular weight genomic DNA was extracted using the TIANamp genomic blood/cell/tissue genomic DNA kit (TIANGEN Biotech, Beijing), according to the manufacturer's instructions.

### Bisulfite modification and methylation-specific polymerase chain reaction (MSPCR)

Sodium bisulfite treatment of DNA converted all unmethylated cytosines to uracils, but the level of methylated cytosines was unaffected. Briefly, 2 µg aliquots of genomic DNA was denatured by adding 6 µL freshly prepared 3 mol/L NaOH and incubating the solution at 37°C for 10 min. For complete denaturation, the samples were incubated at 95°C for 1 min and subsequently cooled on ice. Bisulphate solution was prepared by dissolving 8.1 g sodium bisulphite in 16 mL H<sub>2</sub>O, adding 30 µL 10 mmol/L hydroquinone solution and adjusting the pH to 5.0 with 520 µL 3 mol/L NaOH. Bisulphate solution (0.5 mL) was mixed with the denatured DNA, overlaid with mineral oil, and incubated at 50°C for 17.5 h in a water bath in the dark. DNA was recovered using the Wizard DNA clean-up system (Promega, Madison, WI, USA) and eluted in 100 µL H<sub>2</sub>O. Following this, 11 µL 3 mol/L NaOH was added and the sample was incubated for 15 min at 37°C. The solution was then neutralized by adding 110 µL 16 mol/L NH<sub>4</sub>OAC (pH 7.0). DNA was ethanol-precipitated, washed with 70% ethanol, dried and resuspended in 50 µL distilled H<sub>2</sub>O. Bisulphite-treated DNA, as a template for MSPCR, was used in each of the PCR assays. Amplification was carried out in a 25 µL reaction volume containing 2 µL 1 × PCR buffer, 2.0 mmol/L MgCl<sub>2</sub>, 2.5 mmol/L dNTPs, 50 mg/mL DMSO, 10 µmol/L each primer, 50 ng DNA template, 2 U hot start Taq polymerase (Finzymes OY, Finland). After heating at 95°C for 5 min, PCR was performed in a thermal cycler for 40 cycles of denaturation at 95°C for 45 s, annealing at 51°C (*CHFR*-UMS) or at 55°C (*CHFR*-MS) for 45 s and extension at 72°C for 45 s. Distilled water without DNA was used as a negative control. The PCR products were separated on 3.0% agarose gels. The following primer sets were used: *CHFR* M forward (5'-TTTT AATATAATATGGCGTCGATC-3'), a *CHFR* M reverse (5'-AACGACAACAAACGAAACCG-3') for methylated *CHFR* sequences, which could amplify a 141-base pair product. *CHFR* U forward (5'-GTTC

TAATATAATGGTGTGATT-3') and CHFR U reverse (5'-AAAACAACAACAAACAAA CCA-3') for unmethylated CHFR sequences, which could amplify a 144-base pair product as described previously<sup>[14]</sup>.

#### **Reverse transcription-PCR (RT-PCR)**

Expression of the *CHFR* gene was analyzed by RT-PCR. Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First-strand cDNA was generated using a first strand cDNA synthesis kit (Qiagen, German), then amplified by a primer set that is specific for the *CHFR* gene. The primer sequences are CHFR S (sense): 5'-TAAAGGAAGTGGTCCCTGTG-3' and CHFR AS (anti-sense): 5'-GGTTGGGCATTCTACGC-3', which resulted in a DNA product of 205 bp. The PCR amplification consisted of 1 cycle at 95°C for 5 min, 35 cycles at 95°C for 30 s, at 58°C for 45 s, and at 72°C for 1 min, and 1 cycle at 72°C for 6 min. The expression of β-actin was used as a control to confirm the success of RT-PCR using the following primer pair: 5'-AGTTGCGTTACACCC TTTCTTG-3' (forward) and 5'-TCACCTTCACCCTCCAGTT-3' (reverse). The PCR products were separated by 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light. Electrophoresis strips were analyzed by BANDSCAN 5.0 software and the optical density ratio of target mRNA to β-actin served as an index for statistical analysis. If the relative optical density value of *CHFR* mRNA expression was decreased more than 50% compared with paired normal gastric mucosa, it was defined as down-regulation of expression. No expression was regarded as loss of mRNA expression.

#### **Western blotting**

Tumor and control tissue samples were homogenized for extract preparations in an ice-cold mild lysis buffer containing 10 mL/L nonidet P-40, 0.15 mol/L NaCl, 0.01 mol/L sodium phosphate (pH 7.2), 2 mmol/L EDTA, 50 mmol/L sodium fluoride, 0.2 mmol/L sodium vanadate, and 1 μg/mL aprotinin. The tissue homogenates were centrifuged at 20 000 r/min for 15 min and supernatants were collected. Protein density was determined by Coomassie brilliant blue, and then 12% SDS polyacrylamide gel electrophoresis was performed. Separated proteins were then transferred onto nitrocellulose membranes, which were blocked in 50 g/L nonfat milk in TBST (TBS buffer containing 1 g/L Tween 20) for 2 h at room temperature, incubated in primary antibodies specific for mouse CHFR (1:400 dilution) for 2 h, washed and probed with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The specific bands were quantified by BANDSCAN 5.0 software and the optical density ratio of target protein to β-tubulin served as an index for statistical analysis. If the relative optical density value of CHFR protein expression was

decreased more than 50% compared with paired normal gastric mucosa, it was defined as down-regulation of expression. No expression was regarded as loss of protein expression.

#### **Immunohistochemical staining**

Tissue chips of GC, precancerous lesions and normal gastric mucosa were immunohistochemically stained by the Envision method. Immuno-bridge kits, mouse anti-human CHFR monoclonal antibody (diluted at 1:75) were bought from Abnova Company. All steps were accomplished in accordance with the instructions. PBS (0.01 mol/L, pH 7.4) was used instead of specific antibodies for negative control. Immunohistochemical staining was graded as positive if the staining signals of CHFR protein were yellow brown granules and located in cytoplasm. For each sample, two representative high power fields were examined. The average positive rate was assessed by the percent of positive cells in the totally counted 100 cells from two representative high power fields. Positive cells ≤ 20% of the totally counted cells were defined as negative while positive cells > 20% of the totally counted cells as positive.

#### **Statistical analysis**

The data were processed by SPSS 13.0 statistical software. Quantitative data were expressed as mean ± SD. Data were analyzed by Fisher's exact test and chi square test, independent sample t-test and Spearman rank related test.  $P < 0.05$  was considered statistically significant.

## **RESULTS**

#### **Aberrant methylation of *CHFR* gene in GC tissue samples**

The representative results of MSPCR for the *CHFR* gene promoter in GC samples are shown in Figure 1 and Table 1. DNA methylation of the *CHFR* gene was detected in 9 (45%) of the 20 GC samples. By contrast, no methylation was detected in the corresponding normal gastric mucosa from these same patients. The difference in GC tissue and normal gastric mucosa samples was significant ( $P < 0.001$ ). A significant difference was observed in the tumor samples ( $P = 0.014$ ), indicating that poorly differentiated GC is more frequently methylated than well-differentiated GC. However, aberrant methylation of the *CHFR* gene in human GC was not significantly correlated with other clinicopathological factors such as gender, age, tumor size, Borrman type, depth of tumor invasion, and status of lymph node metastasis.

#### **Aberrant expression of *CHFR* mRNA in GC tissue samples**

As shown in Table 2, *CHFR* mRNA expression was down-regulated in GC tissue samples ( $0.2186 \pm 0.2113$ ) compared with normal gastric mucosa ( $0.7020 \pm 0.2163$ ) and the difference was significant ( $t = 7.148$ ,  $P < 0.0001$ ).

**Table 1** Clinicopathological features of CHFR promoter methylation in GC

Variable	n	Methylated	Unmethylated	Methylated rate (%)	P value
Total	20	9	11		
Age (yr)					
≤ 50	6	3	3	50.00	1.000
> 50	14	6	8	42.86	
Gender					
Female	12	5	7	41.67	1.000
Male	8	4	4	50.00	
Gastric cancer					
Tumor size (cm)					
< 5.0	9	5	4	55.56	0.653
≥ 5.0	11	4	7	36.36	
Borrmann type					
I + II	10	4	6	40.00	1.000
III + IV	10	5	5	50.00	
Differentiation degree					
Well	6	0	6	0.00	0.014
Poorly	14	9	5	64.29	
Invasive depth					
Within Muscle layer	5	2	3	40.00	1.000
Penetrating muscle layer	15	7	8	46.67	
Lymph node metastasis					
Positive	12	6	6	50.00	0.670
Negative	8	3	5	37.50	

**Table 2** Clinicopathological features of CHFR mRNA and protein expression in GC

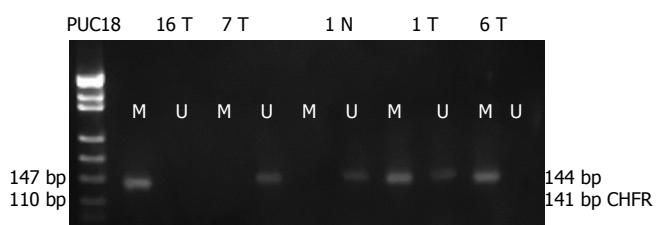
Variable	n	Relative expression density of CHFR mRNA	t	P	Relative expression density of CHFR protein	t	P
Age (yr)							
≤ 50	6	0.1400 ± 0.1572	1.189	0.176	0.3300 ± 0.3809	0.965	0.347
> 50	14	0.2800 ± 0.2187			0.2064 ± 0.1989		
Gender			0.608	0.551		0.648	0.525
Female	12	0.1942 ± 0.2179			0.2125 ± 0.2618		
Male	8	0.2537 ± 0.2094			0.2913 ± 0.2734		
Gastric cancer							
Tumor size (cm)							
< 5.0	9	0.1722 ± 0.1780	0.872	0.395	0.3289 ± 0.3360	1.346	0.195
≥ 5.0	11	0.2555 ± 0.2364			0.1736 ± 0.1678		
Borrmann type							
I + II	10	0.2560 ± 0.2312	0.797	0.436	0.2310 ± 0.2772	0.208	0.838
III + IV	10	0.1800 ± 0.1934			0.2560 ± 0.2602		
Differentiation degree							
Well	6	0.4106 ± 0.1574	3.276	0.004	0.5447 ± 0.2573	5.162	0.001
poorly	14	0.1364 ± 1.1772			0.1143 ± 0.1224		
Invasive depth							
Within muscle layer	5	0.2480 ± 0.1620	0.296	0.907	0.3678 ± 0.3346	1.243	0.230
Penetrating muscle layer	15	0.2147 ± 0.2320			0.2020 ± 0.2320		
Lymph node metastasis							
Positive	12	0.1500 ± 0.1839	1.892	0.075	0.1817 ± 0.2353	1.318	0.204
Negative	8	0.3200 ± 0.2190			0.3363 ± 0.2879		

Among them, CHFR mRNA expression in 14 poorly differentiated GC tissue samples ( $0.1364 \pm 1.1772$ ) was significantly lower than that ( $0.4106 \pm 0.1574$ ) in 6 well-differentiated GC samples ( $t = 3.276$ ,  $P = 0.004$ ). The representative RT-PCR results for the CHFR mRNA expression in GC samples are shown in Figure 2.

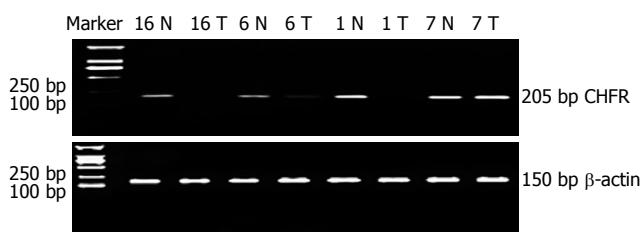
#### CHFR protein expression levels in GC tissue samples

Western blotting analysis showed that the down-regulation and loss rate of the CHFR gene-coded protein was 70.00% (14/20) in GC tissue samples. The

difference in relative optical density value between GC tissue and normal gastric mucosa samples was significant ( $0.2435 \pm 0.2620$  vs  $0.5955 \pm 0.2196$ ,  $t = 4.605$ ,  $P < 0.0001$ ). The level of CHFR protein expression in poorly differentiated GC tissue samples was significantly lower than that in well-differentiated GC tissue samples ( $t = 5.162$ ,  $P = 0.001$ ). The CHFR protein expression level was correlated with the other clinicopathological features of GC tissue samples and paired normal gastric mucosa samples (Table 2). The representative Western blotting results for the CHFR protein expression in GC samples



**Figure 1** Representative results of MSPCR in human GC tissue samples. Lanes U and M: Products derived from unmethylated and methylated alleles, respectively. Methylation of the *CHFR* gene was detected in 16 and 6 poorly-differentiated adenocarcinoma tissue samples and 1 mucinous adenocarcinoma tissue sample, while unmethylation of the *CHFR* gene was detected in 7 well-differentiated adenocarcinoma tissue samples. PUC18: Marker; N: Normal gastric mucosa corresponding to tumors; T: Gastric cancer.



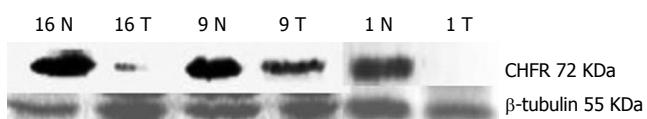
**Figure 2** Representative results of RT-PCR in human GC tissue samples.  $\beta$ -actin was used as an internal control. *CHFR* mRNA expression level in 16 and 6 poorly differentiated adenocarcinoma tissue samples and 1 mucinous adenocarcinoma tissue sample was significantly lower than that in paired normal gastric mucosa samples from the same patients, while no difference was found in 7 well-differentiated adenocarcinoma tissue samples. Marker, D2000: Marker; N: Normal gastric mucosa corresponding to tumors; T: Gastric cancer.

are shown in Figure 3.

In addition, the *CHFR* protein expression in 69 GC tissue samples was analyzed by immunohistochemical staining of paraffin-embedded sections. Negative staining was observed in 55.07% of GC tissue samples (38/69). Positive staining was observed in 36.73% of poorly differentiated GC tissue samples (18/49), which was significantly lower than 65.00% (13/20) of well-differentiated GC tissue samples ( $\chi^2 = 4.586$ ,  $P = 0.032$ ). However, the *CHFR* protein expression in human GC tissue samples was not correlated to the other clinicopathological factors such as gender, age, tumor size, Borrmann type, depth of tumor invasion and status of lymph node metastasis. The example of immunohistochemical staining is shown in Figure 4.

#### Correlation of aberrant *CHFR* methylation with mRNA and protein expression level

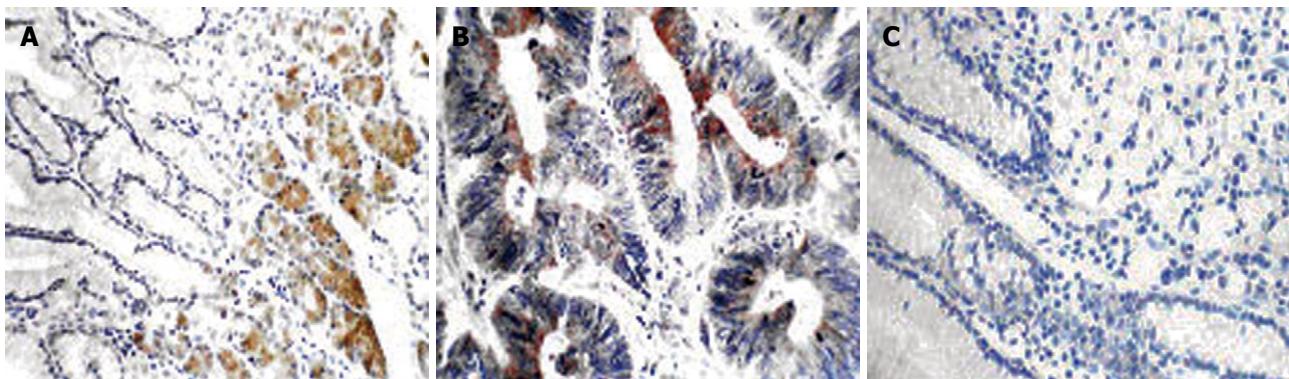
The level of *CHFR* mRNA and protein expression in 9 GC tissue samples with *CHFR* methylation was down-regulated or lost. The level of mRNA and protein expression was down-regulated only in 5 of the 11 GC tissue samples with an unmethylated *CHFR* gene. The *CHFR* mRNA and protein expression was inversely correlated with promoter methylation ( $r = 0.592$ ,  $P = 0.006$ ).



**Figure 3** Representative results of Western blot in human GC tissue samples.  $\beta$ -tubulin was used as an internal control. *CHFR* protein expression level in GS tissue samples was significantly lower than that in paired normal gastric mucosa samples. Aberrant methylation of *CHFR* and down-regulation or loss of *CHFR* mRNA expression were detected in 16 poorly differentiated adenocarcinoma tissue samples and 1 mucinous adenocarcinoma tissue sample, while positive protein expression of *CHFR* was detected in 9 moderately-differentiated adenocarcinoma tissue samples without *CHFR* methylation. N: Normal gastric mucosa corresponding to tumors; T: Gastric cancer.

It is well known that both carcinogenesis and tumor progression evolve from genetic and epigenetic alterations of several genes<sup>[15]</sup>. Epigenetic alteration refers to the heritable phenotypic alteration in the absence of DNA sequence changes, and DNA methylation is one of the extensively studied epigenetic alterations<sup>[16]</sup>. In human beings and other mammals, CpG island methylation is an important physiological mechanism. The inactivated X-chromosome in female silenced alleles of imprinted genes or inserted viral genes and repeat elements are inactivated through promoter methylation<sup>[17]</sup>. Aberrant CpG methylation is common in cancer development and may play an important role in the carcinogenic process<sup>[18-25]</sup>. Methylation changes occurring in cancer include global hypomethylation in genomic DNA and gene-specific promoter hypermethylation. Whereas global hypomethylation increases mutation rates and chromosomal instability, promoter hypermethylation usually results in transcriptional gene inactivation. Thus, promoter hypermethylation, by silencing anti-cell-proliferation genes, anti-apoptosis genes, anti-angiogenesis genes, DNA repair genes, and anti-metastasis genes, plays an important role in carcinogenesis<sup>[26,27]</sup>. Recent evidence indicates that epigenetic changes might "addict" cancer cells to altered signal-transduction pathways during the early stages of tumor development. Dependence on these pathways for cell proliferation or survival allows them to acquire genetic mutations in the same pathways, providing the cells with selective advantages that promote tumor progression<sup>[28]</sup>. Processes that regulate gene transcription are directly under the influence of genome organization. DNA methylation of CpGs constitutes an epigenetic mark generally correlated with transcriptionally silent condensed chromatin. Replication of methylation patterns by DNA methyltransferases maintains genome stability through cell division. Periodic, strand-specific methylation and demethylation occur during transcriptional cycling of the *pS2/TFF1* gene promoter activated by estrogens. DNA methyltransferases exhibit dual actions during these cycles and are involved in CpG methylation and active demethylation of 5mCpGs through deamination. Inhibition of this process precludes demethylation of the *pS2* gene promoter and its subsequent transcriptional activation. Cyclical changes in the methylation status of

## DISCUSSION



**Figure 4** Immunohistochemical staining for CHFR protein expression in GC tissue samples and normal gastric mucosa samples. Positive expression of CHFR in normal gastric mucosa tissue samples (A), in well-differentiated adenocarcinoma tissue samples (B), and in signet-ring cell carcinoma tissue samples (C).

promoter CpGs may thus represent a critical event in transcriptional achievement<sup>[29]</sup>.

However, aberrant methylation of CpGs is not a random event, but an event with gene-specific or tissue-specific differentiation. According to quantitative DNA methylation patterns at 4600 *Not I* sites and more than 150 differentially methylated regions in several C57BL/6J mouse tissue samples, comparative analysis between mice and human beings suggests that some, but not all, tissue-specific differentially methylated regions are conserved. A deeper understanding of cell-type-specific differences in DNA methylation might lead to a better illustration of the mechanisms behind tissue-specific differentiation in mammals<sup>[16]</sup>.

As a tumor suppresser gene, aberrant methylation of CHFR promoter region associated with gene silencing has been reported in several primary tumors as followsings<sup>[30-35]</sup>.

The aberrant hypermethylation rate of CHFR was 12.3% (2/14) in cervical adenocarcinoma samples<sup>[30]</sup>. Thirty-six percent of patient samples showed a low or negative CHFR protein expression or staining. In addition, lack of CHFR detection was associated with increased tumor size and weakly correlated with estrogen receptor-negative tumors, suggesting that decreased CHFR expression results in the acquisition of many phenotypes associated with malignant progression, including accelerated growth rates, higher mitotic index, enhanced invasiveness, increased motility, greater aneuploidy, and amplified colony formation in soft agar, further supporting the role of *CHFR* as a tumor suppressor in breast cancer<sup>[31]</sup>. An aberrant methylation of the *CHFR* gene was detected in 25 out of 98 (26%) primary colorectal cancers and no methylation was detected in the corresponding normal tissue specimens<sup>[32]</sup>. In 46 patients with GC, 24 (52%) had aberrant CHFR methylation. By contrast, aberrant methylation was detected in only 2 samples (4%) of normal gastric mucosa and CHFR methylation status did not correlate with gender, sex, and clinicopathological features, such as tumor size, histological type, and stage. In cell lines, aberrant CHFR methylation correlated with the loss of mRNA expression, and treatment with the methyltransferase inhibitor 5-aza-dC induced re-

expression of the gene, indicating that loss of CHFR expression due to aberrant methylation may be a cancer-specific event, which frequently occurs in primary GC<sup>[33]</sup>. CHFR staining was lost in 33% (57/174) of GC tissue samples, and there was a significant difference between staining in diffuse and intestinal histology. Loss of CHFR expression was found more commonly in the diffuse-type GC ( $P = 0.001$ ), but no correlation was observed with age, location or tumor stage<sup>[34]</sup>. The aberrant methylation rate of CHFR was 41.1% (23/56) in GC samples. The mean age of patients with CHFR methylation was significantly higher than that of patients without CHFR methylation ( $P = 0.040$ ). However, no significant correlation was observed with the other clinicopathologic factors<sup>[35]</sup>.

In this study, the methylation rate of *CHFR* genes in GC tissue samples was significantly higher than that in paired normal gastric mucosa, suggesting that aberrant methylation of *CHFR* gene may be involved in carcinogenesis and development of GC. In addition, a significant difference was observed in GC ( $P = 0.014$ ), indicating that poorly differentiated GC is more frequently methylated than well-differentiated GC and that aberrant methylation of the *CHFR* gene may participate in histological differentiation of GC and is associated with tumor malignant behavior. Its exact mechanism needs to be further studied. However, aberrant methylation of the *CHFR* gene in human GC was not significantly correlated with other clinicopathological factors such as gender, age, tumor size, Borrmann type, depth of tumor invasion and status of lymph node metastasis. Moreover, the *CHFR* mRNA or protein expression level in 9 GC tissue samples with CHFR methylation was down-regulated or lost, while the level of the mRNA or protein expression was down-regulated only in 5 of 11 GC tissue samples with an unmethylated *CHFR* gene. These findings show that aberrant methylation of CHFR promoter in GC tissue samples is the main cause of down-regulation or loss of its mRNA or coded protein expression. On the other hand, CHFR staining was lost in 55.07% (38/69) of GC tissue samples, and there was a significant difference between staining in poorly differentiated and well-differentiated GC tissue samples. Because such a loss

was found more commonly in GC tissue samples, CHFR probably acts as a tumor suppressor in development of GC.

In conclusion, aberrant methylation of the *CHFR* gene is a frequent event in human GC and the principle mechanism underlying gene silencing, down-regulation or loss of CHFR. Since aberrant methylation of *CHFR* gene is closely correlated with tumor pathobiological behavior, the detection of the *CHFR* gene may be helpful in predicting the prognosis of GC and *CHFR* may become a novel target of gene therapy for GC in the future.

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## COMMENTS

### Background

*CHFR* is a novel tumor suppressor gene and its encoding product is a ubiquitous ligase of Plk1. In cellular response to mitotic stress induced by microtubule inhibitors, CHFR activation delays chromosome condensation during prophase and increases the cells' ability to survive the stress. CHFR prevents errors in chromosome segregation that can lead to neoplasia. CHFR is ubiquitously expressed in normal human tissues while loss of CHFR expression due to aberrant methylation has been observed in human tumors, suggesting that it may be involved in carcinogenesis and development of gastric cancer. To date, few studies have addressed whether aberrant methylation of CHFR promoter is a common event in gastric cancer (GC).

### Research frontiers

The pathogenesis of GC is poorly understood. DNA methylation is an epigenetic modification. This control mechanism has received increasing attention in carcinogenesis research. As a tumor suppressor gene, aberrant methylation of CHFR promoter associated with gene silencing has been reported in several primary tumors including lung, esophageal, colorectal and hepatocellular cancers. In this study, we found that aberrant methylation of CHFR promoter played an important role in gastric carcinogenesis.

### Innovations and breakthroughs

Our study showed that aberrant methylation of CHFR promoter was a frequent event in Chinese GC tissue samples and the principle mechanism underlying gene silencing, down-regulation or loss of CHFR. Aberrant methylation of CHFR promoter was closely correlated with tumor pathobiological behavior. Results of the present study may further our understanding of the molecular mechanism of DNA methylation, which is an epigenetic modification.

### Applications

The data obtained from this study demonstrate that aberrant methylation of *CHFR* gene is a frequent event in human GC and the principle mechanism underlying gene silencing, down-regulation or loss of CHFR, suggesting that CHFR probably acts as a tumor suppressor in development of GC. Moreover, aberrant methylation of the *CHFR* gene was found to be closely correlated with tumor malignant behavior, indicating that detection of the *CHFR* gene may be helpful in predicting the prognosis of GC and CHFR may become a novel target of gene therapy for GC in the future.

### Peer review

In this study, the authors showed that methylation of CHFR promoter was significantly increased in GC tissue samples and was higher in poorly differentiated GC tissue samples than in well-differentiated GC tissue samples. Protein expression was found to be inversely correlated with promoter methylation. These findings suggest that aberrant methylation of the *CHFR* gene may be involved in the development of gastric carcinoma. This paper is original and informative.

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## LIVER CANCER

# Positional and expressive alteration of prohibitin during the induced differentiation of human hepatocarcinoma SMMC-7721 cells

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products of oncogenes or tumor repression genes including *c-fos*, *c-myc*, *p53* and *Rb* and its alteration of distributive area in the cells treated by HMBA.

**CONCLUSION:** These data confirm that PHB is a nuclear matrix protein, which is located in the nuclear matrix, and the distribution and expression of PHB and its relation with associated genes may play significant roles during the differentiation of SMMC-7721 cells.

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**Key words:** Prohibitin; Nuclear matrix; SMMC-7721 cells; Hexamethylamine; Bisacetamide; Cell differentiation

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## Abstract

**AIM:** To explore the existence and distribution of prohibitin (PHB) in nuclear matrix and its co-localization with products of some related genes during the differentiation of human hepatocarcinoma SMMC-7721 cells.

**METHODS:** The nuclear matrix of the SMMC-7721 cells cultured with or without  $5 \times 10^{-3}$  mmol/L hexamethylene bisacetamide (HMBA) was selectively extracted. Western blot was used to analyze the expression of PHB in nuclear matrix; immunofluorescence microscope observation was used to analyze the distribution of PHB in cell. LCSM was used to observe the co-localization of PHB with products of oncogenes and tumor suppressor genes.

**RESULTS:** Western blot analysis showed that PHB existed in the composition of nuclear matrix proteins and was down-regulated by HMBA treatment. Immunofluorescence observation revealed that PHB existed in the nuclear matrix, and its distribution regions and expression levels were altered after HMBA treatment. Laser scanning confocal microscopy revealed the co-localization between PHB and the

## INTRODUCTION

Prohibitin (PHB) is a tumor suppressor protein that is expressed in a variety of cell lines. It is not only localized to the inner membrane of mitochondria, where it acts as a chaperone protein, but is also present in the nucleus where it negatively regulates transcription. PHB plays important roles in the regulation of cell growth, proliferation, differentiation, aging and apoptosis. It is also involved in the genesis of tumor and some degenerative diseases<sup>[1-3]</sup>. In cell differentiation, some studies have found that the expression level of PHB was very low in rapidly proliferating cells, whereas it was much higher in cells undergoing differentiation. This indicates that PHB may promote cell differentiation and suppress cell proliferation<sup>[4]</sup>. Heretofore, over-expression of PHB has been found in various tumor cells. This seems to be conflictive with its tumor suppressive function. So far, the mechanism of its subcellular localization, nuclear transportation and regulation of cell proliferation and differentiation are

not well understood. Our previous studies revealed that PHB existed in nuclear matrix extractions of human adenocarcinoma MGc80-3 cells, and its expression level was altered during the differentiation induced by hexamethylene bisacetamide (HMBA)<sup>[5]</sup>. Furthermore, in the differentiation of human osteosarcoma MG-63 cells, we observed similar results<sup>[6]</sup>. This implies that PHB might be a common differentially expressed nuclear matrix protein in some tumor cells. In this study, we further studied the existence, localization, expression alteration of PHB in the nuclear matrix and the relationship between PHB and related products of oncogenes during the differentiation of human hepatocarcinoma SMMC-7721 cells induced by HMBA. This study provides scientific evidence for the function of PHB during cell differentiation and understanding of the mechanism of development of cancer and its reversion.

## MATERIALS AND METHODS

### Materials

Human hepatocarcinoma SMMC-7721 cells were obtained from China Center for Type Culture Collection (Wuhan University). RPMI-1640 was from Gibco Co., newborn calf serum was from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. HMBA was from Sigma Co. The antibodies used are listed in Table 1.

### Cell culture and inducement

Human hepatocarcinoma SMMC-7721 cells were cultured at 37°C in RPMI-1640 medium (pH 7.2) supplemented with 15% newborn calf serum, 100 U/mL penicillin, 100 U/mL streptomycin and 50 µg/mL kanamycin. Cells were passaged in log phase. Twenty-four hours after passaging the cells the experimental group was treated with culture media containing 5 mmol/L HMBA. Fresh culture media was added to the cells every 48-72 h. Cells were harvested at subconfluence.

### Cell selective extraction and sample preparation for light microscope

The cells were selectively extracted as described in our previous article<sup>[7]</sup>. The nuclear matrix-intermediate filament (NM-IF) samples on the cover slip strips after the selective extraction were prefixed in 2% glutaraldehyde at 4°C for 30 min, and rinsed in phosphate-buffered saline (PBS), pH 7.4. The samples were then stained with 0.2% Coomassie brilliant blue for 20 min, washed in distilled water, air dried, clarified by xylene, enveloped in the resin, and observed using an Olympus BH-2 microscope.

### Purification of nuclear matrix proteins

Nuclear matrix proteins of SMMC-7721 cells were purified by using a routine method with a few improvements<sup>[8]</sup>. The SMMC-7721 cells were washed with PBS and extracted with cytoskeleton buffer (CSK100) (10 mmol/L PIPES pH 6.8, 300 mmol/L sucrose, 100 mmol/L NaCl, 4 mmol/L CaCl<sub>2</sub>, 1.0 mmol/L PMSF, 0.5% Triton X-100) at 0°C for 10 min,

**Table 1 Antibodies used in the study**

Antibodies	Source	Antibodies	Source
Mouse anti-human PHB	NeoMarkers Co.	Goat anti-mouse IgG/HRP	Boster Co.
Goat anti-rabbit IgG-FITC	KPL Co.	Goat anti-rabbit IgG/HRP	Boster Co.
Goat anti-mouse IgG-TRITC	KPL Co.	Rabbit anti-p53	Boster Co.
Rabbit anti-human β-actin	Boster Co.	Rabbit anti-pRb	Boster Co.
Rabbit anti-c-Fos	Boster Co.	Rabbit anti-c-Myc	Boster Co.

and subjected to centrifugation for 5 min at 400 g. The pellet was washed twice with CSK50 (10 mmol/L PIPES pH 6.8, 300 mmol/L sucrose, 50 mmol/L NaCl, 4 mmol/L CaCl<sub>2</sub>, 1.0 mmol/L PMSF, 0.5% Triton X-100) and digested for 30 min at 25°C in the same buffer containing 300 U/mL DNase I. One mole per liter ammonium sulfate was added dropwise to a final concentration of 0.25 mmol/L. After incubation for 15 min, the nuclear matrix proteins were pelleted by centrifugation at 1000 r/min for 5 min, and washed once with the CSK50 buffer, then stored at -80°C.

### Western blot of PHB

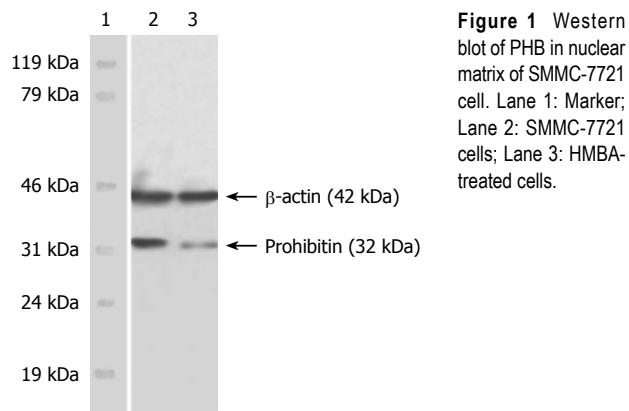
The nuclear matrix proteins were separated by SDS-PAGE and then transferred onto PVDF membranes. Nonspecific reactivity was blocked by incubation at room temperature for 1.5 h in 5% BSA buffer. The membrane was then incubated with PHB (1:2000) primary antibody at room temperature for 2 h. After washing, a horseradish peroxidase tagged secondary antibody was used to detect the primary antibody. Reactive protein was detected by the enhanced chemiluminescence (ECL) detection system (Pierce). As a negative control, the primary antibody was replaced by 5% BSA buffer. β-actin was also detected as an internal control.

### Sample preparation for fluorescence microscopy

The NM-IF samples on the cover slip were prefixed in 4% paraformaldehyde at 4°C for 10 min, rinsed in the TPBS (contain 0.5% Triton X-100) twice, 5 min each, blocked by 5% BSA at room temperature for 1 h, incubated with mouse anti-PHB monoclonal antibody (1:300) at room temperature for 30 min, and 4°C overnight, then washed 3 times with TPBS. Then, the cells were incubated with goat anti-mouse secondary antibody labeled with fluorescence dye TRITC, washed with water and dried by airing. Afterwards, 90% glycerol/PBS was applied and the cells observed using fluorescence microscopy. The entire process after incubation with secondary antibody was performed in the dark. The primary antibody was replaced by 5% BSA buffer as a negative control.

### Sample preparation for LSCM

Cells on the cover slips were rinsed in PBS, submerged in TBS (including 0.5% Triton X-100) for 30 min. After being washed with PBS, the cells were fixed in 4% paraformaldehyde for 10 min, blocked with 5%



BSA at room temperature for 1 h, and then incubated with dual primary antibodies at room temperature for 30 min and then 4°C overnight. The dual primary antibody sets comprised of PHB (1:1000)/c-Fos (1:200), PHB (1:1000)/c-Myc (1:200), PHB (1:1000)/p53 (1:200), PHB (1:1000)/pRb (1:200). After being washed with TTBS, the cells were incubated with different secondary antibody sets (goat anti rabbit or goat anti mouse) which were labeled with FITC and TRITC, respectively, at room temperature for 30 min and then 4°C overnight, washed with TPBS, enveloped with 90% glycerol/PBS after drying and then observed under LSCM (TCS- SP2 MP).

## RESULTS

### Detection of PHB in nuclear matrix by Western blot

Western blot results showed the dominant PHB band located at 32 kDa (PHB) and β-actin positioned at 42 kDa. The immunobead of PHB in the nuclear matrix of SMMC-7721 cells was obviously fuscous, while it was light and thin in HMBA-treated cells. The expression level of hnRNP A2/B1 was significantly decreased. The expression level of β-actin, which was referred to as endogenous control, had no obvious changes (Figure 1).

### Localization and expression of PHB in the NM-IF system of SMMC-7721 cells

Light microscopy observation revealed that the intermediate filaments in SMMC-7721 cells were sparse and arranged irregularly. In HMBA-treated MG-63 cells, the whole framework became more outspread, and the NM-IF system showed characteristics of uniform distribution. The intermediate filaments, which were uniformly stained, spread from the region around the nucleus to the cellular edge and formed a well-distributed and regular network throughout the cytoplasmic region. The nuclear matrix filaments were abundant and evenly distributed (Figure 2A and B).

The observation of immunofluorescence revealed the localization and expression of PHB. PHB was labeled with TRITC (red). The results showed PHB existed in the whole cell, but was weak in nuclei where it was scattered as little particles. It was relatively strong in regions near the nuclear membrane. The distribution of PHB was

uniform in the cytoplasmic region (Figure 2C). After treatment with HMBA, the distribution and expression of PHB in the NM-IF system was significantly altered. The holistic intensity of the fluorescence in nuclear matrix and nuclear lamina region dropped dramatically, while the fluorescence in the cytoplasmic region of HMBA-treated cells strengthened. It displayed a tendency of transferring from NM to lamina and cytoplasm of PHB (Figure 2D).

### Co-localization study of PHB with products of several predominant oncogenes and tumor suppressor genes

The localization of PHB and the opposite proteins including c-Fos, c-Myc, p53 and pRb were observed by LSCM. PHB was labeled with TRITC (red), other proteins were labeled with FITC (green). The co-localization fluorescence was yellow or orange when two different colors of fluorescence overlapped (Figure 3).

In SMMC-7721 cells, PHB mainly distributed in the nucleus, nuclear membrane, cytoplasm and the edge region of cells. In karyoplasm, the fluorescence was relatively weak and scattered unevenly. In SMMC-7721 cells treated with HMBA, PHB expression became decreased or even disappeared in the nuclear regions and was enhanced and scattered broadly in the cytoplasm.

### Co-localization of PHB with c-Fos in SMMC-7721 cells

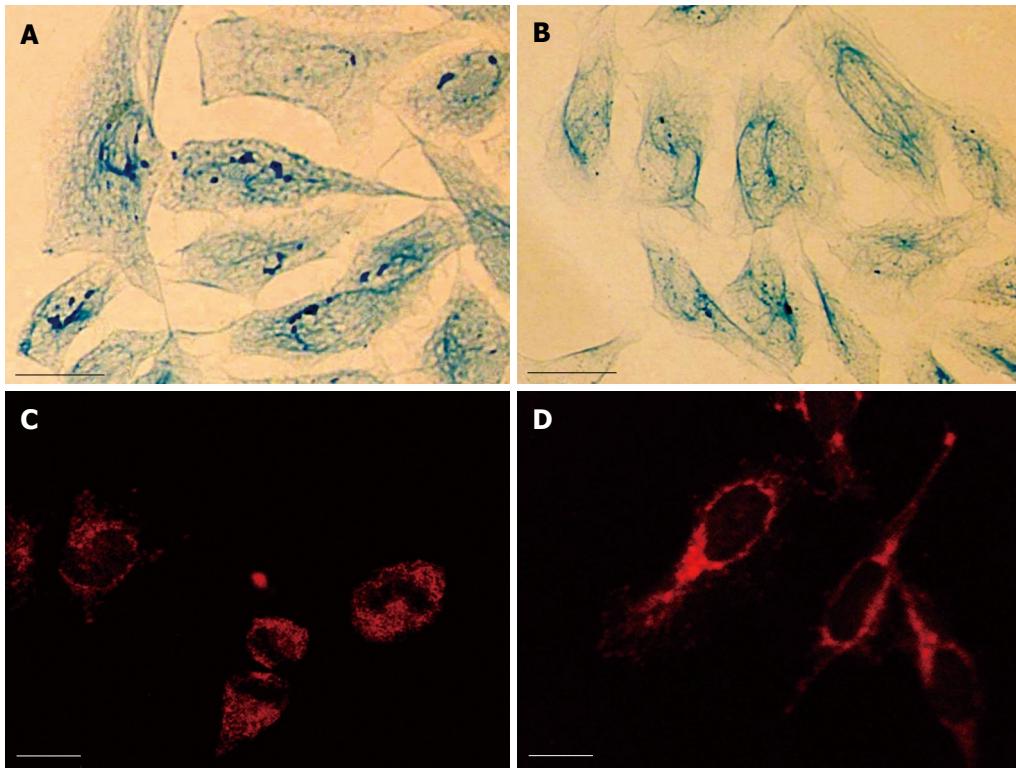
In SMMC-7721 cells, c-Fos was distributed mainly in the nucleolus region. The yellow overlapped fluorescence indicated co-localization between PHB and c-Fos existed mainly in the nucleolus region. In SMMC-7721 cells treated with HMBA, the holistic intensity of PHB and c-Fos fluorescence all weakened. The overlapped fluorescence indicated that the co-localization between PHB and c-Fos in the nucleolus weakened, but the co-localized fluorescence in karyotheca and cytoplasm was enhanced. It seemed that the co-localized region of both proteins transferred from the nucleolus region to karyotheca and cytoplasm (Figure 3A-F).

### Co-localization of PHB with c-Myc in SMMC-7721 cells

In SMMC-7721 cells, the highly-expressed c-Myc distributed mainly in the nucleolus and its peripheral regions. The overlapped fluorescence indicated they were co-localized in karyoplasms and karyotheca. In SMMC-7721 cells induced by HMBA, c-Myc dispersed in the whole cell, and its expression decreased. The overlapped fluorescence of PHB and c-Myc showed that the co-localization of two proteins strengthened in the karyotheca region, and clustered co-localization fluorescence could be seen in some special places. It indicated the tendency of transportation from karyoplasm to the nearby nuclear membrane (Figure 3G-L).

### Co-localization of PHB with p53 in SMMC-7721 cells

Due to the short half-life of wild-p53 in tumor cells (6 min), the p53 detected in this article was mostly its mutant counterpart namely mtp53. In SMMC-7721 cells, the highly-expressed mtp53 concentrated mainly



**Figure 2** **A:** LM observation of NM-IF system in SMMC-7721 cells, coomassie brilliant blue staining, bar = 10  $\mu\text{m}$ ; **B:** LM observation of NM-IF system in SMMC-7721 cells after HMBA treatment, Coomassie brilliant blue staining, bar = 10  $\mu\text{m}$ ; **C:** Immunofluorescence staining of PHB in the NMIF system of SMMC-7721 cells, bar = 10  $\mu\text{m}$ ; **D:** Immunofluorescence staining of PHB in the nuclear matrix-intermediate filament system of SMMC-7721 cells after HMBA treatment, bar = 10  $\mu\text{m}$ .

in the nucleolus and evenly distributed in other regions of the nucleus. The overlapped fluorescence indicated the co-localization distributed mainly in the region of karyoplasm and cytoplasm. In SMMC-7721 cells induced by HMBA, mtP53 decreased and its distribution was much more dispersive than that of untreated cells. The overlapped fluorescence showed the co-localization region of two proteins existed in the cytoplasm and lamina regions, and the co-localization fluorescence weakened remarkably after treatment (Figure 3M-R).

#### Co-localization of PHB with pRb in SMMC-7721 cells

In SMMC-7721 cells, Rb expression in the nucleolus was stronger than in other regions where it was relatively weak and distributed evenly. The overlapped fluorescence indicated they were co-localized in most regions of the nuclear membrane and cytoplasm. After treatment with HMBA, Rb increased remarkably and localized mainly at the nuclear membrane and nucleus regions after HMBA treatment. The overlapped fluorescence indicated the co-localization region of two proteins existed mainly in the nuclear membrane. It also showed their co-localization had a tendency of transferring from the cytoplasm to the nuclear membrane during the differentiation of SMMC-7721 cells (Figure 3S-X).

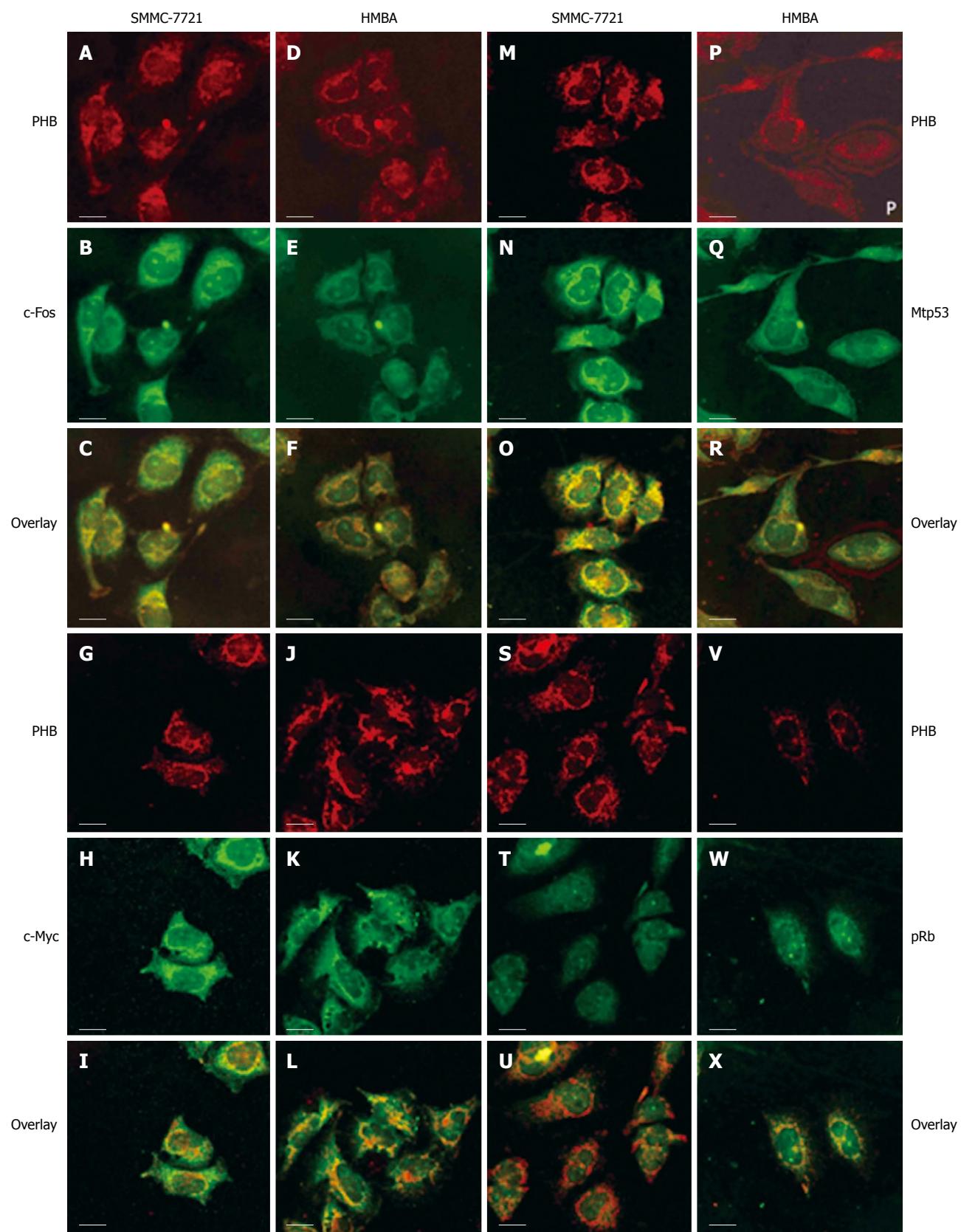
## DISCUSSION

### Changes of the expression and localization of PHB in the nuclear matrix

PHB is an important tumor suppressor protein. It not only mediates signaling of cell proliferation by acting as a membrane receptor, but is also involved in the maintenance of stability and function of

mitochondria. Previous studies have shown that PHB was mainly present in the mitochondria, and could be found in other subcellular organelles rarely<sup>[9,10]</sup>. So far, the localization of PHB in the nuclear matrix has not been reported. In this study, Western blot analysis confirmed that PHB was one of the components of the nuclear matrix and its expression in the nuclear matrix was down-regulated remarkably after treatment with HMBA. Immunofluorescence microscopy revealed that PHB was distributed mainly in the regions of the nuclear membrane and cytoplasm of SMMC-7721 cells. These results indicate that PHB exists not only in the cytoplasm, but also in the nuclear matrix of SMMC-7721 cells. Therefore, our laboratory, for the first time, has discovered the subcellular location of PHB in the nuclear matrix and showed it was a nuclear matrix protein.

PHB has great relevance to the development of cancer. Recent studies have shown that expression of PHB was abnormal in a variety of tumor cell lines<sup>[11-14]</sup>. In this study, using Western blot analysis, we found PHB is expressed highly in the nuclear matrix of SMMC-7721 cells, but in cells treated with HMBA, its expression was down-regulated. Immunofluorescent microscopy showed that the distribution of PHB in the nuclear matrix of SMMC-7721 cells was altered after HMBA treatment. PHB was mainly distributed in the karyoplasms and cytoplasm region, while in the HMBA-treated cells, PHB localized to the nuclear membrane and cytoplasm. PHB might have undergone translocation from nucleus to cytoplasm. Over expression of PHB has been reported in many kinds of tumor cells<sup>[15,16]</sup>. Our previous studies on the nuclear matrix proteins



**Figure 3** LSCM observation of the co-localization of PHB, bar = 10  $\mu$ m. **A-F:** With c-Fos in SMMC-7721 cells; **G-L:** With c-Myc in SMMC-7721 cells; **M-R:** With mtp53 in SMMC-7721 cells; **S-X:** With pRb in SMMC-7721 cells.

of human adenocarcinoma MGc80-3 and human osteosarcoma MG-63 cells showed that PHB existed as a component of the nuclear matrix, and expression

of PHB was down-regulated during differentiation. Therefore, our results further affirmed the alterations of PHB expression, and confirmed the involvement of

PHB in the regulation of tumor cell proliferation and differentiation.

