

# World Journal of *Gastroenterology*

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2010-2013

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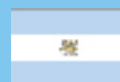
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## HLA class II associated with outcomes of hepatitis B and C infections

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### Abstract

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

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

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

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

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### INTRODUCTION

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

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

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

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

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

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

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

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

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

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

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

### HBV infection

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



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

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

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

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

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

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

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

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

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

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

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

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

### HCV infection

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

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

### FUTURE DIRECTIONS

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

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

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

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

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

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

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

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

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

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

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### Abstract

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

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### INTRODUCTION

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

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

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

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

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

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

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

## IMMUNITY IN NAFLD

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

## IMMUNITY IN IBS

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

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

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

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



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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

## NAFLD AND IBS MAY BE RELATED

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

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

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

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

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

## GUT MICROBIOTA

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

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

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

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

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

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

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

## Obesity and NAFLD

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

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

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

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

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

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

## IBS

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

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

## INTESTINAL PERMEABILITY

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

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

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

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

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

## NAFLD and NASH

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

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

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

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



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

## IBS

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

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

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

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

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

## INTESTINAL MOTILITY

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

Mice infected with *Trichinella spiralis* develop muscle

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

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

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

## Obesity and NAFLD

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

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

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

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

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

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

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

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

## IBS

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

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

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

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

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

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

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

## CNS INVOLVEMENT

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

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

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

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

### Obesity and NAFLD

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

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

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

### IBS

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

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

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

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

### UNANSWERED QUESTIONS

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

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## Current status in the treatment options for esophageal achalasia

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### Abstract

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

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

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

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

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## INTRODUCTION

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

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

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

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

## TREATMENT OF ESOPHAGEAL ACHALASIA

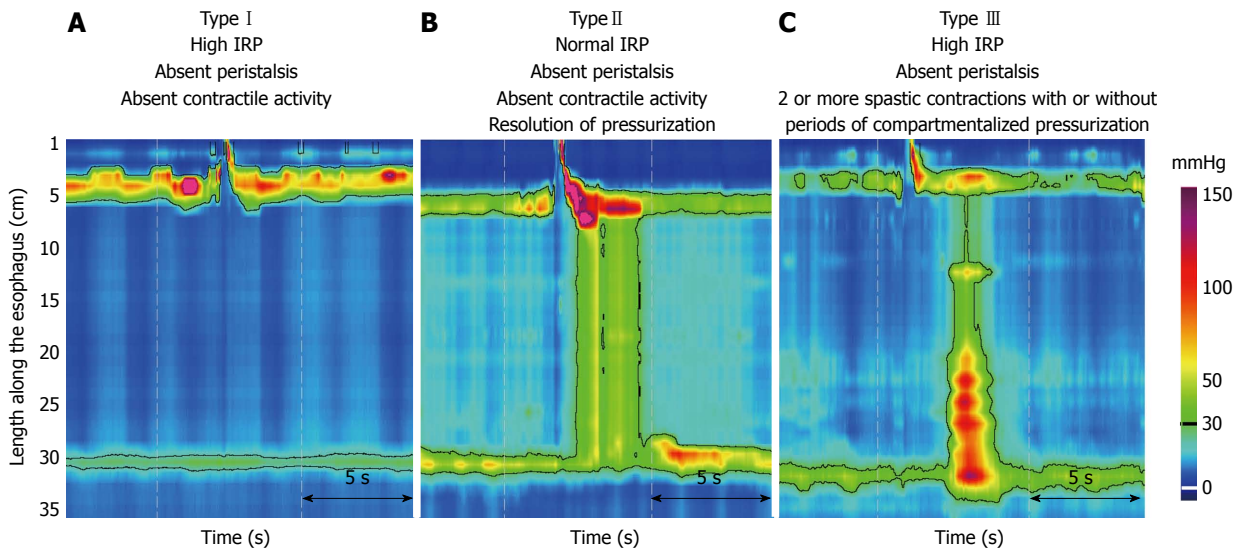
### Pharmacological management

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

### Endoscopic treatment

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

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



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

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

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

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

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

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

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

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

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

BMD: Browne-McHardy dilator.

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

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

### Surgical treatment

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

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

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

LHM: Laparoscopic Heller myotomy.

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

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

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



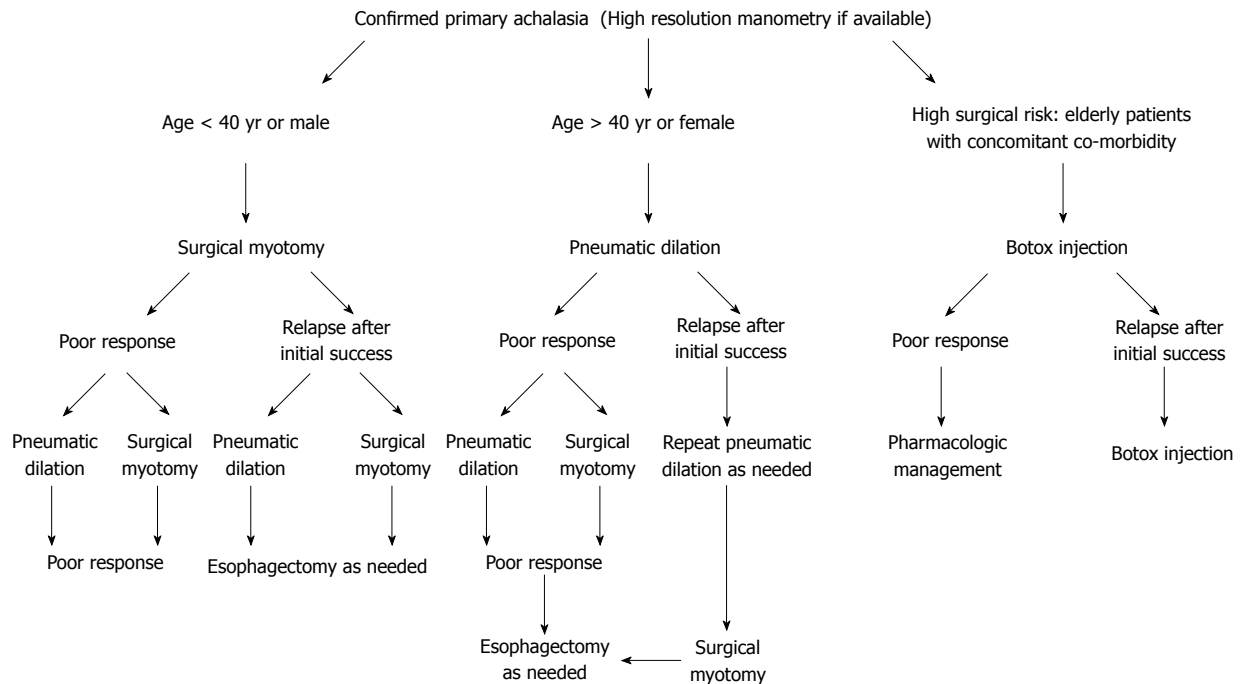


Figure 2 Proposed algorithm for the treatment of esophageal achalasia.

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

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

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

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

## Surgery vs PD

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

Complications and cost-effectiveness, besides the durability of the procedure, are the main concerns for deciding on a treatment option. Perforation of the esophagus occurs in 1%-2% of patients during PD and can be hazardous if left undiscovered<sup>[31]</sup>. Mucosal tears occur in 12% of patients during LHM but can usually be repaired, and the patients recover. However, the main drawback of LHM is the incidence of acid reflux after surgery, which

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

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

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

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

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

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

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

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

## FUTURE PERSPECTIVES

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

## CONCLUSION

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

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

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

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

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

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### Abstract

Primary liver cancer and liver metastases are among

## INTRODUCTION

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

## PURPOSE

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

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

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

## LITERATURE SEARCHES

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

## DESCRIPTION OF MWA

### Mechanism

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

### Technical advantages

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



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

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

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

### Equipment

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

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

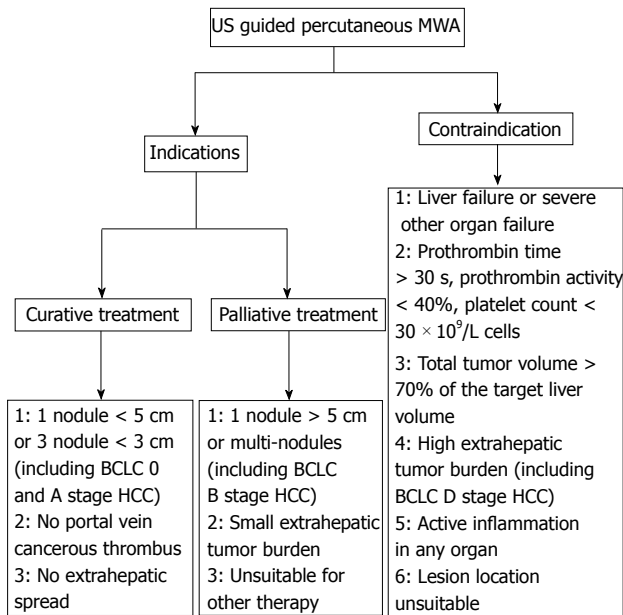
Some types of commercially available radiofrequency devices contain a thermocouple in the nickel-titanium lateral tine of expandable electrode tip to allow temperature recording during the ablation procedure. The aim of temperature monitoring is to ensure that the maximum energy is applied by using the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

## DIAGNOSIS AND INDICATIONS

### Diagnosis

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





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

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

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

### Indications

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

For patients with very early stage and early stage HCC

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

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

### Contraindications

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

## PATIENT PREPARATION AND DATA REQUIRED

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

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

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

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

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

## TECHNIQUES

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

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

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

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

## CARE AFTER MWA

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

## COMBINED TREATMENT WITH OTHER MODALITIES

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

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

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

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

## FOLLOW-UP AND THERAPEUTIC EFFICACY ASSESSMENT

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

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

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

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

## DISCLAIMER

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



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

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

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

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## Epigenetics of hepatocellular carcinoma: Role of microRNA

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

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

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

### Abstract

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

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### INTRODUCTION

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

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

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

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

## ABERRANT EXPRESSION OF MICRORNAS IN HCC

MiRs play a central role in basic biological processes such

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

## CLINICAL SIGNIFICANCE AND TRANSLATIONAL APPLICATIONS OF MICRORNAS IN HCC

### Single nucleotide polymorphism in miRs

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

### miRs as biomarkers in HCC

miRs are prognostic markers of HCC. Down-regulation



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

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

miRs: MicroRNAs; EMT: Epithelial-mesenchymal transition.

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

### miRs as therapeutic targets in HCC

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

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

## CONCLUSION

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

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

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### Abstract

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

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

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

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

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### INTRODUCTION

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

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

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

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

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

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

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

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

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

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

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

## **HBV GENOTYPE H**

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

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

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

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

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

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

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

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

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

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

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

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

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

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

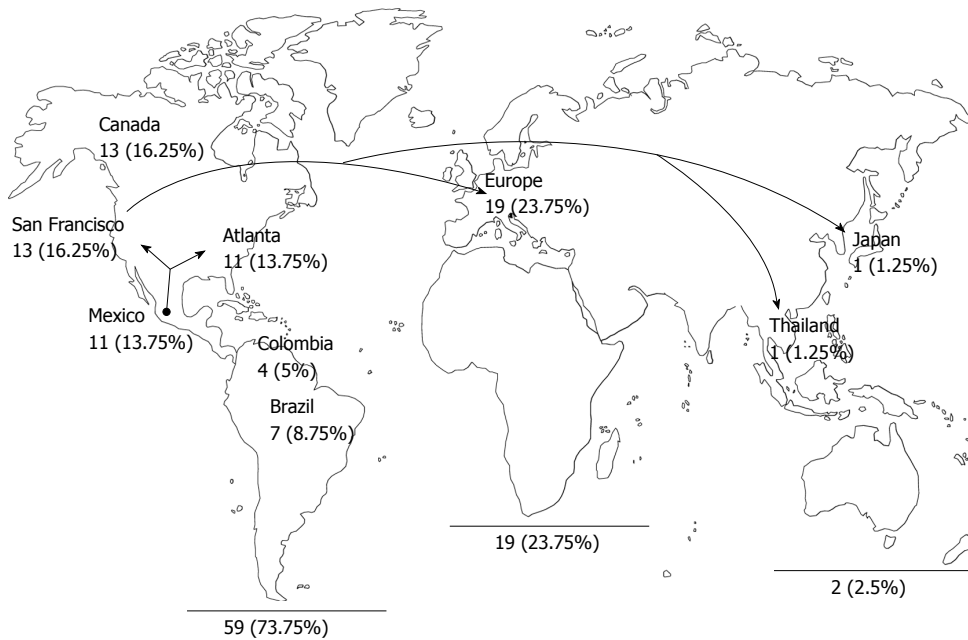
This matter raises a word of caution regarding the strategy for antiviral therapy in Mexico since the main cause of liver disease may be attributed to co-morbidities, such as HCV or HIV infection, alcoholism or obesity, but not to HBV infection exclusively. Therefore, antiviral therapy for HBV/H-infected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

## **HBV GENOTYPE G**

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

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





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

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

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

### Molecular epidemiology of HBV/G in Mexico

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

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

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

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

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

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

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

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

## CONCLUSION

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

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## Biliary phosphatidylcholine and lysophosphatidylcholine profiles in sclerosing cholangitis

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

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

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

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

### Abstract

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

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

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

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

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## INTRODUCTION

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

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

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

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

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

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

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

## MATERIALS AND METHODS

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

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

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

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

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

### Control group

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

### Patients with PSC

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

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

### Patients with SSC

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

### Patients with CCC

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



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

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

PC: Phosphatidylcholine.