### **Co-localization of PHB with products of related genes and its alteration during differentiation of SMMC-7721 cells**

The regulation of PHB in cell proliferation, senescence, apoptosis and differentiation involves complicated molecular mechanisms, including interaction between PHB and associated regulators in signaling and cell cycle, the subcellular localization of PHB in the cell, the phosphorylation level of PHB, and the connection between PHB and related oncogenes<sup>[17-19]</sup>. In this study, the results of laser scanning confocal microscopy revealed that there was a co-localizational relationship between PHB and the products of *c-fos*, *c-myc*, *p53* and *Rb* genes in SMMC-7721 cells, and the co-localization areas changed after HMBA treatment.

*c-fos* and *c-myc* are oncogenes whose expressions are up-regulated frequently in hepatocellular carcinomas (HCC). Our research indicated PHB colocalized with c-Fos, c-Myc in the nuclear region of SMMC-7721 cells. When SMMC-7721 cells were induced into differentiation, the co-localization relationship of PHB with c-Fos and c-Myc strengthened in the regions of lamina and karyotheca, but weakened in the karyoplasms. Other studies showed that PHB was a downstream target gene of *c-myc* and *c-myc* could bind to the promoter region of the *PHB* gene<sup>[20]</sup>. This indicated the close relation between PHB and c-Myc. The direct interaction of PHB and c-Fos has not yet been reported. Our research showed the co-localization between PHB and c-Myc, c-Fos in the region of the nucleus, and its alteration in SMMC-7721 cells after treatment with HMBA. It suggested that PHB might interact directly with the products of *c-myc* and *c-fos* genes. Our study discovered that PHB and p53 were co-localized at the nuclear membrane and karyoplasms region. The co-localization fluorescence transferred from nucleus to cytoplasm after HMBA treatment. This suggested that PHB might have direct connection with p53. Recently, some studies showed that PHB and p53 could cooperate with each other and participated in regulating downstream gene expression. Some studies found co-localization between PHB, p53 and E2F1 in the nucleus of breast cancer cells, and demonstrated PHB could activate transcription mediated by p53 and enhance binding of p53 with the promoter. It is also reported that p53-PHB complex translocated from nucleus to cytoplasm after stimulation by the signal of apoptosis<sup>[21-23]</sup>. Our results were consistent with former studies. It implies that PHB can directly interact with p53 and participate in the regulation of transcription mediated by p53. The co-transferring of p53 and PHB may be one of the mechanisms that mediate regulation of cell proliferation and differentiation. The studies about Rb and PHB showed that PHB could cooperate with products of Rb, a dominant tumor suppressor gene which regulates transcriptional activity of E2F. PHB could also form a triad with Rb and E2F in the nucleus, suppress activity

of E2F, and thereby inhibit cell proliferation<sup>[24,25]</sup>. The results of this study revealed that the co-localizational fluorescence in the region of the nuclear lamina was enhanced after HMBA treatment. It is obvious that the alteration is correlative with the state of proliferation and differentiation of SMMC-7721 cells. Nuclear lamina is the active site of DNA replication and transcription. Because of these, the alteration of PHB transferring and its enhanced combination with Rb in the lamina might repress the activity of E2F which promotes transcription of its downstream genes.

Taken together, these findings suggest that the subcellular localization and altered expression of PHB may have a potential role in the differentiation and phenotype reversion of SMMC-7721 cells by cooperating with products of oncogenes or tumor suppressor genes. Further investigation of the function of PHB in the nuclear matrix will help to clarify the mechanism of cell differentiation, cell cancer development and its reversion.

## **COMMENTS**

### **Background**

Previous studies showed that prohibitin (PHB) played important roles in the regulation of cell growth, proliferation, differentiation and tumorigenesis. However, the existence and expression of PHB in the nuclear matrix of tumor cells have not been well illustrated, and the functions of PHB in the induced differentiation of human hepatocarcinoma SMMC-7721 cells have also not been investigated in detail.

### **Research frontiers**

To identify differentially expressed nuclear matrix proteins and analyze their function in cancer cell differentiation is one of the most interesting hotspots in current studies of nuclear matrix.

### **Innovations and breakthroughs**

The authors revealed for the first time that PHB was a nuclear matrix protein in SMMC-7721 cells, and the distribution/expression of PHB and its relation with associated genes played a significant roles during the differentiation of SMMC-7721 cells.

### **Applications**

PHB can be used as a potential target in both diagnosis and treatment of tumors.

### **Terminology**

Nuclear matrix is the residual protein structure that remains after the nuclei are depleted of the nuclear membranes, histones, soluble nuclear proteins and nucleic acids. The structures that remain in matrix preparations are the nuclear lamina, the residual nucleolus and the fibrillar granular network.

### **Peer review**

The study is about the expression and distribution of proteins. Scientific content is interesting as several protein complexes are involved in cellular processes and identification of a role for PHB in carcinogenesis by association with other oncogenes is intriguing.

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RAPID COMMUNICATION

# Significance of scintigraphy for the localization of obscure gastrointestinal bleedings

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positive scintigraphy was independent of the age of the examined patient. A provocation test for bleeding with heparin resulted in an additional 46% of positive scintigraphies with a reliable localization in primary negative scintigraphies.

**CONCLUSION:** Our results show that scintigraphy and scintigraphy with heparin provocation tests are reliable procedures. They enable a reliable localization in about half of the obscure GI-bleeding cases. Scintigraphy is superior to angiography in locating a bleeding. It is shown that even in the age of video capsule endoscopy and double-balloon enteroscopy, scintigraphy provides a reliable and directed localization of GI bleeding and offers carefully targeted guidance for other procedures.

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**Key words:** Gastrointestinal bleeding; Scintigraphy; Localization; Angiography

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Brünnler T, Klebl F, Mundorff S, Eilles C, Reng M, von Korn H, Schölmerich J, Langgartner J, Grüne S. Significance of scintigraphy for the localization of obscure gastrointestinal bleedings. *World J Gastroenterol* 2008; 14(32): 5015-5019 Available from: URL: <http://www.wjgnet.com/1007-9327/14/5015.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.5015>

## Abstract

**AIM:** To determine the role of scintigraphy in patients with gastrointestinal (GI) bleeding of unknown localization.

**METHODS:** We performed retrospective analyses on 92 patients receiving scintigraphies from 1993 to 2000 in the University of Regensburg hospital, which were done for localization of GI bleeding as a diagnostic step after an unsuccessful endoscopy. In addition to the scintigraphies, further diagnostic steps such as endoscopy, angiography or operations were performed. In some of the scintigraphies with negative results, a provocation test for bleeding with heparinisation was carried out.

**RESULTS:** 73% of all scintigraphies showed a positive result. In 4.5% of the positive results, the source was located in the stomach, in 37% the source was the small bowel, in 25% the source was the right colon, in 4.5% the source was the left colon, and in 20% no clear localization was possible. Only 4% of all scintigraphies were false positive. A reliable

## INTRODUCTION

Gastrointestinal (GI) bleeding is a common GI disorder that requires an exact localization to guarantee adequate treatment. The clinical presentation ranges from asymptomatic or mild symptoms to a life threatening situation with mortality rates of up to 10%-14%<sup>[1-3]</sup>. Most deaths are associated with comorbidities and often occur in elderly patients<sup>[1,2]</sup>. Moreover the incidence of GI bleeding increases with age<sup>[3]</sup>.

GI bleeding is usually categorized by its localization as an upper GI bleeding (originating proximal to the Ligament of Treitz) or a lower GI bleeding (localized distal to the Ligament of Treitz), or must be classified as

obscure bleeding if not defined<sup>[4]</sup>. Dependent on local and on other factors, such as hemodynamic instability or age, different diagnostic steps, including endoscopic, radiological, or nuclear medical methods, must be performed. An example for such a diagnostic algorithm was published by Lee and Laberge 2004<sup>[5]</sup>.

Radionuclide imaging is one diagnostic possibility for the detection of GI bleeding. Its sensitivity as well as its specificity for bleeding localization seems to be high, with results of 93% to 95% at a rate of 0.04 mL/min for red blood cell (RBC) scans<sup>[6]</sup>. Its accuracy rises up from 41% to 97% when the results are verified by endoscopy, angiography or surgery<sup>[7]</sup>. This method offers advantages in being non-invasive, not requiring special preparations for the patient, and detecting both arterial and venous bleeding sites, whereas angiography only detects arterial bleedings. Moreover it offers the capability of imaging over a prolonged period of time. But, as a disadvantage, localization of bleeding sites is often not precise.

In our study we retrospectively evaluated the results of 92 patients requiring scintigraphy with <sup>99m</sup>Tc-labelled red blood cells with special focus on elderly patients, patients with scintigraphy after a provocation test with heparin, and comparison of the results with angiography.

## MATERIALS AND METHODS

### Patient recruitment and review of data

This is a retrospective study performed at a university hospital. By searching the internal medicine databases, 92 patients were identified with the diagnosis GI bleeding of unknown localization who underwent scintigraphy with <sup>99m</sup>Tc-marked red blood cells as a diagnostic procedure. If a patient required a second hospital stay, it was considered as a new case. Demographic (age, sex) and clinical data (length of hospital stay, underlying diseases if related to bleeding) as well as diagnostic and therapeutic approaches performed were collected by reviewing patient flow charts. Thereby we assessed the time of scintigraphy in relation to the time of first bleeding symptoms. Duration of not more than 7 d was classified as acute bleeding; a bleeding persisting for more than 7 d was classified as chronic bleeding. For laboratory values, we assessed the red blood cell count, and if patients required blood products we evaluated the number of red blood cell units and classified the patients as having received one unit, two units, three or more units.

For negative scintigraphies, a provocation test for bleeding with heparinisation was carried out. Comparative diagnostic or therapeutic procedures following scintigraphy, such as gastroscopy, colonoscopy, angiography, laparoscopy, and computed tomography (CT) scan, were assessed.

### Nuclear medicine procedures

For scintigraphy with <sup>99m</sup>Tc labeled red blood cells, a kit from Nycomed Amersham Sorin Italia, containing

24 mg DTPA, 3.6 mg SnCl<sub>2</sub> H<sub>2</sub>O, 22 mg Sodium-acetate and 9 mg Sodium-chloride, was used. The intravenous <sup>22m</sup>Tc pertechnetate activity was 1000 MBq. Imaging procedures were performed dynamically during the first hour (one picture every second during the influx period, then one picture per minute until 1 h) with a static image after exactly 1 h. If further images were needed they were performed up to 29 h after the initial application. For imaging procedures, a Siemens Company camera was used as well as an Icon Processing Work Station for reconstructions.

### Statistical analysis

Statistical analyses were performed with SPSS 12.0 statistical software (SPSS inc., Chicago, IL) and Microsoft Excel. Values are shown as total numbers, average or median with range, or as percentages where necessary. Kind support for statistical analyses was given by Metronomia Clinical Research GmbH (Munich, Germany).

## RESULTS

### Patient population

From our database, we identified 92 patients with GI bleeding who received scintigraphy as a diagnostic procedure and for whom complete data could be collected. Table 1 shows demographic characteristics, underlying diseases, fractions of acute and chronic bleeding, use of red blood cell units and the need of heparin induced provocation as a further diagnostic step for all 92 patients.

### Characteristics of scintigraphy

The details of performed scintigraphy in relation to a positive or negative result are shown in Table 2. In 25 patients (27%), there was no evidence of GI bleeding and no further examination or treatment was necessary in 19 patients. In 67 patients (73%), a positive result was found. Verification of the location was performed with further diagnostic procedures, such as gastroscopy, colonoscopy, angiography, computed tomography, and laparoscopy. 23 results were definitely correct and 7 were definitely false. A positive scintigraphy result was not confirmed by further diagnostic procedures in 37 patients (Figure 1).

Concerning reliable positive scintigraphies, no significant difference between age-groups was found (Figure 2).

### Scintigraphy after provocation with heparin

A provocation test with heparin was performed in 13 patients. Five of these patients had previously received a scintigraphy without heparinisation, which was negative in 2 patients, slightly positive in 2 patients, and positive without localization in one patient. The other 8 patients received a heparin-provoked scintigraphy initially in our hospital because they had a negative scintigraphy outside the university hospital. Six (46%) patients had a positive result with localization of the bleeding, 3 (23%)

**Table 1** Patients characteristics

	Characteristics (n = 92)	
	Average	Range (%)
Age	60	17-88
Male	52	56.5
Gastrointestinal bleeding without underlying disease	47	51.1
Gastrointestinal bleeding with underlying disease	45	48.9
Gastro-intestinal malignancy	11	11.9
Diverticulosis	5	5.4
Acute leukemia	6	6.5
Chronic renal insufficiency	3	3.3
Angiodysplasia	3	3.3
Chronic inflammatory bowel disease	4	4.4
Hepatic cirrhosis	3	3.3
Others	10	11.9
Acute gastro-intestinal bleeding	55	59.8
Chronic gastro-intestinal bleeding	37	40.2
Use of red blood cell concentrates		
≤ 1	70	76.1
2	17	18.5
≥ 3	5	5.4
Provocation test with heparin	13	14.1
Positive	9	9.8
Negative	4	4.4
Intestinoscopies during surgery	8	8.7
With a definite bleeding localization	2	2.2
Without a definite bleeding localization	6	6.5
Mortality	9	10.9

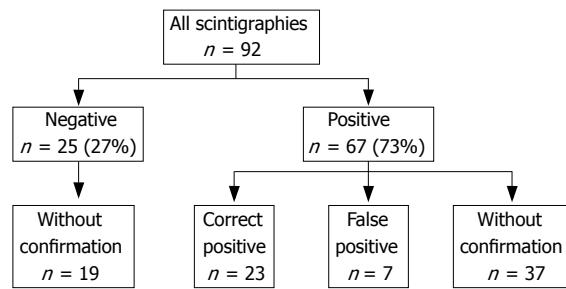
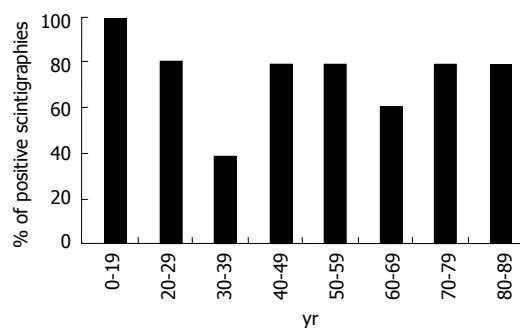
**Table 2** Details of performed scintigraphy

	Negative scintigraphy result	Positive scintigraphy result
Total (n = 92)	n = 25 (27.2%)	n = 67 (72.8%)
Total time of scintigraphy (h)	18 (0.75-25)	19 (1-48)
Radioactivity (MBq)	903 (312-1360)	975 (486-1300)
Gastrointestinal bleeding without underlying disease	11 (44%)	36 (54%)
Gastrointestinal bleeding with underlying disease	14 (56%)	31 (46%)
Acute bleeding	17 (68%)	38 (57%)
Chronic bleeding	8 (32%)	29 (43%)
Surgery required	3 (3.3%)	22 (23.9%)
Laboratory values		
Hemoglobin (mg/dL)	10.1 (6.9-15.5)	8.6 (3.2-15.6)
Platelet count (/dL)	241 (18-643)	268 (40-665)
PTT (s)	36.5 (24.2-6.9)	33.5 (21.2-64.7)
Quick (%)	89 (51-100)	89 (39-100)
Use of red blood cell concentrates		
≤ 1	20	50
2	5	12
≥ 3	0	5

patients showed a positive result without localization, and 4 (31%) patients showed a negative result.

### Comparison in patients receiving angiography

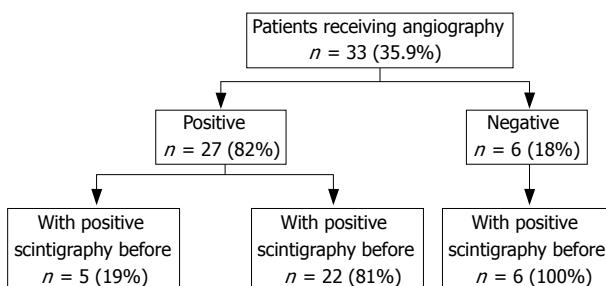
An angiogram was performed in 33 of our 92 patients. 6 patients were negative in both tests. Retrospectively, 1 of these 6 patients showed a false negative result in both of the examinations. Twenty two patients had a negative angiogram, however, 9 of these patients showed a positive scintigraphy result (correct localization in 8 patients and unclear localization in 1 patient). Thirteen patients showed a false positive scintigraphy result. Five

**Figure 1** Scintigraphy results.**Figure 2** Reliable positive scintigraphies by age.

patients showed a positive angiogram with positive scintigrams. The localization was correct in 2 patients, unclear in 2 patients and in 1 patient correct in the angiogram but false positive in the scintigram (Figure 3).

### Relevance of performed scintigraphy

Our results showed a sensitivity of 79% (23/29) for the presence of bleeding with scintigraphy, and a specificity of 30% (19/63). The negative predictive value was 76% (19/25), the positive predictive value 77%, including the patients with a positive scintigraphy result without confirmation 34% (23/67).

**Figure 3** Angiography.

### Outcome

Nine of 92 patients died. Death occurred due to underlying diseases, which was hemato-oncologic malignancy in 6 patients, sepsis by vasculitis and gangrenous leg in one patient, sepsis and cirrhosis of the liver in one patient, and terminal heart insufficiency due to ischemic cardiomyopathy in one patient.

No death was correlated to the performed scintigraphy, and there were no procedure-associated complications documented. In the subpopulation of patients receiving heparinisation, no patient died. Scintigraphy itself, even with heparin-induced provocation, seems to be a safe procedure.

## DISCUSSION

Scintigraphy with  $^{99m}\text{Tc}$  labelled red blood cells is a possible tool in the diagnosis of GI bleeding<sup>[8-13]</sup>. The reported success rates for identifying correct locations of bleeding range from 19% to 96% according to the literature<sup>[14-19]</sup>.

Different reasons may explain the wide range of sensitivities, specificities or accuracies of localizations. In some studies, results were not confirmed by further diagnostic procedures or were confirmed by diagnostic tests, such as barium contrast studies or simple clinical observation, which are not very effective procedures regarding localization of GI bleeding. Therefore, confirmation of scintigraphy results differs in the reported studies making comparisons of results difficult.

In previous studies, focus was on positive results rather than negative results, however, mortality and morbidity due to diagnostic and therapeutic procedures can be controlled by a cautious and well planned surgical approach. It has been shown in this context that a negative result is predictive of a good outcome, and may help in diagnostic risk stratification by avoiding unnecessary emergency care<sup>[20]</sup>. This fact may also be reflected in our population with 3 out of 25 patients dying in the subpopulation of patients with negative scintigraphy results but with no death correlated with the GI bleeding.

For imaging procedures, we used scintigraphy with  $^{99m}\text{Tc}$ -labelled red blood cells. Imaging protocols have recently been well described<sup>[21-23]</sup>. Active bleeding rates greater than 0.3 mL/min can be detected<sup>[24]</sup> and also some evidence exists that bleeding rates lower than 0.1 mL/min can be detected<sup>[25]</sup>. In contrast to the erythrocyte labelled method, there is also the possibility

of using  $^{99m}\text{Tc}$ -labelled sulfur colloid scintigraphy. This method, first described in 1977<sup>[26]</sup>, is also a successful tool for identification of bleeding sites, but may be problematic in the detection of bleeding in the stomach, proximal duodenum, or colonic flexures, due to intense radiotracer activity within the liver and the spleen. It has been shown that there is no practical advantage in the use of  $^{99m}\text{Tc}$ -Technetium labelled red blood cell scintigraphy over  $^{99m}\text{Tc}$ -labeled sulfur colloid scintigraphy<sup>[27]</sup>.

Our study has some limitations. One limitation is the study was retrospective within a single centre. Moreover, the patient cohort was mixed in terms of existing underlying diseases.

Our study shows that scintigraphies, as well as scintigraphies with heparin provocation tests, are safe procedures. They enable a reliable localization in about half of the GI bleeding cases. Scintigraphy is, in our setting, superior to angiography and there is evidence that it seems to be a helpful procedure especially for older patients who are restricted concerning invasive procedures. To our knowledge, no literature exists specifically for older patients receiving blood pool scintigraphy.

Even in the era of video capsule endoscopy and double-balloon enteroscopy, we showed that scintigraphy is a safe and helpful diagnostic tool, which allows for safe detection of GI bleeding sites and therefore carefully targeted use of further procedures.

## COMMENTS

### Background

In 30%-35% of all gastrointestinal (GI) bleedings no clear localization of the bleeding site through endoscopy is possible. For further diagnostic steps scintigraphy is a safe method to detect the possible localizations of a so far unknown bleeding.

### Research frontiers

Blood pool scintigraphy is an established method in the diagnostic of the obscure GI bleeding. Our data show that it is a safe procedure being non-invasive with low-examination-related risks. Further prospective evaluation and correlation with further diagnostic tests as i.e. capsule endoscopy and double-balloon enteroscopy scintigraphy is needed.

### Innovations and breakthroughs

It is shown that even in the time of video capsule endoscopy and double-balloon enteroscopy blood pool scintigraphy enables a reliable and directed localization of a GI bleeding and a carefully targeted use of further diagnostic procedures.

### Applications

These findings may help to optimize the diagnostic approach in patients with an obscure GI bleeding.

### Peer review

The study was a retrospective analysis of 92 patients for patients with unknown intestinal bleeds further leading to unsuccessful endoscopy. The significance of scintigraphy is under evaluation and this paper helps to bring focus on this issue particularly as there is presently a limited ability to detect GI bleeding.

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RAPID COMMUNICATION

## Probiotic effects on intestinal fermentation patterns in patients with irritable bowel syndrome

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### Abstract

**AIM:** To determine whether *Lactobacillus casei* strain Shirota (Yakult<sup>®</sup>) can alter small intestinal bacterial overgrowth (SIBO), as tested by the lactulose breath test, and whether this is associated with changes in symptoms in irritable bowel syndrome (IBS).

**METHODS:** 18 patients with IBS (Rome II criteria), who showed an early rise in breath hydrogen with lactulose (ERBHAL), consumed 65 mL of Yakult<sup>®</sup> daily for 6 wk. Lactulose breath test was repeated at the end of the treatment period. Symptoms were recorded daily using a 10 cm visual analogue scale.

**RESULTS:** 14 patients completed the study, 9 (64%) had reversal of ERBHAL, with the median time of first rise in breath hydrogen increasing from 45 to 75 min ( $P = 0.03$ ). There was no significant improvement in the symptom score with probiotic therapy, except for wind ( $P = 0.04$ ). Patients commencing with at least moderate symptoms and who no longer had ERBHAL at the end of treatment, showed improvement in the overall symptoms scores [median final score 5.3 (IQR 3.9-5.9), 55% reduction;  $n = 6$ ] to a greater extent than those who had had persisting ERBHAL [final score 6.9 (5.0-7.0), 12% reduction;  $n = 5$ ;  $P = 0.18$ ].

**CONCLUSION:** Yakult<sup>®</sup> is effective in altering fermentation patterns in the small bowel, consistent with reducing SIBO. The loss of ERBHAL was associated with reduced symptoms. The true interpretation of these findings awaits a randomised, controlled trial.

### INTRODUCTION

Irritable bowel syndrome (IBS) is the most common gastrointestinal disorder, thought to affect approximately 15% of the population<sup>[1]</sup>. Differences in gut microflora are evident between IBS sufferers and controls, with the former demonstrating significantly lower concentrations of bifidobacteria and lactobacilli, and higher concentrations of *Streptococcus*, *E. coli* and *Clostridia*<sup>[2,3]</sup>. Small intestinal bacterial overgrowth (SIBO) occurs in up to 78% patients with IBS, and may be directly related to the genesis of IBS symptoms<sup>[4,5]</sup>. The mechanism by which symptoms are generated from SIBO is most probably through fermentation, with rapid production of hydrogen, methane and carbon dioxide. This results in distention of the small intestine, leading to discomfort, sensation of bloating and secondary disturbances in motility.

SIBO is difficult to assess, but can be detected indirectly through the use of lactulose breath hydrogen or <sup>14</sup>C-xylose breath tests. In the case of the lactulose hydrogen breath test, a rise in breath hydrogen is expected approximately 90 min after the solution is consumed. An early rise in breath hydrogen after lactulose (ERBHAL) leads to one of two conclusions: either bacterial fermentation is occurring in the small intestine due to bacterial overgrowth, or there is rapid small intestinal transit<sup>[6]</sup>.

The role of SIBO in the genesis of IBS symptoms has been reviewed recently<sup>[7]</sup>. Several clinical trials have played

a key role in examining this association, in which antibiotic use has not only normalized lactulose breath hydrogen tests but has also led to an improvement in the symptoms of IBS<sup>[5,8-10]</sup>. It has also been suggested that malabsorption of fructose and lactose may result from SIBO, which, when corrected with antibiotics (as demonstrated by normalisation of the lactulose breath test), is associated with improved absorption of these sugars<sup>[5,8]</sup>.

Apart from antibiotics, other potential therapies have been suggested for the treatment of SIBO. Dietary restriction of fermentable carbohydrates is promising, as shown by the use of elemental diets<sup>[11]</sup>. However, an elemental diet is not a practical solution. Secondly, prokinetic agents, such as tegaserod, may play a role in promoting clearance of the lumen<sup>[12]</sup>. Finally, probiotics may influence bacterial populations in the small intestinal biofilm, manifesting as reduction in the total count or as a change in the activity, such that symptoms are not induced. It has been well documented that probiotics modify fermentation in the large intestine by increasing the absolute count of probiotic-type bacteria and reducing the *Streptococcus* counts<sup>[13]</sup>. It has also been shown that some probiotic strains improve symptoms of bloating, wind and pain in IBS patients<sup>[14,15]</sup>. However, the impact of probiotics specifically on SIBO in IBS patients has not been formally investigated.

The primary aim of the present study was to address the hypothesis that probiotics reverse SIBO, as defined by the breath hydrogen criteria. The secondary aim was to determine whether changes in the symptoms were associated with changes in breath test patterns. To examine this, an open label, pilot study was performed to assess the effect of *L. casei* strain Shirota (Yakult®) on ERBHAL in patients with IBS and ERBHAL.

## MATERIALS AND METHODS

### **Patients**

Patients referred for breath testing, who were found to have ERBHAL and fulfilled Rome II criteria for IBS, were invited to participate in the study. Patients were excluded if they were currently taking IBS medication, had previous specific dietary therapy, had taken probiotics for greater than 2 wk within the previous six months, or antibiotics in the previous 2 mo, were ingesting aspirin, non-steroidal anti-inflammatory drugs or alcohol in excess of 20 g/d, or had any clinically significant co-morbidity. Eighteen patients were recruited. The mean age (range) was 44 (20-70) years; five subjects were male. All patients fulfilled Rome II criteria for IBS, with 8 (44%) being constipation-predominant, 4 (22%) diarrhoea-predominant, and 6 (33%) having alternating bowel habits.

### **Experimental protocol**

Patients undertook a 1 to 2 wk run in period during which they completed a daily symptom diary. Daily recordings were made regarding symptom severity and, at the end of each week, the patients rated their symptoms overall, including the following specific

symptoms-abdominal pain/discomfort, bloating, satisfaction with stool consistency, passage of wind, tiredness, and nausea-using a 10 cm visual analogue scale (VAS). The symptoms were classified as "mild" if VAS was < 3 cm, "moderate" 3 to < 7 cm and "severe" ≥ 7 cm. The patients were then started on probiotic therapy, comprising of 65 mL (one bottle, 6.5 billion bacteria, 1 g lactose per dose) of *L. casei* strain Shirota in a solution containing sucrose, skim milk powder and dextrose (Yakult®, Yakult Ltd, Melbourne Australia), which was taken each morning prior to breakfast, for 6 wk. The same symptom diary and weekly questionnaire was completed throughout the treatment period. The patients were reviewed after 3 and 6 wk of treatment. During the last week of the study, the lactulose breath test was repeated.

Patients whose symptoms worsened significantly, terminated the study early and, if possible, the lactulose breath hydrogen test was performed prior to the cessation of the probiotic therapy. The symptom scores were considered valid if the patient had completed at least 3 wk of treatment.

### **Breath hydrogen test**

Prior to the breath hydrogen test, dietary restriction (minimising intake of fibre and poorly absorbed short-chain carbohydrates) was advised for 24 h and the subjects fasted overnight. The patients consumed 15 g lactulose, made up to a 100 mL solution with water. Breath hydrogen samples were collected at baseline and every 15 min for at least 2 h.

ERBHAL was defined as a rise of breath hydrogen of 10 ppm or greater above the baseline breath hydrogen on two consecutive 15-min samples before 90 min, following the ingestion of lactulose. The time of the first rise in breath hydrogen of 10 ppm or more was recorded.

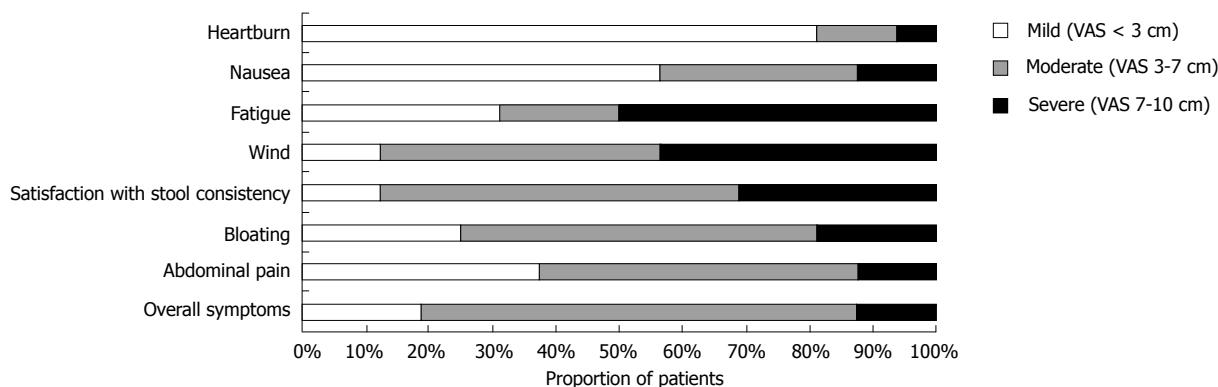
### **Statistical analysis**

Data were expressed as mean and standard error of the mean (SEM), or median and interquartile range (IQR) according to the distribution of the findings. Changes in the end-points were compared using a Wilcoxon matched pairs test. Univariate regression analyses were performed and Pearson's correlation coefficients were calculated. All statistical tests were performed using Microsoft Office® Excel 2003 and GraphPad Prism (version 3.00 for Windows, GraphPad Software, San Diego California USA).  $P \leq 0.05$  was considered statistically significant.

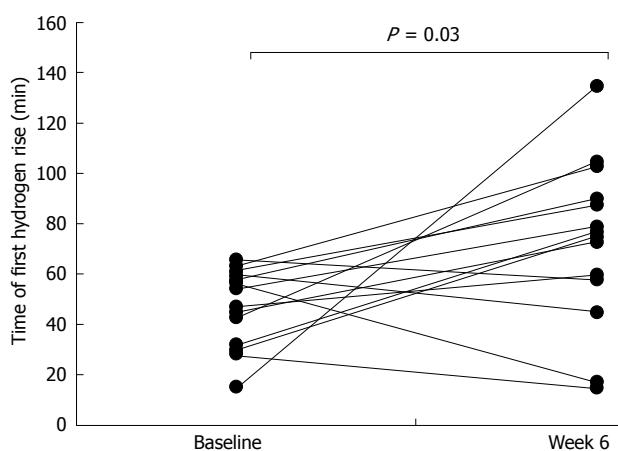
## RESULTS

### **Patient baseline symptom characteristics**

On the self-rated scales, the overall symptom severity varied across the patients, with three patients experiencing very mild symptoms, 11 moderate symptoms and two severe symptoms. However, all patients, rated at least one specific abdominal symptom as ≥ 3 cm, with the passage of wind and dissatisfaction with stool consistency being



**Figure 1** Individual and overall symptom scores at baseline, based on the visual analogue scale (VAS).



**Figure 2** The time of the first rise at or above 10 ppm in breath hydrogen after lactulose during the baseline period, and during treatment with *L. casei* strain Shirota. The time during the treatment period was significantly later compared to at baseline ( $P = 0.03$ , Wilcoxon matched pairs test).

the most frequently reported symptoms. The proportion of patients with mild, moderate or severe baseline symptoms is shown in Figure 1.

#### Adherence to protocol and treatment

Two patients terminated the study after taking Yakult® for < 2 wk due to intolerable symptoms (increasing nausea). They were excluded from the analysis as per protocol. An additional patient terminated after 3 wk, also because of increasing nausea but did not agree for a repeat hydrogen breath test. One patient commenced antibiotics for an unrelated illness after taking Yakult® for 3 wk and was withdrawn from the study due to the confounding influence of antibiotic therapy. Thus, breath test assessment was performed as per protocol in 14 patients and the effect of probiotics on symptoms was evaluable in 16 patients.

Adherence to the study treatment was > 95% in all the participants, as judged by the returned open and unused probiotic bottles.

#### Effect of Yakult® on end-points

Prior to treatment, the median time after ingestion of lactulose when breath hydrogen deviated by  $\geq 10$  ppm from the baseline was 45 min (IQR 30-60) (Figure 2).

During probiotic therapy, the median time for the first breath hydrogen rise increased to 75 min (IQR 60-90) ( $P = 0.03$ ) with 9 patients (64%) no longer demonstrating an ERBHAL, according to the entry criterion (Figure 2).

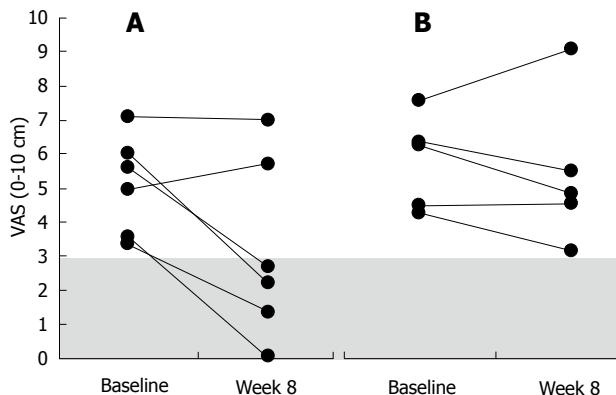
There was no significant difference in the VAS score for the overall abdominal symptoms from baseline ( $4.8 \pm 0.56$  cm) to the final week of treatment ( $4.2 \pm 0.68$  cm) ( $P = 0.33$ ). The effect of probiotic treatment was examined for each individual symptom. Only the VAS score for the passage of wind improved significantly with Yakult® ( $6.1 \pm 0.5$  cm to  $4.4 \pm 0.56$  cm;  $P = 0.04$ ), while none of the symptoms worsened significantly. There was no association of change in the symptoms with the subtype of IBS (data not shown).

#### Relationship between final ERBHAL status and change in symptoms

Patients were divided into two groups: those who no longer had ERBHAL at the end of 6 wk's therapy ("no-ERBHAL" group;  $n = 9$ ), and those with continuing ERBHAL ("ERBHAL" group;  $n = 5$ ). The change in VAS for the overall symptoms in the two groups is shown in Figure 3. Seven of the 9 patients in the no-ERBHAL group had moderate overall symptoms prior to treatment; 5 improved by at least 2 cm on the VAS, compared with none of 5 patients in the ERBHAL group ( $P = 0.06$ ; Fisher's exact test). The change in the overall symptom score in patients with at least moderate symptoms prior to treatment was greater in those who no longer had ERBHAL [median final score 5.3 (IQR 3.9-5.9), 55% reduction;  $n = 6$ ] compared to patients with continuing ERBHAL [final score 6.9 (5.0-7.0), 12% reduction;  $n = 5$ ;  $P = 0.18$ ; Mann Whitney *t*-test].

## DISCUSSION

This is the first study on the effect of a probiotic agent in patients with IBS and ERBHAL. The findings show that Yakult®-induced change in the fermentation pattern resulted in 64% patients no longer fulfilling the definition of ERBHAL at the end of the treatment period. While such a change may represent regression towards the mean due to the repetition of the breath test<sup>[16]</sup>, it more likely reflects a physiological change. This could conceivably be due to slower oro-caecal transit,



**Figure 3** The overall abdominal symptom score at baseline in patients with more than minimal symptoms (VAS < 3 cm), compared with scores at week 6 of treatment, with and without ERBHAL at the conclusion of the treatment. **A:** Without ERBHAL; **B:** With continuing ERBHAL. Shading indicates the minimal symptom score category. These changes were statistically not significant.

however, there are no reports of *L. casei* altering gastric emptying or small intestinal transit time. Alternatively, the effect may represent a more distal intestinal shift in the initial fermentation of lactulose. Formal radionuclide transit tests could address this issue in future studies, but the findings that ERBHAL represents SIBO in the majority of patients has been observed previously, along with the findings of reduction in functional gut symptoms, with the use of antibiotics and elemental diet<sup>[5,8,9,11]</sup>.

The criteria used for the diagnosis of SIBO by the lactulose breath test have varied with different investigators. Much of the earlier work used bacterial cultures of the proximal small bowel as the gold standard<sup>[6,17]</sup>. However, such methodology is not helpful in detecting an increase in bacterial biofilm, if it occurs in the distal small bowel. The presence of findings such as a double peak in hydrogen production after lactulose, or high basal breath hydrogen are not well validated in patients with IBS, and are no longer considered appropriate diagnostic features. Glucose breath hydrogen test is useful for the detection of proximal bacterial overgrowth, but since glucose is readily absorbed in the proximal small intestine, SIBO occurring more distally is not detected<sup>[18]</sup>. The most consistent and accepted measure is the time of the first significant rise (usually  $\geq 10$  ppm) in breath hydrogen after lactulose, which therefore was applied in the present study.

No consistent effect of treatment with Yakult® was observed on abdominal symptoms or fatigue; some patients worsened whereas others improved. Three patients terminated the study early because of increasing nausea. All three complained of nausea at baseline and only one had previously been tested for lactose intolerance (that person was not lactose intolerant). However, regardless of whether these patients were lactose intolerant, it is unlikely that the small amount of lactose in 1 bottle of Yakult® (1 g) was the cause of these symptoms, given that up to 7 g of lactose in one sitting is well tolerated even in true lactose intolerant patients<sup>[19]</sup>.

The loss of ERBHAL resulted in improvement in

the overall abdominal symptom VAS score by at least 2 cm in 71% of the patients with moderate to severe baseline symptoms compared to 0% who maintained the early rise. However, one patient who lost ERBHAL developed more severe symptoms compared to the baseline findings. It cannot be expected that all patients will improve with the correction of ERBHAL, since only 35% improved symptomatically when ERBHAL was corrected with the use of antibiotics<sup>[9]</sup>. Previous studies on IBS have identified a particular ability of probiotics to improve bloating and wind<sup>[20-22]</sup>. While bloating did not improve with Yakult®, a significant improvement was seen in the passage of wind. A pathogenic link between the passage of wind, bacterial overgrowth and fermentation can be postulated. The present study was not designed to specifically address the effect on symptoms. However, our findings raise the possibility that symptomatic benefit can be obtained when ERBHAL is corrected with the use of Yakult®.

In conclusion, the hypothesis that treatment with Yakult® alters the fermentation pattern, exhibited by hydrogen breath testing after lactulose was supported in this uncontrolled, proof-of-concept study. A small dose of *L. casei* strain Shirota led to a statistically significant shift in the time of early rise of breath hydrogen after lactulose, suggesting a reduction in SIBO. Furthermore, the overall abdominal symptoms tended to improve when ERBHAL was corrected. Our findings suggest that probiotics may have a beneficial effect, but further work is required to determine the dose effects and clinical relevance with well powered, double blind, placebo-controlled trials including the measurement of transit time to support the interpretation of ERBHAL.

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## COMMENTS

### Background

Small intestinal bacterial overgrowth (SIBO) is a common feature of irritable bowel syndrome (IBS) and may be directly related to the genesis of IBS symptoms. An early rise in breath hydrogen after lactulose (ERBHAL) may indicate SIBO. The use of antibiotics and elemental diets has been shown to be effective in treating SIBO, but the efficacy of probiotics is untested.

### Research frontiers

This was a pilot study designed to determine the effect of *Lactobacillus casei* strain Shirota (Yakult®) on the intestinal fermentation pattern in IBS, using the lactulose breath test. The results indicate that Yakult alters the fermentation pattern suggesting a reduction in SIBO. ERBHAL may also indicate rapid transit and therefore, in order to confirm the effect of Yakult on SIBO, future studies should include monitoring of transit time in addition to a placebo control group.

### Innovations and breakthroughs

The most promising treatments to date for SIBO are antibiotics and elemental diets, the side effects and practicalities of which make them undesirable

options. This is the first study evaluating the effect of a probiotic on SIBO. The shift of the time of the first rise of hydrogen following lactulose indicating SIBO was no longer present at the end of 6 wk of treatment. These results offer a promising alternative treatment for SIBO in IBS. A placebo controlled trial is required to confirm these findings.

### Applications

The results indicate that Yakult® may be an effective treatment for SIBO in IBS. Our findings have important implications in the treatment of IBS if these results are supported by a placebo controlled trial.

### Peer review

The shift in fermentation patterns and the trend towards improvement of symptoms indicates the need for further work to examine the dose effects and clinical relevance with well powered, double blind, placebo controlled trials including the measurement of transit time to support the interpretation of ERBHAL. This is a well written paper with a nice literature review.

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S- Editor Li DL L- Editor Anand BS E- Editor Lin YP



RAPID COMMUNICATION

# Hepatocytes isolated from neoplastic liver-immunomagnetic purging as a new source for transplantation

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**Author contributions:** Gunasegaram A, Akhter J and Johnson LA contributed equally to this work; Akhter J and Morris DL designed research; Gunasegaram A, Akhter J and Johnson LA performed research; Gunasegaram A and Akhter J analysed data; Gunasegaram A, Akhter J, Yao P, Johnson LA and Riodan SM wrote the paper.

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tumors in Balb/C nude mice. Co-transplantation of hepatocytes with tumor cells did not increase tumorigenesis of the tumor cells.

**CONCLUSION:** Immunomagnetic depletion appears to be an effective method of purging contaminating tumor cells to below threshold for likely tumorigenesis. Along with improved techniques for isolation of large numbers of viable hepatocytes, normal liver resected for neoplasia has potential as another clinically useful source of hepatocytes for transplantation.

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**Key words:** Hepatocyte transplantation; Immunomagnetic purging; Isolation of hepatocytes

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Gunasegaram A, Akhter J, Yao P, Johnson LA, Riodan SM, Morris DL. Hepatocytes isolated from neoplastic liver-immunomagnetic purging as a new source for transplantation. *World J Gastroenterol* 2008; 14(32): 5025-5031 Available from: URL: <http://www.wjgnet.com/1007-9327/14/5025.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.5025>

## Abstract

**AIM:** To investigate whether hepatocytes isolated from macroscopically normal liver during hepatic resection for neoplasia could provide a novel source of healthy hepatocytes, including the development of reliable protocols for malignant cells removal from the hepatocyte preparation.

**METHODS:** Hepatocytes were procured from resected liver of 18 patients with liver tumors using optimised digestion and cell-enrichment protocols. Suspensions of various known quantities of the HT-29 tumor cell line and patient hepatocytes were treated or not with Ep-CAM-antibody-coated immunomagnetic beads in order to investigate the efficacy of tumor-purging by immunomagnetic depletion, using a semi-quantitative RT-PCR method developed to detect tumor cells. Immunomagnetic bead-treated or bead-untreated tumor cell-hepatocyte suspensions were transplanted intra-peritoneally in Balb/C nude mice to assess the rates of tumor development.

**RESULTS:** Mean viable hepatocyte yield was  $9.3 \times 10^6$  cells per gram of digested liver with mean viability of 70.5%. Immunomagnetic depletion removed tumor cells to below the RT-PCR detection-threshold of 1 tumor cell in  $10^6$  hepatocytes, representing a maximum tumor purging efficacy of greater than 400 000-fold. Transplanted, immunomagnetic bead-purged tumor cell-hepatocyte suspensions did not form peritoneal

## INTRODUCTION

While advances in supportive medical care have improved survival, liver failure in its most severe form continues to carry a high mortality rate unless orthotopic liver transplantation (OLT) is performed. Nonetheless, there is a limit to the number of patients that can be treated in this way. The rapidity with which the clinical syndrome often progresses and a worldwide shortage of donor organs are significant limiting factors and many patients listed for OLT die or develop contraindications to transplantation before a donor liver becomes available. Further, the presence of co-morbidities in a substantial number of patients often precludes listing for OLT altogether. Consequently, there is considerable ongoing interest in the provision of other means of liver support, including extracorporeal artificial or bioartificial devices and hepatocyte transplantation (HT).

The feasibility of HT as a therapeutic tool has been demonstrated in studies performed in animals with liver-

based metabolic defects such as analbuminaemic and Gunn rats, hypercholesterolaemic rabbits and dogs with impaired purine metabolism<sup>[1-3]</sup>. Experience with HT in experimental animals with liver failure due to chemically-induced hepatic necrosis, surgical models of hepatic ischaemia or resection has also been favourable, with evidence of improved survival even when small numbers of cells (0.5% to 3% of normal hepatocyte mass) are used<sup>[4-7]</sup>.

Transplantation of human hepatocytes has, to date, been performed in only a small number of paediatric and adult patients with liver failure<sup>[8-11]</sup>. Instances of mostly short-term improvement in encephalopathy and some metabolic parameters have been recorded both in OLT and non-OLT candidates. Potential advantages of HT over OLT include its minimal invasiveness, ease of treatment, substantially lower cost and the possibility of on-demand use following the establishment of a cell bank. Limited availability of primary human hepatocytes for transplantation however, represents a major limitation. Currently, the standard approach is to isolate hepatocytes from livers rejected for liver transplantation due to excessive steatosis, cirrhosis and prolonged ischemia. However, availability of hepatocytes from this source is in increasingly short supply and concerns with the functional capability of such cells have been raised<sup>[12]</sup>.

Our group recently reported the feasibility of harvesting tumor-free hepatocytes from macroscopically normal liver unavoidably removed during hepatic resection for malignancy<sup>[13]</sup>. Here we report further improvements in our hepatocyte isolation and tumor-purging techniques, resulting in the harvest of sufficiently large numbers of viable hepatocytes from resected liver specimens to offer the prospect of effective clinical support and a high degree of tumor-purging efficacy. Furthermore, we demonstrate the safety of transplantation of hepatocytes harvested in this way, in terms of lack of complicating tumor development in an athymic mouse model.

## MATERIALS AND METHODS

### *Hepatocyte isolation and cryopreservation*

The human isolation study was approved by the South East Sydney Area Health Service Ethics Committee (approval No. 01/123). Hepatocytes were harvested from liver resection specimens of consenting patients undergoing partial hepatectomy for neoplasia. Patients with hepatocellular carcinoma were excluded. Patient demographic and disease details are outlined (Table 1). The bulk of specimens (61%) arose from livers with colorectal metastases.

Following resection, the specimen was transferred to a sterile table where the diseased portion of the liver was dissected with a 1cm margin, leaving the remaining macroscopically normal liver for hepatocyte harvest. The tissue was placed immediately into a sterile cooling solution (< 4°C) and 2-4 of the largest vessels were cannulated. Warm ischemic time, defined as clamping time of hepatic inflow and/or outflow during resection

**Table 1 Patient demographic, clinical and operative details**

Parameters	n
Age (yr; mean ± SD, range)	59 ± 11 (33-78)
Male	10
Female	8
Primary tumor	
Colorectal cancer	12
Benign liver lesions	2
Renal carcinomas	1
Prostate carcinoma	1
Cholangiocarcinoma	1
Pseudopapillary pancreas tumor	1
Operation	
Right hemi-hepatectomy	7
Left hemi-hepatectomy	4
Extended right hemi-hepatectomy	2
Right lateral sectorectomy	2
Extended left hemi-hepatectomy	1
Left lateral sectorectomy	1
Non-anatomic resection	1

plus specimen processing time before being cooled, was recorded. Cannulae ranging from 0.95 mm to 2 mm diameter were suture-ligated around the respective vessels and capped with one-way valved bungs to prevent backflow. Histoacryl® (Braun, Germany) was used to seal most of the remaining liver surface, in addition to the cannula entry points. This was done to prevent leakage of perfusate and to maximize perfusion of the microcirculation during isolation, having been previously shown to increase hepatocyte yield and viability<sup>[14,15]</sup>. The specimen was then flushed with 500-1000 mL of heparinized (5000 units per litre) Custodiol® histidine-tryptophan-ketoglutarate (HTK) solution (Kohler Chemie, Germany)<sup>[16]</sup> to prevent obstruction of the hepatic microcirculation by thrombus and facilitate organ preservation. The specimen was then transported under sterile conditions to the laboratory where it was re-warmed and digested by a modified version of Seglen's original 2-step technique<sup>[17]</sup>. Cold ischemic time, defined as time that the specimen had been on ice prior to re-warming, was recorded. Pre-warmed (37°C) buffers were perfused in the following order: (1) Wash buffer-HBSS without Ca<sup>2+</sup>/Mg<sup>2+</sup> (Gibco, Auckland, New Zealand) + 208.1 mg/L EDTA (Sigma, St Louis, MO, USA) + ascorbic acid 50 mg/L (Sigma, St Louis, MO, USA) + bovine serum albumin (BSA) (Sigma, St Louis, MO, USA) 0.5% w/v-10 min perfusion and then discarded. (2) EDTA washout-HBSS + BSA 0.5% w/v; 5 min perfusion and then discarded. (3) Digestion buffer-HBSS + 0.05% w/v Collagenase P (Roche, Germany) + 0.5% w/v BSA-re-circulated for 20-30 min.