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

### Collection and storage of bile specimens

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

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

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

### Bacterial cultures

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

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

### Lipid extraction from bile specimens

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

### Nano-electrospray ionization tandem mass spectrometry

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

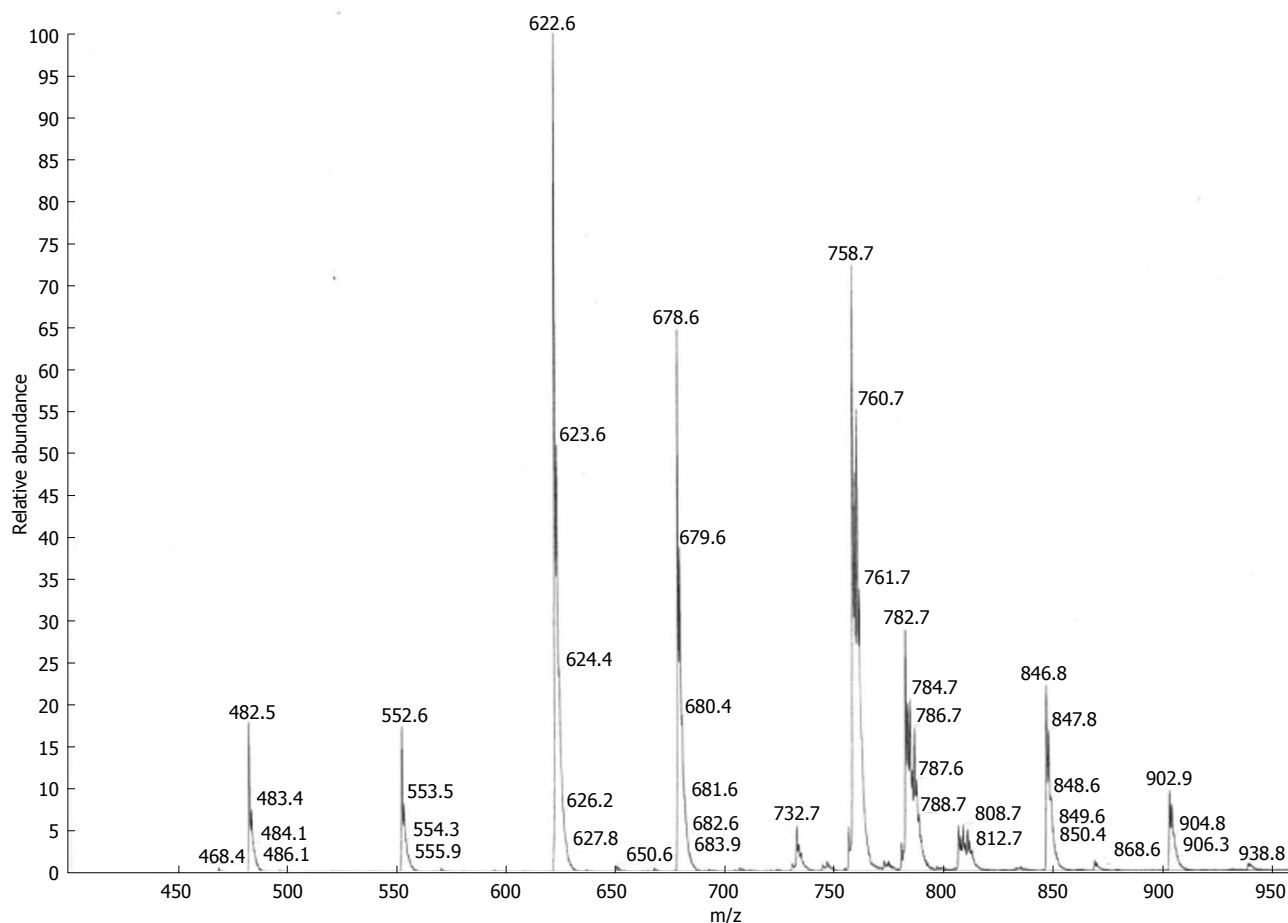
### Statistical analysis

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

## RESULTS

### Clinical data

Statistical differences in the clinical data of the four pa-



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

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

### Biliary phospholipid analysis

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

### Biliary PC species profiles

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

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

### LPC concentrations and LPC/PC ratios

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

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

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

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

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

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

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

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

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

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

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

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

#### Ratios of phospholipids to other bile components

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

#### Bacterial cultures and biliary phospholipids

At certain concentrations, bacteria in hepatic bile are

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

## DISCUSSION

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

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

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

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

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

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

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



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

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

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

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

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

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

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

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

## COMMENTS

### Background

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

### Research frontiers

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

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

### Innovations and breakthroughs

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

### Applications

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

### Terminology

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

### Peer review

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

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

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### Abstract

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

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

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

**RESULTS:** The PVP in the Portal group was significantly lower than that in the Systemic group during the time period from 2 to 49 h PH ( $P < 0.05$ ). Serum alanine aminotransferase (ALT), total bilirubin (TB) and ammonia were significantly reduced in the Portal group compared with the Systemic group from 24 to 48 h PH ( $P < 0.05$ ). The Portal group may have attenuated sinusoidal endothelial injury and decreased the level of HA compared with the Systemic group. In the Systemic group, there was significant sinusoidal dilation, hydropic changes in hepatocytes and hemorrhage into the hepatic parenchyma, and the sinusoidal endothelial lining was partially destroyed and detached into the sinusoidal space. CD31 immunostaining revealed significant destruction of the endothelial lining. In the Portal group, there was no intraparenchymal hemorrhage and the sinusoidal endothelial cells and hepatocytes were well preserved. CD31 immunostaining was mild which indicated less destruction of the endothelial lining. HA was significantly decreased in the Portal group compared with the Systemic group from 2 to 48 h PH. The rate of liver remnant regeneration was elevated, while apoptosis was attenuated in the Portal group compared with the Systemic group. Thymidine kinase activity was much higher in the Portal group than in the Systemic group at 48 h PH. The PCNA index was significantly increased and the apoptotic index was significantly decreased in the Portal group compared with the Systemic group. Bacterial translocation and endotoxin, as well as the inflammatory response, were significantly attenuated in the Portal group compared with the Systemic group. LPS, tumor necrosis factor- $\alpha$  and interleukin-6 levels were all significantly decreased in the Portal



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

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

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

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

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## INTRODUCTION

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

## MATERIALS AND METHODS

### Animal model of PLF and SFSS

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

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

### Study protocol

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

### Postoperative management

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

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

### Plasma extraction

During each extraction, 200–300 mL plasma was obtained from 6 donor pigs *via* the internal jugular vein catheter and immediately frozen at  $-20^{\circ}\text{C}$ .

### PVF, HAF, and PVP measurement

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

### Blood and serum analysis

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

### Histological analysis

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

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

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

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

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

### Measurement of LPS levels

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

### Bacterial translocation

Total bacterial quantification was accomplished by DNA isolation and real-time polymerase chain reaction (PCR). DNA was isolated from blood using the Fast DNA Spin Kit (Cat. 69506; Qiagen, United States) according to the manufacturer’s instructions. Subsequently, total bacterial quantification was performed with 16S rRNA gene-targeted primers. The sequences of the universal primers were as follows: 5’TTCCGGTGTGATCTGCCGGA3’ forward and 5’GGTTACCTTGTACGACTT-3’ reverse<sup>[18,19]</sup>. The serially diluted genomic DNA of selected bacterial isolates was used as a real-time PCR positive control for total bacterial quantification. Bacterial counts are expressed as Log<sub>10</sub> cells per gram tissue (cells/g).

### Quantification of serum cytokine levels

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

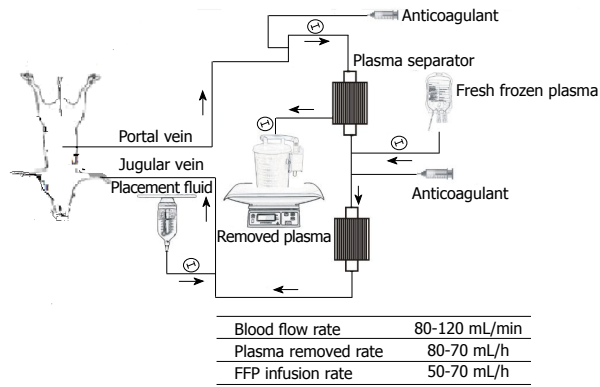
### Statistical analysis

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

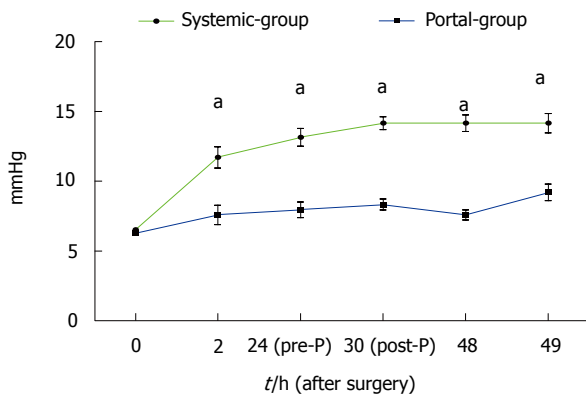
## RESULTS

### Arrangement of the experiment and the hemodynamic studies

Extracorporeal continuous portal diversion (ECPD) plus



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



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

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

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

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

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

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

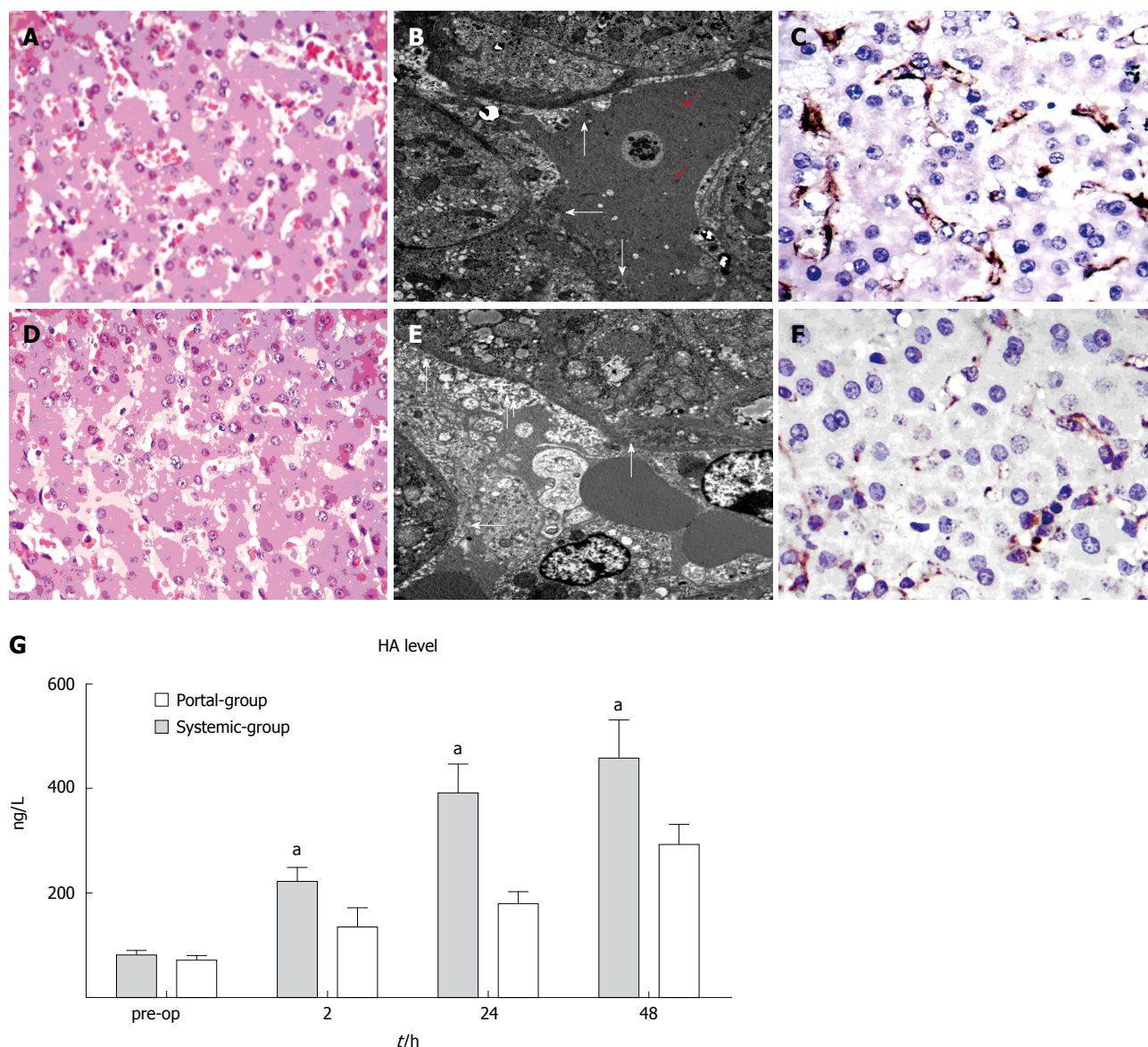
The infusion plasma volume was equal to 1.3 times the plasma volume that had been removed per hour in each pig. The total exchanged plasma volume was 4000-5000 mL each time. The adequacy of anticoagulation was monitored by activated clotting time (ACT), and heparin was administered as required to maintain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

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

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

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





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

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

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

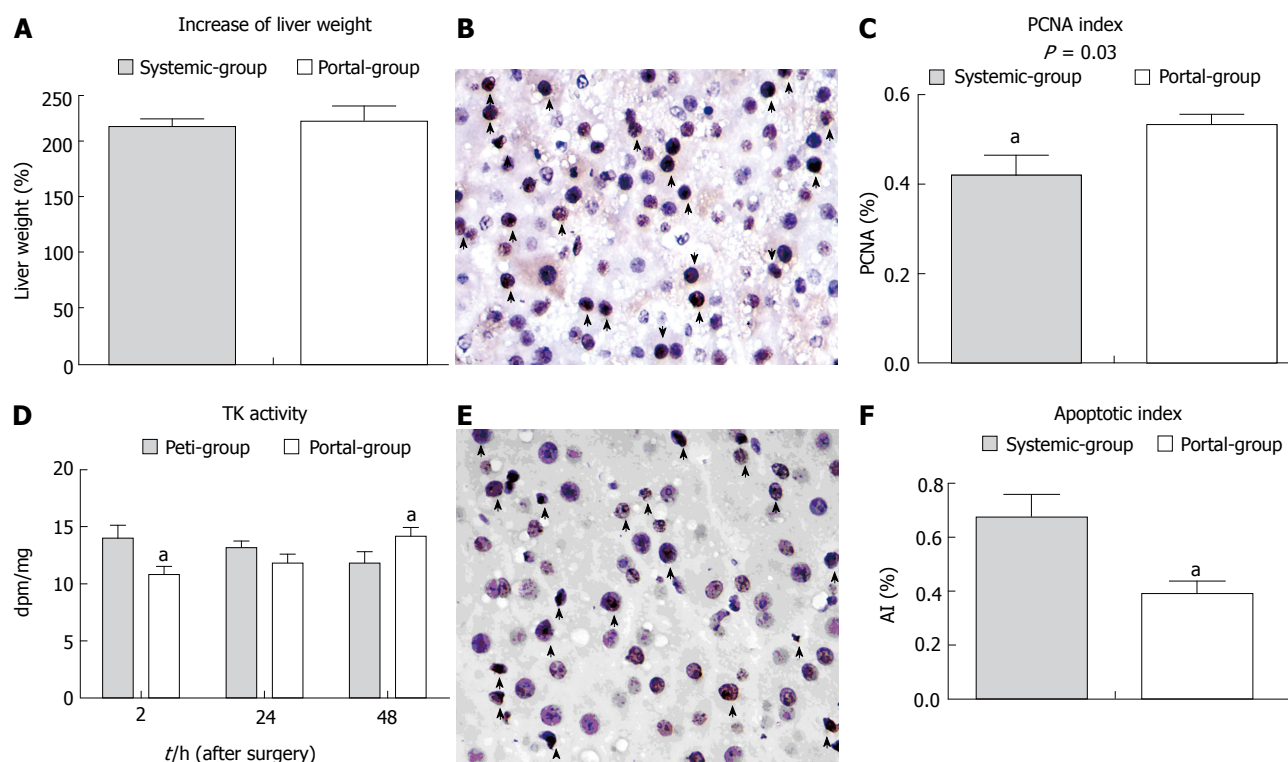
Levels of serum ammonia, ALT and TB collected serially during the follow-up period are shown in Table 2. The results showed that serum ALT, TB and ammonia were significantly reduced in the Portal group compared with the Systemic group from 24 to 48 h PH ( $P < 0.05$ ). In addition, serum ALT and ammonia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

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

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

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





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

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

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

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

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

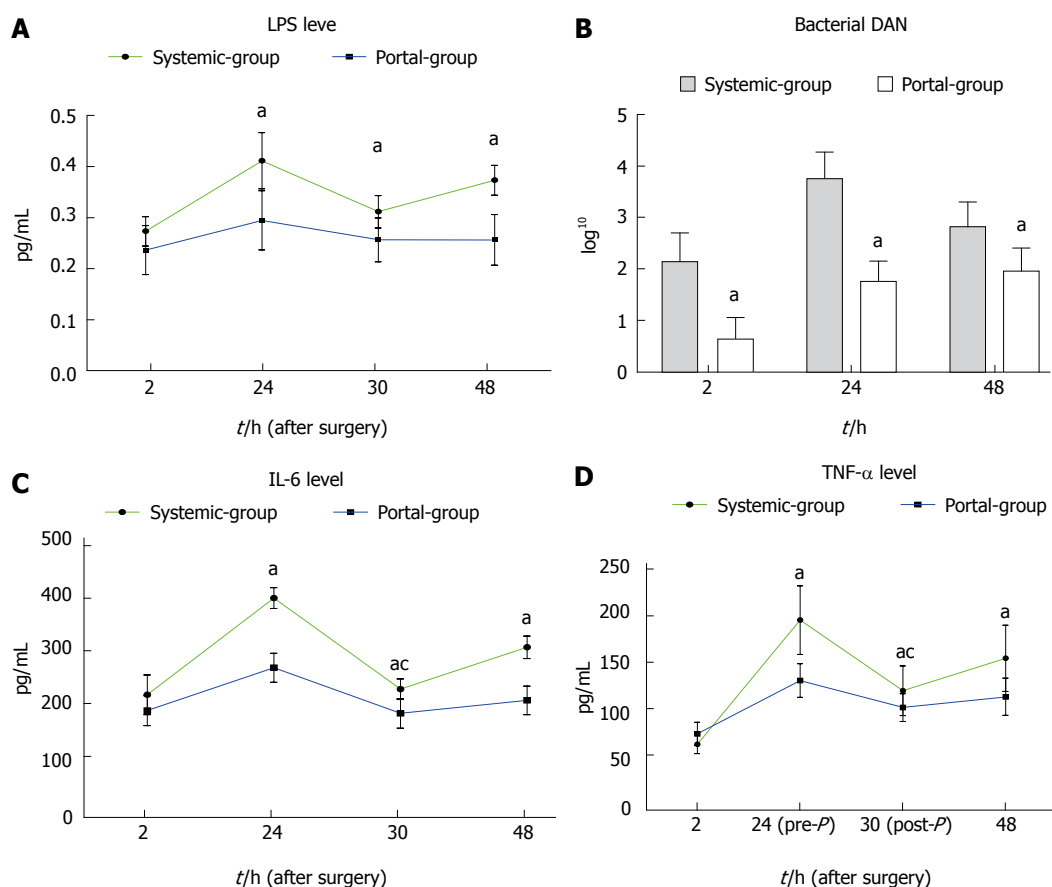
#### Rate of liver remnant regeneration was elevated while apoptosis was attenuated in the Portal group compared with the Systemic group

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

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

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

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



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

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

## DISCUSSION

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

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

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

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

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

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

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

## COMMENTS

### Background

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

### Research frontiers

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

### Innovations and breakthroughs

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

### Applications

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

### Peer review

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

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

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### Abstract

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

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

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

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

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

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

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

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

Yan B, Shi J, Xiu LJ, Liu X, Zhou YQ, Feng SH, Lv C, Yuan XX, Zhang YC, Li YJ, Wei PK, Qin ZF. Xiaotan Tongfu granules contribute to the prevention of stress ulcers. *World J Gastroenterol* 2013; 19(33): 5473-5484 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i33/5473.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i33.5473>

## INTRODUCTION

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

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

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

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

## MATERIALS AND METHODS

### Animals

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

### Drug administration

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

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

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

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

### Induction of stress ulceration

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

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

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

### Measurement of myeloperoxidase activity in the gastric mucosa

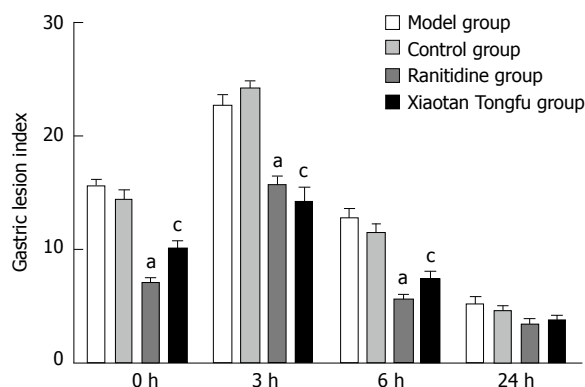
Myeloperoxidase (MPO) activity was determined by the method described by Bradley *et al.*<sup>[34]</sup> with some modifications<sup>[35]</sup>. The gastric mucosa was homogenized in a potassium phosphate buffer containing 0.5% hexadecyl trimethyl ammonium bromide, and the supernatant was assayed for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

### Immunohistochemical staining for PCNA, HSP70 and MIF

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

### Measurement of apoptotic cells in the gastric mucosa

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



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

diaminobenzidine- $H_2O_2$ . Finally, the sections were counterstained with Mayer's hematoxylin. Sections treated with DNase I in buffer solution served as the positive control, whereas the negative control was prepared by omitting the TdT from the buffer solution. The positive cells were counted by the Image Pro Express system (Olympus) at  $\times 400$  magnification (BX51, Olympus). The apoptotic index was defined as the average number from 10 to 25 glands of each mucosal section.