Perfusion was carried out manually due to the varying size of the specimens (range, 55-690 g) at 20-40 mL/min per cannula depending on the weight of the specimen and cannula size. Digestion buffer was perfused until the specimen was soft and friable. A cell suspension was obtained by gentle mechanical dissociation of the digested specimen in 500 mL of ice-cold suspension buffer DMEM (Gibco, Auckland, New Zealand) + 10% (v/v) FCS (Gibco, Auckland, New Zealand) +

1% antibiotic-antimycotic Penicillin 10000 units/mL + streptomycin 25 µg/mL + amphotericin B as Fungizone® (Gibco, Auckland, New Zealand) and sequentially filtered through three sterile stainless steel filters of decreasing pore size (425 µm, 150 µm and 75 µm) (Endecotts, UK). The raw fraction was pelleted by centrifugation at 50 g for 3 min at 4°C and washed three times in the suspension buffer. Cells were counted in quadruplicate and viability assessed by Trypan blue exclusion, using a hemocytometer (Neubauer, Germany). Cells were cryopreserved in suspension medium containing 10% DMSO in 5-mL tubes by freezing to -80°C in 1°C decrements per minute and then transferred to liquid nitrogen after 24 h.

### **Hepatocyte spiking & purging**

*In vitro* and *in vivo* studies were performed using suspensions of isolated human hepatocytes spiked with various numbers of HT-29 human colorectal cell-line tumor cells and then treated or not with immunomagnetic beads (CELLlection® Epithelial Enrich; Dynal AS, Oslo, Norway), in the method described by Kielhorn *et al*<sup>[18]</sup> and Flatmark *et al*<sup>[19]</sup>. Specifically, we used 4.5 µm magnetic beads coated with mouse IgG1 Ber-EP4 antibody against Ep-CAM, an antigen highly expressed on colorectal cancer (CRC) cells (including HT-29 tumor cells), but not on mature hepatocytes<sup>[20]</sup>.

### ***In vitro* hepatocyte and HT-29 cell-line preparation**

Cryopreserved human hepatocytes were thawed in a 37°C water bath, diluted in suspension medium, pelleted by centrifuging at 50 g for 3 min at 4°C and re-suspended in suspension medium. Density gradient centrifugation was performed to purify the thawed hepatocytes. The suspension was then mixed with a Percoll® (Amersham Biosciences, Uppsala, Sweden) density gradient (25% final concentration) medium and centrifuged at 75 g for 5 min at 4°C to separate viable from dead hepatocytes. Pellets were re-suspended in PBS with 0.1% BSA and placed on ice. Cells were counted in quadruplicate, using a hemocytometer (Neubauer, Germany), and their viability assessed by Trypan blue exclusion. HT-29 cells grown in 75 cm<sup>2</sup> culture flasks (Greiner Bio-One, Germany) in 95% O<sub>2</sub> and 5% CO<sub>2</sub> at 37°C were trypsinised, washed in PBS (Invitrogen, Auckland, New Zealand), pelleted by centrifugation at 75 g for 5 min, re-suspended in PBS and counted as above.

### **Determination of RT-PCR sensitivity for tumor cell detection**

One, 10, 50, 100, 1000, 5000 or 10000 HT-29 cells were added to suspensions of 1 million human hepatocytes to establish the lower limit of detection of tumor cells by RT-PCR. Suspensions of 1 million HT-29 cells alone and 1 million hepatocytes alone served as positive and negative controls respectively.

Total RNA was extracted using TRIzol® reagent (15596026, Invitrogen) as per the manufacturer's instructions. Following DNase I treatment (18068-015,

Invitrogen), the total RNA concentration and quantity was assessed by spectrophotometry at 260 nm and the RNA stored at -80°C.

### **RT-PCR**

cDNA was synthesized from total RNA using One-Step SuperScript III® system (12574-026, Invitrogen) with target specific primers as per the manufacturer's instructions. EpCAM primers were as published by Sakaguchi *et al*<sup>[21]</sup> with actin housekeeping primers as follows; antisense 5'-GGAGCAATGATCTTGATCTT-3'; sense 5'-CTTCCTGGGCATGGAGTCCT-3'. The RT-PCR program was as follows; cycle one, 56°C, 30 min, 94°C for 3 min, followed by 40 cycles of 30 s at 94°C, 30 s at 60°C, 30 s at 72°C, with a final extension for 10 min at 72°C. The cDNA products were visualized on a 1% agarose gel, and sequenced to confirm product identity.

### **Immunomagnetic bead treatment of spiked hepatocytes**

Five mL suspensions of 1 × 10<sup>6</sup> hepatocytes spiked with 1000, 10 000 and 50 000 HT-29 cells per mL were prepared in duplicate. Immunomagnetic beads (CELLlection® Epithelial Enrich; Dynal AS; 4 × 10<sup>8</sup> beads/mL), were washed in PBS + 0.1% (w/v) BSA, and added to half the tubes in the ratio of 20 beads to 1 HT-29 cell. The remaining preparations constituted controls and contained no immunomagnetic beads. All tubes were placed in a rotator (15 r/min) at 4°C for 30 min, to allow maximal tumor cell-bead contact and capture. Treated tubes were placed in the magnetic particle concentrator provided by the manufacturer for 2 min, the supernatant transferred to new tubes and the process repeated. One mL from each sample was collected after treatment for RT-PCR analysis to assess the efficacy of immunomagnetic bead-mediated tumor-purging.

### ***In vivo* study**

Male Balb/C athymic nude mice (Animal Resource Centre, Perth, WA, Australia) were housed and fed under specific pathogen-free conditions according to study protocols approved by the Animal Care & Ethics Committee of UNSW (approval No. 02/103). The athymic mouse was chosen due to its minimal cellular immunity, so as to minimize risk of rejection of human hepatocytes and facilitate tumor engraftment. The main aims were to study (a) the tumor load required for tumorigenesis following intraperitoneal transplantation, (b) the effect of co-transplantation of human hepatocytes on tumorigenesis and (c) the effect of our immunomagnetic purging protocol on tumorigenesis. The experimental protocol is described below in Table 2.

Hepatocytes and HT-29 cells were prepared as per the *in vitro* arm, separately and in combination to produce suspensions containing the cell numbers required per mL PBS (cf Table 2). Two hundred µL samples, containing 20% of the cell number in each 1 mL inoculation, were collected for negative control,

**Table 2 Mice & cell transplantation details**

Treatment groups	Mouse group	IP injection	No. of mice
Negative control (Hepatocyte only)	1	$5 \times 10^6$ hepatocytes	3
Positive control 1 (HT-29 only)	2	5000 HT-29 cells	3
	3	100 000 HT-29 cells	3
	4	500 000 HT-29 cells	3
	5	2 million HT-29 cells	3
Positive control 2 (Hepatocytes + HT-29)	6	5 million hepatocytes + 5000 HT-29	3
	7	5 million hepatocytes + 100 000 HT-29	3
	8	5 million hepatocytes + 500 000 HT-29	3
	9	5 million hepatocytes + 2 million HT-29	3
Bead (Hepatocyte + HT-29)	10	5 million hepatocytes + 5000 HT-29	3
	11	5 million hepatocytes + 100 000 HT-29	3
	12	5 million hepatocytes + 500 000 HT-29	3
	13	5 million hepatocytes + 2 million HT-29	3

**Table 3 Tumor growth**

Treatment groups	IP injection	Mouse with tumour	Percentage expression (%)
Negative control (Hepatocyte only)	$5 \times 10^6$ hepatocytes	0/3	0
Positive control 1 (HT-29 only)	5000 HT-29 cells	0/3	0
	100 000 HT-29 cells	1/3 <sup>1</sup>	0 <sup>1</sup>
	500 000 HT-29 cells	2/3	67
	2 million HT-29 cells	3/3	100
Positive control 2 (Hepatocytes + HT-29)	5 million hepatocytes + 5000 HT-29	0/3	0
	5 million hepatocytes + 100 000 HT-29	0/3	0
	5 million hepatocytes + 500 000 HT-29	3/3	100
	5 million hepatocytes + 2 million HT-29	2/3	67
Bead (Hepatocyte + HT-29)	5 million hepatocytes + 5000 HT-29	0/3	0
	5 million hepatocytes + 100 000 HT-29	0/3	0
	5 million hepatocytes + 500 000 HT-29	0/3	0
	5 million hepatocytes + 2 million HT-29	0/3	0

<sup>1</sup>Injection-site tumor in skin only; In italics: Groups in which tumor was found; In standard form: Tumor-free.

positive control and immunomagnetic bead groups, before and after purging for RT-PCR analysis.

Mice were monitored for 28 d post-transplantation after which they were sacrificed with a lethal 6 mg dose of pentobarbital sodium. The abdomen, pelvis and thorax were examined visually for the presence of tumor.

## RESULTS

### Isolation of hepatocytes

The total viable hepatocyte yield averaged at  $9.3 \times 10^8$  cells per isolation (range  $2.0 \times 10^8$ - $36.3 \times 10^8$ ; mean  $\pm$  SD viable hepatocyte yield  $9.33 \times 10^8 \pm 6.0 \times 10^6$  cells/g digested liver tissue), with the five most recent isolations each yielding over  $1.0 \times 10^9$  cells. The mean viability of freshly isolated hepatocytes was  $70.5\% \pm 8.1\%$ . Mean warm ischemic time was  $31 \pm 19$  min (range, 25-60 min); mean cold ischemic time was  $1.5 \pm 0.6$  h (range, 0.5-16 h).

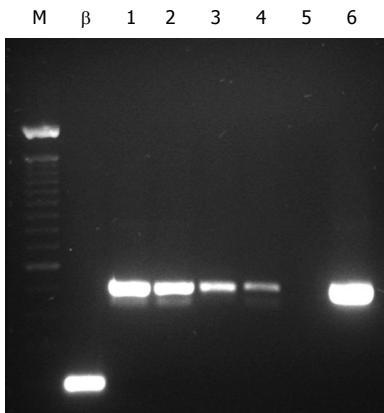
### Tumorigenesis of transplanted hepatocyte and tumor cell suspensions

RT-PCR analysis demonstrated clear single bands at the 515 bp position, indicating Ep-CAM detection, in representative samples of all hepatocyte/HT-29 cell suspensions transplanted into mice belonging to Positive

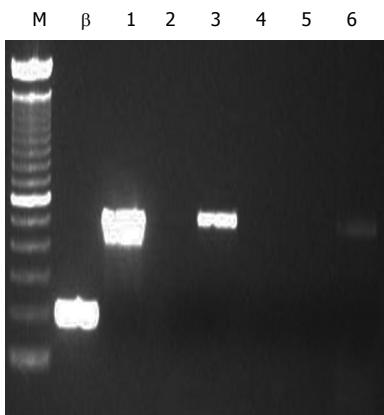
Control 2 and pre-treatment Bead groups. No detectable bands were seen in the samples transplanted into the Negative Control and post-treatment Bead groups (gel images not shown), indicating in the latter case the removal of Ep-CAM positive cells (including HT-29 cells) to below the detection limit of 1 tumor cell in 1 million hepatocytes (Figure 1) and thus, a maximum tumor purging efficacy of immunomagnetic bead treatment of at least 400 000 fold (Figure 2).

In the control groups, all mice injected with 100 000 HT-29 cells and below showed no tumor, except for one animal inoculated with 100 000 HT-29 cells only. The mouse developed a small (0.1 g) skin nodule at the injection site and had no evidence of intra-abdominal tumor on detailed examination. This is probably due to an inadvertent subcutaneous rather than intraperitoneal injection of cells, and could thus be excluded on the basis of technical error. All except two mice inoculated with at least 500 000 HT-29 cells (83%) developed tumor. There was no significant difference in tumor expression between mice injected with or without hepatocytes (Tables 2 and 3). These results would suggest that the minimum tumor load required for engraftment and growth was between 100 000 and 500 000.

There was a complete absence of tumor development in any mouse injected with HT-29 cell-spiked hepatocyte



**Figure 1** Detection sensitivity. RT-PCR detection of HT-29 cells in  $1 \times 10^6$  hepatocytes. Lane M: 100 bp ladder (100 bp to 1 kb); Lane  $\beta$ :  $\beta$ -actin; Lane 1: 100 HT-29 cells; Lane 2: 10 HT-29 cells; Lane 3: 5 HT-29 cells; Lane 4: 1 HT-29 cell; Lane 5: Hepatocytes only; Lane 6: HT29 only.



**Figure 2** Tumor-purging efficacy. RT-PCR detection of EpCAM RNA in HT-29-cell-spiked hepatocytes ( $1 \times 10^6$ ) with and without treatment with Ber-EP4-coated immunomagnetic beads. Lane M: 100 bp ladder (100 bp to 1 kb); Lane  $\beta$ :  $\beta$ -actin; Lane 1: 50000 HT-29 cells; Lane 2: 50000 HT-29 cells treated with immunomagnetic beads; Lane 3: 10000 HT-29 cells; Lane 4: 10000 HT-29 cells treated with immunomagnetic beads; Lane 5: 10 HT-29 cells treated with immunomagnetic beads; Lane 6: 10 HT-29 cells only.

suspensions treated with immunomagnetic beads. All mice survived until day 28 without any significant weight loss/gain or signs of malaise or distress. Where intraperitoneal tumors occurred, these were seen only intra-abdominally, expressed as distinct, scirrhouous, white nodules adherent to the parietal or visceral peritoneum, without evidence of metastasis.

## DISCUSSION

Our results show that clinically relevant numbers of hepatocytes can be recovered from macroscopically normal liver unavoidably removed during hepatic resection for neoplasia. Further, when isolated and subjected to an immunomagnetic cell separation technique, the resulting hepatocytes can be safely transplanted intra-peritoneally in athymic mice without increased risk of development of tumor. Only patients requiring anatomical resection were included in this study. Our technique may be less useful in the case of

patients undergoing small, non-anatomical resection; in this situation the viable hepatocyte yield would be less.

Our mean viable yield of  $9.33 \times 10^6$ /g is a significant improvement from our preliminary study<sup>[13]</sup> and marginally exceeds that reported by Richert *et al*<sup>[22]</sup>. To achieve this, the original isolation protocol was altered to include sealing of cannula points and cut surfaces prior to perfusion and ensure a cold ischemic time of less than 5 h; both of these changes have been independently identified to positively influence viable yield<sup>[22,23]</sup>.

The number of hepatocytes transplanted in clinical experiences to date has ranged between  $2 \times 10^8$  to  $4 \times 10^9$  cells<sup>[24]</sup>. Given a mean viable yield of  $9.3 \times 10^8$  cells per isolation, our technique offers the possibility of one hepatocyte transplant for every hepatocyte isolation. In the order of 80–100 liver resections are performed annually in our unit, of which approximately half will be suitable for hepatocyte isolation. Thus, a minimum of forty hepatocyte transplants could potentially be performed annually.

A comparison of viable hepatocytes yield from resection specimens versus cells obtained from explanted organs rejected for OLT shows that the resected specimens have a consistently higher viable yield<sup>[22]</sup>. Further, recovery from cryopreservation of hepatocytes derived from normal resected liver is significantly higher compared to that of cells obtained from organs rejected for liver transplantation<sup>[25]</sup>, thus improving the potential quality and quantity of bankable cells for use on-demand. Such factors, combined with the opportunity to utilize a hitherto untapped hepatocyte source, further enhance the potential application of hepatocyte transplantation as a clinically relevant treatment modality.

An important concern regarding the use of hepatocytes isolated from liver resections performed for malignancy has been the possibility of co-transplanting contaminating tumor cells. Although immunomagnetic cell separation has been widely utilized to enhance detection of tumor cells in different body compartments, purging has been mainly limited to *ex vivo* removal of tumor cells from autologous stem cell transplants<sup>[18,19,26,27]</sup>. To our knowledge, our centre is the first to propose immunomagnetic purging of any residual, contaminating tumor cells from isolated hepatocyte suspensions.

The Ep-CAM cell-surface antigen is consistently present on both HT-29 colorectal cancer cells and most colorectal metastases<sup>[28]</sup>, the pathology in the majority of our liver resection specimens in this study. The antigen is not expressed by mature hepatocytes<sup>[20]</sup> making differential separation of Ep-CAM-expressing tumor cells by immunomagnetic beads (coated with Ber-EP4) possible. Various other carcinomas, including all the types from our patient cohort, also express Ep-CAM<sup>[29]</sup>. As Ep-CAM has been shown consistently to be absent in hepatocellular carcinoma, patients with such tumors, along with other Ep-CAM-negative lesions, were excluded from our study to avoid undetectable tumor contamination. Whilst hepatocytes were harvested only from patients whose tumors expressed Ep-CAM in this

study, the targeting of additional molecular markers such as CK-20 and CEA enhanced detection of any potential tumour cells without an Ep-CAM phenotype<sup>[30,31]</sup>. Additional surface antigens are currently being studied to potentially increase the tumor-purging efficacy by multiplying the number of target molecules per cell.

In our study, immunomagnetic purging was shown to remove tumor cells by a factor greater than 400 000. This compares favorably with similar large-scale experiments involving breast cancer cells in blood stem cell harvests<sup>[32]</sup>. It also represents a substantial improvement on our preliminary experience<sup>[13]</sup>, attributable to optimization of the sample purging treatment and bead to cell ratio employed. The development of an RT-PCR detection assay, in addition to standard immunohistochemical methods, has enabled a more efficient and sensitive detection of tumor contamination (1 tumor cell per 1 million hepatocytes) enabling confidence that in a typical human hepatocyte transplant of 1 billion cells, a maximum tumor load of no more than 1000 cells could be present in the preparation. This is significantly below the 100 000-500 000 cell threshold for tumor engraftment and growth demonstrated in our athymic mouse model in this study. Further, no additional growth potential was conferred to tumor cells by the co-transplantation of hepatocytes, an important observation that indicates that the presence of hepatocytes does not magnify the risk of tumor cell engraftment.

We have demonstrated that with the use of an optimized cell-isolation protocol, liver resection specimens obtained from patients undergoing resection for neoplasia can offer sufficient viable hepatocytes to potentially provide clinically-relevant liver support. We have further shown both *in vitro* and in a suitable *in vivo* animal model that immunomagnetic purging can confer safety from the potential of tumor contamination of hepatocyte suspensions. We therefore propose that liver resection specimens, by a simple purging step, may provide a safe, alternative hepatocyte source for clinical transplantation.

## COMMENTS

### Background

With a world wide shortage of liver donor organs, adjunct treatment regimes to support patients to orthotopic liver transplantation (OLT) or to replace when OLT is contra-indicated are becoming increasingly important. Hepatocyte transplantation is one such, however, a major limitation to its clinical application is the availability of primary human hepatocytes. Hepatocytes isolated from macroscopically normal liver removed during hepatic resection for neoplasia could provide an additional source of hepatocytes, given the development of strategies to detect and remove residual malignant cells.

### Research frontiers

The liver margins of neoplasia patients, an increasingly common procedure, are normally discarded. The aim was to discover whether clinically relevant numbers of healthy hepatocytes could be recovered from these waste pieces and used for transplantation, thereby adding another source to the traditional sources of hepatocytes.

### Innovations and breakthroughs

It was found that clinically relevant numbers of hepatocytes can be recovered from macroscopically normal liver removed during neoplasia hepatic resection. A protocol based on current immunomagnetic bead technology was developed

to capture and remove cancerous cells from hepatocytes in concert with a novel RT-PCR assay for detection of the tumour cells.

### Applications

An additional source of hepatocytes for transplantation boosts both ongoing research into the efficacy of hepatocyte transplantation and clinically, increases the number of treatable patients. Additionally, hepatocytes prepared correctly can be stored until required, divided amongst multiple patients, or combined with hepatocytes from other donors, increasing further the number of patients that can be treated.

### Peer review

The authors evaluated the efficacy of Ep-CAM antibody-coated magnetic beads in tumor cell removal from hepatocyte suspensions. This is an interesting work that normal liver resected for neoplasia may be potential as another clinically useful source of hepatocytes for transplantation.

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RAPID COMMUNICATION

## Differentiation of malignant and benign proximal bile duct strictures: The diagnostic dilemma

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### Abstract

**AIM:** To identify the criteria for the differentiation of hilar cholangiocarcinoma (HCCA) from benign strictures.

**METHODS:** A total of 68 patients underwent resection of lesions suspicious for HCCA between 1998 and 2006. The results of laboratory investigations, imaging studies and brush cytology were collected. These findings were analyzed to obtain the final diagnosis.

**RESULTS:** Histological examination of the resected specimens confirmed HCCA in 58 patients (85%, group I) whereas 10 patients (15%, group II) were diagnosed to have benign strictures. The most common presenting symptom was obstructive jaundice in 77% patients (79% group I vs 60% group II,  $P = 0.23$ ). Laboratory findings showed greater elevation of transaminase levels in group I compared to group II. The various imaging modalities showed vascular involvement exclusively in the malignant group (36%,

$P < 0.05$ ). Brush cytology was positive for malignant cells in only 50% patients in group I whereas none in group II showed malignant cells.

**CONCLUSION:** Despite improvements in imaging techniques, 10 patients (15%) with a presumptive diagnosis of HCCA were ultimately found to have benign strictures. Except for vascular involvement which was associated significantly with malignancy, there were no conclusive features of malignancy on regular imaging modalities. This uncertainty should be taken into account when patients with a suspicious lesion at the liver hilum are considered for resection.

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**Key words:** Biliary stricture; Hilar cholangiocarcinoma; Benign; Radiological; Vascular involvement

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### INTRODUCTION

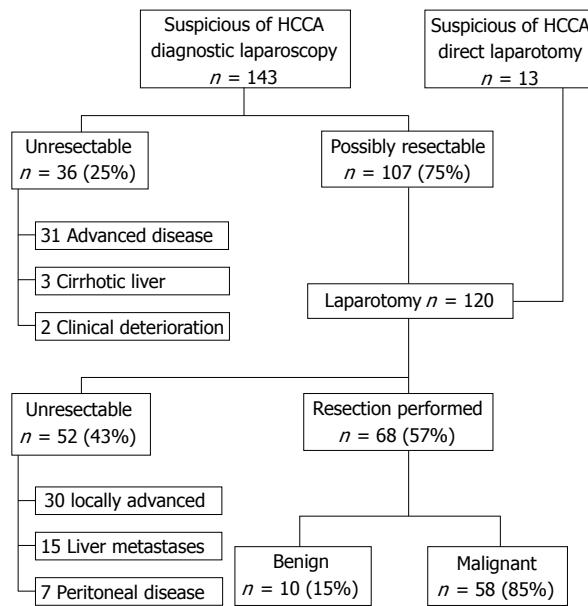
Hilar resection en bloc with liver resection is the only curative treatment option for patients with carcinoma at the hepatic duct confluence. Although mortality rates associated with partial hepatectomy have decreased markedly in the past decades, postoperative morbidity is considerable and can exceed 50 percent<sup>[1-4]</sup>. Undertaking partial hepatectomy for hilar cholangiocarcinoma (HCCA) becomes even more a subject of discussion when histopathology of the resected specimen shows benign disease. A variety of benign lesions at the liver hilum can mimic malignancy. In particular, inflammatory lesions may present with the same clinical and radiological features as a malignant tumor.

In the past decade, imaging techniques have improved and the diagnostic work-up of patients suspected of HCCA usually comprised of ultra-sonography (US), contrast enhanced multi-slice computed tomography (CT) and magnetic resonance cholangiography (MRC). In experienced hands, colour Doppler US is very useful in assessing proximal biliary extension of the tumor and vascular invasion, but has limited value in distinguishing HCCA from inflammatory lesions<sup>[5]</sup>. Contrast enhanced CT and MR imaging (MRI) provide important information regarding resectability and vascular involvement<sup>[6-8]</sup>. Furthermore, these cross-sectional imaging modalities are accurate in detecting a tumor mass and signs of lymphadenopathy, although both features may also be present in benign diseases. Cholangiography in combination with brush cytology has a low sensitivity, and although the specificity is higher, it is not 100% since longstanding stenting of the biliary duct may give rise to false positive cytology results<sup>[9]</sup>. Overall, an extensive work-up with multiple imaging techniques may improve the differentiation between malignant and benign proximal bile duct strictures.

Several studies have noted that approximately 14% to 25% of patients resected for presumed HCCA prove to have a benign lesion at histopathology<sup>[10-14]</sup>. A recent study attempted to identify potential criteria for distinguishing patients with HCCA from those with an alternative diagnosis<sup>[15]</sup>. However, the non-HCCA patients in this series comprised of benign and malignant diseases (for instance gallbladder cancer), which makes any conclusions difficult to interpret. In a previous series of 132 patients who had undergone surgical treatment for suspicious HCCA at our center from 1983 to 1998, 15% (20/132) of the patients had benign lesions<sup>[16]</sup>. In the present study, which covers a more recent period during which improved imaging techniques were used, the rate of mis-diagnosed benign lesions was re-examined and the diagnostic features of benign and malignant lesions was compared. The aim of the study was to identify criteria that can differentiate HCCA from benign proximal bile duct strictures.

## MATERIALS AND METHODS

Between January 1998 and December 2006, a total of 143 patients underwent a diagnostic laparoscopy for staging of HCCA, and 13 patients underwent laparotomy without diagnostic laparoscopy. Unresectable disease was found in 36 patients undergoing laparoscopy, and in the majority of these cases the diagnosis was confirmed by histology (Figure 1). Another 52 patients were found to have unresectable disease during subsequent laparotomy which was confirmed by histology. Finally, 68 consecutive patients underwent resection and are the subject of the present study (Figure 1). These patients had hilar resection with complete lymphadenectomy of the hepato-duodenal ligament usually en bloc with (extended) hemi-hepatectomy including resection of the caudate lobe and left or right portal vein<sup>[17]</sup>. Although in the majority of the patients included in the study,



**Figure 1** Flow chart of patients eligible for resection of hilar cholangiocarcinoma (HCCA) with final histopathological outcome in the period from January 1998 to December 2006.

the initial diagnostic evaluation was performed at the referring hospital, imaging studies such as US, CT and MRC were usually repeated in our center. The medical data of patients obtained from these hospitals and from our center included demographic features, relevant medical history, presenting symptoms, laboratory investigations, results of imaging studies (including cholangiography, CT, MRC, duplex US), radiological and endoscopic interventions including placement of a stent, brush cytology and intra-operative findings.

The results of biliary brush cytology obtained during antegrade percutaneous transhepatic cholangiography (PTC) or endoscopic retrograde cholangiopancreaticography (ERCP) were categorized as follows: highly suspicious for adenocarcinoma, atypical cells/inconclusive, and no malignant cells. Data from imaging studies were collected for staging purposes. The data recorded from these studies included: the presence of a mass lesion, proximal extent of the tumor within the biliary tree according to the Bismuth-Corlette classification system, vascular involvement (hepatic artery, portal vein), liver lobe atrophy, and lymphadenopathy. Ultrasonography, CT and MRI were used to detect a mass lesion, to determine the size of the lesion and the level of the biliary obstruction. Findings on PTC and/or ERCP showing an irregular and eccentric stenosis and/or a blunt end rather than a smooth tapering narrowing of the duct were considered more suggestive of a malignant lesion. Vascular invasion on colour Doppler US was defined as an increase in flow compatible with stenosis or absence of flow compatible with occlusion. Furthermore, on contrast enhanced CT, presence of vascular stenosis or occlusion of the portal vein and encasement of the artery were indicative of vascular involvement.

The final histological diagnosis was correlated with

the preoperative clinical and laboratory findings as well as with the radiological data in an effort to identify criteria which may be useful for the differentiation of HCCA (group I, 58 patients) from benign proximal bile duct stricture (group II, 10 patients). The resected specimens of the benign lesions were re-assessed by a single pathologist, specialized in hepatobiliary pathology (FJ. tK.). The results obtained are expressed as the mean (SEM). The differences between categorical variables were evaluated by chi-square analysis, while Student's *t* test was used for all comparisons among continuous variables. A two-tailed *P* value less than 0.05 was considered to indicate significant differences. All statistics were carried out using the SPSS Base 12.0 for Windows Statistical Package for Social Sciences (SPSS®, Chicago, IL).

## RESULTS

The demographic features, presenting symptoms, and preoperative laboratory findings are shown in Table 1. The mean age and male-female gender ratio were equal in the two study groups. Although 9 patients (groups combined) had a prior cholecystectomy, none of the patients had a complicated procedure with bile duct stricture. In the group with benign lesions, one patient had history of alcohol abuse and related chronic pancreatitis, and another patient had history of inflammatory bowel disease (ulcerative colitis). In the malignant group, one patient had history of alcohol abuse and one patient had Crohn's colitis with primary sclerosing cholangitis (PSC).

There were no statistically significant differences between the two groups with respect to the clinical presentation. Jaundice was present in 79% of the patients in group I and 60% of group II. Abdominal pain, usually located in the right upper abdomen or in the epigastric region was mostly vague and nonpersistent. Weight loss was observed in both groups with a median loss of 6.4 kg in group I (range 2-16) and 7.3 kg (range 2-10) in group II. No differences were observed in the results of the laboratory tests, except for serum transaminase levels which showed higher levels in group I compared to group II (*P* < 0.05, Table 1). Assessment of tumor markers, including carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA 19-9) was not performed routinely.

The majority of patients underwent imaging studies, and interventions such as ERCP, PTC and diagnostic laparoscopy (Table 2). The mean number of imaging procedures was 3.3 per patient in both groups. CT scan was performed most frequently, with an overall rate of 96%. Sixty patients (88%) underwent colour Doppler US, while MRI was performed in 21 patients (31%). Cholangiography (either ERCP or PTC) was performed in 63 patients (93%). Forty-seven patients underwent ERCP and 15 patients underwent PTC with at least one bile duct stent or drainage tube inserted. Five patients underwent both ERCP and PTC to achieve biliary decompression. Examination of brush cytology, shown

**Table 1 Demographic features, presenting symptoms and laboratory findings in patients resected for presumed HCCA**

	Malignant (n = 58)	Benign (n = 10)	P
<b>Demographics</b>			
Gender male/female	35/23	3/7	0.09
Mean age (range)	62 (30-80)	61 (40-71)	0.62
Prior history of cholecystectomy	6 (10%)	3 (30%)	0.12
<b>Presenting symptoms</b>			
Jaundice	46 (79%)	6 (60%)	0.23
Abdominal pain	27 (47%)	6 (60%)	0.51
Weight loss	34 (59%)	4 (40%)	0.32
Fever	2 (3%)	2 (20%)	0.10
<b>Preoperative laboratory findings</b>			
Bilirubin (μmol/L)	144 (± 15)	107 (± 35)	0.36
AP (U/L)	430 (± 61)	371 (± 64)	0.73
AST (U/L)	119 (± 12)	58 (± 15)	0.02
ALT (U/L)	190 (± 21)	88 (± 22)	0.03
GGT (U/L)	675 (± 89)	405 (± 114)	0.32
LDH (U/L)	313 (± 20)	277 (± 30)	0.42
PT (Prolonged-Normal)	3-33 (8%)	1-6 (14%)	0.62

HCCA: Hilar cholangiocarcinoma; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma glutamyl transpeptidase; LDH: Lactate dehydrogenase; PT: Prothrombin time; AP: Alkaline phosphatase.

**Table 2 Imaging studies and interventions in patients resected for presumed HCCA**

	Malignant (n = 58)	Benign (n = 10)	P
<b>No. of imaging studies</b>			
ERCP	51 (88%)	9 (90%)	
PTC	13 (22%)	3 (30%)	
US + colour Doppler	52 (90%)	8 (80%)	
CT	56 (97%)	9 (90%)	
MRC	17 (29%)	4 (40%)	
Mean No. of procedures per patient	3.3	3.3	
<b>Stent placement procedure</b>			
ERCP	40	7	
PTC	12	3	
ERCP and PTC	4	1	
<b>Biliary brushing</b>			
No. of brushings performed	30/58 (52%)	6/10 (60%)	0.74
Highly suspicious	15/30 (50%)	0/6 (0%)	0.03
No malignant cells	9/30 (30%)	4/6 (67%)	0.16
Atypical cells, inconclusive	6/30 (20%)	2/6 (33%)	0.60
<b>Diagnostic laparoscopy</b>			
No. of laparoscopies performed	53 (91%)	8 (80%)	
Intra-operative US performed	15	1	

in Table 2, showed malignant cells in 50% of patients in group I, whereas none in group II showed malignant cells (*P* < 0.05). Furthermore, brush cytology in 9 patients in the malignant group proved false negative. Atypical cells were found in 6 and 2 patients in group I and II, respectively.

The findings obtained with the imaging studies are shown in Table 3. A mass lesion at the hepatic duct confluence was seen in 56 patients (97%) in group I and 8 patients (80%) in group II. The presence and size of a mass could not distinguish between benign or malignant disease. The extent of bile duct involvement classified according to the Bismuth-Corlette system was similar in the two groups. Vascular involvement was

**Table 3 Results of imaging studies and intra-operative findings in patients resected for presumed HCCA**

	<b>Malignant (n = 58)</b>	<b>Benign (n = 10)</b>	<b>P</b>
Findings on preoperative imaging			
Presence of mass	56 (97%)	8 (80%)	0.10
Mean size (range, cm)	2.5 (0.7-7.0)	2.6 (1.0-3.7)	0.62
Bismuth classification			
Type I, II	14	3	0.70
Type IIIa/b, IV	39	6	
Intrahepatic	5	1	
Vascular involvement <sup>1</sup>	21 (36%)	0 (0%)	0.03
Portal vein	19	0	
Hepatic artery	6	0	
Lobar atrophy	10 (17%)	1 (10%)	1.00
Lymph nodes (> 1 cm)	12 (21%)	1 (10%)	0.67
Intra-operative findings			
FS performed <sup>2</sup>	57/58 (99%)	10/10 (100%)	
FS positive for malignancy	20/57 (35%)	0/10 (0%)	0.03
Suspicious <sup>3</sup> -Not suspicious	51-7	5-5	
Type of resection			
Local bile duct resection	17 (29%)	3 (30%)	
Concomitant partial hepatectomy	41 (71%)	7 (70%)	

FS: Frozen section. <sup>1</sup>Non cumulative, patients with simultaneously portal v. and hepatic a. involvement; <sup>2</sup>FS from suspicious lesions, lymph nodes and/or resection margins; <sup>3</sup>A positive FS and/or palpable mass.

observed only in patients with malignant disease ( $P < 0.05$ ), although lobar atrophy was not more frequent in the malignant group. Lobar atrophy was present in one patient in the benign group, probably caused by segmental biliary obstruction. Moreover, the presence of enlarged lymph nodes ( $> 1$  cm, short axis diameter) could not differentiate the two groups. After taking into consideration the clinical presentation, laboratory findings, imaging studies, prior interventions and brush cytology, all 68 patients were diagnosed to have a lesion suspicious of HCCA.

During surgical exploration, frozen sections were obtained of suspicious lymph nodes, tissue suspicious of tumor infiltrating the vessel walls, and the resection margins to ensure radical resection in all patients except one (Table 3). The frozen section examination confirmed malignancy in 35% of the patients in group I, and no false positive results were obtained in group II ( $P < 0.05$ ). A palpable, suspicious hilar tumor was found in 5 patients (50%) in the benign group and in 51 patients (88%) in the malignant group. Eventually, 70% of all patients underwent hilar resection, in combination with partial liver resection; the prevalence of local bile duct excision was equally divided between the two groups. Histological analysis of the resected specimens showed a benign bile duct stricture in 10 patients, diagnosed as chronic fibrosing lesion, erosive inflammation, sclerosing cholangitis or autoimmune cholangitis (IgG4-related) (Table 4). In the remaining 58 resection specimens, histology confirmed HCCA.

## DISCUSSION

Cholangiocarcinoma and benign, inflammatory lesions of

the biliary tract may have a similar clinical presentation. Differentiating the two conditions is complex because on the one hand, HCCA is frequently associated with secondary inflammatory changes, and on the other hand, conditions such as PSC predispose to malignancy of the bile ducts (with a reported prevalence of 30%)<sup>[18]</sup>. A combination of different imaging modalities is usually employed in the diagnosis of hilar bile duct lesions<sup>[8]</sup>. However, to date, no single investigation has been found to reliably differentiate HCCA from benign proximal bile duct strictures.

In the present study, the rate of patients (15%) with benign lesions misdiagnosed as malignancy was similar to the rate (15%) observed in a previous study performed at our center a decade earlier (1983-1997)<sup>[16]</sup>. Despite considerable improvements in imaging techniques (contrast enhanced multi-slice CT and MRC), and an increase in the number of imaging procedures (a mean of 3 modalities in comparison to 2 in the previous study), patients still required unnecessary extensive resections, for achieving adequate biliary drainage. Case series from other groups worldwide, have noted comparable rates of benign lesions in resections performed for presumed HCCA (Table 5). Our observations confirm the findings of these studies and emphasize the difficulty in differentiating benign from malignant lesions at the liver hilum, despite use of state-of-the-art imaging modalities.

Clinical features alone cannot differentiate HCCA from benign proximal bile duct strictures. With regard to laboratory tests, plasma transaminase values were significantly elevated in the malignant group compared to patients with benign hilar lesions. Although raised transaminase values may occur in patients with benign proximal bile duct strictures (usually in conjunction with cholangitis), our results show that this is an uncommon finding. Other laboratory tests failed to identify patients with a malignancy. The diagnostic value of tumor markers such as CA 19-9 and CEA in biliary cancer has been extensively studied. One study showed 100% sensitivity using a combination of CA 19-9 and CEA<sup>[19]</sup>, but these results could not be confirmed by other workers<sup>[9,10]</sup>. Another, potentially useful test is serum IgG4. Recently, IgG4-related lymphoplasmacytic sclerosing disease was observed in patients with strictures of the pancreatic duct mimicking carcinoma<sup>[20]</sup>. In a study from our center, it was shown that IgG4-related sclerosing disease also occurs in patients with benign proximal bile duct strictures<sup>[21]</sup>. In the present series, two of the ten patients with benign proximal bile duct strictures showed infiltration by IgG4-plasma cells with histological features suggestive of an autoimmune disorder. Serum levels of IgG4 have potential to differentiate benign disease from HCCA, although the diagnostic value of this marker and the role of immunomodulatory drugs requires further investigation<sup>[22]</sup>.

The findings obtained with the different imaging studies revealed that only one feature, i.e. vascular involvement, was significantly different between the

**Table 4 Patients with benign proximal bile duct strictures: Preoperative, intra-operative and histological findings**

Number of patient	Age/Gender	Medical history	Bismuth classification	Brush cytology	Intra-operative findings	Treatment	Final histological diagnosis
1	40/F	LC	Intrahepatic	Atypical cells	Suspicious	Hemihepatectomy le	Fibrosing cholangitis
2	54/F	-	Type IIIa	No malignancy	Suspicious	Hemihepatectomy ri <sup>1</sup>	Sclerosing cholangitis
3	56/M	IBD	Type IIIb	-	Not suspicious	Hemihepatectomy le <sup>1,2</sup>	Sclerosing cholangitis
4	60/F	-	Type II	No malignancy	Not suspicious	Local resection	Fibrosing cholangitis
5	63/F	LC	Type II	Atypical cells	Suspicious	Local resection	Erosive inflammation
6	65/F	-	Type IIIa	-	Not suspicious	Hemihepatectomy ri	Autoimmune-like cholangitis
7	68/F	LC	Type IIIa	-	Suspicious	Hemihepatectomy ri	Fibrosing cholangitis
8	69/F	-	Type IIIb	-	Not suspicious	Hemihepatectomy le	Sclerosing cholangitis
9	70/M	-	Type II	No malignancy	Not suspicious	Local resection	Erosive inflammation
10	71/M	CP	Type IIIa	No malignancy	Suspicious	Hemihepatectomy ri <sup>1</sup>	Autoimmune-like cholangitis

CP: Chronic pancreatitis; LC: Laparoscopic cholecystectomy; IBD: Inflammatory bowel disease. <sup>1</sup>Partial liver resection + local resection; <sup>2</sup>Partial liver resection was performed because of atrophic liver lobes.

**Table 5 Incidence of benign lesions in patients resected for presumed HCCA: Review of literature**

Source, yr <sup>1</sup>	Period of inclusion	Number of patients <sup>2</sup>	Number of benign lesions (%)
Hadjis <i>et al</i> 1985 <sup>[34]</sup>	1979-1983	104 <sup>3</sup>	8 (8)
Wetter <i>et al</i> 1991 <sup>[35]</sup>	1985-1990	59 <sup>4</sup>	8 (14)
Verbeek <i>et al</i> 1992 <sup>[36]</sup>	1984-1990	82	11 (13)
Nakayama <i>et al</i> 1999 <sup>[10]</sup>	1990-1997	99	14 (15)
Gerhards <i>et al</i> 2001 <sup>[16]</sup>	1983-1997	132	20 (15)
Knoefel <i>et al</i> 2003 <sup>[12]</sup>	1996-1999	33	6 (18)
Khalili <i>et al</i> 2003 <sup>[13]</sup>	2000-2001	20	4 (20)
Koea <i>et al</i> 2004 <sup>[37]</sup>	1998-2002	49	12 (24)
Corvera <i>et al</i> 2005 <sup>[38]</sup>	1992-2003	275 <sup>5</sup>	22 (8)
Are <i>et al</i> 2006 <sup>[15]</sup>	1997-2001	59	9 (15)
Uhlmann <i>et al</i> 2006 <sup>[39]</sup>	1998-2004	49	7 (14)
Present study	1998-2006	68	10 (15)

<sup>1</sup>Listed chronologically; <sup>2</sup>Indicates only patients undergoing resection;

<sup>3</sup>Patients evaluated of suspected hilar cholangiocarcinoma (not all resected); <sup>4</sup>Patients resected for papillary tumours were excluded (*n* = 5).

benign and malignant groups. Even a mass lesion was not able to differentiate between the two groups. Furthermore, in contrast to the report by Are and co-workers<sup>[15]</sup>, we did not find any difference between the benign and malignant groups with respect to either the level of obstruction or the extent of lesion. Moreover, there was no difference in rate of liver lobe atrophy. Portal vein occlusion of one side of the liver typically gives rise to ipsilateral lobar atrophy and compensatory hypertrophy of the contralateral lobe. However, it is possible to develop lobar atrophy without portal vein occlusion. In benign disease, lobar atrophy can develop secondary to longstanding cholestasis. In a series of 162 patients with intrahepatic cholelithiasis, lobe atrophy was observed in 7% of the patients<sup>[23]</sup>. Overall, the finding of lobar atrophy is uncommon with benign disease and should be considered as a manifestation of vascular involvement and therefore of malignancy. Occlusion of the portal vein obviously suggests malignancy, however two cases have been reported of a benign inflammatory pseudotumor mimicking HCCA with vascular involvement<sup>[24,25]</sup>. Therefore, although uncommon, even vascular involvement may be seen in benign lesions at the liver hilum.

Brush cytology of a biliary stricture is often undertaken during ERCP or PTC. Unfortunately, the diagnostic yield is poor with sensitivity rates around 50%<sup>[9,26]</sup>. Moreover, the specificity is not 100% since longstanding stenting of the biliary duct induces chronic inflammation, which makes it difficult to differentiate benign from malignant cells, resulting in false positive results<sup>[9]</sup>. The present study showed similar rates which is consistent with previous studies. Fluorescence in situ hybridization (FISH) has been increasingly used to facilitate the identification of neoplastic cells in cytologic specimens<sup>[27]</sup>. In one study, 20% of patients with cholangiocarcinoma missed by conventional cytology were identified by FISH without affecting the specificity<sup>[28]</sup>. Several studies have evaluated the diagnostic yield of endoscopic US fine needle aspiration in patients with biliary strictures<sup>[29-32]</sup>. In one of the studies, the sensitivity and specificity of biopsy were 89% and 100%, respectively<sup>[30]</sup>. Moreover, the planned surgical approach was changed in 27 of 44 patients. Therefore, biopsy from either the mass or the surrounding malignant-appearing lymph nodes appears to have a higher sensitivity than ERCP or PTC with brush cytology. More recently, techniques such as intraductal ultrasound (IDUS) and cholangioscopy have been used to obtain direct biopsy specimens. IDUS with biopsy increased the accuracy of ERCP from 58%-60% to 83%-90% in distinguishing benign and malignant strictures<sup>[33]</sup>. These diagnostic modalities appear very promising in differentiating benign and malignant biliary lesions, although they are highly expert-dependent and are still not widely available.

In one-half of the patients diagnosed to have a benign lesion at the liver hilum, the intra-operative findings were consistent with a malignant tumor (i.e. positive frozen section diagnosis and/or evident palpable mass). Furthermore, 7 patients in the malignant group were not found to have a suspicious lesion during laparotomy. Thus, even at surgery it is difficult to determine the nature of a hilar lesion, and resection is the only way to rule out malignancy.

In conclusion, despite improvements in the quality and increase in the number of imaging studies, 10

out of 68 (15%) patients with presumed HCCA, were misdiagnosed. Vascular involvement showed a significant association with malignant lesions. However, there was no feature on imaging studies or laboratory tests that reliably distinguished HCCA from benign proximal bile duct lesions. Therefore, differentiation of benign from malignant lesions at the liver hilum remains difficult and this should be taken into account when considering resection in patients with suspicious hilar lesions.

## COMMENTS

### Background

The main etiology of proximal bile duct stricture is hilar cholangiocarcinoma (HCCA). However, the differentiation of benign and malignant strictures is difficult which has obvious important consequences for management. Extensive work-up including multi-slice computed tomography and magnetic resonance cholangiography may help improve the diagnostic dilemma.

### Research frontiers

The differentiation of benign and malignant lesions at the liver hilum remains a diagnostic dilemma despite improvements in the quality and increase in the number of imaging techniques. Novel diagnostic and imaging techniques are discussed, although none have shown a high rate of accuracy. The most promising diagnostic modality appears to be intraductal ultrasound in combination with cholangioscopic biopsy.

### Innovations and breakthroughs

The authors observed that vascular involvement had a significant association with malignant lesions. No other feature on imaging studies or laboratory tests was able to reliably distinguish HCCA from benign proximal bile duct lesions.

### Applications

Vascular involvement emerged as the most important diagnostic feature.

### Terminology

Vascular invasion on colour Doppler ultrasonography was defined as an increase in the portal and/or hepatic arterial flow compatible with stenosis, or absence of flow compatible with occlusion. Furthermore, on contrast enhanced computed tomography a vascular stenosis or occlusion was considered as vascular involvement.

### Peer review

This is a retrospective study of 68 patients with suspicion of cholangiocarcinoma. Fifteen percent of patients were found to have benign strictures after resection. The authors concluded that despite the use of sophisticated diagnostic tests and imaging studies, differentiation of malignant from benign hilar lesions remains a dilemma.

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RAPID COMMUNICATION

## Accuracy of *Helicobacter pylori* serology in two peptic ulcer populations and in healthy controls

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compared to the serology test. The sensitivity of the serology test was good, but the specificity was low (41%-71%). The association between *H pylori* IgG antibodies and scores of gastric mucosal inflammation and current or previous peptic ulcer were weak.

**CONCLUSION:** The accuracy of C14-UBT to diagnose *H pylori* infection was good, and the clinical utility of a negative *H pylori* serology test was substantial, while the gain in clinical information of a positive test was meagre. Positive *H pylori* titres could not distinguish between subjects with or those without active peptic ulceration.

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**Key words:** C14-urea breath test; Gastric inflammation; *Helicobacter pylori* serology; Peptic ulcers; Test characteristics

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### Abstract

**AIM:** To estimate the test characteristics of *Helicobacter pylori* (*H pylori*) serology and of C14-urea breath test (C14-UBT) in two different peptic ulcer populations and in community controls. Second, the aim was to explore the association between the level of *H pylori* IgG antibodies and severity of inflammation as to active peptic ulceration in the same populations.

**METHODS:** Vagotomized ( $n = 83$ ), medically treated peptic ulcer patients ( $n = 73$ ) and one reference group of community controls ( $n = 88$ ) were gastroscoped. *H pylori* status was determined by histology, bacterial growth, C14-UBT and serology. Based on the updated Sydney System, cumulative scores from biopsies from the prepylorous, incisura angularis, corpus and fundus were calculated.

**RESULTS:** The prevalence of *H pylori* infection varied from 70% to 79%. The C14-UBT had high accuracy

### INTRODUCTION

Serology and C14-urea breath test (C14-UBT) are the most commonly used non-invasive tests of *Helicobacter pylori* (*H pylori*) infection<sup>[1-3]</sup>. Knowledge about the diagnostic validity of particular serological tests is mandatory for inferring its test results<sup>[4]</sup>. In the diagnostics of *H pylori* infection, most commercially available serological tests are reported to have both a high sensitivity and a high specificity<sup>[5]</sup>. The diagnostic characteristics of the tests depend also on the prevalence of *H pylori* infection in the population tested<sup>[5-7]</sup>. Higher prevalence's would imply higher sensitivity and lower specificity<sup>[5-7]</sup>. There are reports suggesting that there is an association between the level of *H pylori* IgG antibodies and the severity of inflammation of the gastric mucosa and also

between antibody level and a current peptic ulcer<sup>[8-10]</sup>. If so, the level, not only positively or not, of *H pylori* IgG antibody tests might be of clinical importance.

The aim of this study was to estimate the test characteristics of *H pylori* serology compared to the urea breath test (C14-UBT) in two different peptic ulcer populations and in a randomly selected group of community controls without known peptic ulcer disease. Second, the aim was to explore the association between the level of *H pylori* IgG antibodies and severity of inflammation as to active peptic ulceration in the same populations.

## MATERIALS AND METHODS

Based upon a questionnaire survey<sup>[11]</sup>, three groups of subjects were invited to participate in an upper endoscopic investigation: one group of vagotomized peptic ulcer patients; one group of medically treated peptic ulcer patients and one reference group of community controls without known peptic ulcer disease.

### Vagotomized peptic ulcer patients

The medical records of all patients operated with a vagotomy for peptic ulcer disease from 1967 to 1990 at Tromsø University Hospital were reviewed, totally 1038 records. Seven hundred and twenty one were alive and received a postal questionnaire with 105 different questions on abdominal and dyspeptic complaints, medications, use of health services, health, life style, diet and social conditions. Two hundred and eighty two answered that they were interested in a gastroscopic examination if offered. By binomial distribution 106 of these 282 vagotomized patients were randomly selected and invited into the study. Sixteen patients were excluded because they had undergone gastric resections in addition to the vagotomy operation and seven due to interrupted endoscopic examination according to the patient's wishes. Accordingly, 83 patients in these groups completed the investigation protocol. Fifty nine had been electively vagotomized, whereas 24 had been vagotomized on emergency indications.