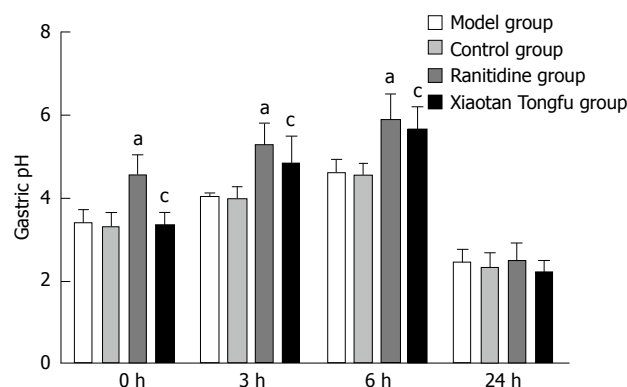
### Statistical analysis

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

## RESULTS

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

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



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

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

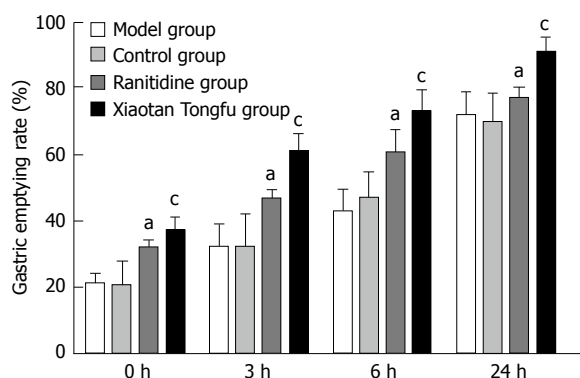
### Gastric pH in the XP and RP groups is significantly lower than in the MP and CP groups

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

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

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





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

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

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

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

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

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

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

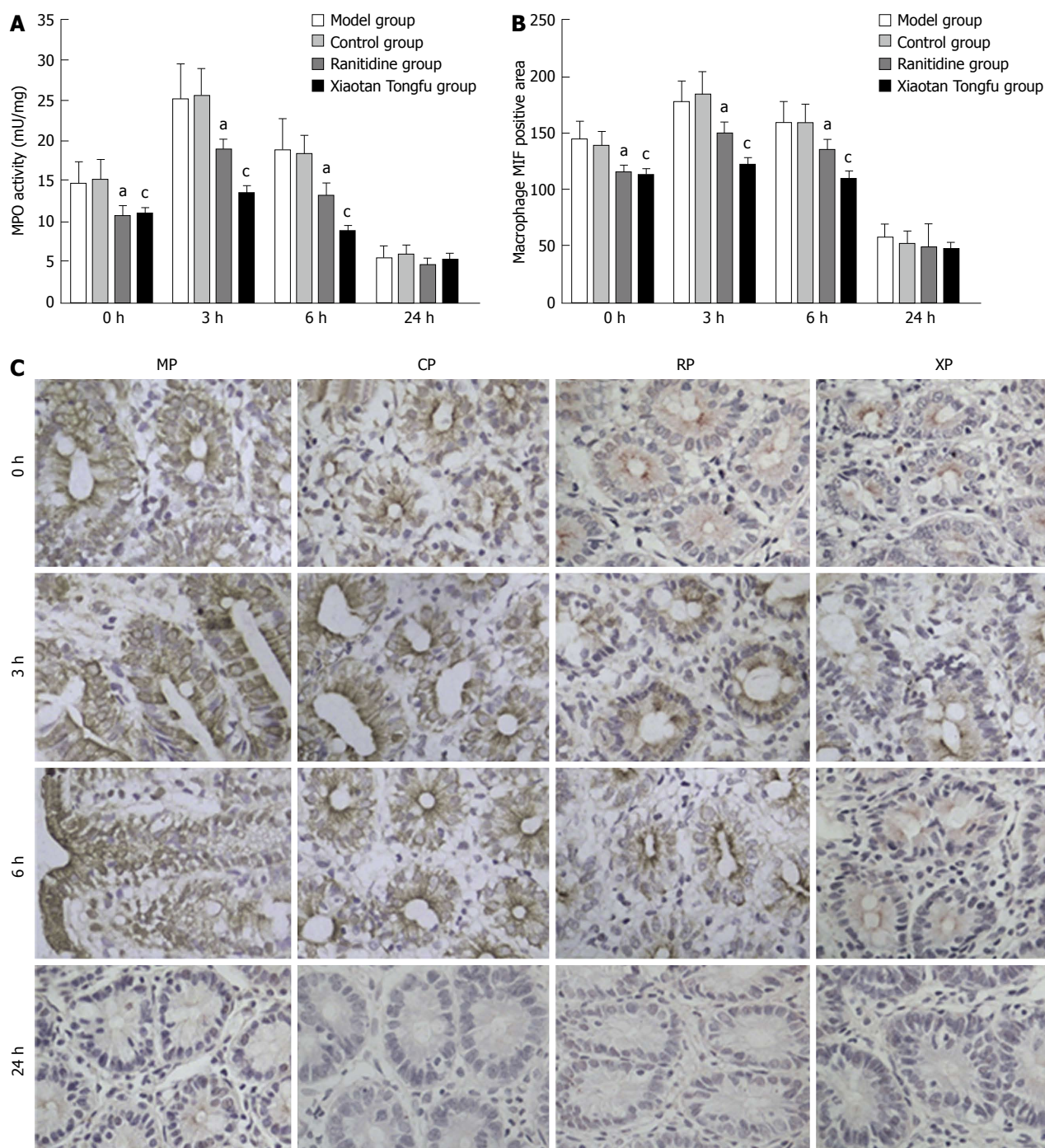
As shown in Figure 6, cell proliferation varied in a time-

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

## **DISCUSSION**

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

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

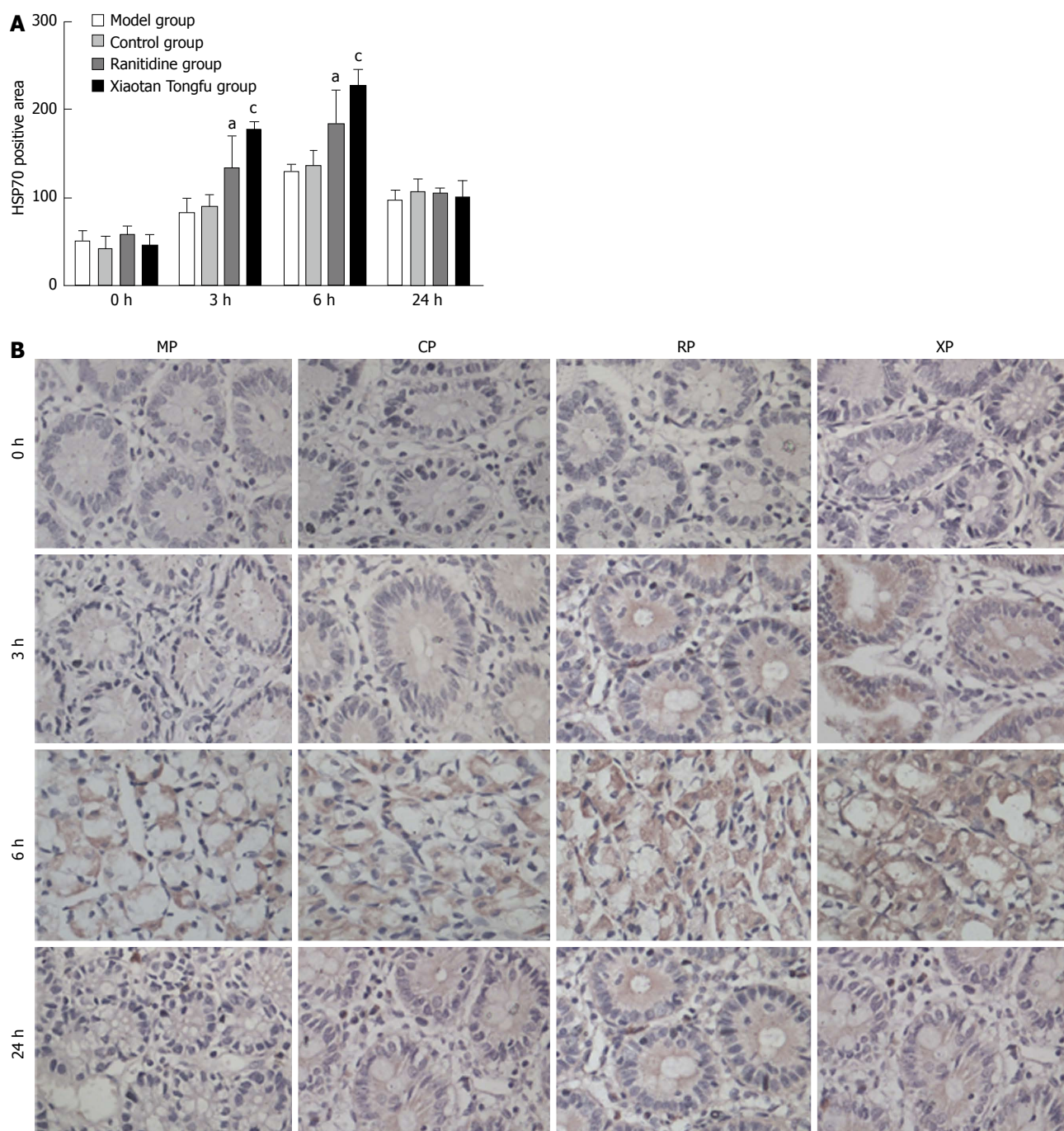


**Figure 4** Measurement of myeloperoxidase activity and macrophage migration inhibitory factor ( $n = 6$  for each group). A, B: The variation of myeloperoxidase activity (MPO) activity and migration inhibitory factor (MIF) was similar. At 0 h, no significant difference was detected between the ranitidine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

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

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

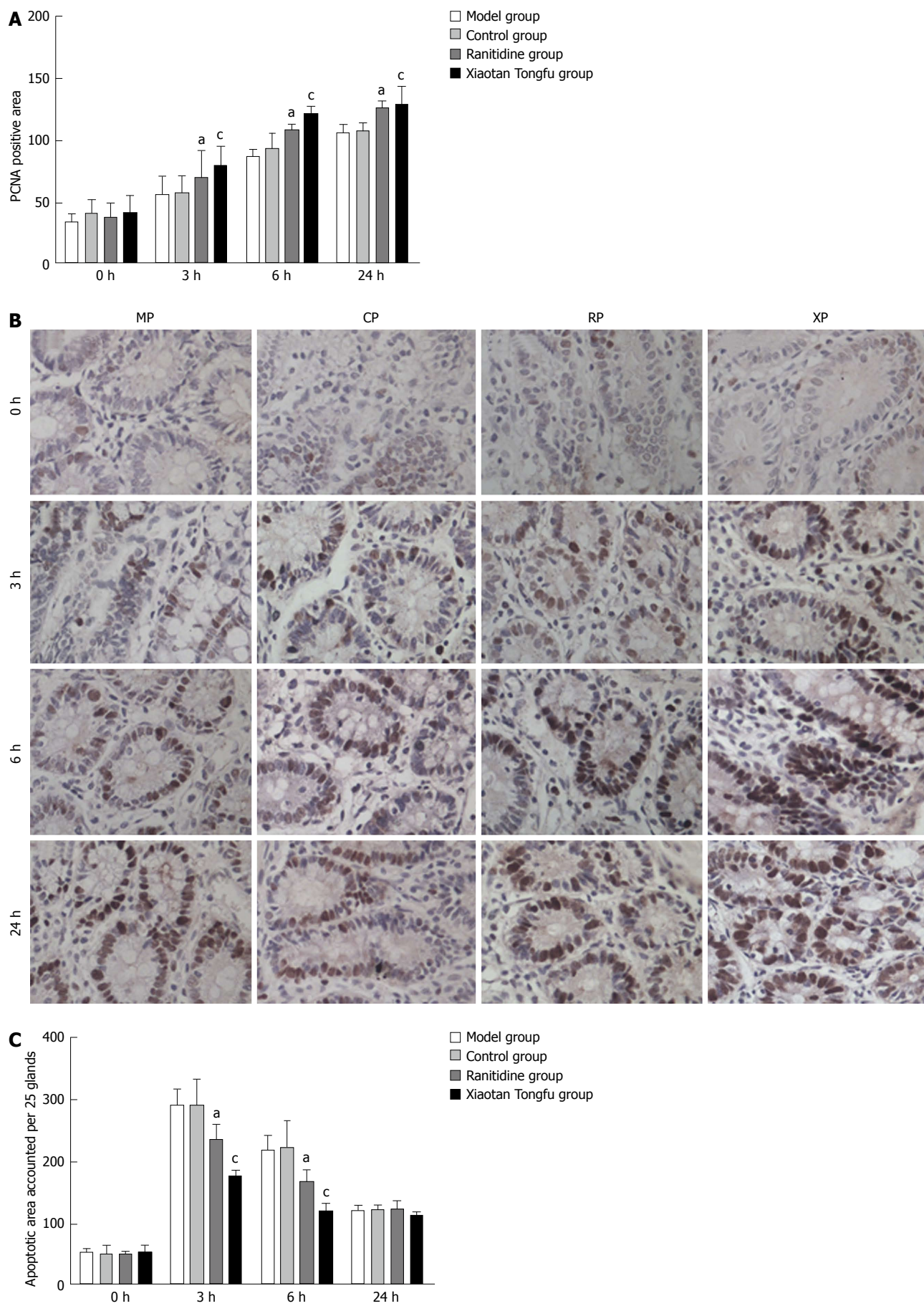




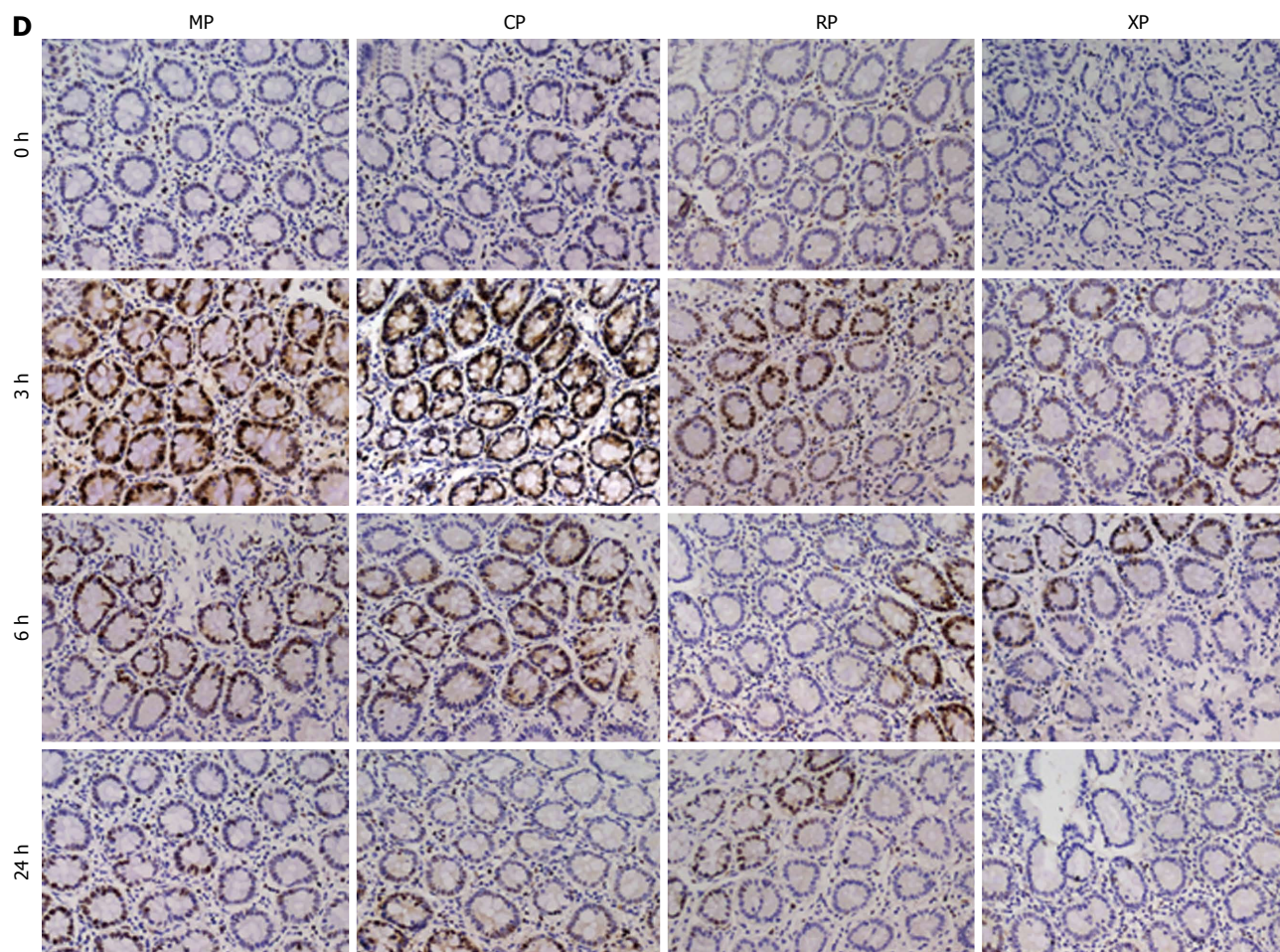
**Figure 5** Immunohistochemical staining results for heat shock protein 70 ( $n = 6$  for each group). A: There was a statistically significant difference in the protein expression levels between the ranitidine group (RP group) and the Xiaotan Tongfu granule group (XP group) groups at 3 and 6 h at the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

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

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







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

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

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

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

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

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

## COMMENTS

### Background

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

### Research frontiers

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

### Innovations and breakthroughs

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

### Applications

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

### Terminology

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

### Peer review

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

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

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### Abstract

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

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

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

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

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

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

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

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

### INTRODUCTION

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

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

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

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

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

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

## MATERIALS AND METHODS

### Cell culture

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

### Drug treatment

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

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

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

### DNA ladder analysis for apoptosis

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

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

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

Apoptosis was evaluated by terminal transferase dUTP nick-end labeling (TUNEL) assay according to the manufacturer's instructions for MIA-PaCa-2 cells treated with 100  $\mu$ mol/L propofol or 0.5 mmol/L Na<sub>2</sub>CO<sub>3</sub> (vehicle control) for 24-72 h; or 10-100  $\mu$ mol/L propofol for 72 h; or 25  $\mu$ mol/L propofol for 24 h followed by 0-100  $\mu$ mol/L of gemcitabine for 72 h. TUNEL-positive cells were colored using diaminobenzidine as chromogen and counterstained with hematoxylin. The percentage of TUNEL-positive cells was assessed in five randomly selected fields per section. All assays were performed in quadruplicate.

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

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

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

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

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

#### **Statistical analysis**

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

## **RESULTS**

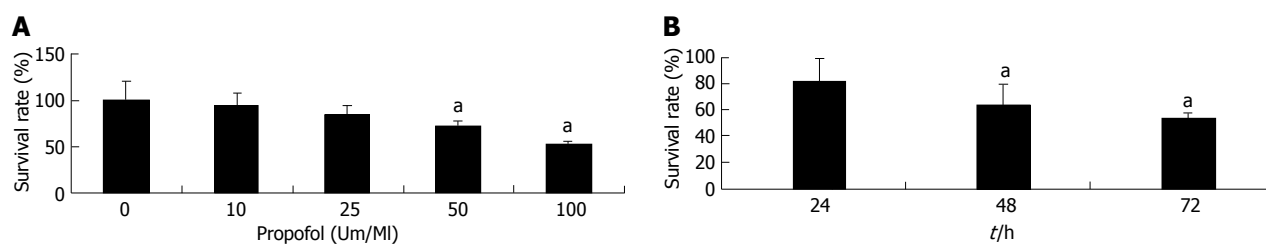
#### **Effect of propofol on cell proliferation**

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

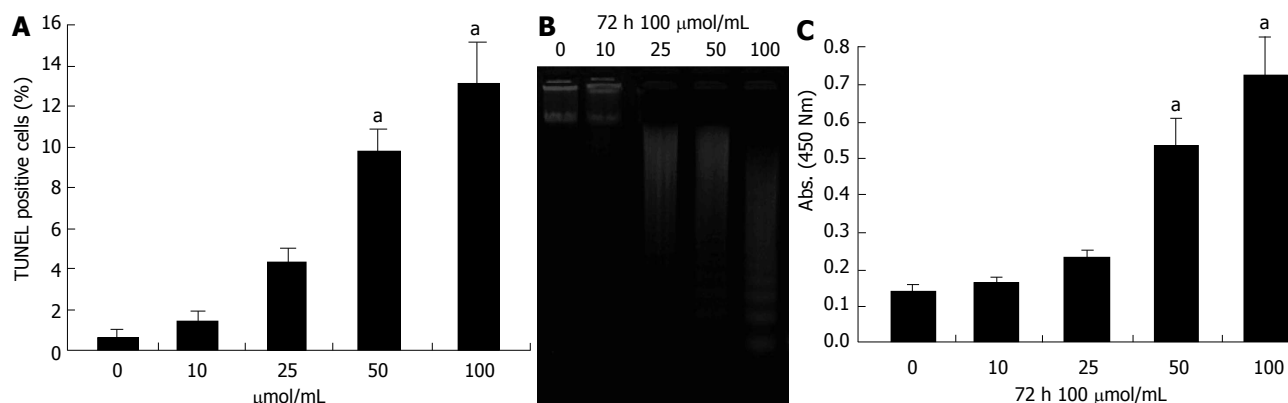
#### **Effect of propofol on apoptosis**

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

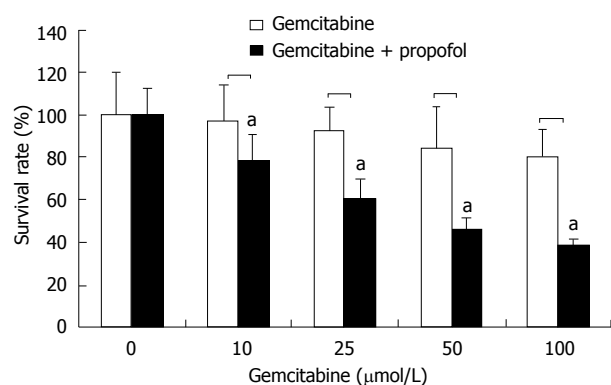




**Figure 1** Evaluation by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay of pancreatic cancer MIA-PaCa-2 cell viability after propofol pretreatment. A: Cells were either untreated or treated with 10-100 μmol/mL propofol for 72 h; B: Cells treated with 100 μmol/mL propofol for 24,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



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



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

assays gave the same results as TUNEL assays.