### Medically treated, non-operated, peptic ulcer patients

Two hundred and thirty one medically treated patients with radiographically (barium meal) or endoscopically verified peptic ulcer disease diagnosed in the period 1979 to 1986 received the same questionnaire as the vagotomized patients. One hundred and five were interested in an endoscopic examination if offered. All of these were invited. Seventy four finally accepted the invitation; one patient failed to swallow the endoscope. Accordingly, 73 patients fulfilled the investigation protocol.

### Community controls

For comparison a group of community controls was included. Seven hundred and sixty two inhabitants of the local municipality were randomly selected from the National Population Registry. They were all without

known peptic ulcer disease and were invited to participate in the same questionnaire survey as the peptic ulcer patients to serve as a community reference group in the comparison of abdominal and dyspeptic complaints. They were group-matched with the vagotomized patients regarding sex distribution and mean age. Two hundred and twenty five persons responded positively to the offered endoscopic examination. By binomial distribution, 105 subjects were randomly selected and invited to the endoscopic study. Ninety six finally accepted the invitation of which 7 were excluded due to interrupted endoscopic examination according to the patient's wishes, and one because of previous gastric surgery. Accordingly, 88 community subjects completed the investigation protocol.

The Regional Ethical Committee for Medical Sciences and the Norwegian Social Science Data Services approved the study design and the data security. There was no financial gain or hints of health benefits associated with participation in the study.

After an overnight fast, all subjects were pre-medicated with a topical anaesthetic spray (lidocaine hydrochloride, 10 mg/dose, Astra, Sweden). No additional sedation was used. The same endoscopist (ROL) examined all patients, and he was unaware of the subjects' peptic ulcer history, any previous treatment or current dyspeptic or abdominal complaints. All endoscopies were recorded by a Sony Hi-8 video-recorder using a Pentax gastroscope EG 2901.

None of the participants in the study received long term or continuous medical acid suppressive treatment prior to the endoscopic examination or any known treatment against *H pylori* infection.

### Detection of *H pylori*

The culture, PCR and the serology tests were performed in the laboratories at the Department of Microbiology, Tromsø University Hospital (accredited according to the ISO 45001 standard).

### Histology

The biopsy specimens for histological detection of *H pylori* infection was taken from the greater curvature of the duodenal bulb, from the greater curvature in the prepyloric antrum 2-3 cm from the pyloric channel, from the angulus (incisura angularis) of the lesser curvature, from the corpus of the greater curvature 10 cm from the cardia, and from the fundic top. Separate biopsy specimens were taken from all lesions. All biopsies were fixed in neutral-buffered 4% formaldehyde. Hematoxylin-eosin stains of paraffin-embedded biopsies were used for histological evaluation<sup>[12]</sup>. Histology was considered positive if *H pylori* like organisms were found in any of the four gastric biopsy sites, and negative if *H pylori* like organisms were not found in any of the biopsies. All histological specimens were evaluated by the same experienced pathologist (TJE) who was blinded for the subjects' medical history and other *H pylori* test results. The histological evaluations were performed according to the updated Sydney System recommendation<sup>[13]</sup>.

**Table 1** Sensitivity, specificity and likelihood ratio of positive test (LR+) of C14-UBT and IgG and IgA antibodies against *H pylori* at cut-off values 300 and 500 in vagotomized (vag) or medically (med) treated peptic ulcer patients (med) and in community controls (con)

	Sensitivity (95% CI)			Specificity (95% CI)			LR+ (95% CI)		
	vag	med	con	vag	med	con	vag	med	con
Method of detection									
C14-UBT	94 (85-99)	92 (81-98)	96 (87-100)	85 (62-97)	80 (56-94)	90 (73-98)	6.3 (2.4-31.5)	4.6 (1.7-17.6)	9.3 (3.0-46.6)
Serology IgG300	95 (86-99)	98 (90-100)	93 (83-98)	41 (21-64)	50 (28-72)	68 (49-83)	1.6 (0.9-3.2)	2.0 (1.0-4.2)	2.9 (1.5-6.4)
Serology IgG500	87 (76-94)	90 (79-97)	83 (70-91)	55 (32-76)	50 (28-72)	71 (52-86)	1.9 (1.0-4.2)	1.8 (0.9-3.9)	2.8 (1.4-6.6)
Serology IgG/IgA <sup>1</sup>	95 (86-99)	98 (90-100)	93 (83-98)	32 (14-55)	50 (28-72)	58 (39-76)	1.4 (0.8-2.7)	2.0 (1.0-4.2)	2.2 (1.2-4.4)

*H pylori* infection detected by a positive histology and/or by a positive culture was defined as reference standard. <sup>1</sup>Serology IgG/IgA: IgG ≥ 300 and/or IgA ≥ 500.

### Gastric inflammation score

Semi-quantitative descriptions of the updated Sydney System were transformed to exact numbers from 1-4 (1 = no/none/no *H pylori*, 2 = slight/mild/*H pylori* in 1-3 pits, 3 = moderate/*H pylori* in more than 3 pits, but not all, 4 = severe/*H pylori* in all pits). The score from the 4 local biopsy sites were added up and the total scores were grouped in order to describe the histologically evaluated gastric inflammation of the total gastric mucosa. The same semi-quantitative terms were used for the chronic gastritis score. A score of 4 denotes no chronic gastritis/no *H pylori*, 5-8 denotes mild chronic gastritis/few *H pylori*, 9-12 denotes moderate chronic gastritis/some *H pylori*, 13-16 denotes severe chronic gastritis/much *H pylori*. The inflammatory activity was described as 1 = no inflammatory activity, 2 = slight/moderate inflammatory activity, 3 = severe inflammatory activity. In the scoring of the inflammatory activity 4 denotes no inflammatory activity, 5-6 slight inflammatory activity, 7-8 moderate inflammatory activity, 9-12 severe inflammatory activity.

### *H pylori* culture growth

Two biopsies taken from the angulus of the lesser curvature were placed in a transport medium (Portagerm pylori, bioMérieux, Lyon, France) and immediately transported to the Department of Microbiology for culture and detection of the *H pylori* DNA by PCR. Culture was performed using selective (Oxoid SR 147E supplement) and nonselective columbia agar (Oxoid CM 331) with 7% lysed horse blood at 37°C. The plates were incubated under a microaerobic atmosphere (5% O<sub>2</sub>, 7% CO<sub>2</sub>, 8% H<sub>2</sub>, 80% N<sub>2</sub>) for at least 7 d. The culture was classified as positive when oxidase-, catalase- and urease positive colonies showed typical *H pylori* morphology on Gram staining.

### C14 urea breath test

Fifty milliliters of tap water with 2.5 µCi carbon-14 labelled urea was given orally to the overnight fasted patient. Double breath samples were taken immediately before and at 10, 20 and 30 min after the ingestion of the urea solution and analysed for <sup>14</sup>CO<sub>2</sub> in a Beta-counter as a measure of *H pylori* urease activity in the stomach. *H pylori* negative patients do not expire <sup>14</sup>C labelled CO<sub>2</sub>. A <sup>14</sup>C

urea breath test was positive when the accumulated <sup>14</sup>CO<sub>2</sub> in the breath samples adjusted for the patients' body-weight and the background radiation was more than 1.5% of the ingested <sup>14</sup>C dose<sup>[14]</sup>.

### Enzyme-linked immunoabsorbent assay (ELISA) detection of IgG and IgA *H pylori* antibodies

ELISA was used for detection of serum IgG and IgA antibodies according to the specifications of the manufacturer (Pyloriset EIA-G®, Orion Diagnostica, Finland). A titre ≥ 300 was interpreted as a positive IgG serology result.

### Reference standard

As reference tests were chosen the combination of histologically detected presence of *H pylori*-like organisms in any of the four biopsy sites<sup>[15]</sup> augmented by *H pylori* culture growth. A patient was considered as *H pylori* positive when having a positive test for *H pylori* by histological examination and/or by culture. The biopsies from the gastric mucosa were taken according to recommended biopsy sites and procedures<sup>[16]</sup>.

### Statistical analysis

The microcomputer software Confidence Interval analysis<sup>[17]</sup> estimated 95% confidence intervals for the various proportions and Epiinfo 6<sup>[18]</sup> was used to analyze dichotomous variables by the Mantel-Haenszel  $\chi^2$ -test with Yates correction.

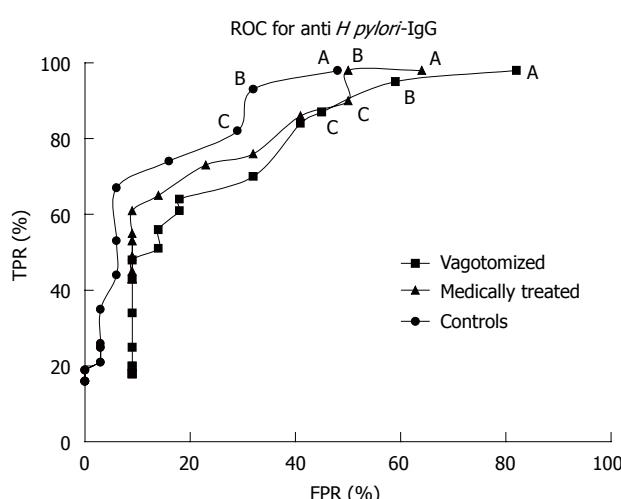
## RESULTS

The prevalence of *H pylori* positive individuals among the vagotomized peptic ulcer patients was 79% (95% CI: 69-88), 75% (95% CI: 63-85) among the medically treated peptic ulcer patients, and 70% (95% CI: 59-80) among the community controls.

Anti *H pylori* IgG at a cut-off value 300 had comparable properties to C14-UBT in detecting the true positive patients in all three groups with sensitivities around 95% (Table 1). When increasing the cut-off value to 500, the sensitivities decreased 8%-10% in all three groups. The combination of anti *H pylori* IgG at a cut-off value 300 and anti *H pylori* IgA at a cut-off value 500 did not improve the sensitivity or specificity of the test in any of

**Table 2** *H pylori* status by histology and inflammatory activity in the gastric angulus (anginflam) in different combinations with culture growth, related to level of IgG antibodies against *H pylori* in each category

	<i>n</i>	Range	Mean (95% CI)	Median
IgG when histology HP neg and growth neg	66	100-12800	700 (290-1120)	200
IgG when histology HP pos and growth neg	9	100-18000	4960 (530-9390)	4000
IgG when histology HP pos and growth pos	143	100-20000	3480 (2740-4220)	1600
IgG when histology HP neg and growth pos	12	200-7000	2660 (1270-4050)	2250
IgG when anginflam neg and growth neg	60	100-18000	690 (90-1290)	200
IgG when anginflam pos and growth neg	8	300-9000	3830 (1420-6240)	3750
IgG when anginflam pos and growth pos	126	100-20000	3500 (2700-4300)	1650
IgG when anginflam neg and growth pos	19	100-13000	2840 (960-4720)	1200



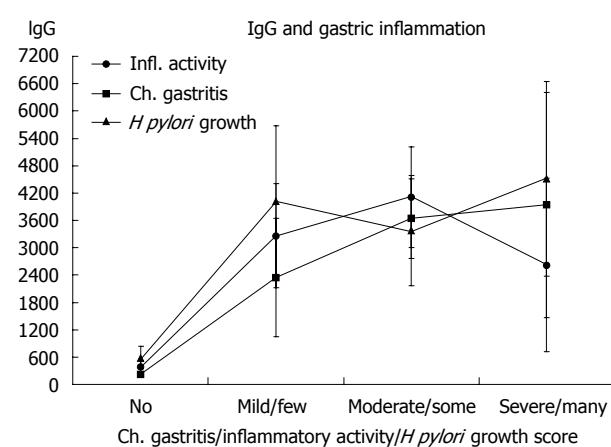
**Figure 1** Receiver operating characteristic (ROC) curve for different cut-off values for *H pylori* IgG antibodies in vagotomized or medically treated peptic ulcer patients and in community controls. Cut-off values were 200, 300, 500, 600 and at intervals of 300 for IgG values above 600 to 4800. TPR = True positive rate (sensitivity), FPR = False positive rate (1-specificity). A: Cut-off 200; B: Cut-off 300; C: Cut-off 500.

the groups. The specificity of anti *H pylori* IgG and IgA were between 32%-50% among the peptic ulcer patients and 58%-71% among the controls, and far lower than C14-UBT with a specificity ranging from 80%-90% in all three groups.

When combining the sensitivity and specificity expressed by the ROC (receiver operating characteristic) curve for anti *H pylori* IgG curves for each group, the area under the curve was largest in the control group (Figure 1).

Incomplete or missing biopsies for *H pylori* detection by both histology and culture growth occurred in 14 cases (*n* = 230, histology HP and growth, Table 2), whereas 215 biopsies were finally included to evaluate inflammation in the angulus. Two of these had missing culture growth biopsies (*n* = 213, anginflam and growth, Table 2).

Anti *H pylori* IgG had a median value of 200 when the reference standard (no *H pylori* like bacteria in any of the four biopsy sites and negative culture growth) was negative. Signs of *H pylori* either by histology or by bacterial growth, or by inflammation in the angulus were associated to elevate anti *H pylori* IgG levels (Table 2).



**Figure 2** Grade of inflammatory activity (no, mild, moderate, severe), grade of chronic gastritis (no, mild, moderate, severe) and semiquantitative numbers of *H pylori* (no, few = *H pylori* like organisms in 1-3 pits, some = *H pylori* like organisms in more than 3 pits but not all, many = *H pylori* like organisms in all pits) detected in gastric biopsies from 4 different biopsy sites (prepylorus, angulus, corpus and fundus) related to the mean value with 95% confidence intervals of IgG antibodies against *H pylori* in each category.

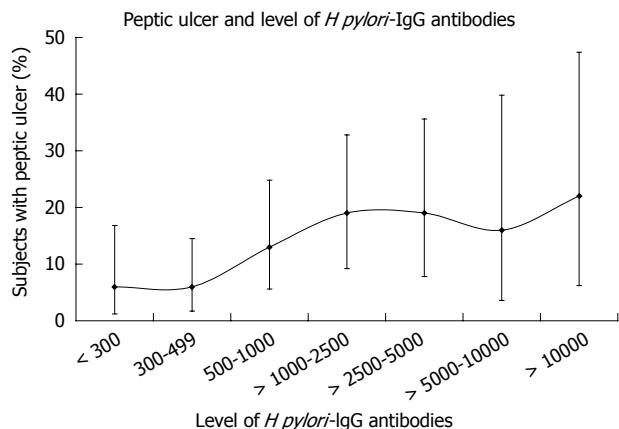
This association was dichotomous and independent of severity of active inflammation or quantitative histological evaluation of *H pylori* (Figure 2).

Increasing levels of *H pylori* IgG antibodies were associated with increasing frequency of subjects with active peptic ulcer (Figure 3). With levels of *H pylori* IgG antibodies above 1000, there was an increase in PU prevalence ( $P = 0.03$ ). Still, 80% of the subjects did not have PU.

## DISCUSSION

The prevalence of *H pylori* in the peptic ulcer patients was 75%-79%. This is somewhat lower than expected in peptic ulcer populations<sup>[19,20]</sup>. Among the vagotomized or medically treated patients are subjects with gastric ulcers that have a lower *H pylori* prevalence than duodenal ulcer patients have<sup>[19]</sup>. In addition, false negative *H pylori* tests or previous antibiotic treatment of other indications than *H pylori* infections might also decrease the prevalence of *H pylori* infection.

In populations with high prevalence of *H pylori* infection the test characteristics of C14-UBT are very good. While the sensitivity of the serology test is excellent too,



**Figure 3** Subjects with peptic ulcer related to level of *H pylori* IgG antibodies. Frequencies are shown with 95% CI.

the specificity is low. The validity varies with the prevalence of *H pylori* infection, illustrating the effects of spectrum bias<sup>[21]</sup>. The test characteristics are, at best, in the control group with the lowest prevalence of *H pylori* infection. Increasing the threshold of a positive test from 300 to 500 or higher did not improve the validity of the test.

The relative low specificity of the anti *H pylori* IgG and/or IgA test indicates a high antibody titre despite no actual presence of *H pylori* in the gastric mucosa. Previous *H pylori* infection or cross-reacting antibodies from closely related bacteria might explain a positive serology test despite no actual *H pylori* infection.

Likelihood ratio is a more clinically relevant method of reporting accuracy than only specificity and sensitivity of a test. The probability of having a disease after a positive or negative test can be calculated and reveals more understandable information in the clinical setting and could also be applied to the clinical problem of dyspepsia management<sup>[22]</sup>.

By comparing the true positive rate to the false positive rate, information about the probability of whether a positive test result is likely to be from a truly *H pylori* positive subject, compared to a similar result to be from a truly *H pylori* negative subject, can be obtained (positive likelihood ratio)<sup>[15]</sup>. By this method C14-UBT was about three times better than Hp serology. 13C-UBT is in full concordance with 14C-UBT and should be preferred because of its lack of radioactivity.

The low specificity reduces the clinical utility of a positive test rest result. When, among medically treated peptic ulcer group, applying the test results of a sensitivity of 98%, a specificity of 50%, and a pre-test probability of 75%, the post-test probability of a positive test barely increases to 85%, while the post-test probability of a negative test enlarges from 25% to 90%.

In 21 subjects, there were discrepancies between the results of histological identification of *H pylori* and growth of the bacteria. As culture growth is highly specific<sup>[2]</sup>, and since none of the 12 subjects with negative histology and positive culture is a false positive, the sensitivity of the histology could not be 100%<sup>[16]</sup>. If there is a misclassification of the reference standard applied

in this study, the direction is towards an overrating of *H pylori* positive subjects. Consequently, the test characteristics, mainly the sensitivities of serology could be somewhat underestimated.

Others have published sensitivity of 100% and specificity of 79% using the same serology Elisa-kit<sup>[23]</sup>. However, the population was on average about 10 years younger than in this study and the *H pylori* prevalence was 82%. The prevalence of *H pylori* is equivalent to our vagotomy group. While the sensitivity is comparable, the populations differ regarding the specificity of the test. The two populations could differ regarding number of case-mix, or people in North Norway might have more infections that could cross-react with *H pylori* serology kits.

An objection to this presentation is the lack of validation of the constructed cumulative scores of inflammatory activity, chronic gastritis and *H pylori* density, based on the histological Sydney System scores at the four different biopsy sites. The chronic gastritis and the subsequent atrophic changes in *H pylori* infected subjects are commonly described as antrum pre-dominant, corpus-predominant or both (pangastritis)<sup>[24]</sup>. However, the objective of this study was to detect any association between the global measurement of *H pylori* serology and the general inflammatory status of the gastric mucosa. Depending on the severity grading, according to the updated Sydney System, the antral predominant chronic gastritis would thus have a relatively high cumulative score, as the condition would be detected in the biopsies from both the pre-pylorus and from the incisura angularis. In addition to the corpus biopsy, the corpus-predominant chronic gastritis should also be reflected in the cumulative scoring system in the transition zone (incisura angularis) as much as the antrum-predominant chronic gastritis. Pangastritis would be reflected by even higher scores by summation from all four biopsy sites.

The percentage of subjects with peptic ulcers was not significantly different at various levels of *H pylori* IgG antibodies above the recommended cut-off titre value of 300. The same tests could neither differentiate between previous peptic ulcer patients and healthy community controls, nor in the severity score of gastric inflammation measured by inflammatory activity, chronic gastritis or histologically evaluated bacterial growth.

In this study, *H pylori* IgG antibodies could not be used to differentiate between previous peptic ulcer patients and healthy community controls, nor between patients with or those without active peptic ulcers.

No association was found between the level of positive IgG titres and the cumulative scores of inflammatory activity and *H pylori* density, according to the updated Sydney System, from the four different biopsy sites in the gastric mucosa. In the clinical setting, this means that the level of positive *H pylori* titres give no diagnostic information about the degree of inflammation in the gastric mucosa, nor cannot distinguish between subjects with or those without active peptic ulceration, nor between previous peptic ulcer patients and community controls. Others have also reported that *H pylori* serology is a poor marker of peptic ulcer disease<sup>[25-28]</sup>.

When using *H pylori* serology tests a negative result is of clinical importance at the recommended cut-off value of IgG titre 300, due to the high sensitivity of the test. A negative serology test result is also reported to almost rule out pre-malignant conditions in the gastric mucosa in screening situations<sup>[29]</sup>. A low specificity, however, reduces the clinical utility of a positive test result. Independent of previous peptic ulcer status among the tested subjects, *H pylori* serology and C14-UBT showed comparable sensitivity.

We could not find any association between gastric mucosal morphology and serology, in contrast to what is published by others<sup>[30]</sup>. However, in that study, a combination of serology tests were used, and a more dichotomous approach to the presence of *H pylori* infection and its morphological consequences were applied.

Uncritical use of *H pylori* serology will represent a considerable overestimation of *H pylori* prevalence in the population tested. *H pylori* serology is, on the other hand, very reliable to exclude *H pylori* infection and thereby useful in screening purposes.

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## COMMENTS

### Background

Serology and C14-urea breath test (C14-UBT) are the most commonly used non-invasive tests of *Helicobacter pylori* (*H pylori*) infection. Knowledge about the diagnostic validity of particular serological tests is mandatory for inferring its test results. In the diagnostics of *H pylori* infection, most commercially available serological tests are reported to have both a high sensitivity and a high specificity. The diagnostic characteristics of the tests depend also on the prevalence of *H pylori* infection in the population tested. Higher prevalences would imply higher sensitivity and lower specificity.

### Research frontiers

Application of test characteristics to illustrate limited value of a commonly used blood test (*H pylori* serology) for detection of *H pylori* gastric infection or peptic ulcer. However, a negative test result rule out *H pylori* infection with high certainty.

### Applications

General practice and gastroenterological specialist practice.

### Terminology

Sensitivity means a test ability to correctly identify a true positive subject with a positive test result (frequency of positive test or true positive rate). Specificity means a test ability to correctly identify a true negative subject with a negative test result (frequency of negative test or true negative rate).

### Peer review

This retrospective study has estimated the test characteristics of *H pylori* serology and of C14-UBT in 83 vagotomized patients, 73 medically treated peptic ulcer patients and 88 gastroscooped community controls in Norway. It is helpful to know the prevalence of the infection in the area of study.

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RAPID COMMUNICATION

# Endoscopic findings in patients with upper gastrointestinal bleeding clinically classified into three risk groups prior to endoscopy

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## Abstract

**AIM:** To investigate in a prospective study whether a simplified clinical score prior to endoscopy in upper gastrointestinal bleeding (UGIB) patients was able to predict endoscopic findings at urgent endoscopy.

**METHODS:** All consecutive UGIB patients referred to a single endoscopic center during a 16 mo period were enrolled. Before endoscopy patients were stratified according to a simple clinical score (T-score), including T1 (high-risk), T2 (intermediate-risk) and T3 (low-risk). Endoscopy was performed in all cases within 2 h, and high-risk stigmata were considered for further analysis.

**RESULTS:** Out of the 436 patients included into the study, 126 (29%) resulted to be T1, 135 (31%) T2, and 175 (40%) T3. Overall, stigmata of recent haemorrhage (SRH) were detected in 118 cases (27%). SRH occurred more frequently in T1 patients than in T2/T3 cases (85% vs 3.2%;  $\chi^2 = 304.5309$ ,  $P < 0.001$ ). Older age ( $t = 3.311$ ;  $P < 0.01$ ) and presence of comorbidities ( $\chi^2 = 14.7458$ ;  $P < 0.01$ ) were more frequently detected in T1 than in T2/T3 patients.

**CONCLUSION:** Our simplified clinical score appeared to be associated with the detection of endoscopic findings which may deserve urgent endoscopy. A further,

randomised study is needed to assess its accuracy in safely scheduling endoscopy in UGIB patients.

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**Key words:** Upper gastrointestinal bleeding; Urgent endoscopy; Timing score; Endoscopic treatment; Oesophageal varices; Peptic ulcer

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## INTRODUCTION

Acute upper gastrointestinal bleeding (UGIB) is a very common condition, with an estimated incidence as high as 40-150 cases per 100 000 annually<sup>[1-3]</sup>. Undeniably, UGIB is a dramatic event resulting in a high mortality rate, ranging from 0.9% to 26.5%<sup>[1-4]</sup>. Moreover, it leads to 50-150 hospitalizations per 100 000 adults each year<sup>[5]</sup>. Upper gastrointestinal endoscopy plays a pivotal role in the diagnosis and therapy of these patients, reducing mortality, rebleeding, requirement for transfusion, hospital stay and health care costs<sup>[6-9]</sup>. Endoscopic haemostasis has been shown to be effective in most of the causes of UGIB, such as peptic ulcer, gastro-oesophageal varices and Mallory-Weiss lesions<sup>[8,10,11]</sup>. For this reason, urgent endoscopy is routinely provided by several hospitals both in Europe and United States. Moreover, the absence of stigmata of recent haemorrhage (SRH), such as an adherent clot or an arterial bleeding, may prompt an early discharge of the patients, resulting in substantial healthcare savings<sup>[12]</sup>. Furthermore, several scoring systems, based on clinical-

endoscopic features, have been extrapolated in order to predict the risk of rebleeding and mortality<sup>[13-17]</sup>. Specifically, haemoglobin level, hemodynamic instability, and the presence of comorbidities have been shown to be clinical variables associated with a poorer outcome. Regarding timing of urgent endoscopy, it is widely accepted that it should be performed within 24 h from the admission<sup>[18]</sup>. However, within this period of time, it is still unclear whether it should be performed either very early-i.e. within 2 h- or in a more delayed interval, such as after 6, 12 or 24 h. In particular, some retrospective series did not show a clear advantage for early versus delayed urgent endoscopy<sup>[19-22]</sup>. However, in clinical practice, the endoscopist may be expected to be called by the emergency department immediately after the hospital admission of the bleeding patient, making it his responsibility to proceed towards an immediate procedure or delaying it up to 12 or 24 h. Legal aspects are clearly entailed in this decisional process, so that, in the absence of clear data, endoscopists may be expected to rush in the endoscopic units, even in low-risk cases. This may be particularly troublesome in large hospitals in which the specialist may be repeatedly called during the night or on non-working days. Moreover, due to lack of clinical evidence, administrative parameters have been shown to be important predictors of the timing to endoscopy in UGIB, dangerously exposing similar patients to different outcomes according to the referral hospital. Therefore, optimal timing for urgent endoscopy in UGIB patients has not been yet established. The aim of this prospective study was to evaluate whether a simplified clinical score prior to endoscopy in UGIB patients was able to predict either active bleeding or SRH that may require an urgent (< 2 h) endoscopy.

## MATERIALS AND METHODS

All consecutive patients referred to a single Endoscopic Unit because of an episode of UGIB during a 16 mo period were enrolled. The Endoscopic Unit belongs to a large hospital in which the physicians are urged to contact the gastroenterologist on call when patients present with an UGIB. For the purpose of the study, urgent endoscopy was always performed within 2 h from the referral. Before the procedure, patients were stratified by the endoscopists according to 4 easily assessable clinical variables, already validated in the UGIB setting: (1) general conditions (poor, intermediate, good), (2) pulse (< 90 beats/min, 90-110 beats/min, > 110 beats/min), (3) systolic blood pressure (< 90 mmHg, 90-110 mmHg, > 110 mmHg), and haemoglobin level ( $\leq 8$  g/dL, 9-10 g/dL, > 10 g/dL)<sup>[13-15]</sup>. General conditions were intended as a measure of the risk of an impending shock or the presence of symptomatic comorbidities (cardiovascular, hepatic, nephropathic, diabetes, malignancy). In detail, “poor condition” included patients with impending shock or with  $\geq 3$  comorbidities, “good condition” included patients with no debilitation and without postural hypotension and  $\leq 1$  comorbidity, and “intermediate condition” those

**Table 1** Numerical values for each parameter of the clinical index adopted in the study

Clinical parameter	Score		
	1	2	3
General conditions	Poor	Intermediate	Good
Pulse (beats/min)	> 110	90-110	< 90
Systolic blood pressure (mmHg)	< 90	90-110	> 110
Haemoglobin levels (g/dL)	$\leq 8$	9-10	> 10

The T-score is the sum of the corresponding values for the 4 parameters. In detail, a sum  $\leq 6$  corresponds to T1 (high-risk), 7-9 to T2 (intermediate-risk), and a cumulative value  $\geq 10$  to T3 (low-risk).

patients with conditions in the middle. As shown in Table 1, a numerical score was created for each of these parameters, the sum of all the parameters resulting in the total score (T-score) for each patient who was thereafter classified according to arbitrarily defined T-score cut-off in 3 categories. In detail, a sum  $\leq 6$  corresponds to T1 (high-risk), a sum of 7-9 to T2 (intermediate-risk), and a cumulative value  $\geq 10$  to T3 (low-risk). Further clinical information were collected for data analysis. Validity of such a classification was tested according to the presence of SRH at endoscopy. In detail, SRH was defined as an adherent clot, a bleeding (oozing or spurting) or nonbleeding visible vessel<sup>[23]</sup>, or gastro-oesophageal varices with active or recent signs of bleeding, such as a fibrin clot. All patients gave their informed consent prior endoscopic examination.

## Statistical analysis

Data analysis was performed by using Chi-square and Student's *t*-test as appropriate, and *P* < 0.05 was considered statistically significant.

## RESULTS

Overall, 436 patients (270 males, 166 females; Mean age:  $65 \pm 13$  years) were included in the study. Regarding the setting, 126 (29%) patients were already hospitalized before the UGIB, whilst the remaining 310 (71%) had been admitted by the emergency department because of the UGIB. Major comorbidities were present in 157 patients (36%). In detail, a cardiac disease was present in 78 (18%) patients, a hepatic disease in 61 (14%) cases, a clotting impairment in 9 (2%) cases, whilst renal or neurological comorbidities were detected in the remaining 9 (2%) cases. The mean time between the request to the endoscopic unit and the performance of the urgent endoscopy was  $1.6 \pm 0.47$  h. No death occurred before or during the endoscopic procedure itself. The main endoscopic findings are provided in Table 2. In detail, SRH were detected in 118 cases (27%), prompting an immediate endoscopic haemostasis in 105 (89%) of these cases. When classifying patients according to T-score, 126 (29%) resulted to be T1, 135 (31%) T2, and 175 (40%) T3. A SRH was detected at endoscopy in 107 (85%) T1-cases. In detail, an active bleeding (oozing or spurting) was reported in 34 (32%) T1-patients, a nonbleeding visible vessel/adherent clot was described

**Table 2 Endoscopic findings and SRH detected in the study population**

	<b>Number of patients (%)</b>
Endoscopic finding	436 cases
Duodenal ulcer	144 (33)
Gastric ulcer	74 (17)
Gastro-oesophageal varices	52 (12)
Erosive esophagitis	44 (10)
Malignancy	35 (8)
Erosive gastritis/duodenitis	80 (18)
Mallory-Weiss syndrome	7 (2)
SRH	118 cases
Active bleeding	37 (31)
Nonbleeding visible vessel	16 (14)
Adherent clot	40 (34)
Gastro-oesophageal varices with active or recent signs of bleeding	25 (21)

in 50 (47%) cases, and gastro-oesophageal varices with active or recent signs of bleeding in 24 (22%) patients. As far as T2-patients are concerned, only in 7 (5%) cases a SRH was detected, being an active bleeding in 2 cases, a nonbleeding visible vessel/adherent clot in 4 cases, and gastro-oesophageal varices in 1. Regarding the 175 patients classified as T3, a SRH was found at endoscopy only in 3 (2%) cases, being a nonbleeding visible vessel/adherent clot in 2 patients and an oozing bleeding in 1 case. When comparing the rate of SRH in T1 patients (85%) with that identified in T2 (5%) and/or T3 (2%), a significant difference emerged ( $P < 0.001$ ). Comparison of further demographic and clinical variables is provided in Table 3. As shown, patients classified as T1 were older ( $69 \text{ vs } 63$  and  $64$  years;  $t = 3.311$  and  $3.443$ , respectively;  $P < 0.01$ ) and more frequently had comorbidities ( $49\% \text{ vs } 20\%$  and  $31\%$ ;  $\chi^2 = 14.7458$  and  $13.4355$ , respectively;  $P < 0.01$ ) than T2 and T3 patient groups.

## DISCUSSION

UGIB incidence may be expected to increase as the proportion of elderly people in the population rises, because of the higher prevalence of gastroduodenal diseases in this subgroup of people<sup>[24]</sup>, particularly those NSAID associated<sup>[1,25]</sup>. Indeed, NSAIDs are widely used in clinical practice and they are the most relevant cause of UGIB<sup>[1,2,26]</sup>, and recent data suggested that even selective COX-2 inhibitors are not risk free<sup>[27]</sup>. Undeniably, UGIB has a relevant economic impact<sup>[28]</sup>. Therefore, optimizing an urgent endoscopy setting is of a paramount importance. Indeed, upper endoscopy plays a pivotal role in UGIB diagnosis and treatment<sup>[29]</sup>. However, although it is widely accepted that urgent endoscopy should be performed within 24 h, the best timing is still unclear<sup>[18]</sup>. In the present study, we evaluated whether a simplified clinical score prior to endoscopy in UGIB patients was able to predict either active bleeding or SRH that may require an urgent (< 2 h) endoscopy. Our data found that within 24 h, different timing of urgent endoscopy for UGIB may

**Table 3 Demographical and clinical characteristics of the UGIB patients according to T-score classes**

<b>Variable</b>	<b>T1 (n = 126)</b>	<b>T2 (n = 135)</b>	<b>T3 (n = 175)</b>	<b>P</b>
Mean age ± SD (yr)	$69 \pm 13$	$63 \pm 16$	$64 \pm 12$	$< 0.01^1$
Male sex (%)	66	62	60	NS
Comorbidities (%)	49	20	31	$< 0.01^1$
Systolic blood pressure (mmHg)	$85 \pm 15$	$106 \pm 22$	$139 \pm 37$	$< 0.01^2$
Hemoglobin level (g/dL)	$7.2 \pm 1.3$	$9.7 \pm 2.3$	$13.8 \pm 3$	$< 0.01^2$

<sup>1</sup>T1 vs T2 or T3; <sup>2</sup>T1 vs T2 or T3 and T2 vs T3.

be proposed. In particular, urgent endoscopy may provide a therapeutic resource for most of the patients in severe clinical conditions (T1 score), whilst it does not appear to be necessary in those patients with more favourable conditions (T2/T3 score). Although there was no evidence of a better clinical outcome-i.e. rebleeding and mortality-after a very early endoscopy in some retrospective series<sup>[19-22]</sup>, our study shows that endoscopic therapy is necessary in most of the clinically severe cases. Since an effective endoscopic haemostasis has been associated with a better outcome for both variceal and non-variceal UGIB<sup>[8,9]</sup>, it may be conservatively advised to perform a very early endoscopy, at least in patients in more severe conditions. Moreover, endoscopy may be also useful to stratify these compromised patients, since endoscopic SRH have been shown to predict the UGIB-associated morbidity and mortality<sup>[13]</sup>. On the other hand, our prospective study clearly shows that urgent endoscopy is useless in the vast majority of those patients in intermediate or good clinical conditions, which account for more than two thirds of all UGIB patients, since only a very few of them really gain some benefit from the endoscopic procedure. Due to the relatively stable clinical conditions, it is foreseeable that even in those few patients with SHR, a delay in the urgent endoscopy to 12 h, sufficient to postpone the endoscopy procedure from the night to the immediate following day endoscopic routine list, would have not changed the overall outcome. Our study suggests that the use of a simple clinical score may predict endoscopic SRH in UGIB patients. In particular, to our knowledge, this is the first time that a simple clinical score has been associated with endoscopic findings at urgent endoscopy in a prospective series. This points out that clinical parameters are not only useful in selecting those who may need an urgent endoscopy from those who may not, but also in selecting, among those who need it, those who need a very early procedure. On the other hand, upper endoscopy in T2/T3 score patients may be delayed until the next routine endoscopic list, in which a more suitable setting, i.e. either a more skilled endoscopist or a prolonged pre-endoscopic proton pump inhibitor therapy may result in a better outcome<sup>[29,30]</sup>. Importantly, no death occurred before endoscopy and in the endoscopic setting, supporting the safety of a very early endoscopy even in severe patients, although previous series described

higher cardiovascular complications as compared to more delayed procedures<sup>[21]</sup>. Although urgent endoscopy is usually defined as a within 24 h-procedure<sup>[18]</sup>, legal aspects may be raised against the on-call physician who prefers a delayed approach, when severe complications or even mortality associated to the UGIB episode occur before endoscopy. For this reason, endoscopists generally prefer to anticipate more than to delay an emergency procedure. We feel that our study allows an immediate and user-friendly clinical stratification, allowing less severe patients to be postponed until the next morning. Some limitations are entailed in the present study. In particular, we did not assess the rebleeding rate and the associated mortality in the post-endoscopic period. Nevertheless, SRH's have been shown to be intimately related with these outcomes, and may therefore be regarded as valid intermediate surrogates. However, further studies specifically addressing these end-points, namely rebleeding rate and associated mortality, are needed before proposing the use of such a pre-endoscopic score in clinical practice. In particular, we cannot exclude that even severe UGIB episodes may be only of marginal clinical interest in patients affected by severe comorbidities, such as renal failure. Moreover, it would be important to further validate in future studies our score with others already available in the literature. Secondly, we did not apply different timings (very early *versus* delayed) in T2/T3 patients. However, after these findings, we feel clinically meaningful to plan a further study in which different timings will be tailored according to clinical conditions. Thirdly, we may not exclude that if the urgent endoscopy had been performed later than 2 h, but still within 24 h, the rate of SRH would have been different. However, it is unlikely that such a wide difference between T1, on one side, and T2 and T3, on the other, would have been significantly affected. Fourthly, we applied our score also to oesophageal variceal bleeding. However, as soon as these patients are correctly diagnosed with the underlying liver disease, they should have a prompt endoscopy, irrespective of the severity of the bleeding.

In conclusion, our study shows that timing of urgent endoscopy following an episode of UGIB could be differentiated according to a simple score purely reflecting the clinical conditions of the patients. This would allow most of the patients with SRH to be effectively treated, whilst delaying most of the purely diagnostic procedures in low risk clinical patients. A future, randomized study is required to validate this clinical score.

## COMMENTS

### Background

Acute upper gastrointestinal bleeding (UGIB) is a very common condition, with an incidence of 40-150 cases per 100 000, resulting in high hospitalization and mortality rates. Upper gastrointestinal endoscopy plays a major role in the diagnosis and therapy of these patients, reducing mortality, rebleeding, requirement for transfusion, hospital stay and health care costs. It is widely accepted that urgent endoscopy for UGIB should be performed within 24 h from the admission. However, within this period of time, it is still unclear whether

it should be performed either very early, i.e. within 2 h, or in a more delayed interval, such as after 6, 12 or 24 h. Therefore, optimal timing for urgent endoscopy in UGIB patients has not been yet established.

### Research frontiers

A simple clinical score prior to endoscopy, purely based on general conditions, pulse and haemoglobin level, was strongly associated with the detection of active bleeding or stigmata of recent hemorrhage. When classifying 436 patients according to this score (T-score), active bleeding or signs of recent hemorrhage was detected in 85% of T1 (most severe) patients and only in 5% and 2% of those T2/T3 (less severe), respectively.

### Innovations and breakthroughs

This study shows that timing of urgent endoscopy following an episode of UGIB may be differentiated according to a simple score purely reflecting the clinical conditions of the patients. This would allow most of the high-risk patients to be effectively treated, whilst delaying most of the purely diagnostic procedures in low risk clinical patients. A future, randomized study is required to validate this clinical score.

### Peer review

This is an interesting study that attempts to stratify the urgency for upper GI endoscopy in a patient presenting with acute bleeding. The choice of the "clinical" parameters and the cut-off values chosen are empiric.

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RAPID COMMUNICATION

## Colonoscopy evaluation after short-term anti-tuberculosis treatment in nonspecific ulcers on the ileocecal area

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"bowel disease group" were finally diagnosed as Crohn's disease or nonspecific colonic ulcers during long-term follow up.

**CONCLUSION:** Follow-up colonoscopy shows a healing stage ulcer or scarring change without an active ulcer with just 2 mo to 3 mo of medication in patients with tuberculous colitis. Colonoscopy follow-up after short term anti-tuberculosis trial in patients with nonspecific ulcers on the ileocecal area is valuable in making early differential diagnosis of tuberculous colitis.

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**Key words:** Colonoscopy; Short-term; Anti-tuberculosis medication; Tuberculous colitis; Ileocecal ulcer

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Park YS, Jun DW, Kim SH, Lee HH, Jo YJ, Song MH, Kim NI, Lee JS. Colonoscopy evaluation after short-term anti-tuberculosis treatment in nonspecific ulcers on the ileocecal area. *World J Gastroenterol* 2008; 14(32): 5051-5058 Available from: URL: <http://www.wjgnet.com/1007-9327/14/5051.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.5051>

### Abstract

**AIM:** To evaluate the efficacy of colonoscopy follow-up after short-term anti-tuberculosis treatment in patients with nonspecific ulcers on ileocecal areas being suspicious of tuberculous colitis.

**METHODS:** We prospectively analyzed the colonoscopic findings before and after short term anti-tuberculosis treatment in 18 patients with nonspecific ulcers on the ileocecal area and compared them with 7 patients of confirmed tuberculous colitis by acid-fast bacilli or caseating granuloma on colonic biopsy.

**RESULTS:** Mean duration for short-term follow-up was 107.3 d with combined chemotherapy containing isoniazid, rifampicin, ethambutol and pyrazinamide. Seven patients with tuberculous colitis showed complete healing of active ulcers after short-term medication. After short-term anti-tuberculosis treatment, follow-up colonoscopy findings divided 18 patients with nonspecific ulcers into two groups by ulcer state. One is the "suspicious tuberculous colitis group" showing healing of ulcers and erosions and another is the "suspicious inflammatory bowel disease group" showing active ulcers with or without aggravation of the lesion. Finally, all 9 of the "suspicious tuberculous colitis group" were diagnosed as tuberculous colitis showing no recurrence of ulcers after termination of 9 mo of anti-tuberculosis medication. Patients of the "suspicious inflammatory

### INTRODUCTION

Worldwide incidence of abdominal tuberculosis has been steadily increasing for the past 20 years<sup>[1-4]</sup>, and 2%-3% of reported patients with abdominal tuberculosis have isolated colonic involvement<sup>[5]</sup>. Particularly in Asia, pulmonary and extrapulmonary tuberculosis have been rare until now. Although both the incidence and prevalence rates of inflammatory bowel disease (IBD) are still relatively low compared to Europe and North America, they are increasing rapidly in many Asian countries, including Korea<sup>[6]</sup>.

Difficult colonoscopic differentiation between tuberculous colitis and Crohn's colitis is caused when both entities can present themselves with mucosal ulcerations and nodularity, aphthous ulcers, edematous mucosal folds, strictures, pseudopolyps and luminal narrowing on the ileocecal area<sup>[7]</sup>.

Although caseating granulomas and acid-fast bacilli

make it easy to differentiate the disease entity, many nonspecific ulcers on the ileocecal area in patients with chronic diarrhea and right lower quadrant pain do not provide confirmative diagnosis between tuberculous colitis and other inflammatory bowel diseases.

Therapeutic trial of anti-tuberculosis treatment has been accepted in the case of high clinical suspicion of tuberculosis, which should be continued if there is a good clinical response<sup>[8]</sup>. Sometimes clinical response is not correlated with disease itself. Generally 9 mo to 12 mo of anti-tuberculous medication is necessary for tuberculous colitis. In patients with IBD, disease will be more aggravated during that long-term therapeutic trial. The clinician needs a diagnostic tool confirming tuberculous colitis as early as possible. The precise colonoscopic features after 2 mo to 3 mo of short-term medication in patients with either tuberculous colitis or suspicious tuberculous colitis have not been documented. Thus, we prospectively evaluated the colonoscopic findings after short-term anti-tuberculosis treatment both in patients with nonspecific ulcers on the ileocecal area and in patients with confirmed tuberculous colitis.

## MATERIALS AND METHODS

### **Patients**

This prospective case-control study was conducted at the Medical Center of Eulji University, from March 2002 to October 2004. Eighteen patients (6 males and 12 females) with chronic diarrhea or right lower abdominal discomfort and showing nonspecific ulcers on the ileocecal area on initial colonoscopy were enrolled. The definition of nonspecific ulcer in this study is colonic ulcer with chronic active inflammation without caseating granuloma on biopsy. As a control, we evaluated colonoscopic features of 7 patients with tuberculous colitis who had either acid-fast bacilli or caseating granuloma on colonic biopsy combined with active pulmonary tuberculosis.

Patients with less than 3 wk of symptoms combined with perianal lesion or skip lesion on either the left side of the colon or proximal small bowel were excluded. Stool exam and culture studies were performed to exclude any parasites and bacterial infection. The anti-tuberculosis medication trial proceeded after full and informed consent was granted by each patient.

### **Methods**

One expert colonoscopist, with more than 10 years experience, performed the colonoscopies throughout this study. Colonoscopy was performed with videocolonoscopes (CF240L, Olympus, Japan) after 4 L polyethylene glycol preparation. The whole colon from rectum to cecal base and terminal ileum was photographed for comparison. Chest X-ray and small bowel double-contrast barium study were performed before medication for evaluation of small bowel lesion and pulmonary lesion. During anti-tuberculosis medication, liver function test results, clinical symptoms and follow-up chest X-ray were regularly evaluated.

To determine the patient's susceptibility to drug, we regularly evaluated the tolerance, upper GI symptoms and allergic symptoms relating to medication on outpatient care. "Very good" meant "no complaints during medication; no side effects", "good" meant "some complaints after taking medication", "poor" meant "difficulty in taking medication" due to GI trouble or side effects.

The regimen for tuberculosis was combined chemotherapy containing isoniazid, rifampicin, ethambutol and pyrazinamide.

### **Study design**

The follow-up colonoscopy was performed after 2-3 mo of anti-tuberculosis medication on all enrolled patients by the aforementioned colonoscopist, and some of the features evaluated were ulcer shape, number and location. Compared with colonoscopic features of patients with tuberculous colitis, we subdivided the patients with nonspecific ulcers into either a "suspicious tuberculous colitis group" or "suspicious inflammatory bowel disease (IBD) group". If there were healing of active ulcers similar to tuberculous colitis on follow-up colonoscopy findings, the patients were classified as "suspicious tuberculous colitis group", and if there were still active ulcers or extension of the lesion, classified as "suspicious IBD group". Finally, we confirmed tuberculous colitis by complete resolution of the whole lesion and clinical symptoms after 10 mo of anti-tuberculosis treatment in the "suspicious tuberculous colitis group". For the "suspicious IBD group", we stopped anti-tuberculosis treatment after a short-term trial and reevaluated.

The human subject committee of our hospital approved this study.

### **Statistical analysis**

Kruskal-Wallis tests (three groups) were used for intergroup comparison of continuous variables, whereas Fisher exact tests were used to compare the categorical variables. Continuous variables are summarized as median  $\pm$  SD, whereas categorical variables are summarized as counts or percentages. Statistical analysis was performed with SPSS 11.0 software.

## RESULTS

### **Clinical findings**

All enrolled patients were performed follow-up colonoscopy after short-term anti-tuberculosis treatment. The mean duration of the trial was 107.3 (62-120) d.

Clinical features for enrolled patients are summarized in Table 1. The median age of patients in the "suspicious IBD group" was younger than patients in the "tuberculous colitis group" or "suspicious tuberculous colitis group". Active lesion or old pulmonary lesion on chest X-ray was significant in helping the diagnosis of tuberculous colitis ( $P < 0.001$ ). On laboratory findings, there are no specific differences between groups.

**Table 1 Clinical characteristics**

Group	Tbc colitis (n = 7)	Nonspecific ulcers (n = 18)		P <sup>1</sup>
	Suspicious tbc colitis (n = 9)	Suspicious IBD (n = 9)		
M/F	4/3	2/7	4/5	NS
Median age (yr)	40.1	44.1	28.1	0.03
Chest X-ray (%)				
Active pulmonary tbc	5 (71.4)	3 (33.3)	0 (0)	
Old pulmonary tbc	0 (0)	2 (22.2)	0 (0)	< 0.001
Normal	2 (28.6)	4 (44.4)	9 (100)	
Lab finding (%)				
ESR rise	7 (100)	8 (88.9)	9 (100)	NS
pANCA (+/-)	Not check	Not check	1/8 (11.1/88.9)	
Small bowel lesion (%)				
No lesion	4 (57.1)	4 (44.4)	3 (33.3)	
Jejunum	0 (0)	0 (0)	0 (0)	
Prox. ileum	0 (0)	0 (0)	1 (11.1)	NS
Terminal ileum	3 (42.9)	5 (55.5)	6 (66.7)	
Anal & rectal lesion (%)	0 (0)	0 (0)	0 (0)	

NS: Not significant. <sup>1</sup>Significant at P < 0.05 by Fisher's exact test and Kruskall-Wallis test.

**Table 2 Colonoscopy features before anti-tuberculosis medication trial**

Group	Tbc colitis (n = 7)	Nonspecific ulcers (n = 18)		P <sup>1</sup>
	Suspicious tbc colitis (n = 9)	Suspicious IBD (n = 9)		
Location of lesion (%)				
Terminal ileum	3 (42.9)	5 (55.6)	6 (66.7)	NS
IC valve	5 (71.4)	8 (88.9)	7 (77.8)	NS
Cecum	2 (28.6)	2 (22.2)	5 (55.6)	NS
Prox. Ascending colon	6 (85.7)	6 (66.7)	6 (66.7)	NS
Shape of lesion				
Geographic ulcer	3 (42.9)	2 (22.2)	2 (22.2)	NS
Irregular ulcer	1 (14.3)	3 (33.3)	5 (55.6)	NS
Aphthous ulcer	0 (0)	2 (22.2)	6 (66.7)	0.01
Transverse ulcer	3 (42.6)	2 (22.2)	2 (22.2)	NS
Stenosis	2 (28.6)	1 (11.1)	2 (22.2)	NS
Pathology (%)				
Granuloma	2 (28.6)	3 (28.0)	4 (44.4)	NS
Caseating granuloma	4 (57.1)	0 (0)	0 (0)	0.01
Acid fast bacilli+	3 (21.7)	0 (0)	0 (0)	0.01
Non-specific inflammation	0 (0)	6 (66.7)	5 (55.6)	0.02

NS: Not significant. <sup>1</sup>Significant at P < 0.05 by Fisher's exact test.