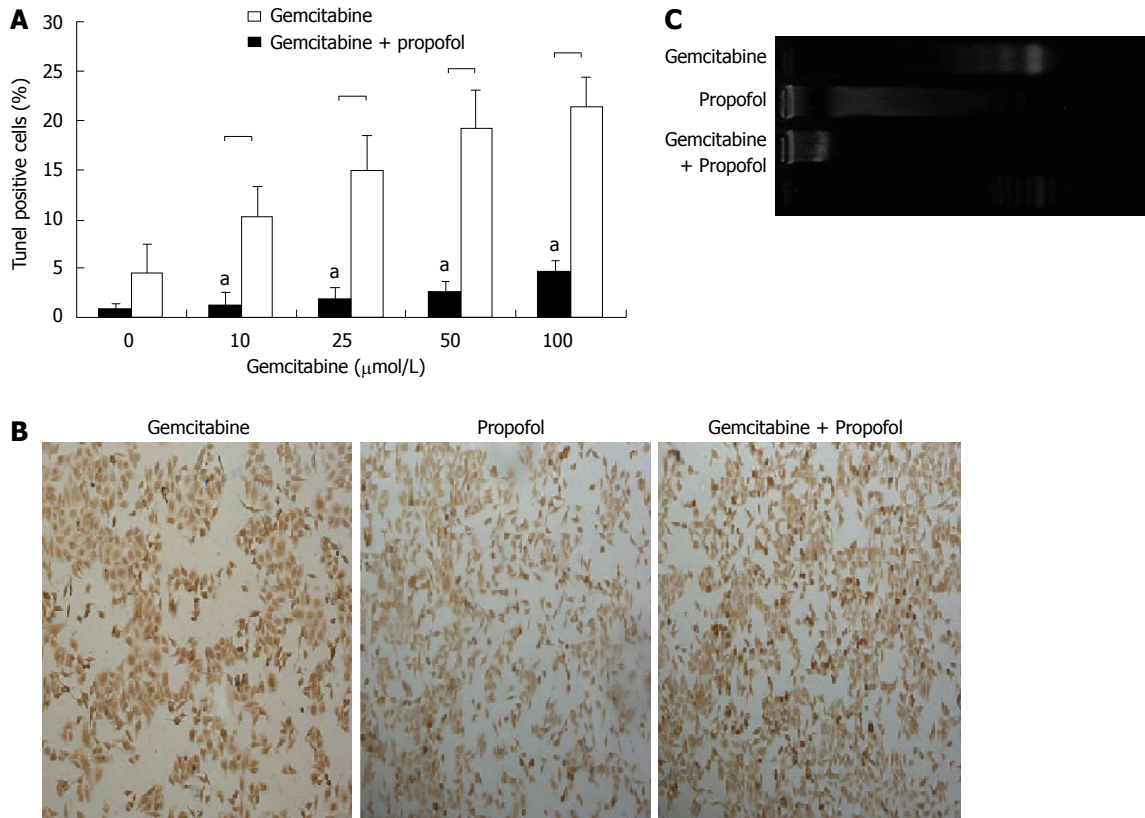
### Propofol potentiates growth inhibition by gemcitabine in MIA-PaCa-2 cells

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

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

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

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



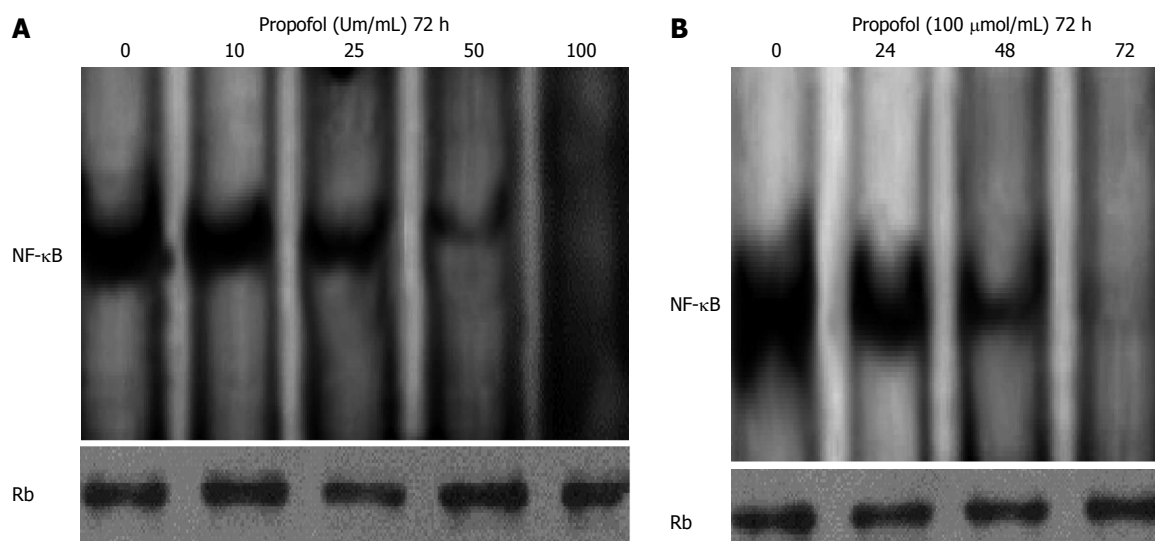
**Figure 4** Evaluation of apoptosis by terminal transferase dUTP nick-end labeling and DNA ladder assays in pancreatic cancer MIA-PaCa-2 cells after propofol pretreatment. A: Sensitization of pancreatic tumor MIA-PaCa-2 cells to propofol- and/or gemcitabine-induced apoptosis measured by terminal transferase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

propofol (25 μmol/mL) for 72 h resulted in only 4.6% apoptosis (Figure 2 A), and treatment with 10, 25, 50 and 100 μmol/L gemcitabine (0-100 μmol/L) for 72 h resulted in 1.2% to 4.6% apoptosis (Figure 4A). Only low levels of apoptosis were detected with single-agent treatment. Figure 4B has representative TUNEL data of MIA-PaCa-2 cells pretreated with propofol (50 μmol/mL) for 24 h followed by coincubation with gemcitabine (25 μmol/L) for 72 h. Apoptosis measured by ELISA and DNA ladder assays gave the same results (data not shown). Figure 4C has Representative DNA ladder assay data of MIA-PaCa-2 cells pretreated with propofol (50 μmol/mL) for 24 h followed by coincubation with gemcitabine (25 μmol/L) for 72 h.

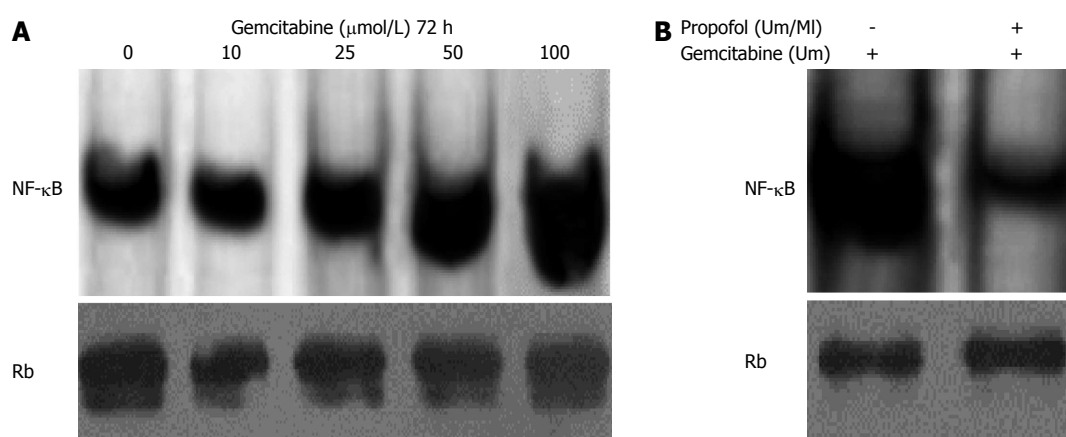
MIA-PaCa-2 cells pretreated with propofol (25 μmol/mL) for 24 h, followed by coincubation with gemcitabine (10, 25, 50 and 100 μmol/L) for 72 h, showed significant cell apoptosis by DNA ladder, TUNEL assay and ELISA assay. These results were consistent with the growth inhibition MTT assays, and suggested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

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

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



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



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

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

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

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

## DISCUSSION

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



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

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

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

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

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

## COMMENTS

### Background

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

### Research frontiers

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

### Innovations and breakthroughs

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

### Applications

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

### Terminology

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

### Peer review

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

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

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### Abstract

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

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

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

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

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

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

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

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## INTRODUCTION

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

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

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

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

patients undergoing screening colonoscopy.

## MATERIALS AND METHODS

### Subjects selection

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

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

### Ethical considerations

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

### Study preparations

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

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

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

### Study design

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

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

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

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

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

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

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

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

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

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

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

Although we used a validated rating scale, the degree

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

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

### Statistical analysis

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

## RESULTS

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

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

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

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

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

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

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

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

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

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

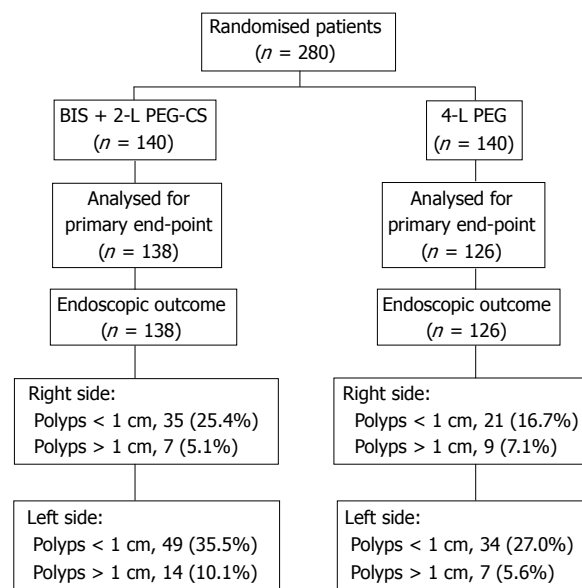
Subjects receiving PEG-CS stated they fully adhered to instructions drinking all the 2-L solution in 97.1% compared with 87.3% in the 4-L PEG ( $P < 0.01$ ) (Table 2).

## DISCUSSION

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

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

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



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

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

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

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

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



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

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

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

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

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

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

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

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

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

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

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

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

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

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

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## COMMENTS

### Background

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

### Research frontiers

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

## Innovations and breakthroughs

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

## Applications

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

## Peer review

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

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

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### Abstract

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

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

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

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

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

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

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

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

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

## INTRODUCTION

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

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

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

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

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

## MATERIALS AND METHODS

### Materials

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

### Cell culture

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

### Measurement of transepithelial resistance

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

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

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

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

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

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

### Immunofluorescence staining of TJ proteins

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

### Statistical analysis

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

## RESULTS

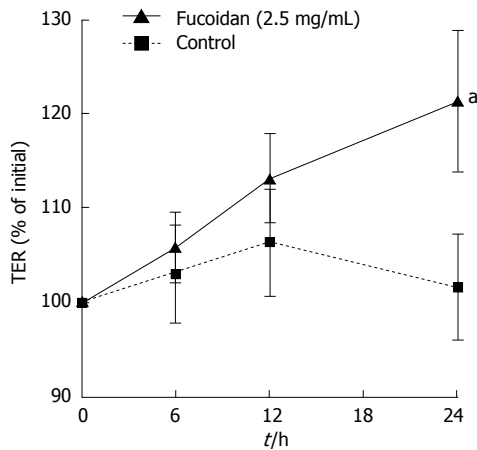
### Fucoidan directly enhanced intestinal epithelial barrier function

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

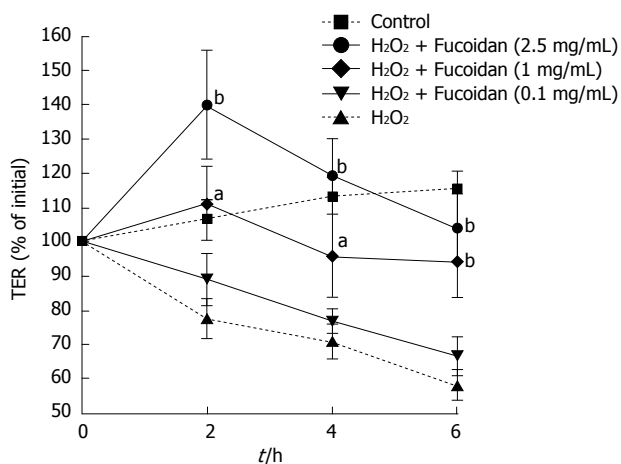
### Fucoidan prevented $\text{H}_2\text{O}_2$ -induced destruction of intestinal epithelial barrier function in a dose-dependent manner

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



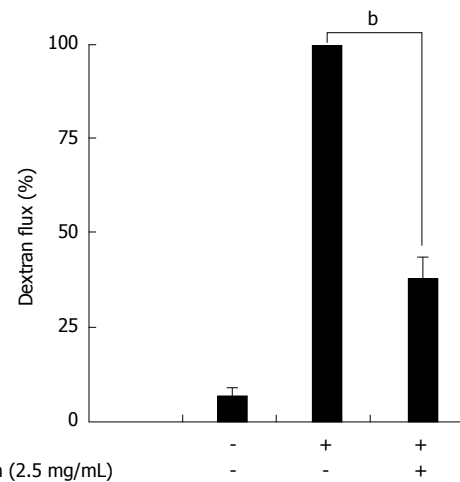


**Figure 1 Fucoidan directly enhanced intestinal epithelial barrier function.** Polarized Caco-2 cell monolayers were incubated in the presence or absence of fucoidan (2.5 mg/mL) for 24 h. Changes in intestinal epithelial barrier function were monitored by measuring the trans-epithelial resistance (TER).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



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

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



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

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

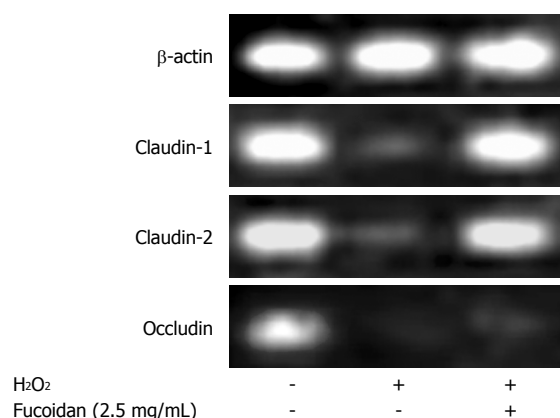
Next, we examined whether H<sub>2</sub>O<sub>2</sub> increased the paracellular permeability of Caco-2 cell monolayers following epithelial injury and whether fucoidan could prevent this effect. For this experiment, an FD4 flux assay was performed. H<sub>2</sub>O<sub>2</sub> markedly increased FD4 flux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

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

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

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

We further examined the effect of fucoidan on the inter-



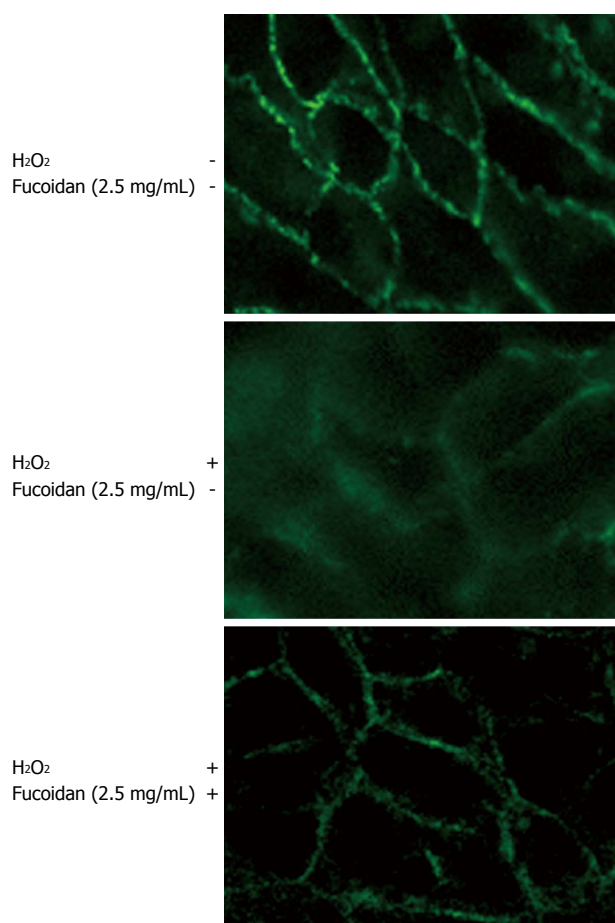
**Figure 4 Fucoidan promoted intestinal epithelial barrier function via direct upregulation of tight junction proteins in intestinal epithelial cells** Polarized Caco-2 cell monolayers injured by H<sub>2</sub>O<sub>2</sub> (500 μmol/L) for 24 h with or without pretreatment of fucoidan (2.5 mg/mL) 30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

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

## DISCUSSION

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

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



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

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

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

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

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

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

## COMMENTS

### Background

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

### Research frontiers

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

### Innovations and breakthroughs

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

### Applications

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

### Terminology

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

### Peer review

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

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

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### Abstract

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

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

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

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

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

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

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## INTRODUCTION

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

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

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

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

## MATERIALS AND METHODS

### Subjects and sample collection

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

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

### Drug sensitivity testing

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

### Statistical analysis

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

## RESULTS

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

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

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

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

## DISCUSSION

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

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

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

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

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

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

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

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## COMMENTS

### Background

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

### Research frontiers

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

### Innovations and breakthroughs

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

### Applications

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

### Peer review

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

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

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

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

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

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

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

### Abstract

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

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

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

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## INTRODUCTION

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

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

## MATERIALS AND METHODS

### Patients and follow-up

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

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

### Endoscopic stenting procedure and laparoscopic resection

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



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

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

TNM: Tumor, node, metastasis.

expansion of the SEMS was assessed using fluoroscopic visualization.

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

### Statistical analysis

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

## RESULTS

### Patient characteristics

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

**Table 2** Characteristics and outcomes

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

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

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

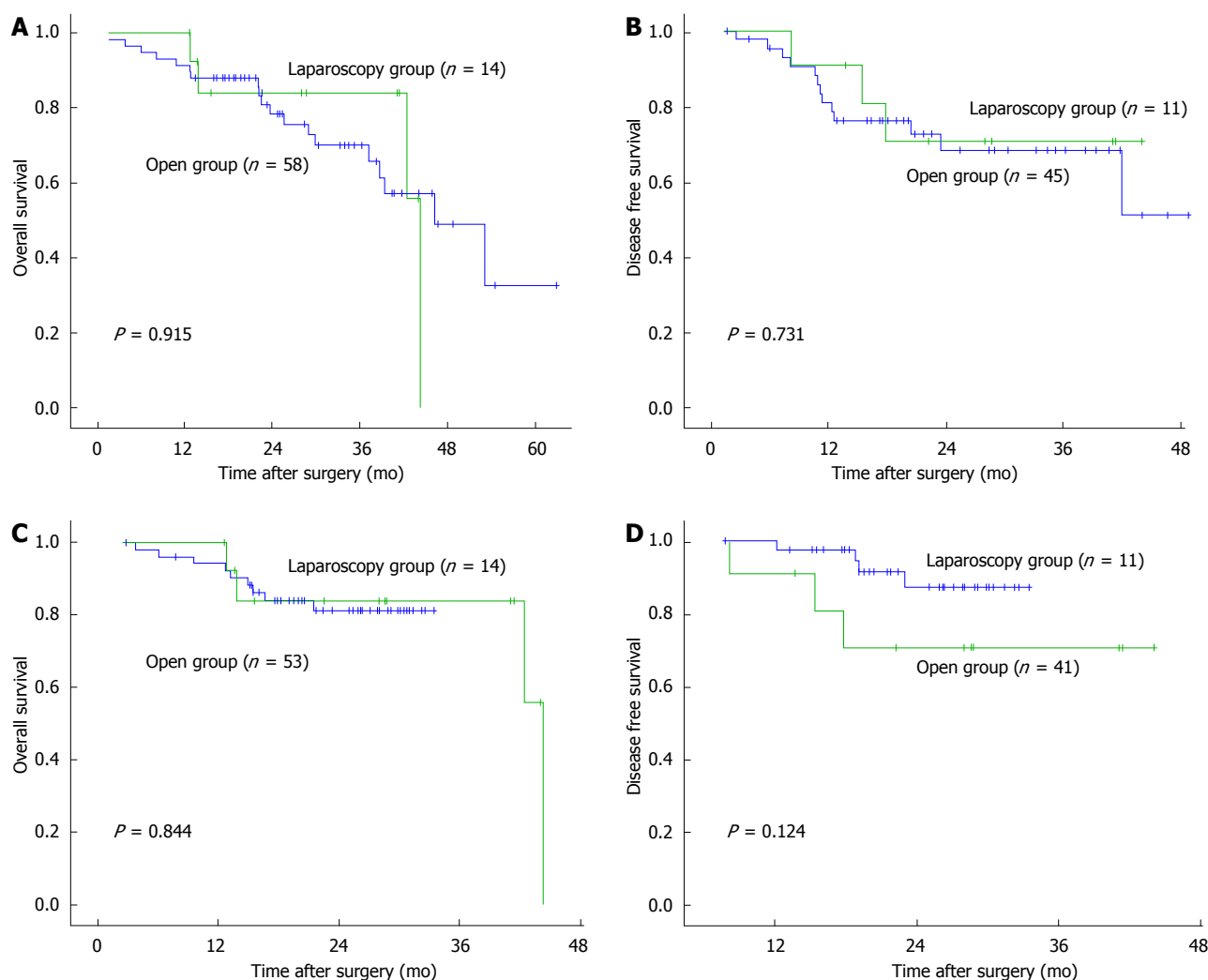
### Comparison of clinical outcomes

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

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

### Comparison of long-term survival

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



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

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

## DISCUSSION

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

**Table 3** Recurrence, metastasis and treatment

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

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

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

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

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

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

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

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

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



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

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

## COMMENTS

### Background

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

### Research frontiers

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

### Innovations and breakthroughs

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

### Applications

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

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

### Terminology

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

### Peer review

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

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

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### Abstract

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

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

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

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

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

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

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

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

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## INTRODUCTION

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

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

## MATERIALS AND METHODS

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

### Patients and treatment

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

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

### DW-MRI

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

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



**Table 1 Patients and treatment characteristics**

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

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

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

### Statistical analysis

Statistical analysis was performed using SPSS 12.0 statistical software. Paired comparisons were performed using the Wilcoxon test. Spearman's correlation was used to assess the significance of differences between groups. A *P* value < 0.05 was considered statistically significant.

## RESULTS

### Treatment characteristics

After neoadjuvant treatment, pCR was observed in 2 patients. Downstaging of the tumor was observed in eight patients. The tumor regression grades after neoadjuvant treatment were grade 0-2 in 6 patients and grade 3-4 in 9 patients.

### DW-MRI data

Diffusion data from 15 patients were obtained prior to

and at constant intervals once weekly during the course of neoadjuvant treatment. The observed ADC values are shown in Table 2. A total of 88 ADC values were obtained in our study, and 2 ADC values were excluded due to measurement errors. Sample T2-weighted and diffusion-weighted images prior to treatment are shown in Figure 1. Sample ADC maps from the images taken weekly during the course of neoadjuvant treatment are shown in Figure 2.

The mean tumor ADC value slightly increased from  $0.749 \times 10^{-3}$  mm<sup>2</sup>/s (95%CI:  $0.641 \times 10^{-3}$ - $0.858 \times 10^{-3}$  mm<sup>2</sup>/s) prior to treatment to  $0.772 \times 10^{-3}$  mm<sup>2</sup>/s (95%CI:  $0.627 \times 10^{-3}$ - $0.918 \times 10^{-3}$  mm<sup>2</sup>/s) after the 1<sup>st</sup> week of treatment. There was also a significant increase at the 2<sup>nd</sup> week to  $0.884 \times 10^{-3}$  mm<sup>2</sup>/s (95%CI:  $0.775 \times 10^{-3}$ - $0.994 \times 10^{-3}$  mm<sup>2</sup>/s). Subsequently, the mean ADC decreased to  $0.800 \times 10^{-3}$  mm<sup>2</sup>/s (95%CI:  $0.675 \times 10^{-3}$ - $0.925 \times 10^{-3}$  mm<sup>2</sup>/s) at the 3<sup>rd</sup> week and  $0.766 \times 10^{-3}$  mm<sup>2</sup>/s (95%CI:  $0.659 \times 10^{-3}$ - $0.872 \times 10^{-3}$  mm<sup>2</sup>/s) at the 4<sup>th</sup> week. ADC increased again at the 5<sup>th</sup> week to  $0.839 \times 10^{-3}$  mm<sup>2</sup>/s (95%CI:  $0.702 \times 10^{-3}$ - $0.976 \times 10^{-3}$  mm<sup>2</sup>/s). We also observed a significant increase in the mean ADC value at the 2<sup>nd</sup> (*P* = 0.004) and 5<sup>th</sup> week (*P* = 0.033) during treatment relative to the values prior to treatment. The mean observed ADC values and *P* values are shown in Table 3.

### Tumor ADC for the prediction of treatment response

We compared the tumor ADC values of the responder and non-responder groups to predict the treatment response based on T downstage and TRG criteria.

Downstaging of the tumor was observed for 8 of the 15 patients (53.3%). The ADC values at the 5<sup>th</sup> week during treatment increased for 6/8 patients with T downstaging and increased for 5/7 patients without T downstaging relative to the mean tumor ADC values before treatment. The mean observed ADC values for patients with and without T downstage are shown in Table 4. The difference between these two groups with respect to the mean ADC values measured at the six timepoints did not reach significance.