Serologic results of patients with nonspecific ulcers are not so helpful in diagnosis. On small bowel study, there are no significant differences between groups. All of the enrolled patients had no ano-rectal symptoms or any other Upper GI tract lesion.

### Colonoscopy findings

Initial Colonoscopy findings before anti-tuberculosis medication are summarized in Table 2. Ulcers were located at the ileocecal valve, cecum, proximal ascending colon and terminal ileum in both groups. There were no rectal and perianal lesion in enrolled patients initially. Ulcers showed geographic, irregular shape and sometimes-transverse array. Significantly, aphthous

**Table 3 Follow-up colonoscopy results and feasibility to anti-tuberculosis**

Group	Tbc colitis (n = 7)	Nonspecific ulcers (n = 18)		P <sup>1</sup>
	Suspicious tbc colitis (n = 9)	Suspicious IBD (n = 9)		
Active ulcer	0/7	0/9	9/9	< 0.001
Improvement of stenosis	2/2	0/1	0/2	NS
No. of inflammatory polyps	Increase	Increase	Increase	NS
Extent of lesion	Decrease	Decrease	Increase	0.01
Feasibility to drug (%)				0.01
Very good	5 (71.4)	4 (44.4)	0 (0)	
Good	2 (28.6)	5 (55.6)	5 (55.6)	
Poor	0 (0)	0 (0)	4 (44.4)	

NS: Not significant. <sup>1</sup>Significant at P < 0.05 by Fisher's exact test.

ulcers were common in the "suspicious IBD group" (P = 0.01). About 30% of patients in each group showed non-caseating granuloma on biopsy. Acid-fast bacilli were noted in only 3 patients of tuberculous colitis even though this is definite evidence of tuberculosis infection.

The follow-up colonoscopy features are summarized in Table 3. After short-term anti-tuberculosis medication, there are only scarring and inflammatory polyps remained in patients with tuberculous colitis (Figure 1).

Nine of 18 patients with nonspecific ulcers also showed dramatic response after short-term trial similar to patients with tuberculous colitis presenting no active ulcer or erosions, irrespective of initial lesion (Figure 2, P < 0.001). We regarded them as the "suspicious tuberculous colitis group" and maintained anti-tuberculosis treatment for 10 mo.

The extent of lesion and stenosis in "tuberculous colitis" and "suspicious" groups were also improved, except in one patient of the "suspicious tuberculous colitis group" who underwent ileocectomy due to obstruction during short-term anti-tuberculosis treatment.

Another 9 patients of nonspecific ulcers who showed active ulcers remaining and sometimes more aggravated after trial (P < 0.001) were regarded as the "suspicious IBD group".

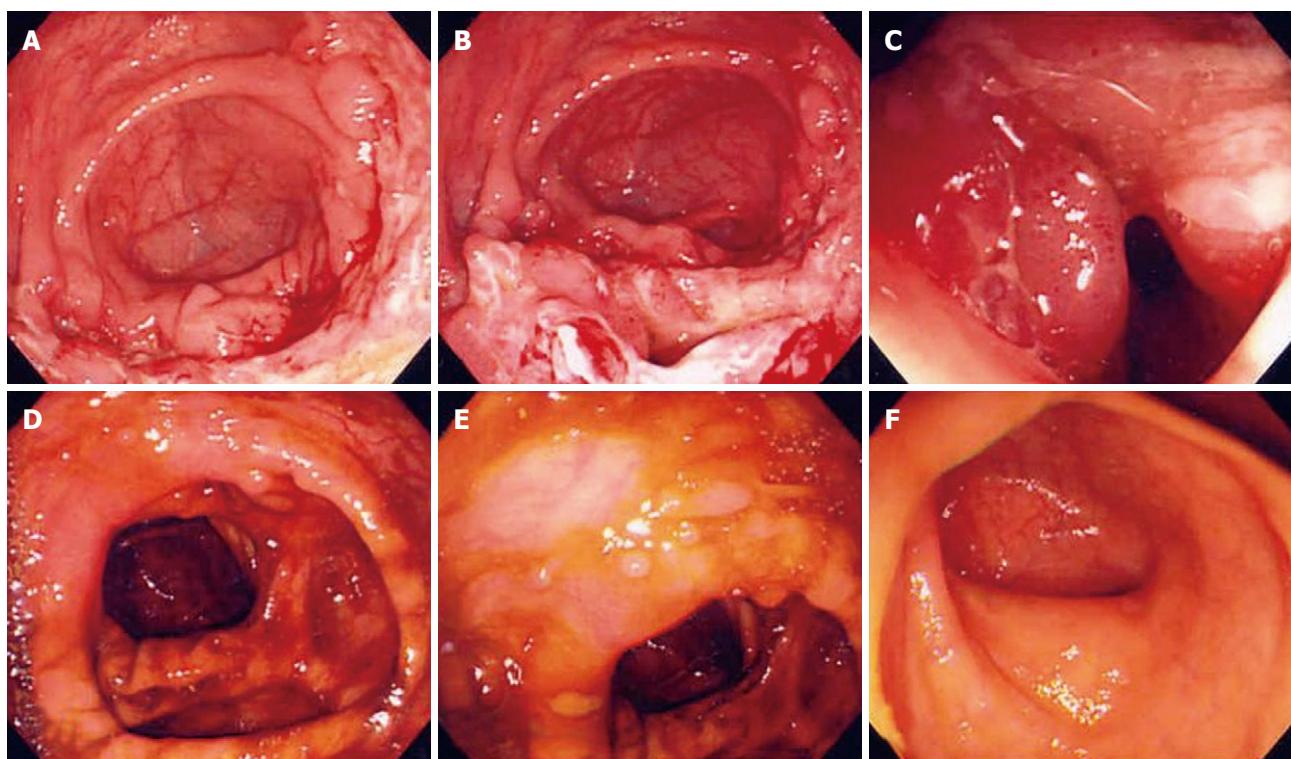
Some patients of the "suspicious IBD group" showed an expansion of the extent of involved colon with aggravation of stenosis (Figures 3 and 4).

### Feasibility of anti-tuberculosis treatment

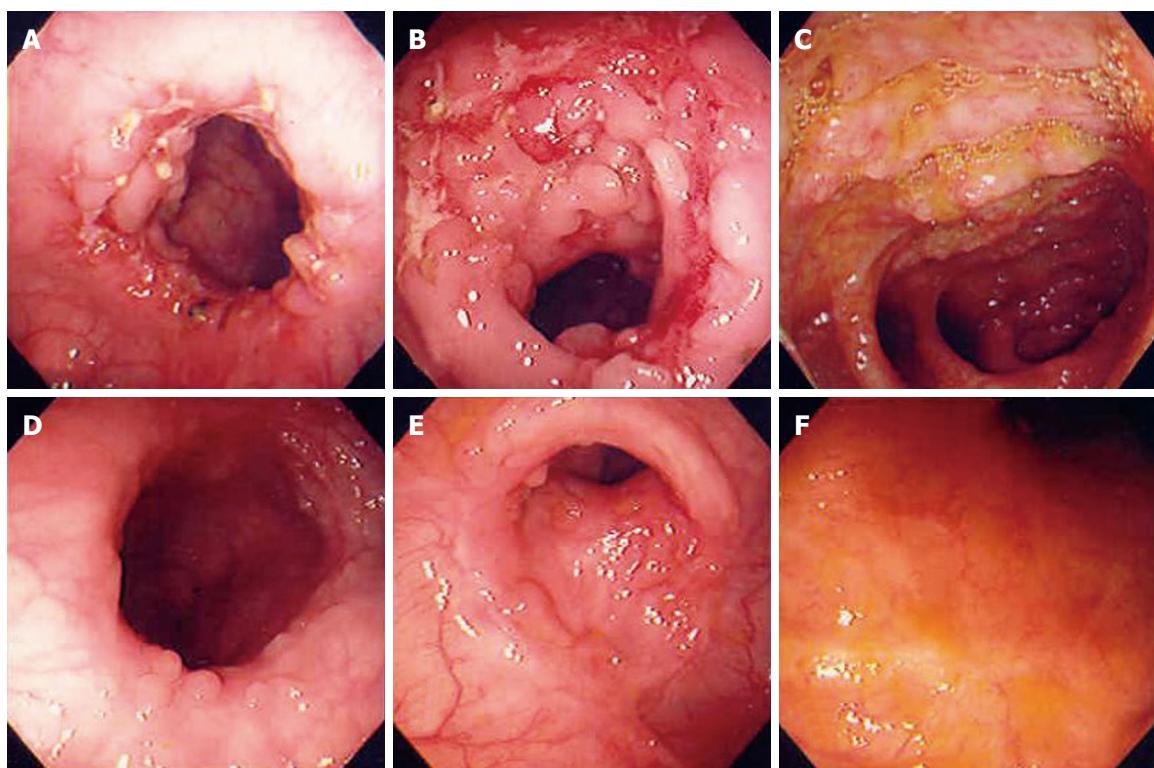
Most of the patients in the "suspicious tuberculous colitis group" showed good tolerance of anti-tuberculosis treatment during the trial, some of the patients in the "suspicious IBD group" showed poor tolerance of anti-tuberculosis treatment (P = 0.01, Table 3). But more than half of the "suspicious IBD group" also showed good toleration and mild symptomatic improvement in spite of aggravating colonoscopic findings.

### Clinical outcome

The 9 patients of the "suspicious tuberculous colitis



**Figure 1** Colonoscopy findings before (A-C) and after (D-F) anti-tuberculosis medication in a 30-year-old male patient with tuberculous colitis on the ileocecal valve which shows acid-fast bacilli on biopsy. After medication, scar and inflammatory polyps were noted.

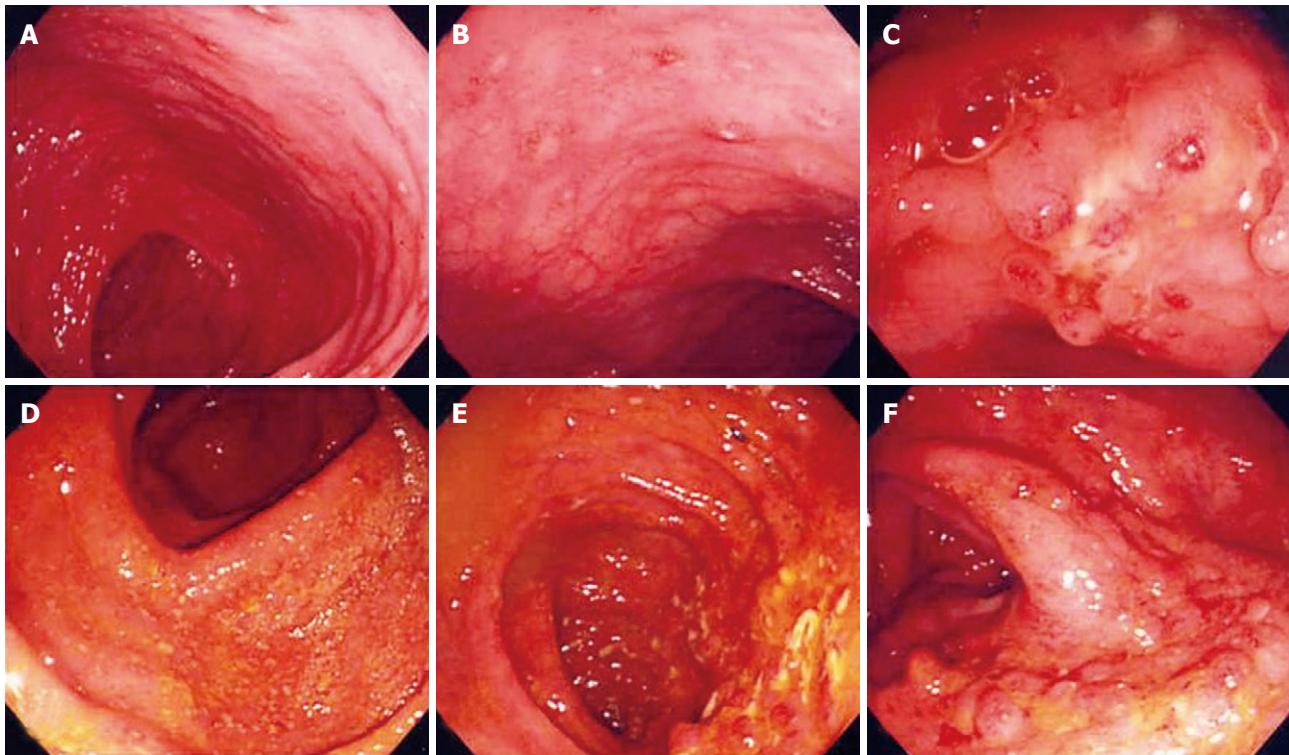


**Figure 2** Colonoscopy findings before (A-C) and after (D-F) anti-tuberculosis treatment in a 37-year-old female patient. It showed transverse arrayed geographic ulcers on ileocecal valve with granuloma on biopsy and presented nodularity on terminal ileum. After medication, only healing stage ulcers were left. We classified this case as "suspicious tuberculous colitis group".

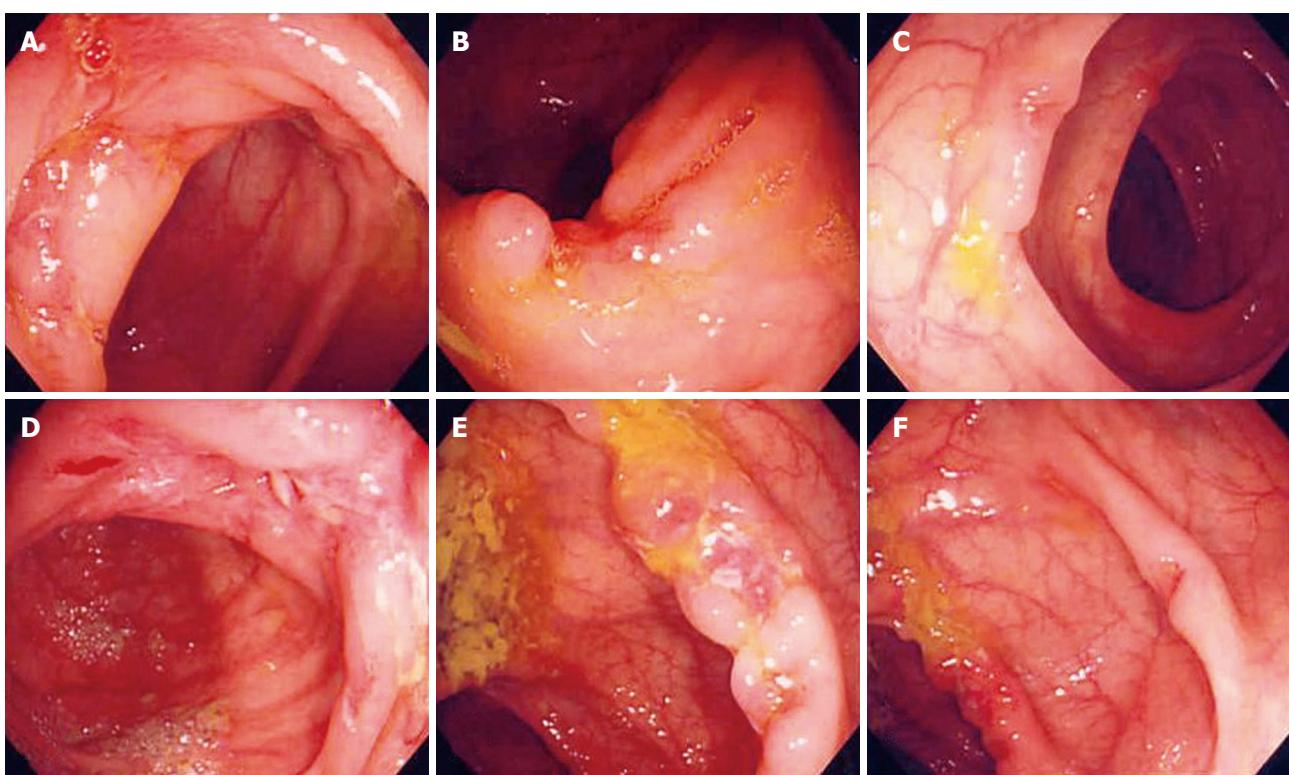
group" showed weight gain and general improvement in well being along with complete resolution of the whole lesion and GI symptoms after 10-mo of anti-

tuberculosis treatment. They were therefore finally confirmed as tuberculous colitis.

The 9 patients in the "suspicious IBD group" who had



**Figure 3** Colonoscopy findings before (A-C) and after (D-F) anti-tuberculosis treatment in a 23-year-old male patient with aphthous ulcers on the proximal ascending colon. After medication it shows aggravating active ulcers and extension of the lesion. We classified this case as the “suspicious IBD group”.



**Figure 4** Colonoscopy findings before (A-C) and after (D-F) anti-tuberculosis medication in a 39-year-old female patient with irregular ulcers on the ileocecal valve and proximal AC. After medication, it showed active ulcers and extension. We classified this case as “suspicious IBD group” and performed mesalazine trial. The patient showed no response to all the medical trials, so we confirmed this case as the “nonspecific colonic ulcers” by surgery.

active ulcers on follow-up colonoscopy were reevaluated; a second biopsy was performed and withdrawn from the anti-tuberculosis treatment trial. They were switched

to mesalazine and/or corticosteroid for inflammatory bowel disease. The new medication was well-tolerated and showed slow clinical improvement by 8 of the “suspicious

**Table 4** Final diagnosis of the patients of “suspicious inflammatory bowel disease group”

Patient	Response	Other manifestation during follow-up	Final Dx
#1 F/32	Response to mesalazine		CD
#2 F/15	Response to steroid	perianal abscess after 2 years	CD
#3 F/20	Response to mesalazine		CD
#4 F/39	No response to mesalazine, active ulcer and symptoms	hemicolectomy	Non-specific ulcers
#5 M/24	Response to mesalazine		CD
#6 M/42	Response to steroid		CD
#7 M/16	Response to mesalazine	Ileocectomy due to ileal perforation after 2 years	CD
#8 F/27	Response to mesalazine	perianal fistula after 2 years	CD
#9 M/26	Response to steroid	perianal abscess after 1 year	CD

IBD group”. However, 1 patient of the “suspicious IBD group” complained of sustained symptoms and weight loss and his 3rd colonoscopy showed more aggravation of lesions, chronic inflammation was found on repeated biopsy. After a 6-mo trial of mesalazine which did not show improvement, it was decided to perform a right hemicolectomy for confirmative diagnosis and treatment (Figure 4). The surgical specimen showed diffuse chronic ulcers on the ileocecal valve and ascending colon with neither granuloma nor any other malignant cells. After operation, the patient was observed for 2 years without further GI problems. During the 2 years of follow-up, 8 patients of the “suspicious IBD group” were improved in diarrhea, abdominal pain and colonic lesion, but 3 patients developed anal problems, and 1 patient underwent an operation due to ileal perforation. Finally 8 patients of the “suspicious IBD group” were diagnosed as Crohn’s disease, one patient as nonspecific simple colonic ulcers after a right hemicolectomy (Table 4).

## DISCUSSION

The Asian Pacific region was previously designated as a low incidence area of IBD, but this could be due to lack of solid population-based studies, low diagnostic awareness, and high incidence of intestinal infections. Current literature and recent information leave no doubt that a true increase of IBD is occurring throughout this region<sup>[9]</sup>. By contrast, the incidence of tuberculosis is rising, both in the United States of America as well as in the United Kingdom<sup>[3,10,11]</sup>. Following the resurgence of tuberculosis, two series of patients with abdominal tuberculosis were recently published in the United States<sup>[12,13]</sup>.

Thus, each side of the world is having to face the task of differentiating between the two diseases, Crohn’s disease and tuberculous colitis, which are similar in both clinical features and colonoscopy findings. In spite of similar clinical findings, the ultimate natural history of the two diseases is different. Appropriate anti-tuberculosis treatment leads in most cases of tuberculous ileocolitis to complete cure, whereas Crohn’s disease is a progressive relapsing illness unaffected by anti-tuberculosis treatment.

In tuberculous colitis, the cecum, Ileocecal valve and terminal ileum are commonly affected sites<sup>[14-17]</sup>, and are also the most common sites of Crohn’s disease. In all

areas of the world where Tuberculosis is endemic high-risk patients in developed countries need differentiation of the two diseases as early as possible.

Typical colonoscopy features described in patients with tuberculous colitis are transverse or linear ulcers, nodules, a deformed ileocecal valve and cecum, presence of inflammatory polyps, and multiple fibrous bands arranged in a haphazard fashion<sup>[14-16,18]</sup>. By contrast, typical Crohn’s disease shows segmental longitudinal ulcers with a cobble stone appearance, stricture, perianal lesion and pseudo polyps<sup>[19]</sup>. In clinical practice, however, nonspecific ulcers on ileocecal valve and cecum without typical features are often seen.

Biopsy during colonoscopy helps in the diagnosis of nonspecific ulcers in the ileocecal area. Caseating granulomas and/or acid-fast bacilli are present only in a small proportion of patients with tuberculous colitis<sup>[20]</sup>. Granulomas, with or without caseation, are usually seen in less than 50% of patients with tuberculous colitis<sup>[3,15,16]</sup>, while clusters of epithelioid cells without well formed granulomas have been reported to occur in 20%-30% of the biopsies obtained<sup>[15,16]</sup>. Our study shows non-caseating granuloma is not significant to the differential diagnosis of tuberculous colitis from Crohn’s colitis.

Acid-fast bacilli have been reported in 50%-100% of specimens from patients with tuberculous colitis<sup>[21-24]</sup>, but there are several reports where acid-fast bacilli could not be detected on histological examination of the biopsy material<sup>[14-16,18,25]</sup>. Acid-fast bacilli were seen in only 3 specimens (21.7%) in our cases. Thus, in a considerable number of tuberculous colitis the biopsies have features of chronic inflammation but no granulomas, caseation or clusters of epithelioid cells<sup>[15,16]</sup>. Culture of the biopsy material may increase the diagnostic yield<sup>[3]</sup>. However, disappointing results with 0% detection of acid-fast bacilli have also been reported<sup>[16]</sup>. Polymerase chain action analyses of biopsy specimens obtained endoscopically has been shown to be more sensitive than culture and acid-fast stains in diagnosing tuberculous colitis<sup>[26]</sup>. Other studies have suggested that an enzyme-linked immunosorbent assay using mycobacterial saline-extracted antigen may increase the yield of correct diagnosis of colonic tuberculosis<sup>[27]</sup>. The symptoms of tuberculous colitis such as chronic diarrhea, RLQ discomfort, and malaise are non-specific. But chest radiographs showing evidence of

active or healed pulmonary infection may be a clue to diagnosis<sup>[15,16,28]</sup>, in our cases, 70% of tuberculous colitis and 33% of suspicious tuberculous colitis cases showed active pulmonary tuberculosis and old scar in 22% of suspicious cases. By contrast, none of the patients of the “suspicious IBD group” showed abnormal chest radiographs ( $P < 0.001$ ).

In clinical practice, therefore, nonspecific ulcers on the ileocecal valve and cecum without any other clue for specific diagnosis are often seen. Efforts to exclude other infection including amoeboma, *Yersinia* infection, GI histoplasmosis, and periappendiceal abscess must be considered<sup>[3]</sup>. If the clinical and colonoscopy features are suggestive of tuberculous colitis and multiple target biopsies do not show evidence of any other disease, then a therapeutic trial of anti-tuberculosis treatment can safely be given to these patients<sup>[29]</sup>. Clinical response to the anti-tuberculosis treatment is usually dramatic and less than 1-year treatment is sufficient for patients with tuberculous colitis compared to life long treatment for inflammatory bowel disease.

The recommended regimen for pulmonary tuberculosis is combined chemotherapy containing isoniazid, rifampicin, ethambutol and pyrazinamide during 9 mo<sup>[30]</sup>. But the exact duration of anti-tuberculosis treatment for tuberculous colitis is not yet established due to difficulties in bacteriological diagnosis and the assessment of response to therapy. As a result, extra-pulmonary tuberculosis has been treated for 12 mo to 24 mo on the basis of warnings of disease recurrence without supportive data. Recent study has shown tuberculosis enterocolitis can be treated with 9 mo of chemotherapy without disease recurrence<sup>[29]</sup>. Our study showed that 10 mo of 4 regimens of chemotherapy were adequate for patients with tuberculous colitis combined with or without pulmonary tuberculosis.

How long a period will be adequate for an empirical trial of undetermined patients? Generally, 2 mo to 3 mo are accepted as adequate for trials based on clinical response. However, the colonoscopy findings are not reported after a 2-mo to 3-mo trial. As our study shows, active ulcers and erosions are completely healed after 2 mo to 3 mo of medication and only leave scarring and inflammatory polyps in all patients with tuberculous colitis and suspicious tuberculous colitis. That means a 2-mo to 3-mo trial of anti-tuberculosis treatment and colonoscopy follow-up are very useful for differential diagnosis of tuberculous colitis with other inflammatory bowel diseases. It is an exciting result that some patients with suspicious tuberculous colitis, who showed no active ulcer and significant resolution on follow-up colonoscopy without any other specific clue for tuberculosis, were also confirmed as tuberculous colitis after 10-mo medication. Likewise, every patient of the “suspicious IBD group” who showed no response to a short-term trial was ultimately confirmed as Crohn’s disease or non-specific ulcer. This study also showed that, in patients with suspicious inflammatory bowel disease, a 2-mo to 3-mo trial was sufficient to exclude tuberculous colitis and adequate medication for

inflammatory bowel disease could be started based on the trial.

Clinical response of symptoms and tolerance to anti-tuberculosis treatment was also important during the trial. In our study, most patients of the “suspicious tuberculous colitis group” tolerated medication well. About 45% of patients in the “suspicious IBD group” showed poor tolerance. But half also showed good tolerance to anti-tuberculosis treatment and mild clinical response during the trial too. Thus, during the anti-tuberculosis treatment trial, the physician’s concern for a patient’s tolerance to drug and clinical improvement is helpful but not definitive to decide maintaining anti-tuberculous treatment. We conclude that in cases of a therapeutic trial on nonspecific ulcers of the ileocecal area, 2 mo to 3 mo of anti-tuberculous medication and colonoscopy follow-up are valuable for making early confirmatory diagnosis.

## COMMENTS

### Background

Differentiation between tuberculous colitis and Crohn’s colitis can be difficult due to nonspecific GI symptoms and similar colonoscopic findings. In Asia, pulmonary and extrapulmonary tuberculosis are not rare until now. Also, the incidence and prevalence rates of inflammatory bowel disease (IBD) are increasing rapidly in many Asian countries, including Korea.

### Research frontiers

There are efforts to differentiate two disease entities through colonoscopic findings. But some of them still could not be confirmed by initial colonoscopic findings. Short-term trial of anti-tuberculosis treatment is necessary before confirmative diagnosis of Crohn’s colitis in the endemic area of tuberculosis.

### Innovations and breakthroughs

The precise colonoscopic features after short-term medication in patients with either tuberculous colitis or suspicious tuberculous colitis have not been documented. This study was performed prospectively and showed that active ulcers and erosions were completely healed after 2-mo to 3-mo of medication and only leave scarring and inflammatory polyps in all patients with tuberculous colitis and suspicious tuberculous colitis. This study showed that, in patients with suspicious inflammatory bowel disease, a 2-mo to 3-mo trial and colonoscopic evaluation would be sufficient to exclude tuberculous colitis and adequate medication for inflammatory bowel disease to be started.

### Applications

We conclude that in cases of therapeutic trial on nonspecific ulcers on the ileocecal area, especially the endemic area of tuberculosis, 2 mo to 3 mo of anti-tuberculous medication and colonoscopy follow-up are valuable for making early confirmatory diagnosis. Duration of the therapeutic trial will be shortened after further clinical trial.

### Peer review

This study provides colonoscopic features after short term anti-tuberculosis medication in patients with tuberculosis colitis and other chronic inflammatory bowel disease.

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RAPID COMMUNICATION

# Cellular DNA repair cofactors affecting hepatitis B virus infection and replication

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## Abstract

**AIM:** To investigate whether hepatitis B virus (HBV) infection activates DNA damage response and DNA repair cofactors inhibit HBV infection and replication.

**METHODS:** Human hepatocyte cell line HL7702 was studied. Immunoblotting was performed to test the expression of ataxia telangiectasia-mutated (ATM)-Rad3-related protein (ATR), p21 and the level of phosphorylation of Chk1, p53, H2AX, ATM in HBV-infected or non-infected-cells. Special short RNAi oligos was transfected to induce transient ATR knockdown in HL7702. ATR-ATM chemical inhibitors caffeine (CF) and theophylline (TP), or Chk1 inhibitor 7-hydroxystaurosporine (UCN01) was studied to determine whether they suppress cellular DNA damage response and MG132 inhibits proteasome.

**RESULTS:** The ATR checkpoint pathway, responding to single-strand breaks in DNA, was activated in response to HBV infection. ATR knockdown cells decreased the HBV DNA yields, implying that HBV infection and replication could activate and exploit the activated DNA damage response. CF/TP or UCN01 reduced the HBV DNA yield by 70% and 80%, respectively. HBV abrogated the ATR-dependent DNA damage signaling pathway by degrading p21, and introduction of the p21 protein before HBV infection reduced the HBV DNA yield. Consistent with this result, p21 accumulation after MG132 treatment also sharply decreased the HBV

DNA yield.

**CONCLUSION:** HBV infection can be treated with therapeutic approaches targeting host cell proteins by inhibiting a cellular gene required for HBV replication or by restoring a response abrogated by HBV, thus providing a potential approach to the prevention and treatment of HBV infection.

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**Key words:** Hepatitis B virus; DNA damage response; Hepatitis B virus infection; Caffeine; RNAi

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## INTRODUCTION

Eukaryotic cells employ multiple strategies of checkpoint signaling and DNA repair mechanisms to monitor and repair damaged DNA. There are two branches in the checkpoint response pathway: ataxia telangiectasia-mutated (ATM) branch and ATM-Rad3-related (ATR) branch<sup>[1,2]</sup>. Many viruses are now known to interact with DNA damage sensing and repair machinery. These viruses have evolved tactics to eliminate, circumvent, or exploit various aspects of the DNA damage response to the host cells. Strategies include activation of repair proteins or targeting of specific cellular factors for degradation or mislocalization<sup>[3-6]</sup>. It is necessary to examine the DNA damage pathway activated by viral replication for generation of antiviral drugs. In human immunodeficiency virus (HIV), it has been clearly determined that prevention of viral integration inhibits viral replication and promotes cellular apoptosis. The ATM-specific inhibitor ku55933 can inhibit HIV replication in primary T cells<sup>[7]</sup>.

Although a safe and efficient vaccine is available at present, chronic hepatitis B virus (HBV) infection

remains a major health problem worldwide. Interferon treatment is effective in only approximately one-third of the patients and produces considerable side effects. Long-term treatment with the second-generation of nucleoside analogue lamivudine (lam) efficiently inhibits HBV replication with frequent viral polymerase mutations. We found that HBV infection could trigger ATR-dependent DNA damage response, resulting in increased ATR and Chk1 phosphorylation levels. However, ATR checkpoint signaling is blocked downstream of the p53-dependent pathway to evade apoptosis by degrading p21. We designed a strategy to select new drug targets that inhibit a cellular gene required for HBV replication or restore a response stalled by HBV in the ATR DNA damage pathway.

The ATM and ATR kinases are targets of a known inhibitor, caffeine (CF), of DNA damage response. CF has shown to inhibit the activity of these kinases *in vitro* and the response to cellular DNA damage controlled by ATM and ATR. Theophylline (TP) is a product of CF metabolism *in vivo* and has been clinically used in treatment of asthma. It has also been demonstrated that TP exhibits CF-like effects on DNA damage response<sup>[8]</sup>.

Originally developed as a protein kinase C (PKC) inhibitor, 7-hydroxystaurosporine (UCN-01) has been subsequently shown to inhibit several other cell survival/cell cycle regulatory proteins, including Chk1 and 3-phosphoinositide-dependent protein kinase-1 (PDK1)/protein kinase B (Akt). By inhibiting Chk1, UCN-01 blocks the proteasome degradation of the cdc25C phosphatase, resulting in dephosphorylation (activation) of p34<sup>cdc2</sup>. In this way, UCN-01 functions as a checkpoint abrogator that potentiates the lethal effects of several DNA-damaging agents, including ara-C18 and camptothecin<sup>[9,10]</sup>.

Proteasome, a multicatalytic enzyme complex, controls the regulation (by means of degradation) of many proteins involved in cell cycle arrest and apoptosis. The proteasome inhibitor MG132 can restore wild-type (WT) p53 levels reduced by E6 and sensitize HPV-positive cervical cancer cell lines to apoptotic stimuli such as rhTRAIL. It was also reported that MG132 could inhibit HSV replication by preventing HSV-1-induced NF-κB activation<sup>[11,12]</sup>.

In this paper, we report that HBV infection activates and exploits DNA damage response to replication stress. We investigated whether inhibition of DNA damage response by CF, TP and UCN01 or restoration of p21 expression by p21 transfection or proteasome inhibition leads to suppression of HBV replication. We set up a chronic HBV infection model by culturing HL7702 liver cells with HBV-positive serum without washing off input virus as conventional. HBV DNA titers inside the infected cells represent the final viral amount including the infected DNA not degraded and the newly synthesized HBV DNA. In this way, we could study the efficacy of DNA damage response inhibitors on HBV infection and replication. In addition, since DNA damage response is an acute response occurring quickly after virus infection, we assume that early intervention

of the DNA damage pathway would function more efficiently, and thus can be used in clinical practice as a HBV infection therapy during its early infectious stage or fulminant HBV infection.

## MATERIALS AND METHODS

### Chemicals

CF, TP, UCN01 and MG132 were obtained from Sigma (St. Louis, MO). The stock concentration was 100 mmol/L. CF and UCN01 were dissolved in water, whereas TP was dissolved in 0.1 mol/L NaOH, and MG132 was dissolved in DMSO.

### Cell cultures, HBV strains, and infections

The human hepatocyte cell line HL7702, isolated from a HBV sera-negative individual, was obtained from Shanghai Biochemistry Institute. HL7702 cells were cultured in RPMI-1640 with 10% heat-inactivated fetal bovine serum (FBS). Serum samples from HBV carriers obtained for infection test were analyzed. The number of serum HBV viral particles was  $7 \times 10^9$  copies/mL, as quantified by FQ-PCR. The sera were stored at -80°C until use.

HBV infection was monitored by culturing  $10^5$  HL7702 cells in a 6-cm plate in 3 mL RPMI-1640 containing  $10^6$  HBV virus particles. Infected cells were simultaneously treated with different types of drugs (CF, TP and UCN01) at various concentrations. The cells were washed thoroughly 8 times to remove excessive viral inputs and drugs before harvesting. Serum from healthy individuals was used as a negative control. All procedures were performed under level P2 biosafety conditions to minimize the possibility of cross-contamination.

### RNA interference

siRNA duplex composed of 21-bp sense and antisense oligonucleotides of ATR was synthesized by Beijing AuGCT Biotechnology Co. Ltd (China). The sequence of siRNA oligos used in this study was (only the sense strand is shown) siATR: 5'-AACCUCCGUGAUGUUGCUGATT-3'. HL7702 cells were transfected with 50 nmol/L siRNA using lipofectamine 2000 (Promega Corp., Madison, WI, USA) according to the manufacturer's protocol. At 24 h after transfection, cells were subjected to HBV infection and then the HBV DNA load was tested at indicated time points post-infection.

### Detection of HBV DNA by FQ-PCR

For FQ-PCR analyses, virus DNA was extracted from the culture medium using an alkaline lysis method. Briefly, HBV DNA was measured using a FQ-PCR diagnostic kit from Da-An Gene Corporation. FQ-PCR was performed using a Lightcycler™ Roche FQ-PCR system with the following amplification cycle: an initial denaturation at 93°C for 2 min, followed by 40 cycles of annealing at 93°C for 5 s, at 57°C for 45 s, and a final extension at 37°C for 10 s.

### Immunoblotting assay

Cell extracts were lysed in ice-cold Tris buffer (50 mmol/L, pH 7.5) containing 5 mmol/L EDTA, 300 mmol/L NaCl, 0.1% Igepal, 0.5 mmol/L NaF, 0.5 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 0.5 mmol/L PMSF, and antiprotease mixture (Roche Molecular Biochemicals) for 30 min and centrifuged at 13 000 × g for 10 min. The supernatant protein concentration was determined with the Bradford procedure (BioRad). The proteins were resolved on a 7%-15% SDS-PAGE gel and transferred onto nitrocellulose membranes. Blots were blocked in TBST containing 5% nonfat dried milk and incubated with the primary antibodies. Antibodies against p21, ATR (Santa Cruz) and tubulin (Sigma) were incubated at room temperature for 1 h, while antibodies against ATM phosphoserine 1981 (ATMp), Chk1 phosphoserine 345 (Chk1p), and p53 phosphoserine 15 (p53p) (cell signaling) were incubated at 4°C overnight. Secondary antibodies were from Jackson Laboratories. Horseradish peroxidase-based detection was performed using a chemiluminescence reagent (Amersham Biosciences), according to the manufacturer's instructions.

## RESULTS

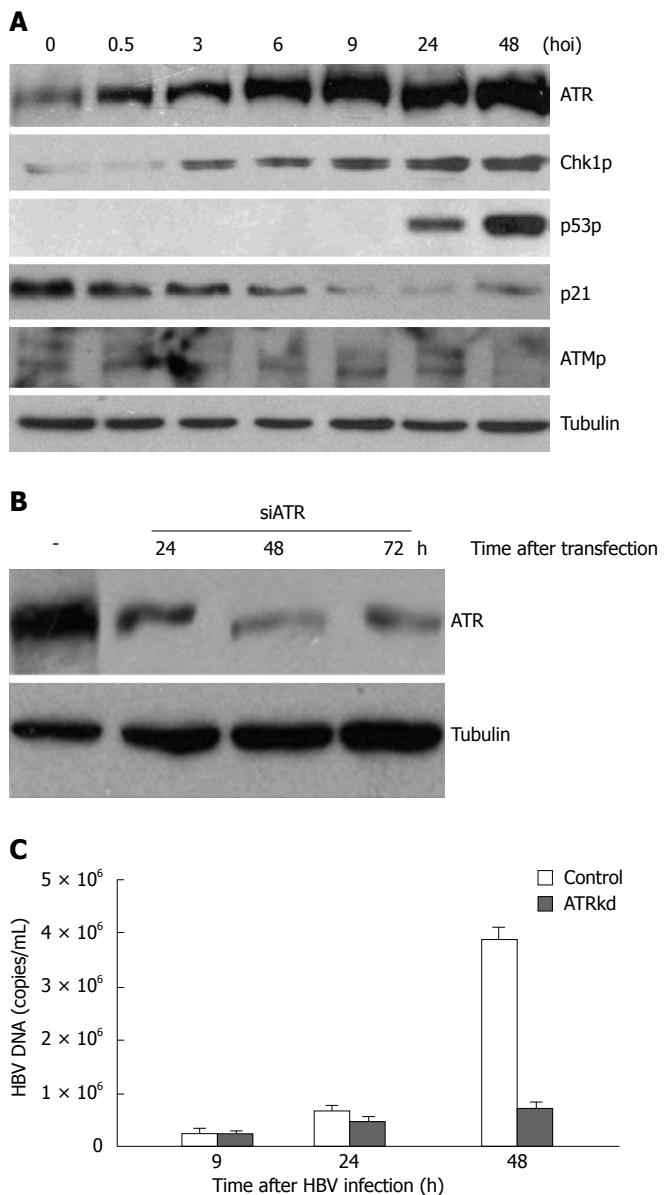
### HBV infection induced an ATR-dependent cellular DNA damage response

To identify whether a cellular DNA damage response was induced upon HBV infection, we set up a HBV infection and replication model by culturing normal HL7702 liver cells with HBV-positive serum<sup>[13]</sup>. A monolayer ( $10^5$ ) of the human HL7702 liver cells in 6-cm plates was infected with  $10^6$  HBV-DNA copies/mL serum at 37°C in an atmosphere containing 50 mL/L CO<sub>2</sub>. HBV infection induced an increase in the steady state levels of the ATR protein and in the phosphorylation levels of its downstream substrates Chk1 and p53 (Figure 1A). Chk1 phosphorylation at Ser-345 was evidently increased at the start of infection with a further increase from 6 to 48 h. p53 phosphorylation at Ser-15 was elevated beginning at 24 hpi and increased considerably at 48 hpi.

In contrast to ATR and its target, the phosphorylated form of ATM at Ser-1981 did not effectively increase upon infection. Furthermore, the phosphorylation of its downstream substrate Chk2 at Thr-68 began to decrease from 3 hpi (data not shown). p53 transcriptional target p21<sub>cip1/waf1</sub>, a cyclin-dependent kinase inhibitory protein, decreased substantially with time after infection, suggesting that p53-dependent downstream signaling can be blocked during HBV infection despite the appearance of phosphorylated p53. Taken together, these results indicate that HBV infection elicited activation of the ATR DNA damage checkpoint pathway that responds to replication stress rather than the ATM DNA damage checkpoint pathway.

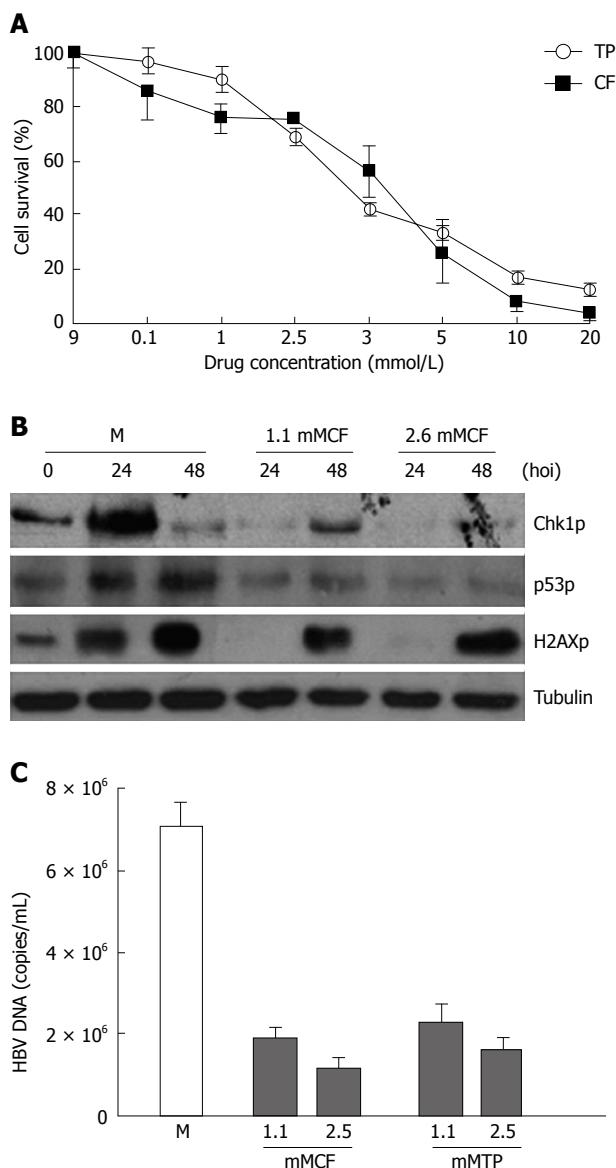
### HBV infection and replication exploited activated DNA damage response

To investigate the effect of the activated DNA damage response on HBV infection and replication, HBV



**Figure 1** An ATR-dependent cellular checkpoint response activated by HBV infection. **A:**  $10^5$  human HL7702 monolayer liver cells in a 6 cm plate were infected with  $10^6$  virus particles from HBV-positive patients at 37°C in an atmosphere containing 50 mL/L CO<sub>2</sub>. Normal serum from healthy individuals was used as non-infected control. Prior to cell harvesting, the cells were washed 8 times thoroughly to remove the excessive viral input. Whole cell lysates were prepared at various time points of infection (hours of infection, hoi) and subjected to immunoblotting assay using antibodies to the indicated proteins. Alpha-tubulin was used as the equal loading control; **B:** Specialized siRNA for ATR was transfected in HL7702 cells using lipofectamine 2000. At the indicated time points, cells were collected for Western blotting testing the ATR level; **C:** HBV-positive serum was added to ATR knockdown cells 24 h after transfection, cells were then harvested for FQ-PCR to test HBV DNA titers at the indicated time points post-infection.

infection was performed in ATR knockdown cells. ATR knockdown was conducted with short RNAi oligos transfection using lipofectamine 2000. RNAi technique diminished total ATR protein from 24 h to 72 h after RNA oligos transfection (Figure 1B). Based on this, HBV-positive serum was added to ATR knockdown cells 24 h after the transfection, cells were then harvested for HBV DNA titer assay at 9, 24 and 48 h, respectively, after infection. HBV DNA load in ATR knockdown

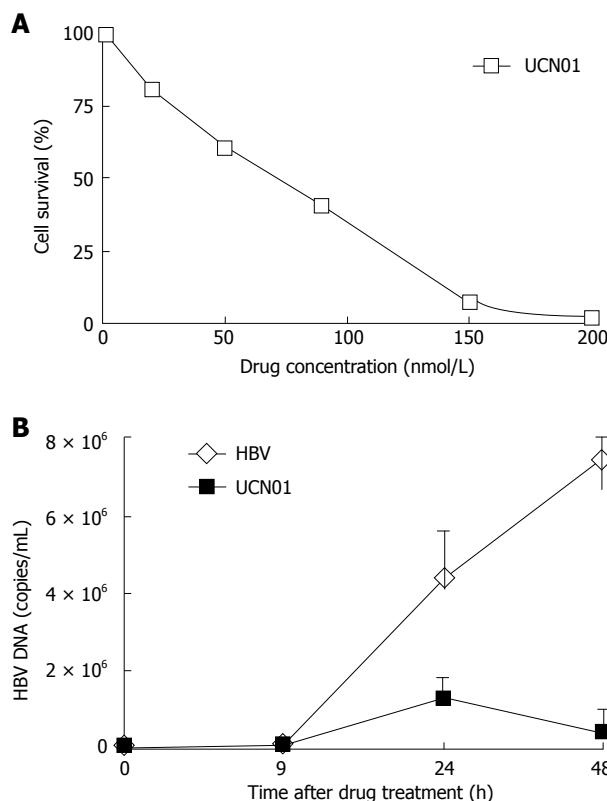


**Figure 2** HBV infection and replication suppressed by CF and TP. **A:** Cells were treated with CF or TP at different concentrations for 48 h, and survived cells were counted with trypan blue staining; **B:** Results of whole cell lysis, Western blotting of infected cells (M) or addition of CF-treated cells (1.1 mol/L CF, 2.5 mmol/L CF) and antibodies at the indicated time points; **C:** FQ-PCR analysis of HBV DNA from the infected cells (M) or addition of both HBV and CF (mMCF) or TP (mMTP).

cells was reduced to about 20% of control (Figure 1C), indicating that HBV replication is dependent on the presence of ATR protein.

#### ATM-ATR kinase inhibitor suppressed HBV infection and replication

To evaluate the influence of the ATR and ATM kinase inhibitor CF and its methylxanthines on HBV replication, the infected cells were simultaneously treated with different types of drugs (CF and TP) at various concentrations. Trypan blue staining was performed and viable cells were counted at different time points after drug addition. The percentage of cells survived was determined by the ratio of the number of treated cells divided by the number of untreated cells. The DNA



**Figure 3** HBV infection and replication suppressed by CF and TP. **A:** HL7702 cells were treated with the indicated concentrations of UCN01, and survived cells were counted 48 h post-treatment with trypan blue staining; **B:** FQ-PCR analysis of HBV DNA from the infected cells (HBV) or addition of both HBV and UCN01 (UCN01) at the indicated time points.

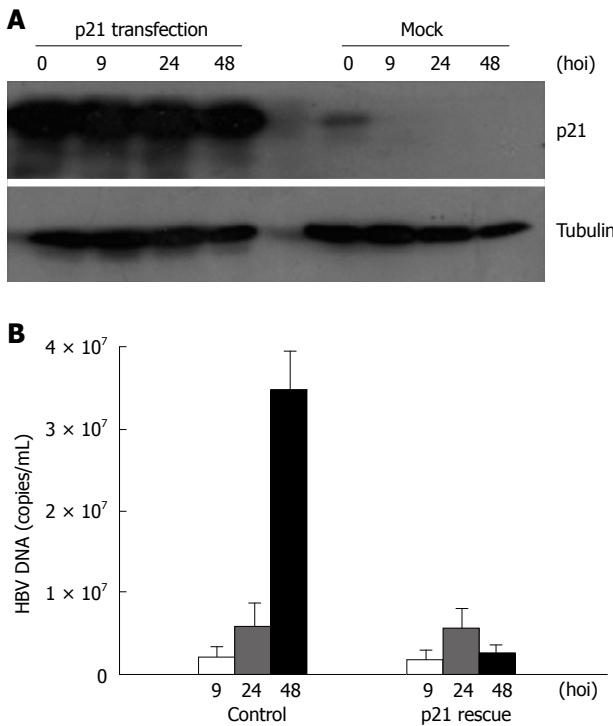
extracted from the infected cells was subjected to FQ-PCR analysis, and the degree of amplification of the viral genome over the time course of infection was determined. We observed a 70% decrease in HBV DNA yields in cells treated with 1.1 mmol/L CF at 48 h during HBV infection (Figure 2C). However, such a dose did not affect cell proliferation (Figure 2A) but abrogated the phosphorylation of target proteins with ATR kinase activity such as Chk1 at Ser-345, H2AX at Ser-139, and p53 at Ser-15 (Figure 2B).

#### Chk1 inhibitor suppressed HBV infection and replication

Chk1 phosphorylation was greatly increased during HBV infection. We therefore investigated whether Chk1 inhibitors would have an effect on HBV replication. Infected cells were simultaneously treated with various concentrations of UCN01. The percentage of survived cells was calculated as described above. The DNA extracted from infected cells was subjected to FQ-PCR analysis. We observed a 90% decrease in HBV DNA yields in cells treated with 50 nmol/L UCN01 (Figure 3B), which did not significantly affect the cell survival was not significantly affected in 48 h (Figure 3A).

#### p21 introduction suppressed HBV infection and replication

In contrast to increased levels of sustained ATR and Chk1 (ATR substrate) phosphorylation during HBV

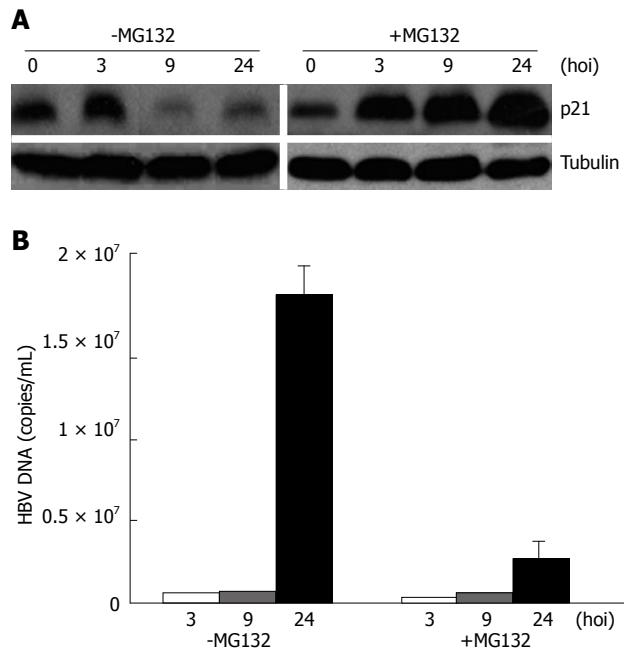


**Figure 4** HBV infection and replication suppressed by introduction of p21. **A:** Result of whole cell lysis, Western blotting of p21-transfected cells and control cells (Mock) at the indicated time points; **B:** FQ-PCR analysis of HBV DNA from the infected cells (control) or both HBV and p21-transfected cells (p21 rescue) at the indicated time points.

infection, HBV abrogated p53-dependent cell cycle checkpoint signaling by degrading p21 (Figure 1A). We thus introduced p21 into host cells to investigate its effect on HBV replication. WT p21 cDNA was transfected into HL7702 cells, which were infected with HBV-positive serum 24 h later, harvested at 9, 24 and 48 h respectively after HBV infection, and subjected to FQ-PCR and immunoblotting. p21 expression decreased with time after infection, and myc-tagged p21 expression was higher in the transfected host cells than in the mock-transfected cells (Figure 4A). We then investigated the effects of p21 transfection on HBV infection and replication. Figure 4B showed that the yield of HBV DNA in the p21-transfected cells was only approximately 8% of that in the cells infected only with HBV, indicating that recovery of p21 destroyed by HBV infection has a detrimental effect on replication of the virus.

#### MG132 inhibited HBV replication

Previous studies have established that HIV-1 infection can be enhanced by treatment of infected cells with proteasome chemical inhibitors<sup>[7,8]</sup>. It is likely that such inhibitors protect the viral core from degradation in target cells. We have proved that introduction of p21 into infected cells leads to decreased HBV replication. Therefore, we tested whether the proteasome inhibitor MG132 would have an effect on HBV replication in the presence of p21 accumulation. To determine p21 levels and the effect of proteasome inhibitors on cell susceptibility to HBV infection, we pretreated cultures



**Figure 5** HBV replication inhibited by MG132. **A:** Result of whole cell lysis, Western blotting of MG132-treated cells (+MG132) and control cells (-MG132) at the indicated time points; **B:** FQ-PCR analysis of HBV DNA from the infected cells (-MG132) or both HBV and MG132-treated cells (+MG132) at the indicated time points.

of HL7702 cells with MG132 (10 μM) for 2 h and then monitored HBV infection. HBV-infected cells were harvested at 0, 9, 24, 48 h after extensive washes 8 times with PBS, and then subjected to immunoblotting and FQ-PCR. The level of p21 expression decreased with time after infection, the addition of MG132 resulted in a substantial increase in the p21 expression levels (Figure 5A). To investigate whether treatment with MG132 can also effectively inhibit virus replication, we analyzed the effect of MG132 on HBV replication. The yield of HBV in the presence of proteasome inhibitors was only approximately 10% of that in control cells 48 h after HBV infection (Figure 5B), indicating that proteasome inhibitors can reduce HBV replication by up-regulating p21.

## DISCUSSION

Several viruses, including DNA virus herpes simplex virus (HSV)<sup>[14,15]</sup>, Kaposi's sarcoma-associated herpesvirus<sup>[16]</sup>, human cytomegalovirus (HCMV)<sup>[17]</sup>, Epstein-Barr virus (EBV)<sup>[18]</sup>, papillomavirus<sup>[19,20]</sup>, simian virus 40 (SV40)<sup>[21]</sup> and retrovirus HIV<sup>[22-24]</sup>, trigger cellular signaling cascades that are characteristic of a DNA damage response. Infection and replication of HBV have been achieved in human hepatoma cell lines and primary fetal human hepatocytes using transfected HBV genomes or HBV serum. In this study, HL7702 cells were inoculated with HBV serum consistently ( $10^6$  particles per  $10^5$  cells), mimicking the HBV infection process. Serum from healthy individuals was used as the non-infected control. By using FQ-PCR and southern blotting, we demonstrated that human hepatocytes

could maintain HBV infection *in vitro* and support the replication of HBV DNA (data not shown), suggesting that HBV infection activates the DNA damage response to replication stress accompanying increased ATR and Chk1 (ATR substrate) phosphorylation. Furthermore, HBV abrogated the checkpoint signaling by degrading p21. The results of this study show that the ATM and ATR inhibitor CF and its methylxanthine TP, the Chk1 inhibitor UCN01, the proteasome inhibitor MG132 and p21 up-regulation could suppress HBV replication. These findings suggest that some DNA damage responsive proteins are implicated in modulating HBV infections and therefore can be used in treatment of drug-resistant viral strains. In addition, targeting cellular proteins with a low mutation rate may not lead to the rapid emergence of HBV strains that are resistant to inhibitors of these proteins.

The effect of CF is mediated by inhibiting its cellular target, the ATR kinase involved in retroviral integration. The precise role of ATR in HBV replication is unclear. The integration of HBV DNA into chromosomes has been reported<sup>[13]</sup>. Interestingly, treatment with TP has an inhibitory effect on HBV replication even at a concentration as low as 0.1 mmol/L, which is too low to impact ATM/ATR kinase activity. Therefore, the antioxidant function of TP or CF may have a great inhibitory effect on HBV infection process, and further experiments should be done on this issue.

Chk1 is activated following HBV infection and the Chk1 inhibitor UCN01 can inhibit HBV replication; however, the precise mechanism underlying this inhibition remains to be determined. The resultant S-G2 phase-like cellular condition appears to favor replication of viral DNA.

In cervical carcinogenesis, the p53 suppressor pathway is disrupted by HPV E6-mediated proteasome degradation. MG132 could restore WT p53 levels and thus may sensitize papillomavirus (PV) positive human cervical cancer cells to apoptosis<sup>[11,25]</sup>. Several studies have recently demonstrated that proteasome is a suitable neoplastic target with a clinical potential<sup>[26-28]</sup>. We report here that MG132 can be used to suppress HBV replication by up-regulating p21. As a latency virus, HBV abrogates the checkpoint signaling controlled by ATR to prevent triggering of apoptotic signals. The mechanism underlying the regulation of apoptosis by HBV is *via* both p53-dependent pathways. The p53-dependent cell cycle checkpoint pathway involves p21-mediated inactivation of cdk2/cyclinE. HBV abrogates p53-dependent checkpoint activation by degrading p21. Our results demonstrate that MG132 could up-regulate p21 and thus suppress HBV replication, possibly due to the partial recovery of the p53-dependent checkpoint signaling pathway.

In summary, CF and its related methylxanthines can suppress replication of HIV as previously reported<sup>[8]</sup>. However, since DNA damage response is acute, DNA repair cofactors against HBV infection and replication should be used in early stage HBV infection. Further studies are necessary to determine the mechanism by

which DNA damage response inhibitors down-regulate HBV infection and replication.

## COMMENTS

### Background

Eukaryotic cells employ multiple strategies of checkpoint signaling and DNA repair mechanisms to monitor and repair damaged DNA. There are two branches in the checkpoint response pathway: ataxia telangiectasia-mutated (ATM) branch and ATM-Rad3-related (ATR) branch. Many viruses are now known to interact with DNA damage and repair machinery. These viruses have evolved tactics to eliminate, circumvent, or exploit various aspects of DNA damage response to the host cells. Strategies include activation of repair proteins or targeting of specific cellular factors for degradation or mislocalization.

### Research frontiers

In human immunodeficiency virus (HIV), prevention of viral integration inhibits viral replication and promotes cellular apoptosis. Thus, the ATM-specific inhibitor ku55933 can inhibit HIV replication in primary T cells.

### Innovations and breakthroughs

In this paper, we first report that (hepatitis B virus) HBV infection activates and exploits DNA damage response to replication stress. We investigated whether the inhibition of DNA damage response by CF, TP and UCN01 or the restoration of p21 expression by p21 transfection or proteasome inhibition would lead to suppression of HBV replication. We set up a chronic HBV infection model by culturing HL7702 liver cells with HBV-positive serum without washing off input virus as conventional. HBV DNA titers inside the infected cells represent the final viral amount including the infected DNA not degraded and the newly synthesized HBV DNA. In this way, we can study the efficacy of DNA damage response inhibitors on HBV infection and replication.

### Applications

Since DNA damage response is an acute response occurring quickly after virus infection, we assume that early intervention of the DNA damage pathway would function more efficiently and thus can be used in clinical practice as a HBV infection therapy during its early infectious stage or fulminant HBV infection.

### Peer review

This is an interesting article that reports data on the effect of DNA repair cofactors on HBV replication and infection. The study is well designed. The data or findings are reliable.

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RAPID COMMUNICATION

## Treatment of polycystic liver disease with resection-fenestration and a new classification

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### Abstract

**AIM:** To evaluate outcomes in patients with autosomal dominant polycyst liver disease (APLD) treated by combined hepatic resection and fenestration. A new classification was recommended to presume postoperative complications and long outcome of patients.

**METHODS:** Twenty-one patients with APLD were treated by a combined hepatic resection and fenestration technique. All patients were reviewed retrospectively, and clinical symptoms, performance status and morbidity were recorded. A new classification of APLD is recommended here.

**RESULTS:** All patients were discharged when free of symptoms. The mean follow-up time was 55.7 mo and three patients had a recurrence of symptoms at 81, 68 and 43 mo after operation, respectively. The overall morbidity rate was 76.2%. Two patients with Type B-II and Type B-I developed biliary leakage. Four patients had severe ascites, including three with Type B-III and one with Type B-II. Nine patients had pleural effusion, including one with Type A-I; one with Type B-I; five with Type B-II; one with Type A-III and one with Type B-III. Three patients with Type B had recurrence of symptoms, while none with Type A had severe complications.

**CONCLUSION:** Combined hepatic resection and fenestration is an acceptable procedure for treatment of APLD. According to our classification, postoperative complications and long outcome can be predicted before surgery.

### INTRODUCTION

Autosomal dominant polycyst liver disease (APLD) is a genetic heterogeneous disorder characterized by progressive development of multiple fluid-filled, biliary epithelial liver cysts<sup>[1-5]</sup>, and is frequently associated with autosomal dominant polycystic kidney disease<sup>[5-7]</sup>. Most cases of APLD are asymptomatic and do not require surgical treatment. However, a number of patients with highly symptomatic cystic hepatomegaly or with a complicated presentation would benefit from surgical decompression of the hepatomegaly<sup>[8]</sup>. Currently, there is controversy over what is the most appropriate therapeutic approach for APLD. Transient improvement with non-surgical treatment such as sclerosing agents has been reported<sup>[9,10]</sup>. Surgical therapies include laparoscopic fenestration<sup>[11-14]</sup>, open fenestration<sup>[15-18]</sup>, liver resection and fenestration<sup>[19-22]</sup>, and liver transplantation<sup>[23-26]</sup>. In 1997, Giot *et al*<sup>[1]</sup> recommended a stratification scheme for the most appropriate surgical approach. In the present study, we evaluated outcomes in patients with APLD treated by combined hepatic resection and fenestration, and provided a new classification to presume the postoperative complications and long-term outcome of patients with APLD.

### MATERIALS AND METHODS

Between May 1995 and May 2007, 21 patients with APLD-associated severe symptoms were referred to Eastern Hepatobiliary Surgery Hospital of the Second Military Medical University in Shanghai. They were

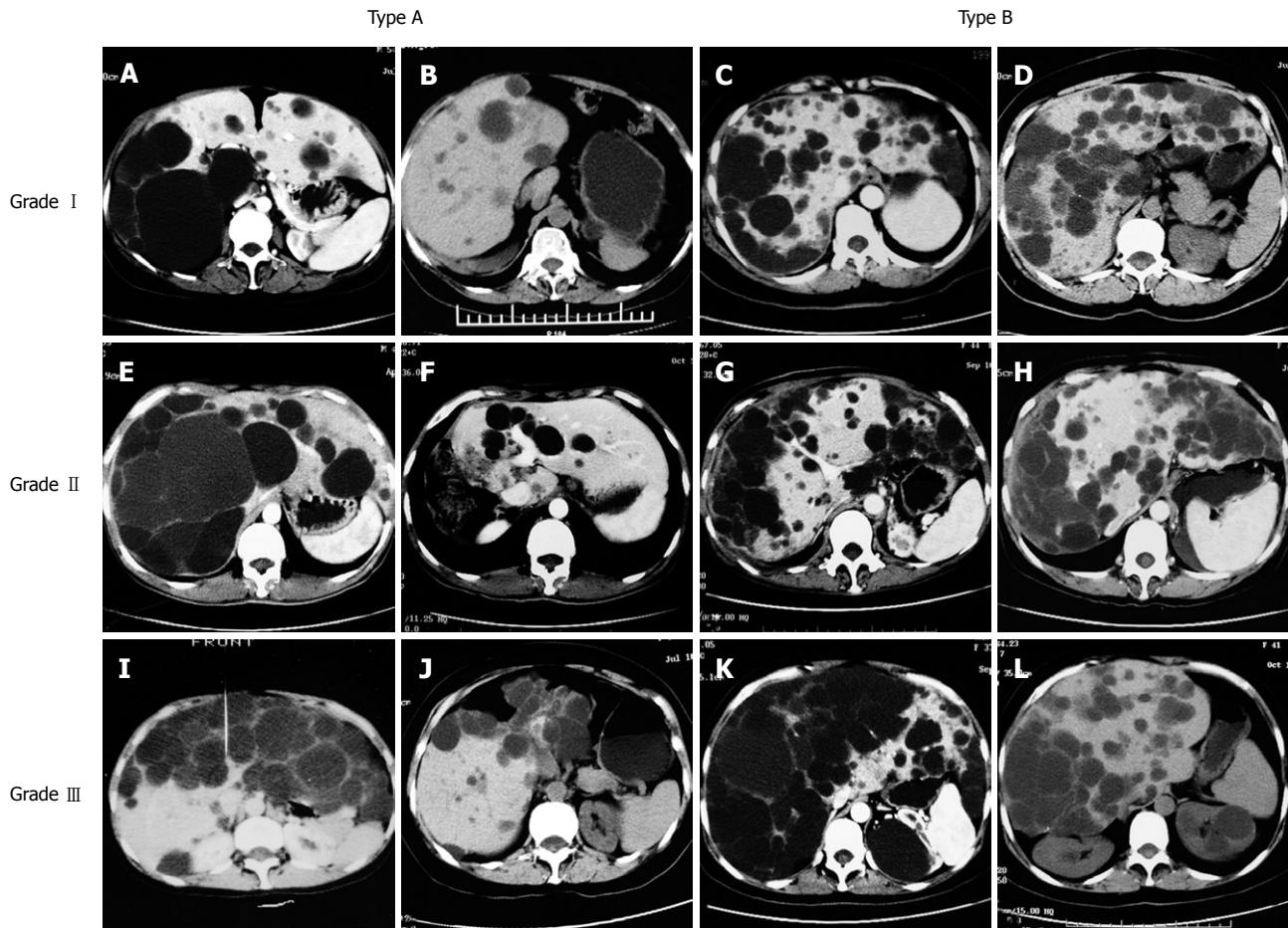
treated by a medical team headed by Dr. Yang *et al.*, using the combined hepatic resection and fenestration technique. The patients included 19 women and two men ranging in age from 34 years to 63 years with a mean of 44.7 (median, 43) years. Of the 21 patients, 12 patients (57.1%) had a familial history of polycystic liver disease (PLD), and 17 patients (81.1%) had associated autosomal dominant polycystic kidney disease. The mean time lag between the diagnosis of APLD and surgery was 65.3 mo (median, 60; range, 1-240 mo). The mean duration of symptoms was 30.6 mo (median, 24; range, 1-168 mo). Specific symptoms included massive abdominal distention (20/21, 95.2%), early satiety (11/21, 52.4%), chronic abdominal pain (9/21, 42.9%), hypertension (8/21, 38.1%), ascites (5/21, 23.8%), supine dyspnea (3/21, 14.3%), elevated temperature related to superinfected cysts (2/21, 9.5%), regurgitation (2/21, 9.5%) and pleural effusion (2/21, 9.5%). Abdominal examination revealed hepatomegaly in all patients. Eleven patients had received treatment previously, including percutaneous cyst aspiration with alcohol sclerotherapy in eight patients, laparoscopic fenestration in two patients, and laparoscopic fenestration in one patient. Liver function tests were essentially normal, except for mild elevation of serum alkaline phosphatase in two patients, glutamyltranspeptidase in five patients, hypoalbuminemia and hyperbilirubinemia in four patients. All patients underwent an abdominal computed tomography (CT) scan in order to delineate cyst distribution, assess portal vein patency, and provide baseline measurement for follow-up comparison in each patient. Since some of the patients in our series were not appropriate for Giot classification<sup>[1]</sup>, a new classification of APLD patients that is different from Giot classification was recommended. This new classification involved a preoperative evaluation of the number of deep cysts in the liver parenchyma that could not be treated during operation. Based on the preoperative evaluation, APLD patients were classified into two types: Type A, with a small number of cysts that could not be treated and left in the deep site of the liver parenchyma (usually  $\leq 15$ ), and Type B, with a large number of such cysts (usually  $\geq 15$ ). For each type, according to the location of cysts diffused in the liver, they were further classified into three grades: Grade I included patients with diffused cysts occupying less than one lobe of the liver; Grade III was a severe form of APLD with liver parenchyma involving fewer than 3 segments; and Grade II was the grade between Grade I and III, where liver parenchyma was limited in the right lobe or left lobe of the liver, or involving 3-4 segments, but not limited in one lobe of the liver (Figure 1).

Preoperative preparations of each patient included: electrocardiography (ECG), gastroscopy and pulmonary function, major medical contraindications for surgical resection were identified, and specific hepatic factors were evaluated. In 13 patients, prothrombin time (PT) was prolonged (within 3 s above the normal reference). Venous catheterization was also important with these

patients. The abdomen was explored through a bilateral subcostal incision. The hepatoduodenal ligament was explored to provide access to vascular clamping areas and to identify major vascular and biliary structures. The liver was mobilized by the division of hepatic peritoneal attachments, which was facilitated by sequential fenestration of accessible cysts according to the Lin technique<sup>[15]</sup>. Liver segments spared of cystic involvement were identified to define limits of resection. No anatomic segmental or lobar planes were removed even if cysts distorted the normal anatomy. Liver segments spared of cystic involvement were identified prior to parenchymal transaction to define limits of resection. Significant islands of functional liver parenchyma were preserved when possible. Two to five segments were resected during operation. After resection of the major cyst segments, extensive fenestration of the residual cysts in the parenchyma was addressed by excision of the cyst walls. Using cautery, the transection plane was developed further by division and excision of the inter-cystic septa<sup>[27]</sup>. Vascular and biliary radicals that coursed through the cyst septa were ligated as indicated. Finally, cyst cavities exposed to the peritoneum were fulgurated by argon beam coagulation (Bard Electromedical Systems, USA) in 15 patients in an attempt to ablate secretory epithelium and reduce postoperative peritoneal fluid losses<sup>[28]</sup>. The portal vein, hepatic vein and small bile duct were identified and carefully protected in order to avoid hemorrhage and bile leakage. Cholecystectomy was performed in seven patients. The hepatic resection beds were drained by two large closed suction drains. Eighteen patients were monitored in the intensive care unit. Both colloid and crystalloid fluids were used for volume replacement to compensate for expected postoperative fluid losses and excessive peritoneal drainage. Fluid maintenance and hemodynamic balance were aided by central venous pressure. Postoperative blood gas analysis was examined in all patients. All patients were followed-up through either telephone calls or at clinical visits. Special data included CT scan and current hepatic and renal function test. SPSS 10.0 was used for statistical analysis.

## RESULTS

No patient died during the operation. All 21 patients were discharged from the hospital free of the preoperative symptoms. The mean postoperative hospital stay was 15.5 d (median, 13; range, 6-60 d). The mean operation time was 247.6 min (median, 225 min; range, 150-435 min). Component transfusion was given intraoperatively and during hospitalization in 16 patients. The median transfusion of packed red blood cells was 4.5 units (range, 1-20 units). The median transfusion of blood plasma was 1400 mL (range, 200-4600 mL). Fifteen patients had ascites. The mean duration of drainage was 8.29 d (median, 5 d; range, 2-57 d). Needle aspiration was attempted to prevent ascites from infection. Seven patients received continuous needle aspiration to relieve ascites, with a



**Figure 1** CT examinations of our classification of APLD. **A** and **B**: Type A-I, preoperative and postoperative CT examinations; **C** and **D**: Type B-I, preoperative and postoperative CT examinations; **E** and **F**: Type A-II, preoperative and postoperative CT examinations; **G** and **H**: Type B-II, preoperative and postoperative CT examinations; **I** and **J**: Type A-III, preoperative and postoperative CT examinations; **K** and **L**: Type B-III, preoperative and postoperative CT examinations.

mean duration of 7.54 d (median, 4 d; range, 3-24 d). Eight patients were successfully managed with diuretics and drainage was achieved within one week. Nine patients had pleural effusion, of which 6 patients needed thoracentesis due to chest distress. Bile leakage occurred in two patients. Postoperative hemorrhage occurred in one patient. The overall morbidity rate was 76.2%. There was a morbidity rate of 38.1% for complications that required special treatment. These included severe ascites, bile leakage, hemorrhage, and pleural effusion. The mean follow-up duration was 60.7 mo (median, 59; range, 10-155 mo). Two patients died of renal failure due to polycystic kidney disease at 137 and 85 mo after operation. Three patients had recurrence of the symptoms at 81, 68 and 43 mo after operation with a recurrence rate of (3/21) 14.3%. Total bilirubin was slightly elevated in two patients. The symptoms (occasional abdominal swelling and abdominal pain) of three patients were not severe, therefore they received outpatient treatment.

Follow-up CT data were available in 19 patients, and lost in the remaining two patients who died. Six patients were classified as Type A, including three with Grade I, one with Grade II and two with Grade III. Thirteen patients were classified as Type B APLD, including one with Grade I, eight with Grade II and four with

Grade III. The mean age, mode of presentation, duration of symptoms, mean operation time, intraoperative component transfusion and hospital stay were not significantly different between the two types.

Of the six patients with Type A in our series, three had mild ascites and one had pleural effusion after operation. None of the patients with Type A had severe complications. In patients with Type B in our series, one with Grade II who received laparoscopic treatment 4 years earlier had massive hemorrhage after operation. Biliary complications occurred in two patients, including one with Grade II and one with Grade III. Severe ascites occurred in three patients with Grade III and one patient with Grade II. Eight patients had pleural effusion, including one with Grade I, six with Grade II, and one with Grade III. Eleven patients had mild ascites, including two with Grade I, seven with Grade II, and two with Grade III. Symptoms reoccurred in three patients with Type B, including one with Grade I, and two patients with Grade II. The follow-up ranged from 12 mo to 155 mo with a mean of 61.2 mo for Type A patients, and from 10 mo to 141 mo with a mean of 58.2 mo for Type B patients. There was no significant difference in follow-up duration between the two types. Postoperative complications and symptom recurrence based on our APLD classification are presented in Table 1.

**Table 1** Postoperative complications and symptom recurrence based on our APLD classification

Classification	<i>n</i>	Severe ascites	Pleural effusion	Bile leakage	Mild ascites	Hemorrhage	Recurrence
Type A	I	3	-	1	-	1	-
	II	1	-	-	-	1	-
	III	2	-	-	-	1	-
Type B	I	1	-	-	-	1	-
	II	8	1	6	1	6	1
	III	4	3	1	1	1	-
Total	19	4	8	2	11	1	3

**Table 2** Review of the literature: mortality, morbidity and outcome of patients with APLD treated by combined hepatic resection and cystic fenestration

Authors <sup>ref</sup>	Number of patients	Mortality (%)	Morbidity (%)	Mean follow-up (mo)	Rate of symptom recurrence (%)	Re-operation (%)
Turnage <sup>[18]</sup>	3	2 (67)	2 (67)	9.6	33	0
Vauther <sup>[19]</sup>	5	0	5 (100)	14	0	0
Henne-Brunns <sup>[35]</sup>	8	0	3 (38)	15	50	0
Que <sup>[20]</sup>	31	1 (3)	18 (58)	28	3	0
Soravia <sup>[36]</sup>	10	1 (10)	2 (20)	68	33	11
Koperna <sup>[11]</sup>	5	0	NR	NR	0	0
Ours	21	0	16 (76)	60.7	14	0

NR: No report.

## DISCUSSION

APLD is a rare disorder usually associated with autosomal dominant kidney disease<sup>[7,29]</sup>. An increasing prevalence is associated with age and female sex<sup>[30]</sup>. Symptoms usually arise from liver enlargement and compression of adjacent organs. Most symptomatic patients complained of an increasing abdominal girth and chronic abdominal dull pain. Liver enlargement may cause early satiety, respiration compromise, and physical disability. Complications such as portal hypertension, pleural effusion, esophageal varices, obstructive jaundice, hepatic failure and malignant degeneration are rare<sup>[31-33]</sup>. In patients with APLD, the optimal treatment is still under dispute. Percutaneous aspiration with alcohol sclerotherapy seems valuable for solitary cysts but not for patients with APLD because cyst collapse may not be sufficient<sup>[9,10]</sup>. In 1968, Lin *et al*<sup>[15]</sup> reported the use of a more extensive cyst fenestration procedure in three patients in whom successively deeper cysts were unroofed and drained through more superficial ones, resulting in a long-term favorable outcome. Kabbej *et al*<sup>[13]</sup> reported in a recent study that 13 patients with APLD underwent laparoscopic fenestration, with a symptom recurrence rate of 72% during a mean follow-up of 26 mo. In patients with the majority of cysts in segments VI, VII, VIII, and in patients with deeply seated cysts that are difficult to visualize and fenestrate with laparoscopy, the postoperative recurrence rate is usually high due to inadequate fenestration of all cysts<sup>[12,14,34]</sup>. Armitage *et al*<sup>[27]</sup> described a patient with APLD who was treated by partial hepatic resection and cyst fenestration in 1984. This procedure allowed for excision of most prominent cysts with minimal resection of the liver tissue, and the liver parenchyma was preserved as much

as possible. Other reports have shown the feasibility of such an approach<sup>[11,18-20,35,36]</sup>(Table 2).

We have already reported seven patients with APLD who were treated by hepatic resection and cyst fenestration during a follow-up of 20.4 mo<sup>[22]</sup>. All patients were relieved of the symptoms after operation. Mild symptoms recurred in three patients, and symptoms were relieved after treatment in the ambulatory clinic at 6-mo intervals. The long-term benefit of combined hepatic resection and fenestration depends on whether there is a progressive increase in size of deep residual untreated liver cysts rather than new cyst development<sup>[1]</sup>. Our experience with the treatment of Type B patients also supports this conclusion. Current data of the natural history of autosomal dominant polycystic kidney disease (APKD) and APLD suggest that progression of cystic disease is slow, affording the possibility of prolonged benefit. Our experience with combined hepatic resection and fenestration shows that some patients with massive polycystic liver benefit markedly from this procedure. No patients with polycystic kidney disease had kidney failure during operation. Two patients in our series died of renal failure during the follow-up period due to polycystic kidney disease. Whether this operation technique affects renal function is still unknown.

According to our classification of APLD patients with Type A seemed less likely to have severe complications after operation. None in Type A had severe ascites or bile leakage in our studies. Patients in every grade with Type A, had more cysts to be resected than cysts to be fenestrated during operation. In addition, their cysts in the liver parenchyma were relatively superficial, and could be treated easily. After fenestration there was little difference in cyst epithelium areas exposed to the abdominal cavity in each grade of patients with Type

A, so their ascites and pleural effusion occurrence rates were seemly no different after operation. Some Type A patients had small bile leakage or hemorrhage during the operation, but these were found easily during operation. In different grades, operative technical difficulties to repair these leakages and hemorrhages were different. Patients in Grade III had large numbers of cysts to be resected or fenestrated, and vascular and biliary radicals coursing through the cyst septa were more difficult to recognize than those of patients in Grade II or Grade I. Operative risk factors increased with increasing grades. Vascular and biliary radicals should be recognized and protected during operation to avoid injury, especially for patients in Grade III.

In Type B patients, a proportion of cysts needed resection, but most cysts in the liver parenchyma could not be resected in order to preserve liver parenchyma as much as possible. Therefore, Type B patients had more cysts requiring fenestration, and cyst epithelium areas that were exposed to the abdominal cavity were also larger. Since cystic epithelium functions in secretion<sup>[37]</sup>, severe ascites occur more in Type B. Severe ascites was found in four patients in Type B, including three with Grade III and one with Grade II. Patients with Grade III had more cysts to be fenestrated than patients with Grade II, and patients with Grade II more than patients with Grade I during operation. Patients with Grade III also had larger cyst epithelium areas exposed to the abdominal cavity than Grade II, and patients with Grade II more than patients with Grade I after operation. We presume that patients with Grade III are more likely to develop ascites than patients with Grade II, and patients with Grade II are more likely to develop ascites than patients with Grade I. Bile leakage occurred in two patients in our series with Type B, including one with Grade III and one with Grade II. Patients with Type B had more cysts in the deep liver parenchyma. We attempted to fenestrate the cysts when possible, though it was dangerous to fenestrate deep interseptal cysts without sufficient exposure. Two risk factors may contribute to biliary complications. One is the possible injury to small biliary radicals on the surface of the cyst lining, when the cyst lining was fulgurated by argon beam coagulation to ablate secretory epithelium. The other is that biliary ducts distorted in polycystic liver anatomy are usually difficult to identify, and likely to be injured within cystic septa during a fenestration procedure especially of deep cysts<sup>[1]</sup>. These two patients with peritoneal closed suction drainage had spontaneous resolution 27 d and 56 d after operation as identified by subsequent radiologic examination. Therefore we recommend a clearly routine examination on the wound surface to avoid missing the biliary ducts and veins which are possibly injured by the end of the operation.

Our classification of APLD applied the postoperative course which was uncomplicated in six patients with Type A, including three patients with mild ascites and one patient with pleural effusion. No patient in Type A had symptom recurrence in the follow-up period. In Type B, two patients had bile leakage, four had severe ascites,

seven had pleural effusion, and three had symptom recurrence. Type A APLD patients may have good immediate and long-term outcomes, so we recommend a combined hepatic resection and fenestration. Symptom recurrence was related to a progressive increase in size of deep residual untreated liver cysts. Patients in Grade I and in Grade II with Type B had more cysts in deep sites within the liver parenchyma, which could not be treated during operation, therefore those patients had a high rate of symptom recurrence. Cysts in Grade III patients can be resected or fenestrated effectively during operation, leading to less symptom recurrence. So we recommend a new classification to predict the postoperative complications and long-term outcome of patients with APLD. This classification still needs to be validated in the future.

We had two patients who received laparoscopic fenestration and one patient received laparoscopic fenestration previously. They had symptom reoccurrence 99, 67 and 53 mo after operation, and received combined liver resection and fenestration. They are living well asymptotically. But in these patients, more intense intra-abdominal adhesions posed technical difficulties<sup>[28]</sup>. Their complications were severe after operation, including one that had abdominal hemorrhage, and one that had severe ascites and bile leakage. The patient who underwent laparoscopic fenestration previously had an abdominal hemorrhage in the right lobe, due to tight adhesion of the cyst wall with the diaphragm, where a small artery was not ligated after stripping. Hemorrhage occurred when blood pressure was returned to normal after operation. For these patients, combined fenestration with resection still can be considered as the choice of treatment, but their operation should be performed by experienced surgical teams due to their severe postoperative complications. Liver transplantation as a treatment for APLD may be another good way to treat APLD, but it has a limited role due to the shortage of liver donors and severe postoperative complications. Two large studies reported by Lang *et al*<sup>[24]</sup> and Pirenne *et al*<sup>[25]</sup> reported the outcomes of liver transplantation in 17 and 16 patients respectively. The former reported 5 deaths (29%) and the latter reported 2 deaths (12.5%). The mortality they reported is higher than that in patients who received combined fenestration procedures. Patients with APLD who receive liver transplantation are placed on immune suppression therapy, and therefore do not have a "normal immune system." Immune suppressants have many side effects on patients with liver transplantation, and after operation they still need much attention to sustain the state of immune suppression to avoid graft rejection. Starzl and his colleagues described the "syndrome of lethal exhaustion" as the major indication to consider when offering transplantation to these patients<sup>[38]</sup>. We fully agree that liver transplantation should be used for patients with cachexia, weight loss, portal hypertension, refractory ascites, liver insufficiency or associated with severe kidney disease<sup>[24,25,39,40]</sup>. Liver resection and fenestration of the remnant liver are more effective than

fenestration and less invasive than liver transplantation, combined liver resection and fenestration should be considered as the choice for treatment for APLD.

## COMMENTS

### Background

Autosomal dominant polycystic liver disease (APLD) is a rare disorder usually associated with autosomal dominant kidney disease with an increasing prevalence associated with age and female sex. Symptoms usually arise from liver enlargement and compression of adjacent organs. Surgical therapies include laparoscopic fenestration, open fenestration, liver resection and fenestration, and liver transplantation, what is the optimal treatment is still under dispute.

### Research frontiers

The study analyzed and summarized the author's clinical experiences in recent 12 years in the treatment of APLD and in an attempt to propose some practicable guidelines, and suggested a new classification to presume postoperative complications and long outcome of each patient.

### Innovations and breakthroughs

Combined hepatic resection-fenestration is a good choice for the treatment of highly symptomatic adult polycystic liver disease. According to the author's classification, postoperative complications and long outcome of each patient can be predicted before surgery.

### Applications

The study provides some practicable guidelines for the treatment of APLD, and according to the author's classification, postoperative complications and long outcome of each patient can be predicted before surgery.

### Peer review

The paper analyzed and summarized some practicable experience for the treatment of APLD and recommended a new classification. It is valuable to see the actual results from various therapies and the new classification by the authors over the last 12 years.

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RAPID COMMUNICATION

# Treatment of upper gastrointestinal fistula and leakage with personal stage nutrition support

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## Abstract

**AIM:** To investigate the feasibility of treatment for upper gastrointestinal fistula and leakage with personal stage nutrition support.

**METHODS:** Forty-three patients with upper gastrointestinal fistula and leakage were randomly divided into two groups. Patients in group A were treated with personal stage nutrition support and patients in group B were treated with total parenteral nutrition (TPN) in combination with operation. Nutritional states of the candidates were evaluated by detecting albumin (Alb) and pre-Alb. The balance between nutrition and hepatic function was evaluated by measurement of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and total bilirubin (Tbill) before and after operation. At the same time their complications and hospitalized time were surveyed.

**RESULTS:** Personal stage nutrition support improved upper gastrointestinal fistula and leakage. The nutrition state and hepatic function were better in patients who received personal stage nutrition support than in those who did not receive TPN. There was no significant difference in the complication and hospitalized time in the two groups of patients.

**CONCLUSION:** Upper gastrointestinal fistula and leakage can be treated with personal stage nutrition support which is more beneficial for the post-operation recovery and more economic than surgical operation.

## INTRODUCTION

The primary causes of death secondary to gastrointestinal fistulæ and leakage include malnutrition, electrolyte imbalance, and sepsis. Without help of an experienced nutrition team, patients with gastrointestinal fistulæ and leakage may not tolerate nutrition support and would have more severe pain due to the doctor's negligence. Nutritional support can improve wound healing<sup>[1]</sup> and gastrointestinal permeability<sup>[2]</sup>, decrease catabolic response to injury<sup>[3]</sup> and bacterial translocation<sup>[4]</sup>, and achieve better clinical outcomes, including a decrease in complications and hospital stay time and cost saving<sup>[5-8]</sup>. It is necessary to find a perfect treatment for gastrointestinal fistulas. More studies have been done on the gastrointestinal fistula, especially on upper gastrointestinal fistula and leakage. Absolute diet has been recognized as a routine diet during treatment of digestive fistula and leakage, and total parenteral nutrition (TPN) has been accepted by many doctors. We have successfully treated acute pancreatitis with "personal stage nutrition support" in the past 10 years. On this basis, we wonder if we can develop a new way of "personal stage nutrition support" for upper gastrointestinal fistula and leakage.

## MATERIALS AND METHODS

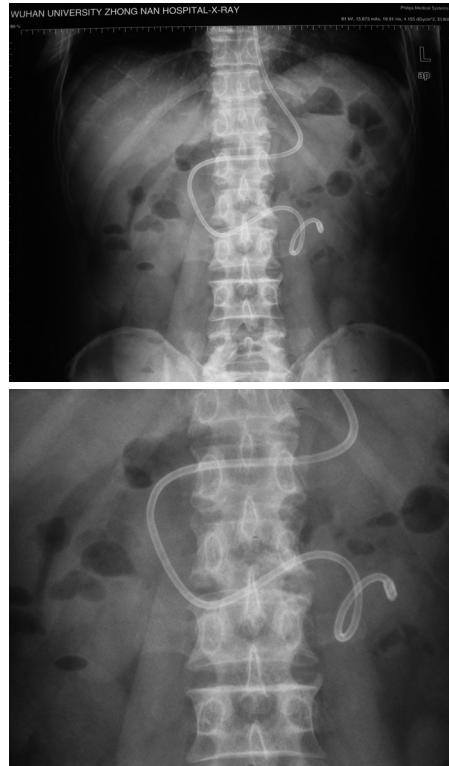
### Patients

Forty-three patients (27 males and 16 females) with upper gastrointestinal fistula and leakage were selected

from Zhongnan Hospital Wuhan University from January 2005 to November 2007. Their age was 20 to 72 years with a mean age of  $51.3 \pm 2.6$  years. The occurrence of upper gastrointestinal fistula was due to emergency of trauma in 6 patients, post-operation for gastric ulcer in 13 patients, excision of duodenum ulcer in 8 patients. Sixteen patients had leakage after gastric perforation neoplasty. Patients with malnutrition or liver dysfunction induced by cancer, tuberculosis, severe anemia, malabsorption, hepatitis, hepatic cirrhosis and other severe cardiovascular disease were excluded. Endoscopy revealed the position of the fistula and leakage above the Vater ampulla. All patients had no severe abdominal cavity infection before surgery and were divided into group A and group B. Group A, that received personal stage nutritional support, served as an experimental group. Group B that received TPN served as a control group. Those who did not finish the treatment plan were excluded. All patients were numbered randomly. During the treatment, all data including electrolyte, liver function, renal function, blood analysis and complications were submitted to professor Yue-Ming He to decide whether it was necessary for the patients to quit from or receive the treatment plan.

### Treatment intervention

Nutrition support for group A was carried out in three steps: TPN stage, parental nutrition (PN) + enteral nutrition (EN) stage, and total enteral nutrition (TEN) stage. (1) TPN stage lasted 4-10 d (mean  $7.8 \pm 2.2$  d). All the patients were asked to take absolute diet and supplied with 10-20 g/d albumin (Alb) and somatostatin. (2) PN + EN stage lasted 8-15 d (mean  $11.6 \pm 3.0$  d). When the function of stomach and intestine recovered with the signal of outgas or defecation, EN was performed. In brief, one spiral nasojejunal feeding tube (Nutricia Company, Holland) was placed in the intestine below Treitz ligament (Figure 1) with the help of intestine enterokinesia after removal of the spiral coil guide wire. The distance from the tip of the tube to the orificium fistula was greater than 30 cm. The position of the nasojejunal tube should be confirmed by X-ray after 8-12 h since auscultation and pH aspiration techniques are inconclusive<sup>[9]</sup>. Nutrient substance was injected into jejunum through the spiral nasojejunal feeding tube. At the same time, we provided the patients with part parental nutrition as supplements. Chinese folk foods such as rice-water, fruit juice, vegetable soup, gravy soup, bean juice, as well as milk and peptison, could be used as nutrient substances and must be discontinued as soon as complications including stomachache, abdominal distension or diarrhea occurred. After we changed the speed and quantity of injection, all patients finished the stage. During this stage, somatotropin was also used. (3) TEN stage lasted 6-8 d (mean  $7.5 \pm 1.2$  d). After completion of the PN + EN stage, we started the third stage, namely TEN stage which was started with closure of the fistula by endoscopic examination. After 35 d, the patients were asked to drink fresh water and take food. Gastrointestinal



**Figure 1** Placement of a spiral nasojejunal feeding tube in the intestine below Treitz ligament, 48 h after X-ray examination.

decompression and drain were performed through the spiral nasojejunal feeding tube in the first stage. At the same time, water and electrolyte disturbances must be prevented by changing ingredient and quantity of nutrient substances. Antibiotics (ceftriaxone sodium) and gastric acid secretion inhibitors (omeprazole) were given by intravenous drip in the first stage.

Patients in group B were given TPN through a central venous catheter which was inserted into the superior vena cava via one of the subclavian veins and advanced into the superior vena cava before and after operation. Basic heat energy was calculated depending on Harris-Benedict's formula, the mean heat energy was 10460kJ (2000-2500 kcal) per day. Nutritional liquids containing glucose and amino acids were supplied with 8368 kJ (2000 kcal). Amino acids (9.4-14.1 g) were provided every day and 500-1000 mL of 10% intralipid was given to meet the need of fat. No hyperlipidemia occurred in our patients. Gastrointestinal decompression and drain were carried out for 7 d. Antibiotics (ceftriaxone sodium) and gastric acid secretion inhibitors (omeprazole) were given by intravenous drip for 7 d.

The electrolyte, liver and renal function, and blood analysis were monitored for preventing electrolyte disturbances and infection. Fluid needs could usually be met by giving 30-35 mL/kg body weight although allowance must be made for excessive losses from drains and fistulae, etc. The feeds contain adequate electrolytes to meet the daily requirement of sodium, potassium, calcium, magnesium and phosphate, although specific requirements may vary greatly.

**Table 1** Levels of Alb, TSF and pre-Alb at different time points in the two groups (mean  $\pm$  SD)

Time	Alb (g/L)			TSF (g/L)			Pre-Alb (mg/L)		
	A	B	P	A	B	P	A	B	P
1 d before treatment	40 $\pm$ 2.1	39 $\pm$ 3.2	0.11	3.51 $\pm$ 0.17	3.49 $\pm$ 0.14	0.78	340 $\pm$ 51	341 $\pm$ 28	0.64
1 d after treatment	39 $\pm$ 3.6	37 $\pm$ 2.5	0.06	3.27 $\pm$ 0.26	3.18 $\pm$ 0.21	0.26	327 $\pm$ 43	308 $\pm$ 19	0.15
15 d after treatment	37 $\pm$ 2.7	35 $\pm$ 3.5	0.05	3.20 $\pm$ 0.33	2.98 $\pm$ 0.25	0.45	321 $\pm$ 37	271 $\pm$ 21	0.001
30 d after treatment	38 $\pm$ 3.8	33 $\pm$ 3.2	0.001	3.28 $\pm$ 0.35	2.77 $\pm$ 0.22	0.001	325 $\pm$ 24	236 $\pm$ 17	0.001

**Table 2** Nitrogen balance between the two groups at different time points (mean  $\pm$  SD)

Time	Nitrogen balance (g)		
	A	B	P
1 d before treatment	-10 $\pm$ 3.6	-8 $\pm$ 4.1	0.17
1 d after treatment	-11 $\pm$ 2.1	-9 $\pm$ 4.5	0.81
15 d after treatment	2 $\pm$ 2.9	2 $\pm$ 3.1	0.95
30 d after treatment	6 $\pm$ 4.2	5 $\pm$ 4.8	0.51

### Plasma protein measurement and liver function evaluation

Five hundred microliter serum segregated from peripheral blood was analyzed by chromatometry to measure the levels of Alb, TSF and pre-Alb. Liver function analysis was completed by Department of Laboratory, Zhongnan Hospital, Wuhan University

### Examination of nitrogen balance

Twenty four-hour urine was collected to determine the level of nitrogen output as previously described<sup>[10]</sup> 1 d before operation and 1, 15 and 30 d after operation, respectively. Total nitrogen was calculated with the following formula: total nitrogen = nitrogen input-(nitrogen output + 3).

### Comparison between complications, hospitalized time and expenditure

The complications, hospitalize time and expenditure were compared after recovery and discharge of the patients.

### Statistical analysis

Data were analyzed with unpaired *t* test using SPSS 13.0. *P* < 0.05 was considered statistically significant.

## RESULTS

Two patients were excluded from our study because they had to accept abdominal cavity drainage for severe abdominal cavity infection. As one of them could not tolerate nasojejunal feeding tube, his data were not included in our statistical analysis. The remaining 40 patients were divided into group A and group B, 20 in each group. Patients in group A received personal stage nutrition support, patients in group B underwent operation and received TPN. We found that there was no significant difference in the levels of Alb, TSF and pre-Alb between the two groups of patients before and after operation. The levels of Alb (g/L), TSF (g/L) and

pre-Alb (mg/L) in patients of group B were lower than those in patients of group A (Table 1). No significant difference in nitrogen balance was found between the two groups (Table 2). Thirty days after operation, the levels of aspartate aminotransferase (AST, U/L), alanine aminotransferase (ALT, U/L) and total bilirubin (Tbill, mol/L) were much higher in group B than in group A (Table 3), indicating that personal stage nutrition support can alleviate the impairment of liver function caused by extended TPN. The complications and the mean hospital stay time of the patients in group A were less than those of the patients in group B (Table 4). The mean cost for the patients in group A was lower than that for the patients in group B.

## DISCUSSION

The traditional treatment of gastrointestinal fistula is surgical operation in combination with prolonged nutrition support. So it is very important to find the best way to support patients with enough nutrition which may achieve good results. Gastrointestinal fistula, especially upper gastrointestinal fistula and leakage are hard to recover. TPN has been widely used in treatment of gastrointestinal fistula. Priorities of the management of gastrointestinal fistulas include restoration of blood volume and correction of fluid, electrolyte and acid-base imbalance, control of infection and sepsis with appropriate antibiotics and drainage of abscesses, as well as TPN and TEN<sup>[11]</sup>.

TPN has many advantages. For example, it is accepted by patients more easily, the supplement of water and electrolyte are more convenient, nutrient substances are infused through veins, etc<sup>[12]</sup>. TPN is a form of nutritional support most suitable to patients with gut failure<sup>[13]</sup>. On the other hand, TPN provides sufficient heat and nutrient substances to meet the need of metabolism. Glyconeogenesis can be inhibited, thus keeping a positive nitrogen balance. In our research, there was no significant difference in nitrogen balance between the two groups. All the patients were switched from negative nitrogen balance to positive nitrogen balance. At the same time many disadvantages were discovered such as metabolic disturbance, catheter septicemia alteration in liver function and cholestasis<sup>[14]</sup>, especially when patients received prolonged TPN, which is consistent with our findings. The levels of AST, ALT and Tbill were much higher in group B than in group A 30 d after TPN. In the stress condition, proper nutritional support may provide necessary nutrients, reduce clinical complications

**Table 3** Liver function of patients in the two groups at different time points (mean  $\pm$  SD)

Time	AST (U/L)			ALT (U/L)			T bill (mol/L)		
	A	B	P	A	B	P	A	B	P
1 d before treatment	26 $\pm$ 2.2	27 $\pm$ 1.9	0.27	27 $\pm$ 1.9	26 $\pm$ 1.6	0.85	15 $\pm$ 1.6	14 $\pm$ 1.8	0.68
1 d after treatment	28 $\pm$ 2.4	29 $\pm$ 2.6	0.31	29 $\pm$ 2.3	30 $\pm$ 2.4	0.24	18 $\pm$ 2.1	19 $\pm$ 2.4	0.14
15 d after treatment	42 $\pm$ 3.5	44 $\pm$ 3.1	0.07	52 $\pm$ 1.8	60 $\pm$ 1.5	0.001	30 $\pm$ 1.4	32 $\pm$ 2.6	0.004
30 d after treatment	35 $\pm$ 3.3	63 $\pm$ 3.6	0.001	36 $\pm$ 2.7	65 $\pm$ 2.3	0.001	26 $\pm$ 3.7	48 $\pm$ 3.1	0.001

**Table 4** Complications, hospitalized time and cost of the two groups

	A (n = 20)	B (n = 20)	P
Stoma fistula	0	2	
Infection of incisional wound	0	3	
Abdominal distension	2	0	
Diarrhoea	1	0	
Infection of abdominal cavity	1	1	
Total incidence rate (%)	20%(4/20)	30%(6/20)	0.53
Average stay (d)	40 $\pm$ 2.6	50 $\pm$ 3.7	0.001
Average expenditure(10 thousand yuan)	2.3 $\pm$ 0.5	3.9 $\pm$ 0.8	0.001

and promote patients' recovery from illness<sup>[12]</sup>. TPN provides significant benefits to patients<sup>[15]</sup>. In the stress stage, all of our patients were given TPN. When the stress condition of patients improved, they should be supported with EN followed by TEN. Gramlich *et al*<sup>[16]</sup> reported that EN could significantly decrease the incidence of infectious complications and may cost less, and they believe that EN should be the first choice of nutritional support. Goonetilleke<sup>[17]</sup> holds that all patients should accept EN after operation. Zaloga<sup>[18]</sup> and Marik<sup>[19]</sup> believe that the gastrointestinal tract is the preferred route for nutritional support in hospitalized patients. Patients with a functioning gastrointestinal tract should be fed enterally. Patients with upper gastrointestinal fistula and leakage have a functioning intestine and can receive EN through a spiral nasogastric feeding tube which can arrive at the upper jejunum enterokinesia. In our study, patients with upper gastrointestinal fistula could accept EN after the stress condition was over. EN has many advantages. For instance, it can relieve the burden on the liver and protect the liver against damage and infection and maintain the balance of visceral blood flow compared with TPN. Enteral nutrition after stress can maintain immunocompetence and gut barrier integrity, and promote wound healing and reduce septic complications<sup>[20]</sup>. Early enteral feeding can prevent enterogenic infection in severely burned patients<sup>[21]</sup>. However, EN also has many disadvantages leading to abdominal distension, diarrhea, nausea and vomiting, etc, which are consistent with the findings in our study. It was reported that TPN can substantially improve the prognosis of gastrointestinal fistula patients by increasing the rate of spontaneous closure and improving the nutritional status of patients requiring repeat operations<sup>[11]</sup>. In the stress stage, EN is not suggested because a lot of digestive fluid is secreted in the gastrointestinal tract and pancreas which may destroy gastrointestinal fistula. Therefore, TPN was

used in all of our patients in the first stage. However, the length of stress stage, nutrition state and recovery speed were all different in each patient. This is why we established the "personal stage nutrition support". As soon as the stress stage was over, we could give the patients EN together with PN in the PN + EN stage. Satinsky<sup>[22]</sup> reported that all patients should be supported with PN + EN after operation. We suggest that even surgical patients could be provided with personal stage nutrition support including PN + EN. We should provide nutrition support in the three stages for patients with upper gastrointestinal fistula and leakage. Since TPN and EN have their advantages and disadvantages, we should make use of their advantages as much as we possibly can. Significant differences were found in complications, hospitalized time and cost between the two groups. When EN and PN are applied, strategies should be adopted to optimize their benefits and minimize their potential harms. This is why we used personal stage nutrition support but not operation in treatment of patients with upper gastrointestinal fistula. We believe that it is feasible to cure upper gastrointestinal fistula with personal stage nutrition support.

## COMMENTS

### Background

Much research work has been done on gastrointestinal fistula, especially on upper gastrointestinal fistula and leakage, but there are many discussions about how to provide nutrition for patients. Absolute diet has been accepted as a routine diet in the treatment of digestive fistula and leakage, and total parenteral nutrition (TPN) is widely used by many doctors. We tried to find a better treatment modality for upper gastrointestinal fistula and leakage in this study.

### Research frontiers

Many randomized controlled clinical trials as well as meta-analysis and systematic reviews have been performed to compare enteral nutrition (EN) with parenteral nutrition (PN). However, no definite conclusion is available. Some people considered that EN with PN should be employed according to the state and development of disease.

### Innovations and breakthroughs

In the present study, we, for the first time, put forward the concept of personal stage nutrition support and applied it in the treatment of upper gastrointestinal fistula and leakage in combination with operation.

### Applications

We have successfully treated acute pancreatitis with "personal stage nutrition support" in the last 10 years. On this basis, we wonder if we can develop a new treatment modality with "personal stage nutrition support" for patients with upper gastrointestinal fistula. A spiral nasojejunal feeding tube has been used for provision of enteral nutrition for several years outside of China.

### Peer review

The research provides a new treatment modality for upper gastrointestinal fistula and leakage with personal stage nutrition support. The new concept is interesting and informative. The paper is well organized except for correction of some errors in writing and punctuation.

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RAPID COMMUNICATION

## Polymorphisms of alcohol dehydrogenase 2 and aldehyde dehydrogenase 2 and colorectal cancer risk in Chinese males

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genotypes were identified by PCR and denaturing high-performance liquid chromatography (DHPLC). Information on smoking and drinking was collected and odds ratio (OR) was estimated.

**RESULTS:** The *ADH2* A/A and *ALDH2* G/G genotypes showed moderately increased CRC risk. The age- and smoking-adjusted OR for *ADH2* A/A relative to G/A and G/G was 1.60 (95% CI=1.08-2.36), and the adjusted OR for *ALDH2* G/G relative to G/A and A/A was 1.79 (95% CI=1.19-2.69). Significant interactions between *ADH2*, *ALDH2* and drinking were observed. As compared to the subjects with *ADH2* G and *ALDH2* A alleles, those with *ADH2* A/A and *ALDH2* G/G genotypes had a significantly increased OR (3.05, 95% CI= 1.67-5.57). The OR for CRC among drinkers with the *ADH2* A/A genotype was increased to 3.44 (95% CI= 1.84-6.42) compared with non-drinkers with the *ADH2* G allele. The OR for CRC among drinkers with the *ALDH2* G/G genotype was also increased to 2.70 (95% CI= 1.57-4.66) compared with non-drinkers with the *ALDH2* A allele.

**CONCLUSION:** Polymorphisms of the *ADH2* and *ALDH2* genes are significantly associated with CRC risk. There are also significant gene-gene and gene-environment interactions between drinking and *ADH2* and *ALDH2* polymorphisms regarding CRC risk in Chinese males.

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**Key words:** Alcohol dehydrogenase 2; Aldehyde dehydrogenase 2; Gene polymorphisms; Alcohol drinking; Colorectal cancer

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Gao CM, Takezaki T, Wu JZ, Zhang XM, Cao HX, Ding JH, Liu YT, Li SP, Cao J, Matsuo K, Hamajima N, Tajima K. Polymorphisms of alcohol dehydrogenase 2 and aldehyde dehydrogenase 2 and colorectal cancer risk in Chinese males. *World J Gastroenterol* 2008; 14(32): 5078-5083 Available from: URL: <http://www.wjnet.com/1007-9327/14/5078.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.5078>

### Abstract

**AIM:** To evaluate the relationship between drinking and polymorphisms of alcohol dehydrogenase 2 (*ADH2*) and/or aldehyde dehydrogenase 2 (*ALDH2*) for risk of colorectal cancer (CRC) in Chinese males.

**METHODS:** A case-control study was conducted in 190 cases and 223 population-based controls. *ADH2* Arg47His (G-A) and *ALDH2* Glu487Lys (G-A)

## INTRODUCTION

There is epidemiological evidence that alcohol intake is associated with increased colorectal cancer risk<sup>[1]</sup>. The oxidative metabolites of ethanol, acetaldehyde, is recognized to be carcinogenic in animals and suspected to have similar effects on human beings<sup>[2]</sup>. Since acetaldehyde accumulates in the blood causing uncomfortable symptoms of facial flushing, palpitation and headache, even when a small amount of alcohol is consumed, greater alcohol consumption is often limited in sensitive individuals.

Ethanol is oxidized to acetaldehyde and then to acetate by alcohol dehydrogenase (*ADH*) and aldehyde dehydrogenase (*ALDH*) in the liver. Most of the acetaldehyde generated during alcohol metabolism *in vivo* is promptly eliminated by *ALDH2*, a low-K<sub>m</sub> mitochondrial *ALDH*<sup>[3]</sup>. The gene for the homotetrameric enzyme *ALDH2* has a polymorphism and its mutant *ALDH2*\*2 allele (Glu487Lys, Lys or A allele) encodes a catalytically inactive subunit<sup>[4]</sup>. *ADH2* is also polymorphic and its mutant *ADH2*\*2 allele (Arg47His) encodes a superactive subunit of *ADH2*<sup>[3,4]</sup>. The *ALDH2* Glu487Lys and *ADH2* His47Arg polymorphisms thus have a strong impact on alcohol metabolism. Inactive *ALDH2* and superactive *ADH2* are considered to contribute to alcohol flushing and prevent people from developing alcoholism<sup>[5-7]</sup>.

Our previous studies have shown that males with a habit of drinking are at a significantly higher risk for colorectal cancer<sup>[8,9]</sup>. In this study, we attempted to define the role of *ADH2* and *ALDH2* polymorphisms and drinking habit in the development of colorectal cancer.

## MATERIALS AND METHODS

### Study subjects

We recruited colorectal cancer patients from the Cancer Registries in Huian and Jintan Cities of Jiangsu Province of China, and also recruited patients who visited Jiangsu Provincial Cancer Hospital from August 2000 to September 2002. All patients were histopathologically diagnosed as having a primary colorectal cancer. Physicians at the hospital asked eligible patients to participate in our study, and doctors or nurses interviewed the subjects and collected blood samples from a peripheral vein after obtaining informed consent. Population-based controls were selected from healthy residents in eight villages or towns of Huian and Jintan Cities. Doctors of the public health centers randomly selected one or two controls for each patient, after matching for ethnicity, sex and age within 2 years using the records of residents at the local governmental office, and then asked eligible residents for their participation. Interviews and blood collection were performed as for the cancer patients. A few patients and residents refused to participate in our study, but the response rate was 97% for patients and 93% for controls, respectively. The Ethics Committee of Jiangsu Provincial Institute of Cancer Research

approved this study. Associations could not be assessed in women because of sparse drinking habits.

### Environmental factors

Smoking and drinking habits were covered in our questionnaire. Each subject was asked whether he had ever smoked at least one cigarette per day for six months or longer. If he answered yes, he was further asked about the age at which he started to smoke cigarettes regularly, the average number of cigarettes smoked per day, and the number of years he had smoked. If the subject had quit smoking at least one year ago, the age at which he stopped smoking was recorded. Each subject was asked whether he had ever drunk alcoholic beverages at least once a month for one year or longer. If his answer was yes, he was asked to provide the age at which he started to drink regularly, frequency and usual amount of hot wine, beer and grape wine consumed separately every time. If the subject had quit his drinking habit at least one year ago, the age at which he stopped drinking was recorded. Consumption of ethanol every month was calculated according to 25 g/100 g of hot wine, 3.5 g/100 g of beer and 12 g/100 g of grape wine. In the present study, smoking status was categorized as never and ever-smokers (including both current and former smokers) and alcohol consumption as drinkers/non-drinkers (the latter including individuals whose alcohol intake was less than 30 g/mo).

### DNA extraction and genotyping

Whole blood was collected into EDTA-coated tubes and centrifuged for 15 min. The buffy coat layer was isolated. Genomic DNA was extracted from 200 μL of buffy coat using a Qiagen QIAamp DNA blood mini kit (QIAGEN Inc., Valencia, CA).

Genotyping of *ADH2* and *ALDH2* was determined by polymerase chain reaction (PCR) and denaturing high-performance liquid chromatography (DHPLC). The sequences of primers used in this study are F: 5'-GGCTTAGACTGAATAACCTTGG-3' and R: 5'-AGGGAAAGAGGAAACTCCTGAA-3' for *ADH2* Arg47His, and F: 5'-TGCTATGATGTGTTGGAGCC-3' and R: 5'-GGCTCCGAGGCCACCA-3' for *ALDH2* Glu487Lys. Reactions were carried out in a total volume of 25 μL containing 20 pmol of each primer, 0.25 mmol/L each dNTPs, 2.0 mmol/L MgCl<sub>2</sub>, 2.5 μL 10 × buffer, 1 IU hotTag polymerase and 0.5 μL genomic DNA. PCR conditions were as follows: denaturation at 95°C for 7 min, followed by 35 cycles at 95°C for 30 s, at 62°C for 30 s, at 72°C for 30 s, and a final extension at 72°C for 5 min. The products were denatured at 94°C for 4 min, and their temperature was declined to 25°C step by step according to 0.1°C/s.

Transgenomic WAVE DNA fragment analysis system (WAVE-300, Transgenomic, USA) and associated WAVEMAKER software were used for genotyping. An aliquot (5 μL) of the PCR products was directly injected into a DNAsep column. The column mobile phase for sample elution consisted of a mixture of buffer A

[0.1 mol/L triethylammonium acetate (TEAA)] and buffer B (0.1 mol/L TEAA with 25% acetonitrile). Samples were eluted at a linear gradient of buffer B over a 4.5-min period at a constant flow rate of 0.9 mL/min. For each DNA region, DHPLC conditions were established by a titration analysis at 1–3°C above and below the mean melting temperature predicted by software simulation. There were three genotypes: namely G/G, G/A, and A/A, for *ADH2* Arg47His and *ALDH2* Glu487Lys, respectively.

### Statistical analysis

Associations between the *ADH2* and *ALDH2* polymorphisms and colorectal cancer risk were estimated by OR, using the unconditional logistic regression model. The procedure LOGISTIC from the statistical package SAS was employed for the calculations. The probability of Hardy-Weinberg equilibrium was assessed by the  $\chi^2$  test.

## RESULTS

The numbers of 190 patients with colorectal cancer and 222 controls are listed in Table 1. The proportional distributions of age and smoking did not significantly differ in patients and controls, but the proportional distributions of alcohol drinkers (including current and former drinkers) were significantly higher in colorectal cancer patients than in controls.

The frequencies of *ALDH2* G/G, G/A and A/A genotypes were 68.95%, 28.42% and 2.63% in patients and 55.41%, 40.54% and 4.05% in controls, respectively (Table 2). The distribution of *ALDH2* genotypes was significantly different in controls and patients ( $\chi^2=7.938$ ,  $P = 0.019$ ). The frequencies of *ADH2* A/A, A/G and G/G genotypes were 53.68%, 38.42% and 7.89% in patients and 41.89%, 49.10% and 9.01% in controls, with no significant difference ( $\chi^2=5.786$ ,  $P = 0.055$ ). The allelic distribution of *ADH2* and *ALDH2* polymorphisms in controls was in the Hardy-Weinberg equilibrium ( $P > 0.05$ ). Therefore, the controls from the general population could be considered as a representative.

The *ADH2* A/A and *ALDH2* G/G genotypes showed a moderately increased risk for colorectal cancer. The age- and smoking-adjusted OR relative to G/A and G/G was 1.60 (95% CI=1.08–2.36), and the adjusted OR for *ALDH2* G/G relative to G/A and A/A was 1.79 (95% CI=1.19–2.69).

The ORs and 95% CIs for association of alcohol drinking with colorectal cancer risk are shown in Table 3. Compared with non-drinkers, the age-adjusted and smoking-adjusted OR for colorectal cancer among alcohol drinkers was 2.04 (95% CI=1.36–3.08). Furthermore, a significant increase trend in the risk of colorectal cancer with amount of alcohol intake was also observed ( $P = 0.0001$ ).

The results of multivariate analysis of smoking, alcohol drinking, *ALDH2* and *ADH2* genotypes and risk of colorectal cancer are shown in Table 4. After

**Table 1 Background characteristics of male colorectal cancer patients and controls**

	<b>Controls</b>		<b>Patients</b>		$\chi^2_{\text{MH}}$	$P$
	No.	%	No.	%		
Age (yr)						
< 40	25	11.26	21	11.05	4.467	0.346
40-49	33	14.86	39	20.53		
50-59	76	34.23	50	26.32		
60-69	62	27.93	53	27.89		
> 70	26	11.71	27	14.21		
Total	222		190			
Smoking status						
Nonsmoker	87	39.19	67	35.26	3.794	0.150
Current smoker	122	54.95	102	53.68		
Former smoker	13	5.86	21	11.05		
Drinking status						
Nondrinker	124	55.86	73	38.42	15.952	0.001
Current drinker	91	40.99	99	52.11		
Former drinker	7	3.15	18	9.47		
Kinds of alcoholic beverage						
Hot wine	68	69.39	77	40.53	3.453	0.327
Beer	0	0	2	1.05		
Wine	3	3.06	1	0.53		
All kinds	27	27.55	37	31.62		
Alcohol consumption (g/mo)						
0-29	124	55.86	73	38.42	15.437	0.001
30-299	20	9.01	14	7.37		
300-599	17	7.66	20	10.53		
≥ 600	61	27.46	83	43.68		

**Table 2 Adjusted odds ratio (OR) and 95% confidence interval (CI) for colorectal cancer with reference to *ALDH2* and *ADH2* polymorphisms**

	<b>Controls</b> <i>n</i> (%)	<b>Cases</b> <i>n</i> (%)	<b>OR<sup>1</sup></b> (95% CI)	<b>OR<sup>2</sup></b> (95% CI)
<i>ALDH2</i> genotype				
G/G	123 (55.41)	131 (68.95)	1.00	1.00
G/A	90 (40.54)	54 (28.42)	0.56 (0.37–0.86)	0.56 (0.37–0.86)
A/A	9 (4.05)	5 (2.63)	0.52 (0.15–1.76)	0.52 (0.17–1.60)
G/A + A/A	99 (44.59)	59 (31.05)	0.56 (0.37–0.86)	0.56 (0.37–0.84)
A/A + A/G	99 (44.59)	59 (31.05)	1.00	1.00
G/G	123 (55.41)	131 (68.95)	1.79 (1.19–2.68)	1.79 (1.19–2.69)
<i>ADH2</i> genotype				
A/A	93 (41.89)	102 (53.68)	1.00	1.00
A/G	109 (49.10)	73 (38.42)	0.61 (0.41–0.92)	0.62 (0.41–0.93)
G/G	20 (9.01)	15 (7.89)	0.68 (0.33–1.41)	0.68 (0.33–1.40)
A/G + G/G	129 (58.11)	88 (46.32)	0.62 (0.41–0.94)	0.63 (0.42–0.93)
G/G + G/A	129 (58.11)	88 (46.32)	1.00	1.00
A/A	93 (41.89)	102 (53.68)	1.61 (1.09–2.38)	1.60 (1.08–2.36)

<sup>1</sup>Crude OR, <sup>2</sup>OR was adjusted by age and smoking status.

adjustment of all these variables for each other, *ADH2* A/A, *ALDH2* G/G genotypes and alcohol drinking were associated with an increased risk for colorectal cancer, but smoking was not.

As compared to the subjects with *ADH2* G and *ALDH2* A alleles, those with *ADH2* A/A and *ALDH2* G/G genotypes had a significantly increased OR (3.05, 95% CI=1.67–5.57, Table 5). The OR for CRC among alcohol drinkers with *ADH2* A/A genotype was markedly increased to 3.44 (95% CI=1.84–6.42) compared to non-drinkers with *ADH2* G allele. The OR for colorectal cancer among alcohol drinkers with

**Table 3** Adjusted OR and 95% CI for colorectal cancer with reference to alcohol drinking

	Controls	Cases	OR <sup>1</sup> (CI)	OR <sup>2</sup> (CI)
Alcohol drinking status				
Non-drinker	124	73	1.00	1.00
Current drinker	91	99	1.85 (1.21-2.83)	1.84 (1.20-2.82)
Former drinker	7	18	4.37 (1.62-12.17)	4.19 (1.64-0.71)
Current and former	98	107	2.03 (1.37-3.01)	2.04 (1.36-3.08)
Alcohol consumption (g/mo)				
0-29	124	73	1.00	1.00
30-299	20	14	1.19 (0.53-2.65)	1.22 (0.58-2.58)
300-599	17	20	2.00 (0.93-4.30)	1.98 (0.96-4.09)
≥ 600	61	83	2.31 (1.46-3.68)	2.33 (1.47-3.71)
<i>P</i> for trend = 0.0001				

<sup>1</sup>Crude OR, <sup>2</sup>OR was adjusted for age and smoking status.

**Table 4** Multivariate analysis of smoking, alcohol drinking, *ALDH2* and *ADH2* genotypes and risk of colorectal cancer

	OR <sup>1</sup>	95% CI	χ <sup>2</sup>	<i>P</i>
<i>ALDH2</i>	1.62	1.06-2.47	5.0035	0.0253
<i>ADH2</i>	1.73	1.15-2.58	7.0548	0.0079
Smokers	0.97	0.63-1.50	0.0138	0.9063
Alcohol drinkers	1.95	1.27-2.98	9.3884	0.0022

<sup>1</sup>Logistic regression model included age (continuous), smoking (nonsmokers, current + former), alcohol drinking (nondrinkers, current + former drinkers), *ALDH2* (A/A+A/G, G/G) and *ADH2* genotype (G/G + G/A, A/A).

*ALDH2* G/G genotype was markedly increased to 2.70 (95% CI=1.57-4.66) compared to non-drinkers with *ALDH2* A allele (Table 6).

## DISCUSSION

Our previous studies showed that drinking is associated with increased colorectal cancer risk<sup>[8-10]</sup>. We also found that a polymorphism of cytochrome P450 2E1 (*CYP2E1*), an alcohol metabolizing enzyme, could influence susceptibility to colorectal cancer and *CYP2E1* C2/C2 genotype and alcohol drinking have a coordinated effect on the development of colorectal cancer<sup>[9]</sup>. In the present study, polymorphisms of the *ADH2* and *ALDH2* genes were significantly associated with the risk of colorectal cancer in Chinese males. Significant gene-gene and gene-environment interactions were also found between alcohol drinking and *ADH2* and *ALDH2* polymorphisms.

Colorectal carcinogenesis, as with other cancers, is complex and due to the coordinated effects of environmental and genetic factors. Thus, the exposure dosage to chemical carcinogens could vary with enzyme activity. In theory, when *ADH2* activity is increased or *ALDH2* activity is decreased, the blood acetaldehyde level also increases in drinkers, thus increasing the risk of developing colorectal cancer. However, inconsistent results have been reported in many studies on the relation between *ADH2* and *ALDH2* gene polymorphisms and cancer susceptibility<sup>[11-18]</sup>. Yokoyama et al<sup>[11]</sup> found *ALDH2* G/A genotype encoding inactive

**Table 5** Interaction between *ALDH2* and *ADH2* genotypes and OR for colorectal cancer

<i>ALDH2</i>	<i>ADH2</i>	Controls	Cases	OR <sup>1</sup> (95% CI)
A/A + A/G	G/G + G/A	55	25	1.00
A/A + A/G	A/A	44	34	1.64 (0.85-3.18)
G/G	G/G + G/A	74	63	1.87 (1.05-3.35)
G/G	A/A	49	68	3.05 (1.67-5.57)

<sup>1</sup>OR was adjusted for age and smoking in a logistic regression model.

**Table 6** Interaction between alcohol drinking and polymorphisms of the *ALDH2* and *ADH2* genes, and the odds ratio (OR) for colorectal cancer

Genotypes	Drinker	Controls	Cases	OR <sup>1</sup> (95% CI)
<i>ALDH2</i>				
A/A + A/G	No	64	32	1.00
G/G	No	60	41	1.36 (0.76-2.43)
A/A + A/G	Yes	35	27	1.39 (0.69-2.81)
G/G	Yes	63	90	2.70 (1.57-4.66)
<i>ADH2</i>				
G/G + G/A	No	65	34	1.00
A/A	No	59	39	1.27 (0.71-2.26)
G/G + G/A	Yes	64	54	1.65 (0.94-2.91)
A/A	Yes	34	63	3.44 (1.84-6.42)

<sup>1</sup>OR was adjusted for age and smoking status.

*ALDH2* and *ADH2* G/G genotype encoding the low-activity form of *ADH2* can enhance the risk of esophageal cancer in Japanese alcoholics. For those individuals with both *ALDH2* G/A and *ADH2* G/G genotypes, the risk of esophageal cancer is increased in a multiplicative fashion. It was reported that after adjustment for age, daily alcohol consumption and amount of cigarette smoking, the risk of developing oropharyngolaryngeal, esophageal, stomach, colon, lung and multiple primary cancers is significantly increased in the presence of *ALDH2* A allele<sup>[12]</sup>. In a study on the association between genetic polymorphisms of tobacco- and alcohol-related metabolizing enzymes and esophageal squamous cell carcinoma susceptibility, Hori et al<sup>[13]</sup> showed that there is a significant difference in the distribution of *ADH2* and *ALDH2* genotypes between healthy controls and esophageal cancer patients, and that *ADH2* G/G and *ALDH2* G/A genotypes are significantly higher in esophageal cancer patients than in healthy controls. Furthermore, persons with combined genotypes of *ADH2* G/G and *ALDH2* G/A are at a high risk of developing esophageal squamous cell carcinoma. Chao et al<sup>[14]</sup> reported that Chinese alcoholic patients with *ADH2* G and *ALDH2* A alleles are more susceptible to esophageal cancer. However, Ohhira et al<sup>[15]</sup> showed that 10 patients with hepatocellular carcinoma (HCC) associated with pure alcoholic liver disease all have *ALDH2* G/G genotype. Takeshita et al<sup>[16]</sup> also examined associations between *ADH2* and *ALDH2* polymorphisms, alcohol drinking and HCC development in Japanese, and showed that a greater cumulative amount of alcohol consumption is significantly associated with HCC development but

not with *ADH2* and *ALDH2* genotypes, indicating that ethanol is directly involved in the development of HCC rather than acetaldehyde. The latest evaluation in 2007 by the International Agency for Research on Cancer (IARC) confirmed that alcoholic beverages are carcinogenic to human beings (Group 1), thus increasing the occurrence of cancer in many sites, such as oral cavity, pharynx, larynx esophagus, liver, colorectum and female breast<sup>[17,18]</sup>.

Our previous study showed that *ADH2* and *ALDH2* polymorphisms are not significantly associated with the risk of developing liver, stomach and esophageal cancer<sup>[19-21]</sup>. However, those with *ALDH2* G allele drinking a greater amount of alcohol significantly elevates the risk of developing HCC and those with *ALDH2* G/G genotype drinking a larger amount of alcohol are at a significantly increased risk of developing gastric cancer. Matsuo *et al*<sup>[22]</sup> evaluated the relationship between genetic polymorphisms of *ADH2* His47Arg and *ALDH2* Glu487Lys and occurrence of colorectal cancer in Japan, and showed that *ADH2* Arg allele is associated with an increased risk of CRC. However, no significant association was found with the *ALDH2* polymorphism itself, a significant interaction between *ALDH2* and *ADH2* polymorphisms was observed in their study.

In the present study, *ADH2* A/A and *ALDH2* G/G genotypes moderately increased the risk of developing colorectal cancer in Chinese males. Significant gene-gene and gene-environment interactions were also observed between alcohol drinking and *ADH2* and *ALDH2* polymorphisms regarding colorectal cancer risk. However, further study is needed to confirm our results with a large sample size.

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## COMMENTS

### Background

Colorectal cancer (CRC) is the fifth most common cancer in China. There is epidemiological evidence that alcohol intake is associated with an increased CRC risk. Alcohol dehydrogenase 2 (*ADH2*) and aldehyde dehydrogenase 2 (*ALDH2*) have a strong impact on alcohol metabolism. To evaluate the relationship between habitual drinking and genetic polymorphisms of *ADH2* and *ALDH2* with reference to the risk of CRC in Chinese males, we conducted a population based case-control study of CRC in Jiangsu Province of China.

### Research frontiers

Susceptibility to cancer is generally thought to be the sum of complex interactions between environmental and genetic factors. Therefore, interaction between environmental and genetic factors is a hotspot of cancer epidemiology. We studied interactions between *ADH2* and/or *ALDH2* and habitual alcohol drinking in CRC development.

### Innovations and breakthroughs

The present study showed that *ADH2* A/A and *ALDH2* G/G genotypes were correlated with the increased risk of CRC. Furthermore, a significant cooperative role of *ADH2* A/A and/or *ALDH2* G/G genotypes and alcohol

consumption was also observed in the development of CRC.

### Applications

This research showed the genetic risk factors and the role of gene environment interactions in identifying individuals at risk of CRC, which have certain theoretical and application values for studying the etiology of CRC and its prevention.

### Peer review

The correlation between habitual drinking and genetic polymorphisms of *ADH2* and *ALDH2* was studied with reference to the risk of CRC in Chinese males. Furthermore, significant gene-gene and gene-environment interactions between alcohol drinking and *ADH2* and *ALDH2* polymorphisms were also found regarding the CRC risk. The experiments contained appropriate controls and data about the age, smoking status and alcohol consumption. This research also reported the genetic risk factors and the role of gene-environment interactions in identifying patients at risk of developing colorectal cancer in Chinese males.

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RAPID COMMUNICATION

# Ivor Lewis subtotal esophagectomy with two-field lymphadenectomy for squamous cell carcinoma of the lower thoracic esophagus

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with two-field (total mediastinum) lymphadenectomy is a safe and appropriate operation for squamous cell carcinoma of the lower thoracic esophagus.

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**Key words:** Esophageal neoplasm; Ivor Lewis approach; Two-field lymphadenectomy

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## Abstract

**AIM:** To evaluate the clinical outcome of Ivor Lewis subtotal esophagectomy with two-field lymphadenectomy for patients with squamous cell carcinoma of the lower thoracic esophagus.

**METHODS:** From January 1998 to December 2001, 73 patients with lower thoracic esophageal carcinoma underwent Ivor-Lewis subtotal esophagectomy with two-field lymphadenectomy. Clinicopathological information, postoperative complications, mortality and long term survival of all these patients were analyzed retrospectively.

**RESULTS:** The operative morbidity and mortality was 15.1% and the mortality was 2.7%. Lymph node metastases were found in 52 patients (71.2%). Nodal metastases to the upper, middle, lower mediastini and upper abdomen were found in 13 (17.8%), 15 (20.5%), 30 (41.1%), and 25 (34.2%) patients, respectively. Postoperative staging was as follows: stage I in 5 patients, stage II in 34 patients, stage III in 32 patients, and stage IV in 2 patients, respectively. The overall 5-year survival rate was 23.3%. For N0 and N1 patients, the 5-year survival rate was 38.1% and 17.3%, respectively ( $\chi^2 = 22.65, P < 0.01$ ). The 5-year survival rate for patients in stages IIa, IIb and III was 31.2%, 27.8% and 12.5%, respectively ( $\chi^2 = 29.18, P < 0.01$ ).

**CONCLUSION:** Ivor Lewis subtotal esophagectomy

## INTRODUCTION

Radical esophagectomy with lymphadenectomy remains the mainstay of curative therapy for esophageal carcinoma. Complete resection of esophagus and regional lymph node ( $R_0$  resection) are essential to improve long-term survival<sup>[1-3]</sup>. For carcinomas of the lower thoracic esophagus, different tumor characteristics between the Western and Eastern countries cause various attitudes on their surgical management<sup>[4]</sup>. Depending on the attending surgeon's philosophy, surgical approaches to carcinomas of the lower thoracic esophagus vary from the conventional left thoraco-abdominal approach<sup>[5,6]</sup> to the transhiatal approach without thoracotomy<sup>[7-9]</sup>, Ivor Lewis approach<sup>[10-12]</sup>, and recently reported thoracoscopic approach<sup>[13-15]</sup>. On the other hand, controversy continues over the optimal extent of lymphadenectomy to be performed with esophagectomy. Some authors do not favor lymphadenectomy considering lymph node involvement as systemic disease with no hope for cure, and the primary goal of surgical intervention is palliative, with a low surgical morbidity and mortality<sup>[8]</sup>. However, some authors prefer two-field lymphadenectomy and regard it as a standard surgical procedure<sup>[10,12,16]</sup>. What is more, other authors, mainly from Japan, advocate three-field lymphadenectomy in order to obtain accurate staging,

control locoregional recurrence and improve long-term survival<sup>[17-20]</sup>.

At our hospital, the main surgical approach to lower thoracic esophageal carcinoma is the Ivor Lewis approach, instead of left thoraco-abdominal approach that is widely adopted in China, because Ivor Lewis approach provides a wider extent of lymphadenectomy than left thoraco-abdominal approach, and the chance of obtaining R<sub>0</sub> resection is greater. The aim of this study was to retrospectively assess our results of Ivor Lewis subtotal esophagectomy with two-field lymphadenectomy for patients with squamous cell carcinoma of the lower thoracic esophagus.

## MATERIALS AND METHODS

### **Patients**

From January 1998 to December 2001, 73 patients with squamous cell carcinoma of the lower thoracic esophagus underwent Ivor Lewis subtotal esophagectomy with two-field lymph node dissection at Zhejiang Cancer Hospital, Hangzhou, China. There were 65 men and 8 women. Median age was 57 years (range, 39 to 78 years). Preoperative evaluation was performed for all patients with a barium swallow examination, endoscopy with biopsy, ultrasonography of the neck and upper abdominal compartment. Cardiac and pulmonary functions were also routinely performed for these patients to determine their ability to withstand the planned surgical procedure. Before treatment, diagnosis of all patients was established histologically. No patient received chemotherapy or radiotherapy before operation. Postoperative staging was based on the 2002 UICC-TNM classification<sup>[21]</sup>.

Duration of surgery, operative blood loss, and the number of lymph nodes were recorded. Postoperative complications were also recorded. Operative mortality was defined as any death during the first 30 d after operation or during the same hospital stay.

### **Surgical technique**

All patients underwent Ivor Lewis subtotal esophagectomy with two-field lymphadenectomy. In brief, all resections were carried out by initial abdominal exploration through an upper midline laparotomy. The stomach was mobilized on the right gastric and gastroepiploic arteries. The left gastric artery was divided at its origin, and all lymph nodes along the celiac axis and its three branches along the left aspect of the portal vein, in front of the inferior vena cava, along the diaphragmatic pillars, and in front of the left adrenal gland were *en bloc* resected. A pyloromyotomy was performed routinely for all the patients. No drainage procedure was done. After the abdominal stage, a right posterolateral thoracotomy was performed. The thoracic dissection included removal of azygous arch with its associated lymph nodes, thoracic duct, and the low paratracheal, subcarinal, paraesophageal, and parahiatal nodes in continuity with the resected esophagus. In addition, upper mediastinal, paratracheal lymphatic tissue

**Table 1 Postoperative pathological staging in 73 patients with esophageal carcinoma**

Stage	TNM	Patients, n (%)
I (n = 5)	T1N0M0	5 (6.8)
II a (n = 16)	T2N0M0	7 (9.6)
	T3N0M0	9 (12.3)
II b (n = 19)	T1N1M0	3 (4.1)
	T2N1M0	15 (20.5)
III (n = 32)	T3N1M0	29 (39.7)
	T4N1M0	3 (4.1)
IV (n = 2)	T3N1M1	2 (2.7)

including lymph nodes of the left and right recurrent laryngeal nerves were also removed. Denudation of the lesser curvature was usually performed in the pleural cavity. After resection of the specimen, an anastomosis was constructed between the stomach and esophagus. The anastomosis was located in the apex of the chest in all patients.

After surgery, all patients returned to the intensive care unit for 2 d on average. On day 5 after surgery, patients underwent contrast X-ray study for assessment of esophagogastric anastomosis and then received enteral feeding.

### **Follow-up**

Postoperative data were collected at the outpatient clinic. Follow-up data were obtained by telephone. The follow-up time ranged 0-88 mo (median 47 mo). Survival time was defined as the period from the date of surgery until death or the most recent follow-up investigation (September 2005), with none lost to follow-up.

### **Statistical analysis**

Survival analysis was carried out with the Kaplan-Meier method, and the log-rank test was used for comparisons. Estimates and 95% confidence intervals (CI<sub>95</sub>) were given for 5 years. Fisher's exact two-tailed test was used to compare categorical data. P < 0.05 was considered statistically significant.

## RESULTS

### **Pathological findings**

All the 73 patients acquired complete resection (R<sub>0</sub> resection). The postoperative stage and TNM classification are shown in Table 1. Of the 8 patients with pathological T1 tumors, 2 had muscularis mucosae tumors and 6 had submucosal tumors. Nodal involvement was found in 3 of the 6 patients with submucosal tumors. Of the 3 patients with pathological T4 tumors, 2 had liver involvement and 1 had lung involvement. Combined resections were performed for the 3 patients. Metastases were found in celiac nodes of 2 patients with M1 disease.

### **Operative outcomes**

The operation time was 243 ± 40 min. The operative blood loss was 365 ± 230 mL. The number of lymph

**Table 2 Postoperative complications in 73 patients with esophageal carcinoma n (%)**

Complications	Patients	Patients died
Pneumonia	4 (5.5)	1 (1.4)
Vocal cord palsy	3 (4.1)	0 (0)
Arrhythmia	3 (4.1)	0 (0)
Anastomotic leakage	2 (2.7)	1 (1.4)
Postoperative bleeding	1 (1.4)	0 (0)
Wound infection	1 (1.4)	0 (0)

**Table 3 Relation between depth of tumor infiltration (T) and lymph node metastasis (N)**

T	N0	N1	Prevalence of nodal metastases (%)
T1 (n = 8)	5	3	37.5
T2 (n = 22)	7	15	68.2
T3 (n = 40)	9	31	77.5
T4 (n = 3)	0	3	100

P = 0.888.

nodes resected was  $31 \pm 11$  (median, 27). Postoperative complications are listed in Table 2. Complications occurred in 11 patients (15.1%, CI<sub>95</sub> 6.9%-23.3%). Of the 73 patients, 2 (2.7%, CI<sub>95</sub> 0%-6.5%) died due to pneumonia and anastomotic leakage, respectively, within 30 d of the operation.

### Tumor depth and lymph node metastases

Lymph node metastases were found in 52 patients and the prevalence of nodal metastasis was 71.2% (CI<sub>95</sub> 60.8%-81.6%). As shown in Table 3, the prevalence of nodal metastases increased with increasing tumor depth, but the difference was not statistically significant. The prevalence of nodal metastases was 37.5% in T1 stage patients, 77.5% in T3 stage patients, which was statistically significant ( $\chi^2 = 5.06$ , P = 0.025). Other differences in the two stages were not statistically significant (T1 vs T2, T1 vs T4, T2 vs T3, T2 vs T4, T3 vs T4).

### Distribution of metastatic lymph node

The frequencies of nodal metastases in different anatomical regions are shown in Table 4, which were statistically significant (P = 0.004). The frequency in lower mediastinum (41.1%) was higher than that in upper (17.8%,  $\chi^2 = 9.53$ , P = 0.007), and middle mediastini (20.5%,  $\chi^2 = 7.23$ , P = 0.007). The frequency in upper abdomen (34.2%) was higher than that in upper mediastinum (17.8%) ( $\chi^2 = 5.12$ , P = 0.024). Other differences in frequencies between the two regions were not statistically significant. Among the 52 patients with lymph node involvement, 13 (25.0%, CI<sub>95</sub> 13.3%-36.7%) had skip nodal metastases without invasion of peritumoral nodes. Isolated lymph node involvement was observed in the upper mediastinum of 6 patients (4 with positive left recurrent laryngeal nerve nodes, 2 positive right left laryngeal nerve nodes) and in the upper abdomen of 7 patients with positive left gastric arterial nodes, respectively. These patients (17.8%, CI<sub>95</sub>

**Table 4 Distribution of lymph node metastatic locations**

Metastatic location	Patients with negative lymph node (n)	Patients with positive lymph node (n)	Frequency of nodal metastases (%)
Upper mediastinum	60	13	17.8
Middle mediastinum	58	15	20.5
Lower mediastinum	43	30	41.1
Upper abdomen	48	25	34.2

P = 0.004.

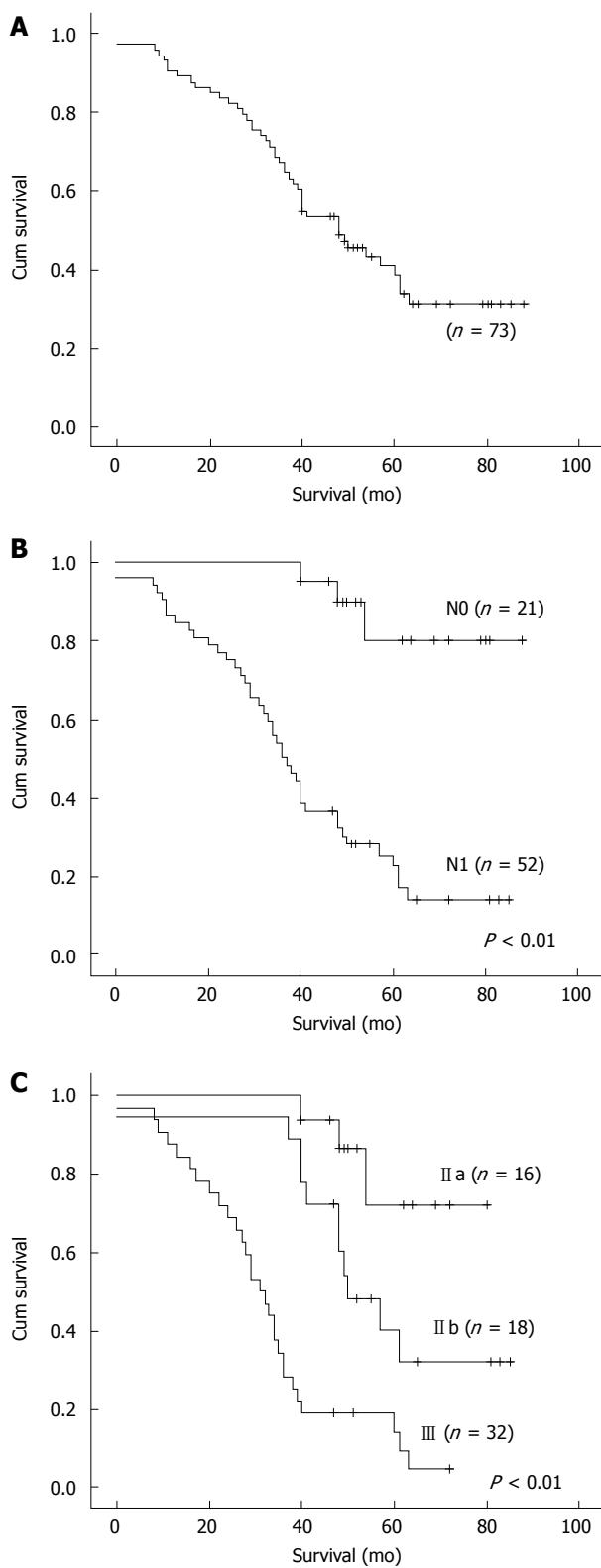
9.0%-26.6%) did not undergo two-field lymphadenectomy but received incomplete resections (R<sub>1</sub> resection of microscopically residual tumors or R<sub>2</sub> resection of macroscopically residual tumors). Had upper mediastinal lymphadenectomy not been performed, six patients (8.2%, CI<sub>95</sub> 1.9%-15.5%) would not have undergone upper mediastinal lymphadenectomy but received inaccurate staging.

### Survival rate

The 5-year survival rate was 23.3% (CI<sub>95</sub> 13.5%-32.9%) for all patients (Figure 1A). The 5-year survival rate was 38.1% (CI<sub>95</sub> 17.4%-58.8%) and 17.3% (CI<sub>95</sub> 7.0%-27.6%) for N0 and N1 patients, respectively ( $\chi^2 = 22.65$ , P < 0.01, Figure 1B). The 5-year survival rate for patients in stages II a, II b, and III was 31.2% (CI<sub>95</sub> 8.9%-53.5%), 27.8% (CI<sub>95</sub> 7.1%-48.5%) and 12.5% (CI<sub>95</sub> 1.0%-24.0%), respectively ( $\chi^2 = 29.18$ , P < 0.01, Figure 1C).

## DISCUSSION

Studies demonstrated that extensive submucosal lymphatic drainage to the esophageal wall causes a unique pattern of nodal metastases<sup>[1,2,4]</sup>. It was reported that the prevalence of nodal metastases of lower thoracic esophageal carcinoma is up to 70%<sup>[1-3]</sup>. Lymphatic dissemination is an early event of esophageal carcinoma, and involved nodes are found in 30%-40% of submucosal tumors<sup>[2,3]</sup>. These data are consistent with our results (71.2% and 50%, respectively). It has been documented that the likelihood of nodal metastases of esophageal carcinoma depends on the depth of tumor invasion of the esophageal wall<sup>[2,3]</sup>. The prevalence of nodal metastases increased with the increasing tumor invasion depth in this study. The prevalence of nodal metastases had no difference between the two groups according to their T status, suggesting that it may be related to the small sample size. Unlike squamous cell carcinoma of the upper thoracic esophagus, which shows lymphatic flow is mainly in the upward direction along the esophageal wall, squamous cell carcinoma of the lower thoracic esophagus, which shows lymphatic spread is mainly in the downward direction along the esophageal wall<sup>[2,4]</sup>, which is consistent with our findings. Although to some degree, the frequencies of lower mediastinal and upper abdominal metastases were higher than those of upper and middle mediastinal metastases, the frequency of upper mediastinal metastases (17.8%) was still not as low as that of middle mediastinal



**Figure 1** Survival curves for the 73 patients with squamous cell carcinoma of the lower thoracic esophagus (A), according to the N status (B), and the TNM stage (C).

metastases (20.5%) in the patients with lower thoracic esophageal carcinoma. An even higher frequency of upper and/or middle mediastinal metastases (30%-40%) has been reported<sup>[2,4]</sup>. In our study, skip nodal metastases were found in 25% of nodal positive patients. Of the 52 patients, 6 (11.5%) and 7 (13.5%) had positive nodes in

the upper mediastinum and abdomen, respectively. Hosch *et al*<sup>[22]</sup> reported that skip metastases nodal positive patients and a frequent event in esophageal cancer. Based on these nodal metastatic features, in order to obtain R<sub>0</sub> resection, lymphadenectomy with upper mediastinal dissection should be performed for squamous cell carcinoma of the lower esophagus.

The greater the number of lymph nodes is removed, the greater the chance of obtaining a R<sub>0</sub> resection, and the more precisely the disease can be staged<sup>[1-3,11]</sup>. The extent of lymphadenectomy for a cancer in the thoracic esophagus has been classified by the Consensus Conference of the International Society for Diseases of the Esophagus (ISDE) as a standard, extended, total, or three-field lymphadenectomy<sup>[23]</sup>. The optimal lymphadenectomy for squamous cell carcinoma of the lower thoracic esophagus is still debatable. Of the 4 types of lymphadenectomy, three-field lymphadenectomy can, most likely, achieve a R<sub>0</sub> resection and accurate staging. The question is whether it definitely improves the long-term survival of patients. However, most data are available from nonrandomized retrospective historical studies in Japan. Igaki *et al*<sup>[4]</sup> reported that three-field lymphadenectomy could prolong the survival time of patients with squamous cell carcinoma of the lower thoracic esophagus compared to 2-field lymphadenectomy for nodal metastases present in the upper and/or middle mediastinum. Fujita *et al*<sup>[24]</sup> reported that there is no difference in survival rate for patients with lower thoracic esophageal cancer between the two procedures of lymphadenectomy. Three-field lymphadenectomy has its obvious advantages and disadvantages<sup>[16,18]</sup>. Hence, two field lymphadenectomy seems to be a more reasonable choice of treatment for squamous cell carcinoma of the lower thoracic esophagus. This viewpoint is far out weighed by the fact that the emphasis on three-field lymphadenectomy has shifted to lymphadenectomy along the recurrent laryngeal nerve chains, where lymph nodes could be dissected through two-field lymphadenectomy<sup>[16]</sup>. Among the other three types of lymphadenectomy, total (mediastinal) lymphadenectomy which was applied in this study, provides more chances to obtain a R<sub>0</sub> resection and accurate staging than the other two types of lymphadenectomy. As our data show, if a standard lymphadenectomy was performed, positive nodes would be left intact, thus only R<sub>1</sub> or R<sub>2</sub> resection could be achieved, leading to stage migration from N0 to N1 in 6 patients (8.2%). If an extended lymphadenectomy was performed, stage migration from N0 to N1 would occur in 4 patients (5.5%), which can be explained by the fact that the extent of standard lymphadenectomy does not cover the upper and left upper mediastini. Total (mediastinal) lymphadenectomy is therefore an alternative for squamous cell carcinoma of the lower thoracic esophagus. Then, which surgical approach could satisfy the demand for total lymphadenectomy? Transhiatal approach without thoracotomy is unlikely to be chosen. In the left thoraco-abdominal approach we used previously, upper mediastinal lymph nodes are

not available. In the McKeown approach (anterolateral right thoracotomy), a radical lymphadenectomy is more difficult to achieve than the Ivor Lewis approach<sup>[5,12]</sup>. Thoracoscopic approach to esophageal carcinoma remains to be investigated and no systemic data are available to support its advantages over the minimally invasive approach to esophagectomy<sup>[25]</sup>. Ivor Lewis approach which allows complete visualization of mediastinum, especially upper mediastinum, seems to be most appropriate for squamous cell carcinoma of the lower thoracic esophagus.

Morbidity increases with the extent of lymphadenectomy but does not lead to a higher mortality<sup>[11]</sup>. Pneumonia was the most common complication in this study, which is consistent with previous reports<sup>[10-12]</sup>. Vocal cord palsy caused by lymphadenectomy around recurrent laryngeal nerves also occurs occasionally. Functional mediastinal lymphadenectomy can preserve the bronchial artery and bronchial branches of the vagus nerve, thus reducing the respiratory complications<sup>[20,26]</sup>. To prevent vocal cord palsy, electrocautery close to the recurrent laryngeal nerve and drawing the nerve with a vessel tape should be avoided during lymphadenectomy<sup>[19]</sup>.

The overall 5-year survival rate of our patients was only 23.3%, which may be due to the small sample size in our study. On the other hand, the survival of patients was ascertained in September 2005 when 15 patients were alive at the most recent follow-up. Accordingly, this overall survival rate could not completely explain the effect of treatment since we did not make comparisons with others. However, there was a significant difference in the survival rate between the two groups of patients with different N status and different stages. This series were proved to be more homogenous with regard to the clinical variables, such as tumor site and pathological type. The surgical procedure performed in this series revealed more R<sub>0</sub> resection and staging information than other surgical procedures (except three-field lymphadenectomy). Compared to other surgical procedures, Ivor Lewis esophagectomy with two-field (total mediastinal) lymphadenectomy could achieve better results in patients with squamous cell carcinoma of the lower thoracic esophagus. Because of the small sample size used in this study, further studies are needed to confirm our results.

In conclusion, Ivor Lewis subtotal esophagectomy with two-field (total mediastinal) lymphadenectomy is a safe and appropriate surgical procedure for squamous cell carcinoma of the lower esophagus.

## COMMENTS

### Background

Radical esophagectomy with lymphadenectomy is still the mainstay of curative therapy for esophageal carcinoma. This study retrospectively analyzed the clinical outcome of Ivor Lewis subtotal esophagectomy with two-field lymphadenectomy for squamous cell carcinoma of the lower thoracic esophagus.

### Research frontiers

Adenocarcinoma of the lower thoracic esophagus is a common pathological type in Western countries, while squamous cell carcinoma is the main type in Eastern countries. The attitudes to the surgical approach and the extent of the lymphadenectomy for the lower thoracic esophageal carcinoma are different in

Western and Eastern countries. Western general thoracic surgeons prefer the transhiatal approach and limited extent of lymphadenectomy, whereas Eastern general thoracic surgeons prefer the transthoracic approach and 2-field or 3-field lymphadenectomy.

### Innovations and breakthroughs

In China, the left thoracoabdominal approach and standard 2-field lymphadenectomy are widely performed for lower thoracic esophageal carcinoma. In our clinical practice, however, they are widely performed as upper mediastinal lymphadenectomy. Furthermore, the prevalence of upper mediastinal nodal involvement is not low. 3-field lymphadenectomy is still controversial. This type of lymphadenectomy is not routinely performed in our institution. We prefer the Ivor Lewis approach with total mediastinal 2-field lymphadenectomy in the treatment of this disease.

### Applications

Ivor Lewis subtotal esophagectomy with 2-field lymphadenectomy is a safe surgical procedure for squamous cell carcinoma of the lower thoracic esophagus.

### Terminology

Ivor Lewis subtotal esophagectomy is a laparotomy followed by a right thoracotomy. Two-field total mediastinal lymphadenectomy involves resection of bilateral upper mediastinum in addition to a standard lymphadenectomy.

### Peer review

The paper is scientific, innovative and readable, showing the advanced level of clinical research in gastroenterology both at home and abroad.

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RAPID COMMUNICATION

## **Lactobacilli inhibit interleukin-8 production induced by *Helicobacter pylori* lipopolysaccharide-activated Toll-like receptor 4**

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**CONCLUSION:** *H pylori*SS1-LPS induces IL-8 production through activating TLR4 signaling in SGC-7901 cells and viable LBG or LBG-s prevents *H pylori*SS1-LPS-mediated IL-8 production via inhibition of the TLR4 pathway.

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**Key words:** *Lactobacillus*; *Helicobacter pylori*; Lipopolysaccharide; Toll-like receptor 4; Interleukin-8

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### **Abstract**

**AIM:** To investigate the effect of *Lactobacillus bulgaricus* (LBG) on the Toll-like receptor 4 (TLR4) pathway and interleukin-8 (IL-8) production in SGC-7901 cells treated with *Helicobacter pylori*Sydney strain 1 lipopolysaccharide (*H pylori*SS1-LPS).

**METHODS:** SGC-7901 cells were treated with *H pylori*SS1-LPS in the presence or absence of pretreatment for 1 h with viable LBG or supernatant recovered from LBG culture MRS broth (LBG-s). Cellular lysates were prepared for Western blot with anti-TLR4, anti-transforming growth factor  $\beta$ -activated kinase 1 (TAK1), anti-phospho-TAK1, anti-nuclear factor  $\kappa$ B (NF- $\kappa$ B), anti-p38 mitogen-activated protein kinase (p38MAPK), and anti-phospho-p38MAPK antibodies. The amount of IL-8 in cell culture medium was measured by ELISA.

**RESULTS:** *H pylori*SS1-LPS up-regulated the expression of TLR4, stimulated the phosphorylation of TAK1, subsequently enhanced the activation of NF- $\kappa$ B and the phosphorylation of p38MAPK in a time-dependent manner, leading to augmentation of IL-8 production in SGC-7901 cells. Viable LBG or LBG-s pretreatment attenuated the expression of TLR4, inhibited the phosphorylation of TAK1 and p38MAPK, prevented the activation of NF- $\kappa$ B, and consequently blocked IL-8 production.

### **INTRODUCTION**

Infection with the human gastric pathogen *Helicobacter pylori* (*H pylori*) can develop into chronic gastritis, peptic ulcer and gastric cancer. Some studies<sup>[1-5]</sup> demonstrated that *H pylori* can stimulate interleukin-8 (IL-8) production in gastric mucosal epithelia, which induces accumulation of neutrophilic granulocytes in mucosa. Chemotactic response initiates inflammatory damage to gastric mucosa, which plays a crucial role in the pathogenesis of *H pylori*. However, signal transduction through which *H pylori* modulates IL-8 production from gastric epithelia is not fully understood.

The product of particular significance for the virulent action of *H pylori* is its cell wall lipopolysaccharide (LPS). The effects of *H pylori* lipopolysaccharide (*H pylori*-LPS) have been manifested by the marked increase of nitric oxide and proinflammatory cytokines including IL-8 in gastric mucosa<sup>[6,7]</sup>, abrogation of proliferation and induction of apoptosis in gastric epithelia<sup>[8]</sup>. Mammalian Toll-like receptors trigger the signaling pathways involved in innate immune responses to microbial challenge after recognizing pathogen-associated molecular patterns.

*H pylori*-LPS is the natural ligand for Toll-like receptor 4 (TLR4) in gastric epithelia. It has been proposed that *H pylori*-LPS induces IL-8 production in gastric epithelia through activating the TLR4 signaling pathway<sup>[7,9]</sup>.

Probiotics are living microorganisms with no or low pathogenicity, which exert beneficial effects on the host. *Lactobacillus bulgaricus* (LBG), a bacterium used in the production of yogurt, is one of the best-studied probiotics. There is increasing evidence<sup>[10,11]</sup> that LBG has therapeutic effects on *H pylori*-related diseases, including enhanced eradication of *H pylori*, amelioration of resistance to antibiotics, down-regulated side effects of antibiotic-based therapy, decreased recurrence of *H pylori* infection, and inhibition of *H pylori*-induced apoptosis. The mechanisms underlying these effects include inhibition of *H pylori* growth and attachment to epithelial cells, inactivation of virulent factors such as urease, and decrease in production of *H pylori*-induced proinflammatory cytokines<sup>[12-16]</sup>. However, the signaling pathways which are modulated by LBG in gastric epithelia have not been well elucidated.

In this experiment, we demonstrated that viable LBG inhibited the activation of the TLR4 signaling pathway and IL-8 production induced by *H pylori*Sydney strain 1 lipopolysaccharide (*H pylori*SS1-LPS) in SGC-7901 cells. Furthermore, supernatant recovered from the LBG culture MRS broth (LBG-s) also exerted these effects on SGC-7901 cells treated with *H pylori*SS1-LPS. These observations provide the novel insight into the rationale for LBG as a potential treatment for *H pylori*-related diseases.

## MATERIALS AND METHODS

### *H pylori*SS1 culture and *H pylori*SS1-LPS preparation

*H pylori*SS1 was kindly offered by Professor Qian Yu (School of Public Health, Sichuan University). *H pylori*SS1 was incubated in Brucella broth (bioMérieux Corporate, La Balme-Les Grottes, France) supplemented with 10% fetal calf serum (FCS; Invitrogen GIBCO, Carlsbad, California, USA), 10 mg/L vancomycin, 10 mg/L amphotericin and 2500 U/L polymyxin B in a shaking incubator (100 r/min) at 37°C in an atmosphere containing 50 mL/L O<sub>2</sub>, 100 mL/L CO<sub>2</sub> and 850 mL/L N<sub>2</sub> for 48 h. *H pylori*SS1 was precipitated from Brucella broth by centrifuging at 10 000 r/min for 10 min at 4°C and washed twice with PBS. Then the concentration of *H pylori*SS1 in PBS was adjusted to 10<sup>8</sup> colony forming units (CFU)/mL with optical density determined as 1 at  $\lambda_{660}$ . The *H pylori*SS1-containing PBS was used to prepare *H pylori*SS1-LPS with the LPS extraction kit (bioMérieux) following guidelines from its manufacturer. *H pylori*SS1-LPS concentrations were determined with the kinetic Limulus amebocyte lysate assay kit (Cambrex, Walkersville, Maryland, USA) according to the manufacturer's instructions.

### LBG culture and LBG-s preparation

LBG, kindly offered by Professor Qian Yu (School of

Public Health, Sichuan University), was incubated in MRS broth (bioMérieux) in a candle jar at 37°C for 24–48 h, precipitated from MRS broth by centrifuging at 5000 r/min for 10 min and washed twice with PBS. Then the concentration of LBG in PBS was adjusted to 10<sup>7</sup> CFU/mL with optical density determined as 0.5 at  $\lambda_{660}$ . LBG was precipitated from PBS by centrifuging at 5000 r/min for 10 min and resuspended with an equivalent volume of RPMI 1640 medium (Invitrogen GIBCO) for pretreatment of SGC-7901 cells.

LBG-s was generated by centrifuging at 1000 × g for 15 min and filtering (0.2 µm) LBG culture MRS broth, then the filtrate was concentrated using Centricon Plus-20 (5-100 kDa; Millipore, Bedford, Massachusetts, USA) by centrifugation at 4000 × g for 1 h following guidelines from the manufacturer. Protein concentrations were determined with the Pierce protein assay kit (Pierce, Rockford, Illinois, USA) using MRS broth as the control.

### Cell culture

SGC-7901 cell line was established from human gastric adenocarcinoma cells. Though the characteristics of cell apoptosis and proliferation are different from the cell line derived from normal gastric epithelia, SGC-7901 cells have been widely used as models for investigations on *H pylori*-induced gastric epithelial inflammatory responses because their inflammatory responsibility is similar to normal gastric epithelia. Therefore, SGC-7901 cells were used in our experiment. They were grown in RPMI 1640 medium supplemented with 10% FCS, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in an atmosphere containing 50 mL/L CO<sub>2</sub>. After 3–4 times of passage, SGC-7901 cells were seeded to generate 1 × 10<sup>6</sup> cells per 6 cm culture dish and incubated in RPMI 1640 medium containing 10% FCS, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in an atmosphere containing 50 mL/L CO<sub>2</sub> for 24 h. Then all cells were serum-starved (0.5% FCS) for 24 h before experimentation.

### Treatment of cell line

SGC-7901 cells were treated with 25 endotoxin units (EU)/mL *H pylori*SS1-LPS for 0, 30, 60 min or 120 min in the absence or presence of pretreatment for 1 h with 10<sup>7</sup> CFU/mL viable LBG or 10<sup>-2</sup> mg/mL LBG-s. At the end of each time point, cells were collected for Western blot, and RPMI 1640 medium was collected for ELISA. Each experiment was in triplicate.

### Preparation of cellular lysates and Western blot analysis

Nuclear and cytoplasmic extraction from SGC-7901 cells was performed using the nuclear-cytosol extraction kit (Cell Signaling Technology, Danvers, Massachusetts, USA) following guidelines from the manufacturer. Protein concentrations of all extracts were determined with the Pierce protein assay kit. Each extract was mixed with an equal amount of 2 × loading buffer and heated at 100°C for 5 min. Thirty micrograms of protein was loaded in each lane, resolved in sodium dodecyl

sulphate-polyacrylamide gels and electrotransferred to polyvinylidene difluoride membrane ( $1.2 \text{ mA/cm}^2$ , 1 h). After blocked with 5% fat-free dried milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h, the membrane was incubated overnight at 4°C with the following primary antibodies: anti-TLR4, anti-transforming growth factor  $\beta$ -activated kinase 1 (TAK1), anti-phospho-TAK1 (p-TAK1), anti-nuclear factor  $\kappa$ B (NF- $\kappa$ B), anti-p38 mitogen-activated protein kinase (p38MAPK), anti-phospho-p38MAPK (p-p38MAPK; all at the dilution of 1:1000, Cell Signaling Technology). Anti- $\beta$ -actin and anti-lamin B1 (both at the dilution of 1:1000, Santa Cruz Biotechnology, Santa Cruz, California, USA) were used for the control of equal protein loading. After washed three times in TBST, the membrane was incubated with horseradish peroxidase-conjugated IgG (1:5000, Santa Cruz Biotechnology) as the secondary antibody at room temperature for 1 h. The photographic film was exposed to bands visualized with the Supersignal West Pico chemiluminescent substrate kit (Pierce). The integrated optical density (IA) of each band was quantified using Quantity One software 4.5.0 (Bio-Rad Laboratories, Hercules, California, USA). Each value for TLR4 band was normalized as the ratio of IA of TLR4 band to that of  $\beta$ -actin band. Each value for p-TAK1 band was normalized as the ratio of IA of p-TAK1 band to that of TAK1 band. Each value for p-p38MAPK band was normalized as the ratio of IA of p-p38MAPK band to that of p38MAPK band. Each value for NF- $\kappa$ B band was normalized as the ratio of IA of NF- $\kappa$ B band to that of lamin B1 band. Each Western blot analysis of extract samples was performed in triplicate.

#### Detection of IL-8 production

The concentration of IL-8 in RPMI 1640 medium was determined with a commercially available ELISA kit (R&D Systems, Minneapolis, Minnesota, USA) following guidelines from the manufacturer. Each sample was detected thrice.

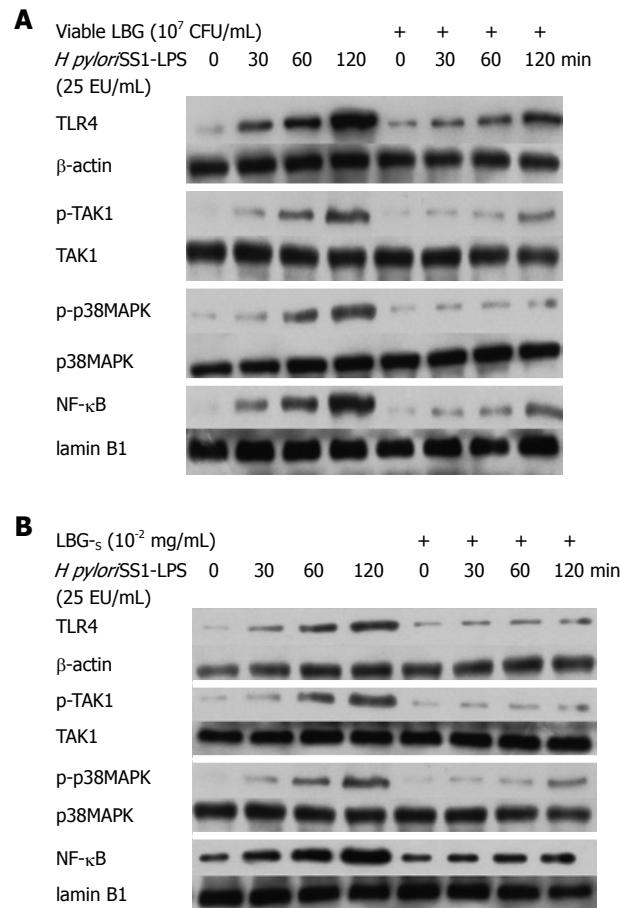
#### Statistical analysis

The data were expressed as mean  $\pm$  SD, and analyzed by SPSS13.0 software (SPSS, Chicago, Illinois, USA) for One-Way ANOVA test.  $P < 0.05$  was considered statistically significant.

## RESULTS

#### Viable LBG or LBG-s inhibited *H pylori*SS1-LPS-induced activation of TLR4 signaling pathway

Twenty-five EU/mL *H pylori*SS1-LPS up-regulated the expression of TLR4, enhanced the phosphorylation of TAK1 and p38MAPK, and induced the translocation of NF- $\kappa$ B into nuclei in a time-dependent manner (Figure 1A and B, Table 1). These results demonstrate that *H pylori*SS1-LPS could activate the TLR4 signaling pathway. Pretreatment for 1 h with  $10^7$  CFU/mL viable



**Figure 1** Expression of TLR4 and activation of TAK1, p38MAPK and NF- $\kappa$ B in SGC-7901 cells treated with *H pylori*SS1-LPS in the absence or presence of pretreatment with viable LBG (A) and LBG-s (B).

LBG (Figure 1A) or  $10^2$  mg/mL LBG-s (Figure 1B) significantly inhibited these effects of *H pylori*SS1-LPS on the TLR4 pathway in SGC-7901 cells to a great extent (Table 1).

#### Viable LBG or LBG-s inhibited *H pylori*SS1-LPS-induced IL-8 production

The production of IL-8 in SGC-7901 cells treated with 25 EU/mL *H pylori*SS1-LPS for 0, 30 or 60 min in the absence or presence of pretreatment for 1 h with  $10^7$  CFU/mL viable LBG or  $10^2$  mg/mL LBG-s was almost undetectable (Table 2). No significant difference in these data was possibly attributed to their extremely small value. Thirty or 60 min of treatment with *H pylori*SS1-LPS might be too short for SGC-7901 cells to produce enough IL-8 for ELISA, because cytokine production usually is posterior to the process of corresponding signal transduction. If we detected IL-8 mRNA in SGC-7901 cells using the retro-transcriptional polymerase chain reaction, results at 30 min or 60 min should have been significantly higher than those at 0 min. Only 120 min of treatment with 25 EU/mL *H pylori*SS1-LPS augmented IL-8 production in SGC-7901 cells (Table 2), which was, however, down-regulated by pretreatment for 1 h with  $10^7$  CFU/mL viable LBG or  $10^2$  mg/mL LBG-s (Table 2).

**Table 1** Effects of viable LBG, LBG<sub>s</sub> and *H pylori*SS1-LPS on the expression of TLR4 and activation of TAK1, p38MAPK and NF- $\kappa$ B in SGC-7901 cells

	LPS 0 min	LPS 30 min	LPS 60 min	LPS 120 min	LBG + LPS 0 min	LBG + LPS 30 min	LBG + LPS 60 min	LBG + LPS 120 min	LBG <sub>s</sub> + LPS 0 min	LBG <sub>s</sub> + LPS 30 min	LBG <sub>s</sub> + LPS 60 min	LBG <sub>s</sub> + LPS 120 min
TLR4/ $\beta$ -actin	0.014 ± 0.003	0.21 ± 0.03 <sup>1</sup>	0.43 ± 0.05 <sup>1</sup>	1.21 ± 0.11 <sup>1</sup>	0.016 ± 0.003	0.08 ± 0.01 <sup>2</sup> 0.15 ± 0.02 <sup>2</sup>	0.40 ± 0.05 <sup>2</sup>	0.016 ± 0.003	0.088 ± 0.013 <sup>2</sup>	0.12 ± 0.02 <sup>2</sup>	0.18 ± 0.03 <sup>2</sup>	
<sup>1</sup> t		2.588	3.068	3.502	<sup>2</sup> t	2.328	2.425	2.302		2.182	2.733	2.901
<sup>1</sup> P		0.012	0.003	< 0.001	<sup>2</sup> P	0.031	0.021	0.033		0.041	0.01	0.007
p-TAK1/TAK1	0.008 ± 0.002	0.075 ± 0.009 <sup>1</sup>	0.17 ± 0.03 <sup>1</sup>	0.49 ± 0.06 <sup>1</sup>	0.008 ± 0.001	0.025 ± 0.003 <sup>2</sup>	0.051 ± 0.006 <sup>2</sup>	0.15 ± 0.02 <sup>2</sup>	0.007 ± 0.002	0.031 ± 0.005 <sup>2</sup>	0.086 ± 0.011 <sup>2</sup>	0.13 ± 0.02 <sup>2</sup>
<sup>1</sup> t		2.445	2.871	3.421	<sup>2</sup> t	2.408	2.502	2.530		2.278	2.108	2.682
<sup>1</sup> P		0.018	0.005	< 0.001	<sup>2</sup> P	0.022	0.018	0.017		0.035	0.044	0.012
p-p38MAPK/ p38MAPK	0.010 ± 0.002	0.079 ± 0.011 <sup>1</sup>	0.18 ± 0.03 <sup>1</sup>	0.48 ± 0.08 <sup>1</sup>	0.009 ± 0.002	0.031 ± 0.004 <sup>2</sup>	0.058 ± 0.008 <sup>2</sup>	0.16 ± 0.03 <sup>2</sup>	0.013 ± 0.003	0.029 ± 0.004 <sup>2</sup>	0.079 ± 0.012 <sup>2</sup>	0.12 ± 0.02 <sup>2</sup>
<sup>1</sup> t		2.401	2.819	3.403	<sup>2</sup> t	2.261	2.445	2.506		2.289	2.093	2.704
<sup>1</sup> P		0.019	0.006	< 0.001	<sup>2</sup> P	0.036	0.02	0.018		0.034	0.046	0.011
NF- $\kappa$ B/lamin B1	0.012 ± 0.002	0.18 ± 0.03 <sup>1</sup>	0.32 ± 0.05 <sup>1</sup>	1.02 ± 0.13 <sup>1</sup>	0.012 ± 0.002	0.042 ± 0.005 <sup>2</sup>	0.084 ± 0.009 <sup>2</sup>	0.26 ± 0.04 <sup>2</sup>	0.014 ± 0.002	0.091 ± 0.012 <sup>2</sup>	0.17 ± 0.02 <sup>2</sup>	0.23 ± 0.04 <sup>2</sup>
<sup>1</sup> t		2.523	2.968	3.458	<sup>2</sup> t	2.796	2.690	2.711		2.088	2.068	2.787
<sup>1</sup> P		0.014	0.004	< 0.001	<sup>2</sup> P	0.009	0.012	0.011		0.046	0.048	0.009

<sup>1</sup>t and P value of each group vs corresponding LPS 0 min group; <sup>2</sup>t and P value of each group vs corresponding LPS group at the same time point.

**Table 2** Inhibitory effects of viable LBG or LBG<sub>s</sub> on *H pylori*SS1-LPS-induced IL-8 production (pg/mL)

<i>H pylori</i> SS1-LPS	Viable LBG+LPS	LBG <sub>s</sub> +LPS
0 min	2.62 ± 0.43	2.56 ± 0.46
30 min	2.78 ± 0.38	2.67 ± 0.42
60 min	3.07 ± 0.35	2.93 ± 0.51
120 min	40.39 ± 3.0 <sup>1</sup>	24.12 ± 3.05 <sup>1,2</sup>
<sup>1</sup> t	2.832	2.506
<sup>1</sup> P	0.006	0.015
<sup>2</sup> t	2.121	2.175
<sup>2</sup> P	0.047	0.044

<sup>1</sup>t and P value of each group vs *H pylori*SS1-LPS group at 0 min; <sup>2</sup>t and P value of each group vs *H pylori*SS1-LPS group at 120 min.

## DISCUSSION

*H pylori* have recently been considered an indigenous biota of human stomach and a dominant niche in gastric microecology including *Lactobacilli* and *Sacharomyces* with the capability of cross-species communication<sup>[17-19]</sup>. There is evidence that Helicobacter species are ancient inhabitants of human stomachs for at least 60000 years which have co-evolved with the host and developed their excellent adaption to humans<sup>[20,21]</sup>. Though the vast majority of people in developing countries carry *H pylori*, most of them have no clinical manifestations at all<sup>[22]</sup>. More and more observations are consistent with the hypothesis that *H pylori* have both pathogenetic and symbiotic features, thus relatively balance their cost and benefit<sup>[23,24]</sup>. Changes in life style and sanitation conditions cause a probable disturbance of gastric microecology, which plays a more important role in the pathogenetic mechanism of *H pylori* in the modern era<sup>[25,26]</sup>. Therefore, eradication of *H pylori* does not seem justified for all individuals, especially children. These findings lead to the speculation that we are supposed to domesticate *H pylori* through restoring the homeostasis

of the gastric microecosystem. It was reported that administration of exogenous *Lactobacilli* has therapeutic effects on *H pylori*-associated diseases by interfering with the pathogenetic progress of *H pylori*<sup>[27-30]</sup>. Inhibition of *H pylori*-induced proinflammatory factor production by *Lactobacilli* is a very important aspect. It has been demonstrated that *Lactobacilli* abrogate *H pylori*-mediated IL-8 release *in vitro* and *in vivo*<sup>[31,32]</sup>. A large body of evidence has shown that *H pylori*-LPS-induced inflammation in gastric mucosa has nearly the same pathological characteristics as the mucosal inflammation initiated by *H pylori* infection<sup>[6,7]</sup>. Bhattacharyya *et al*<sup>[33]</sup> reported that pretreatment with LPS inhibitor greatly attenuated *H pylori* extract-mediated gastric mucosal inflammation, suggesting that *H pylori*-LPS may be a major virulent factor for *H pylori*-associated mucosal inflammation, which urged us to research the effect of *Lactobacilli* on *H pylori*-LPS-induced IL-8 production. It has been documented that *H pylori*-LPS induces mucosal inflammation including IL-8 production via TLR4 signaling. In brief, *H pylori*-LPS is recognized by TLR4 of the gastric epithelium, and then activates interleukin-1 receptor-associated kinase, tumor necrosis factor receptor-associated factor-6, TAK1 and TAK1-binding protein 1/2, p38MAPK and NF- $\kappa$ B at last in a cascade mechanism<sup>[7,9]</sup>. However, whether *Lactobacilli* have the capability of inhibiting *H pylori*-LPS-activated TLR4 pathway through interacting with gastric epithelia directly has not been extensively researched. Our findings demonstrate that viable LBG and LBG<sub>s</sub> can prevent TLR4 signaling activation and IL-8 production mediated by *H pylori*SS1-LPS in SGC-7901 cells, which strongly supports the hypothesis that some soluble proteins secreted by LBG and (or) somatic constituents of LBG exert inhibitory effects on the TLR4 pathways in SGC-7901 cells treated with *H pylori*SS1-LPS. Yan *et al*<sup>[34]</sup> reported that *Lactobacillus rhamnosus* GG (LGG) or supernatant recovered from LGG culture MRS

broth (LGG-s) ameliorated apoptosis of young adult mouse colon cells treated with tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interferon- $\gamma$  or interleukin-1 $\alpha$  through blocking p38MAPK and stress-activated protein kinase/c-Jun amino-terminal kinase pathway. In addition, they have identified two proteins in LGG-s with molecular sizes of 80 and 42 kDa, which may be possible substantial effectors in LGG-s<sup>[34]</sup>. In a recent study, they purified the two proteins from LGG-s again, ultimately determined their molecular weight as 75 and 40 kDa, and named them p75 and p40 respectively<sup>[35]</sup>. Their results demonstrate that both p75 and p40 can inhibit TNF- $\alpha$ -induced apoptosis of intestinal epithelia and promote cell growth through activating Akt<sup>[35]</sup>. LBG-s in our experiment probably contained the similar or even same proteins, which could intervene in *H pylori* SS1-LPS-activated TLR4 signaling through modulating other pathways in SGC-7901 cells. Further study is needed to evaluate the hypothesis. In the purification and characterization of the aforementioned potential soluble proteins secreted by LBG, we also detected the effect of heat-killed LBG (hk-LBG) on *H pylori* SS1-LPS-activated TLR4 signaling for evaluating the presumption that some somatic constituents of LBG may inhibit the effect of *H pylori* SS1-LPS. The incomplete data indicate that hk-LBG could also disrupt the *H pylori* SS1-LPS-activated TLR4 pathway. Nevertheless, the effect of hk-LBG was obviously smaller than that of viable LBG at the same concentration.

In conclusion, our evaluation of LBG as a model probiotic organism reveals an important and novel relationship between *H pylori*-LPS-activated TLR4 signaling and selective microflora, and further our understanding of the signal pathways in gastric epithelia involved in inflammatory responses that are regulated by probiotics and pathogenic bacteria composing the gastric microecosystem.

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## COMMENTS

### Background

Some studies have demonstrated that *Helicobacter pylori* (*H pylori*) can stimulate the release of interleukin-8 (IL-8) from gastric epithelia, which initiates inflammatory damage to gastric mucosa and plays a crucial role in the pathogenesis of *H pylori* infection. *H pylori* lipopolysaccharide (*H pylori*-LPS) is a major virulent factor for *H pylori*-associated mucosal inflammation, which induces IL-8 production via activation of the Toll-like receptor 4 (TLR4) signaling pathway in gastric epithelia. Since *H pylori* is an indigenous biota in gastric microflora including *Lactobacilli* and disturbance of the gastric microecosystem plays a more important role in pathogenetic mechanisms of *H pylori*, eradication of *H pylori* in all individuals does not seem justifiable. The gastric microecosystem was restored after treatment with exogenous *Lactobacilli*.

However, whether *Lactobacilli* inhibit *H pylori*-LPS-induced IL-8 production through blocking *H pylori*-LPS-activated TLR4 pathway needs further study.

### Research frontiers

Though there is more and more evidence that *Lactobacilli* have beneficial effects on gastrointestinal diseases, the molecular mechanisms are not well understood, especially in *H pylori*-associated diseases. Whether certain soluble proteins secreted by *Lactobacillus bulgaricus* (LBG) and somatic constituents of LBG directly interact with some signaling pathways in gastric epithelia has not been clearly demonstrated. Our study indicated that *Lactobacillus rhamnosus* GG secreted p75 and p40, two proteins with a molecular size of 75 and 40 kDa respectively, could ameliorate apoptosis of intestinal epithelia treated with tumor necrosis factor  $\alpha$ , interferon- $\gamma$  or interleukin-1 $\alpha$  and promote cell growth through activating Akt, blocking p38 mitogen-activated protein kinase and stress-activated protein kinase/c-Jun amino-terminal kinase signaling. The supernatant recovered from LBG culture MRS broth (LBG-s) in our experiment may contain the similar or even same proteins, which could intervene in *H pylori* Sydney strain 1 lipopolysaccharide (*H pylori* SS1-LPS)-activated TLR4 signaling through modulating other pathways in SGC-7901 cells, suggesting that probiotic bacterial components may be useful in preventing *H pylori*-associated diseases.

### Innovations and breakthroughs

Viable LBG or LBG-s blocked the *H pylori* SS1-LPS-activated TLR4 pathway in SGC-7901 cells, leading to their inhibitory effects on IL-8 production induced by *H pylori* SS1-LPS.

### Applications

This study implied that certain soluble proteins secreted by *Lactobacilli* could directly interact with some signaling pathways in gastric epithelia and partly block the noxious effects of *H pylori*. This may benefit the exploration of new drugs for the treatment of *H pylori*-associated diseases.

### Peer review

The results of this study indicate that *H pylori* SS1-LPS could increase IL-8 production through TLR4 signaling in gastric epithelia and probiotic bacteria attenuate IL-8 production via inhibition of the TLR4 pathway. The manuscript contains some interesting data, viable probiotics and their extracts against *H pylori*-LPS cytotoxicity.

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CASE REPORT

## Large solitary ovarian metastasis from colorectal cancer diagnosed by endoscopic ultrasound

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### INTRODUCTION

Approximately 148 000 new cases of colorectal cancer are detected yearly in the USA<sup>[1]</sup>. Ovarian metastasis has been reported in various studies to occur in 3%-14% of patients<sup>[2-5]</sup>. Endoscopic ultrasound (EUS) has been shown to have high accuracy in staging rectal cancer<sup>[6]</sup>. The use of EUS for detecting ovarian metastasis from colorectal cancer has not been established. We describe a case of a patient with ovarian metastasis from a rectal cancer that was not detected on computer tomography (CT) scan but detected as a peri-rectal mass on preoperative EUS.

### CASE REPORT

#### Abstract

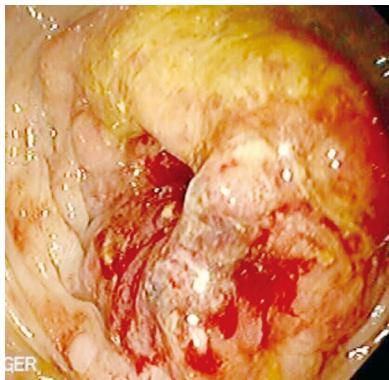
A case is presented of rectal carcinoma in which during staging by endoscopic ultrasound (EUS) a second large extrarectal mass was seen not otherwise visualized on computer tomography (CT) that was a solitary ovarian metastasis. The surgeon was alerted to the EUS finding prior to the planned laparoscopic colectomy. On retrospective review of the CT pelvis after surgery, the radiologist could still not diagnose the ovarian lesion separated from the primary rectal tumor due to their close proximity. However, on EUS we were able to clearly see on real-time imaging that there was a distinct peri-rectal mass apart from the primary rectal tumor.

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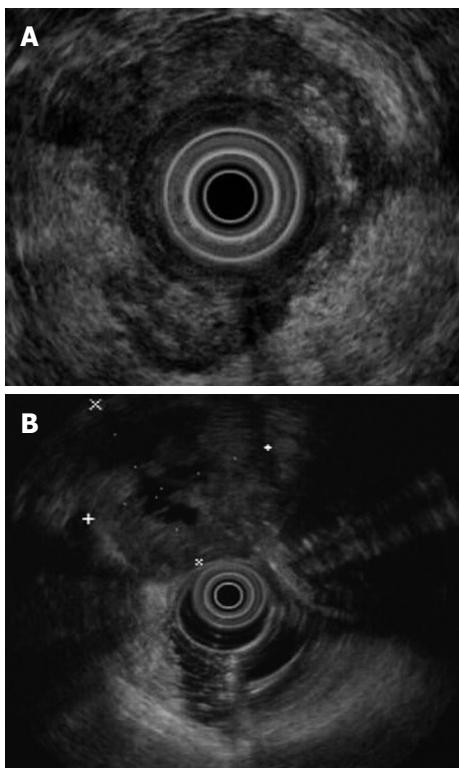
**Key words:** Colorectal cancer; Endoscopic ultrasound; Ovary; Ovarian metastasis; Endoscopic ultrasound; Endosonography

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Moparty B, Gomez G, Bhutani MS. Large solitary ovarian metastasis from colorectal cancer diagnosed by endoscopic ultrasound.



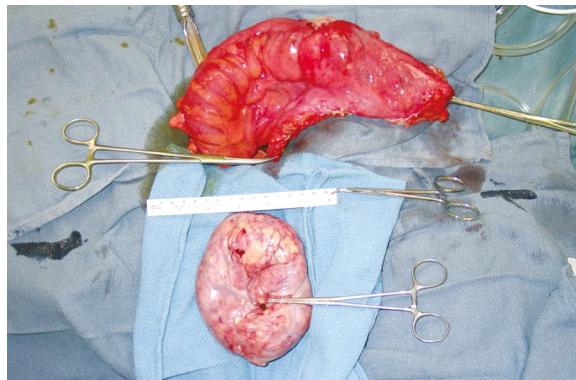
**Figure 1** Colonoscopic view of an obstructing rectal cancer.



**Figure 2** Radial EUS. A: Cancer in Figure 1 demonstrating it to be a T3 lesion; B: Revealing an extra-rectal hypoechoic round mass (between the calipers) measuring 5 cm in size, inferior to and clearly distinct from the primary rectal mass shown in Figures 1 and 2A.

## DISCUSSION

Colorectal cancer with ovarian metastasis has been reported in multiple studies<sup>[2-5]</sup>. Patients generally present with vague symptoms<sup>[7]</sup>. Colonoscopy or barium enema can help identify an intrinsic colonic lesion. If a rectal cancer is detected, EUS is performed to stage the cancer by assessing the extent of infiltration, and the presence/absence of lymph nodes<sup>[6]</sup>. EUS also allows for visualization of adjacent organs such as bladder or prostate. CT abdomen/pelvis is generally performed to evaluate metastasis of the colorectal cancer to the liver and other organs. It may be difficult at times to assess on CT, concomitant adjacent pelvic organ metastasis due to the close proximity to the bowel or to be able to differentiate the origin of a pelvic mass, whether



**Figure 3** Gross pathologic findings at surgery showing the resected rectal carcinoma and the solitary ovarian metastases correlating with the findings in Figure 2.

colorectal or perirectal.

Our case demonstrates the utility of EUS in detecting peri-rectal lesion, which in our patient was difficult to detect even on retrospective review of the CT. Combining information from imaging modalities such as CT and EUS may be even more important when minimally invasive surgical techniques are employed for cancer surgery to provide the surgeon with the maximal amount of pre-operative information. If a solitary ovarian lesion is noted, oophorectomy is generally performed. There has been debate on whether routine bilateral oophorectomy should be performed routinely in those undergoing surgery for colorectal cancer<sup>[8]</sup>. Some recommend discussing this option with the patient prior to surgery since reports suggest that ovarian metastasis occurs in 3%-14%<sup>[2-5]</sup>.

In conclusion, for patients who present with a pelvic mass, and are found to have a colorectal cancer, EUS may be performed preoperatively to detect adjacent perirectal masses such as ovarian metastatic lesions.

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CASE REPORT

## Serious drug-induced liver disease secondary to ezetimibe

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intestinal uptake of dietary and biliary cholesterol. It was approved by the FDA in 2002 for hypercholesterolemia alone or associated with statins. Since then, the use of ezetimibe has increased, especially in the United States<sup>[1]</sup>. Some guidelines suggest that ezetimibe may become the first choice for patients who do not tolerate statins<sup>[2]</sup>. Randomized clinical trials showed that ezetimibe caused significant liver function abnormalities in 1% of the treatment group. These changes were asymptomatic and entirely reversible. We report a case of serious hepatitis secondary to ezetimibe.

### CASE REPORT

A 56-year-old woman was admitted because of painless jaundice and pruritus. Her past medical history revealed essential hypertension, uterine myoma and a hiatal hernia. She never consumed more than 1 alcoholic drink per day. In the last three years, her medication was unchanged and consisted of bisoprolol 10 mg/d and omeprazol 20 mg/d. Because of persistent hypercholesterolemia, her family doctor prescribed ezetimibe 10 mg/d for 4 mo before admission. She was referred to our unit because of apparition of jaundice, a progressive scratching and liver test abnormalities.

At physical exam, there was intense jaundice and some scraping lesions. Her liver was not palpable and there were no further abnormalities. Laboratory investigation showed the following results: aspartate transaminase, 16.64 kat/L (normal, < 0.5 µkat/L); alanine transaminase, 26.26 µkat/L (normal, < 0.56 µkat/L); γ-glutamyl transferase, 1.15 µkat/L (normal, < 0.43 µkat/L); alkaline phosphatase, 1.9 µkat/L (normal, < 1.5 µkat/L); total bilirubin, 602 µmol/L (normal, < 18 µmol/L). Blood count, prothrombin time and kidney function test were normal. Viral serology tests were negative (hepatitis A, B, C, Epstein-Barr virus, Herpes virus, HIV and cytomegalovirus). Autoimmune serology (antinuclear, antimitochondrial, anti-liver-kidney microsomal and anti-smooth muscle) was all negative. At ultrasoundography, the liver showed no abnormality and biliary obstruction was disclosed.

Ezetimibe was stopped. Jaundice and pruritus improved slowly as well as laboratory results. Four weeks later a laboratory exam was completely normal.

### Abstract

Ezetimibe is the first member of a new family of lipid-lowering drugs that inhibits uptake of dietary and biliary cholesterol. It was approved by the FDA in 2002 for hypercholesterolemia alone or in combination with statins. Its use has been spreading over the last years. Ezetimibe was considered a safe drug. We report a case of a woman who developed a serious hepatocellular drug-induced liver disease after 4 mo therapy with 10 mg daily of ezetimibe. After withdrawal of the drug, the patient recovered slowly. Ezetimibe may produce serious toxic hepatitis and prompt withdrawal is mandatory in case of a significant abnormality in liver testing after beginning or during treatment with ezetimibe.

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**Key words:** Ezetimibe; Hepatitis; Drug-induced liver disease

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Castellote J, Ariza J, Rota R, Girbau A, Xiol X. Serious drug-induced liver disease secondary to ezetimibe. *World J Gastroenterol* 2008; 14(32): 5098-5099 Available from: URL: <http://www.wjgnet.com/1007-9327/14/5098.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.5098>

### INTRODUCTION

Ezetimibe is the first lipid-lowering drug that inhibits

### DISCUSSION

The temporal association, the improvement after

cessation of the drug and the exclusion of alternative possible causes permit us to diagnose ezetimibe-induced hepatotoxicity with confidence. Although rechallenge with the same drug could give us a definitive diagnosis, the clinical severity of the hepatitis made us exclude this possibility. Liver biopsy can suggest hepatotoxicity or exclude alternative diagnoses but it was not performed in this case.

Ezetimibe does not affect the cytochrome P450 system; it undergoes extensive glucuronidation in the wall of the small intestine and liver to form the active metabolite ezetimibe glucuronide. Ezetimibe does not induce or inhibit enzyme systems in the liver but undergoes enterohepatic circulation and is exposed to the liver and bile.

Statins rarely cause clinically significant liver injury, although asymptomatic elevations in aminotransferases are common. It has been suggested that this laboratory abnormality may result from muscle damage and not by hepatic injury<sup>[3]</sup>. Ezetimibe does not interfere with plasma levels of HMG-coA reductase inhibitors such as atorvastatin and simvastatin. Association of ezetimibe and statins does not lead to an apparent increase of hepatic side effects. Ezetimibe was expected to be even less hepatotoxic than statins and has been considered a safe drug. Nevertheless, it should be kept in mind that in a newly marketed drug, finding hepatotoxicity with an incidence of 1:10000 (the approximate incidence of most idiosyncratic reactions) would require 30 000 patients treated with this drug<sup>[4]</sup>.

The pattern of drug-related liver injury can be classified in hepatocellular, cholestatic or mixed according to laboratory data<sup>[5]</sup>. Until now, four cases of significant drug-induced liver injury associated with ezetimibe have been reported<sup>[6-8]</sup>, three of them associated with atorvastatin. One case was a cholestatic type, another one was hepatocellular and the last two were drug-induced acute autoimmune hepatitis. Our patient presented as serious hepatocellular hepatitis<sup>[9]</sup>. Patients with acute toxic hepatocellular damage are at high risk of acute liver failure. Mortality, or its surrogate marker, liver transplantation, is superior to 10%; this is known as "Hy's rule".

Although causes of ezetimibe toxicity are unclear,

apart from an idiosyncratic drug reaction, it could be hypothesized that a conjugation defect leads to accumulation of toxic levels of ezetimibe or their metabolites. As hepatotoxicity by ezetimibe has been reported in different types according to laboratory data, several mechanisms may be implied.

The current recommendation to monitor liver function after beginning therapy with statins is controversial with some experts calling for its re-examination. Ezetimibe, alone or associated to statins, may produce serious toxic hepatitis. Caution should be taken and the necessity of analytical follow-up after beginning or during the treatment with ezetimibe is unknown. A prompt withdrawal of the drug is mandatory in case of a significant abnormality in liver tests.

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## Meetings

### Events Calendar 2008-2009

FALK SYMPOSIA 2008  
January 24-25, Frankfurt, Germany  
Falk Workshop: Perspectives in Liver Transplantation

International Gastroenterological Congresses 2008  
February 14-16, Paris, France  
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies  
[www.easl.ch/hepatitis-conference](http://www.easl.ch/hepatitis-conference)

February 14-17, Berlin, Germany  
8<sup>th</sup> International Conference on New Trends in Immunosuppression and Immunotherapy  
[www.kenes.com/immuno](http://www.kenes.com/immuno)

February 28, Lyon, France  
3<sup>rd</sup> Congress of ECCO - the European Crohn's and Colitis Organisation Inflammatory Bowel Diseases 2008  
[www.ecco-ibd.eu](http://www.ecco-ibd.eu)

February 29, Québec, Canada  
Canadian Association of Gastroenterology  
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK  
British Society of Gastroenterology Annual Meeting  
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China  
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea  
Asian Pacific Association for the Study of the Liver  
18<sup>th</sup> Conference of APASL: New Horizons in Hepatology  
[www.apaslseoul2008.org](http://www.apaslseoul2008.org)

March 29-April 1, Shanghai, China  
Shanghai-Hong Kong International Liver Congress  
[www.livercongress.org](http://www.livercongress.org)

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco  
OESO 9<sup>th</sup> World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas  
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA  
SAGES 2008 Annual Meeting - part of Surgical Spring Week  
[www.sages.org/08program/html/](http://www.sages.org/08program/html/)

April 18-22, Buenos Aires, Argentina  
9<sup>th</sup> World Congress of the International Hepato-Pancreato Biliary Association  
Association for the Study of the Liver  
[www.ca-ihpba.com.ar](http://www.ca-ihpba.com.ar)

April 23-27, Milan, Italy  
43<sup>rd</sup> Annual Meeting of the European Association for the Study of the Liver  
[www.easl.ch](http://www.easl.ch)

May 2-3, Budapest, Hungary  
Falk Symposium 164: Intestinal

### Disorders

May 18-21, San Diego, California, USA  
Digestive Disease Week 2008

May 21-22, California, USA  
ASGE Annual Postgraduate Course Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion  
E-mail: education@sgo.org

June 4-7, Helsinki, Finland  
The 39<sup>th</sup> Nordic Meeting of Gastroenterology  
[www.congrex.com/ngc2008](http://www.congrex.com/ngc2008)

June 5-8, Sitges (Barcelona), Spain  
Semana de las Enfermedades Digestivas  
E-mail: sepd@sepdi.es

June 6-8, Prague, Czech Republic  
3<sup>rd</sup> Annual European Meeting: Perspectives in Inflammatory Bowel Diseases  
E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey  
ESGAR 2008 19<sup>th</sup> Annual Meeting and Postgraduate Course  
E-mail: fca@netvisao.pt

June 11-13, Stockholm, Sweden  
16<sup>th</sup> International Congress of the European Association for Endoscopic Surgery  
E-mail: info@aes-eur.org

June 13-14, Amsterdam, Netherlands  
Falk Symposium 165: XX International Bile Acid Meeting, Bile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic  
Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management  
E-mail: idca2008@guantar.cz

June 25-28, Barcelona, Spain  
10<sup>th</sup> World Congress on Gastrointestinal Cancer  
Imedex and ESMO  
E-mail: meetings@imedex.com

June 25-28, Lodz, Poland  
Joint Meeting of the European Pancreatic Club (EPC) and the International Association of Pancreatology (IAP)  
E-mail: office@epc-iap2008.org  
[www.e-p-c.org](http://www.e-p-c.org)  
[www.pancreatology.org](http://www.pancreatology.org)

June 26-28, Bratislava, Slovakia  
5<sup>th</sup> Central European Gastroenterology Meeting  
[www.ceurgem2008.cz](http://www.ceurgem2008.cz)

July 9-12, Paris, France  
ILTS 14<sup>th</sup> Annual International Congress  
[www.ilts.org](http://www.ilts.org)

September 10-13, Budapest, Hungary  
11<sup>th</sup> World Congress of the International Society for Diseases of the Esophagus  
E-mail: isde@isde.net

September 13-16, New Delhi, India  
Asia Pacific Digestive Week  
E-mail: apdw@apdw2008.net

APDW 2008  
September 13-16, New Delhi, Indian Organized: Indian Society of Gastroenterology

III FALK GASTRO-CONFERENCE

September 17, Mainz, Germany  
Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany  
Falk Symposium 166: GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic  
Prague Hepatology Meeting 2008  
[www.czech-hepatology.cz/phm2008](http://www.czech-hepatology.cz/phm2008)

September 20-21, Mainz, Germany  
Falk Symposium 167: Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France  
Third Annual Meeting European Society of Coloproctology  
[www.escp.eu.com](http://www.escp.eu.com)



October 8-11, Istanbul, Turkey  
18<sup>th</sup> World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists  
E-mail: orkun.sahin@serenas.com.tr

October 18-22, Vienna, Austria  
16<sup>th</sup> United European Gastroenterology Week  
[www.negf.org](http://www.negf.org)  
[www.acv.at](http://www.acv.at)

October 22-25, Minnesota, USA  
Anstralian Gastroenterology Week 2008  
E-mail: gesa@gesa.org.au

October 22-25, Brisbane, Australia  
71<sup>st</sup> Annual Colon and Rectal Surgery Conference  
E-mail: info@colonrectalcourse.org

October 31-November 4, Moscone West Convention Center, San Francisco, CA  
59<sup>th</sup> AASLD Annual Meeting and Postgraduate Course  
The Liver Meeting Information: [www.aasld.org](http://www.aasld.org)

November 6-9, Lucerne, Switzerland  
Neurogastroenterology & Motility Joint International Meeting 2008  
E-mail: ngm2008@mci-group.com  
[www.ngm2008.com](http://www.ngm2008.com)

November 12, Santiago de Chile, Chile  
Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

November 28-29, Cairo, Egypt  
1<sup>st</sup> Hepatology and Gastroenterology Post Graduate Course  
[www.egyptgastrohep.com](http://www.egyptgastrohep.com)

December 7-9, Seoul, Korea  
6<sup>th</sup> International Meeting Hepatocellular Carcinoma: Eastern and Western Experiences  
E-mail: sglee@amc.seoul.kr

INFORMATION FOR ALL  
FALK FOUNDATION e.V.  
E-mail: [symposia@falkfoundation.de](mailto:symposia@falkfoundation.de)  
[www.falkfoundation.de](http://www.falkfoundation.de)

Advanced Courses - European

Institute of Telesurgery EITS - 2008 Strasbourg, France  
January 18-19, March 28-29, June 6-7, October 3-4

N.O.T.E.S  
April 3-5, November 27-29  
Laparoscopic Digestive Surgery

June 27-28, November 7-8  
Laparoscopic Colorectal Surgery

July 3-5  
Interventional GI Endoscopy Techniques  
Contact address for all courses:  
E-mail: [info@eits.fr](mailto:info@eits.fr)

International Gastroenterological Congresses 2009  
March 23-26, Glasgow, Scotland  
Meeting of the British Society of Gastroenterology (BSG)  
E-mail: [bsg@mailbox.ulcc.ac.uk](mailto:bsg@mailbox.ulcc.ac.uk)

May 17-20, Denver, Colorado, USA  
Digestive Disease Week 2009

November 21-25, London, UK  
Gastro 2009 UEGW/World Congress of Gastroenterology  
[www.gastro2009.org](http://www.gastro2009.org)



### Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London, The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.



## Instructions to authors

### GENERAL INFORMATION

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- 3 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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- 4 Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

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- 5 Vallancien G, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

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*Personal author(s)*

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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- 13 **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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- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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- 15 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/eid.htm>

*Patent (list all authors)*

- 16 **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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