For the eight patients with tumor downstaging, there was a significant increase (*P* = 0.011) in the mean tumor ADC at the 2<sup>nd</sup> week of treatment relative to the ADC before treatment, whereas for the seven patients without tumor downstaging, there was no significant change in the ADC at any timepoint during treatment relative to the ADC values before treatment (Figure 3A, Table 3).

Good regression (TRG 3-4) was observed in 9 of the 15 patients (60%), and poor regression (TRG 0-2) was observed in 6 patients (40%). The ADC values at the 5<sup>th</sup> week during treatment were increased in 7/9 patients with good regression and 5/6 patients with poor regression relative to the mean tumor ADC values before treatment. The mean observed ADC values in patients with good regression and poor regression are shown in Table 4. Before treatment and at the 3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> week during treatment, significant differences in the mean ADC values between the two groups were obtained.

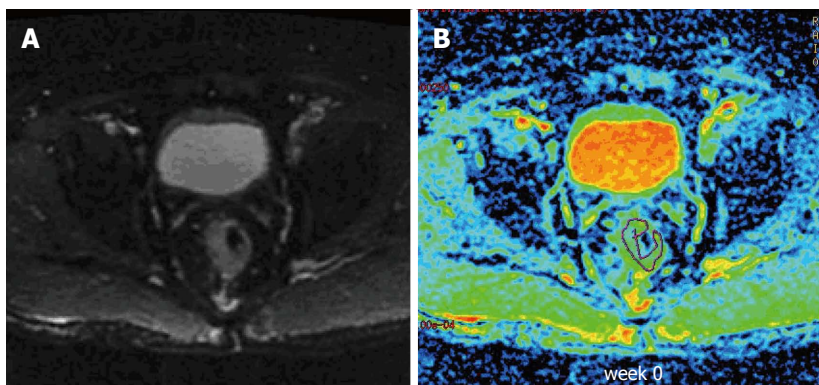
For the nine patients with good regression, there

**Table 2** Apparent diffusion coefficient values at six measurement times

No.	Apparent diffusion coefficient values ( $\times 10^{-3} \text{ mm}^2/\text{s}$ )					
	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5
1	0.723	0.743	0.893	0.756	0.606	0.793
2	0.583	0.458	0.786	0.385	0.540	0.793
3	0.752	0.883	0.655	0.711	0.683	0.772
4	0.883	0.887	0.887	0.945	0.923	0.972
5	0.995	0.853	0.995	0.950	0.832	1.120
6	0.813	0.965	1.133	0.964	0.893	0.686
7	0.518	0.416	0.998	0.539	0.527	0.473
8	0.659	0.747	0.825	0.858	0.631	0.746
9	0.814	0.791	0.809	0.894	0.821	0.798
10	0.628	0.637	0.625	0.703	0.784	0.930
11	0.562	0.575	0.806	0.742	0.677	0.515
12	0.616	0.595	0.834	0.850	0.907	1.050
13	0.851	0.825	0.865	<sup>1</sup>	0.831	0.882
14	0.592	<sup>1</sup>	0.734	0.622	0.574	0.605
15	1.255	1.435	1.420	1.282	1.256	1.450
95%CI	0.641-0.858	0.627-0.918	0.775-0.994	0.675-0.925	0.659-0.872	0.702-0.976

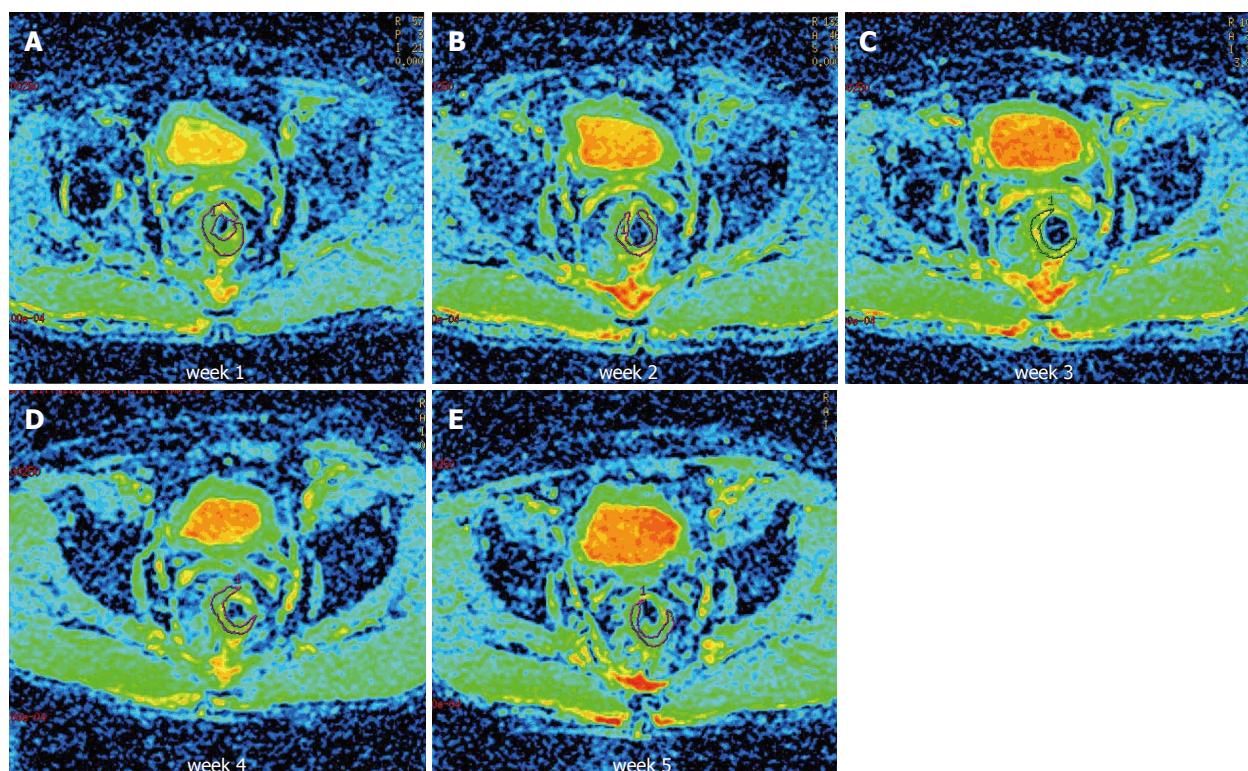
<sup>1</sup>No data due to measurement error.**Table 3** Mean tumor apparent diffusion coefficient values and the *P* values for the comparisons with the pretreatment values

	Apparent diffusion coefficient values ( $\times 10^{-3} \text{ mm}^2/\text{s}$ )					
	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5
All ( <i>n</i> = 15)	0.749	0.772	0.884	0.800	0.766	0.839
<i>P</i> value	-	0.672	0.004	0.077	0.586	0.033
T downstage ( <i>n</i> = 8)	0.703	0.720	0.890	0.724	0.691	0.744
<i>P</i> value	-	0.964	0.011	0.406	0.578	0.284
No T downstage ( <i>n</i> = 7)	0.803	0.824	0.878	0.876	0.851	0.948
<i>P</i> value	-	0.617	0.185	0.117	0.430	0.074
Good regression ( <i>n</i> = 9)	0.659	0.671	0.852	0.696	0.674	0.714
<i>P</i> value	-	0.909	0.004	0.212	0.617	0.251
Poor regression ( <i>n</i> = 6)	0.886	0.907	0.933	0.938	0.904	1.027
<i>P</i> value	-	0.669	0.372	0.264	0.785	0.086

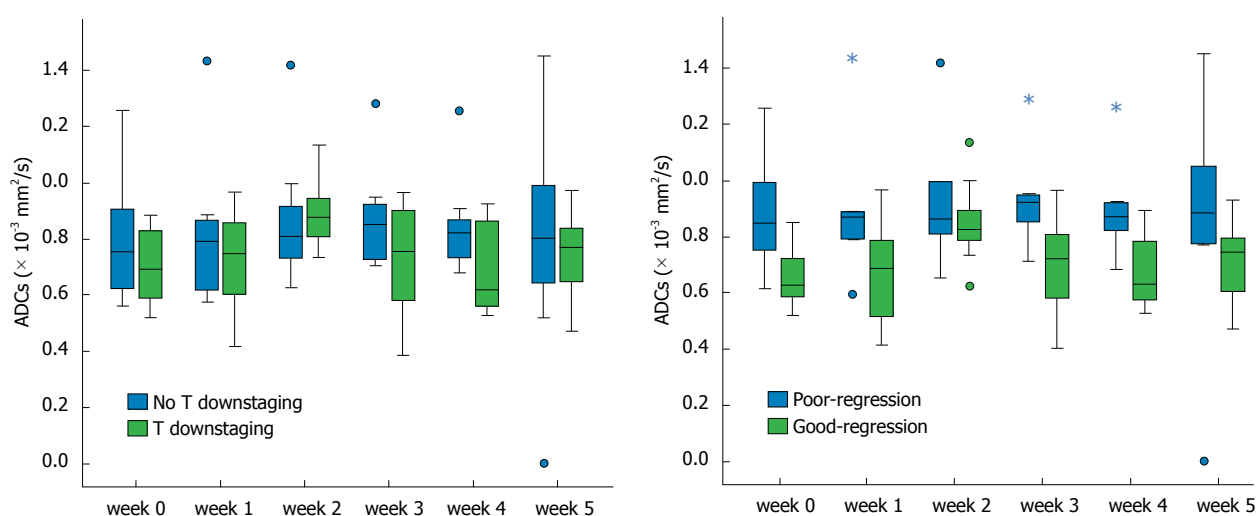
**Figure 1** Sample T2-weighted and diffusion-weighted images from a patient with locally advanced rectal cancer prior to treatment. A: T2-weighted transaxial image through the pelvis prior to treatment; B: Corresponding apparent diffusion coefficient map of the patient obtained from the diffusion-weighted images. A region of interest was drawn around the tumor.

was a significant increase ( $P = 0.004$ ) in the mean tumor ADC value at the 2<sup>nd</sup> week of treatment relative to the ADC values before treatment, whereas for the six patients with poor regression, no significant change in ADC was observed at any timepoint during treatment (Figure

3B, Table 3). The two patients with pCR demonstrated lower ADC ( $0.659 \times 10^{-3} \text{ mm}^2/\text{s}$  and  $0.813 \times 10^{-3} \text{ mm}^2/\text{s}$ ) before treatment but significantly increased tumor ADC ( $0.825 \times 10^{-3} \text{ mm}^2/\text{s}$  and  $1.133 \times 10^{-3} \text{ mm}^2/\text{s}$ ) at the 2<sup>nd</sup> week of treatment.



**Figure 2** Apparent diffusion coefficient maps of one patient obtained at constant intervals once weekly during the course of neoadjuvant treatment from the diffusion-weighted images. A-E: Axial apparent diffusion coefficient maps obtained during treatment (weeks 1-5). A region of interest was drawn around the tumor.



**Figure 3** Mean tumor apparent diffusion coefficient values in patients at six measurement points. A: With and without T downstaging; B: With good regression and poor regression. Circle: Outlier; Star: Extreme value.

## DISCUSSION

The recent trend toward patient-tailored treatment for LARC has highlighted the need for a reliable method for the early assessment of treatment response. DWI-MRI may be a promising functional imaging tool for the prediction of treatment response. In our study, DW-MRI was investigated as a potential clinical tool to predict or assess the response of rectal tumors to neoadjuvant concurrent CRT at an early timepoint.

Our results show that CRT induced a significant increase in mean tumor ADC in LARC. Because the ADC values obtained from DWI measurements reflect tumor cellularity and anti-tumor treatment decreases tumor cellularity, CRT should increase the ADC value. The administration of CRT results in cell swelling, necrosis, and apoptotic cell death. When CRT is initiated, the ADC may rapidly decrease over several hours due to cell swelling, followed by an increase over several days concurrent with cell death. Increased ADC values have also been



**Table 4 Mean tumor apparent diffusion coefficient values and *P* values for the comparisons between groups**

Group	Apparent diffusion coefficient values ( $\times 10^{-3} \text{ mm}^2/\text{s}$ )					
	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5
T downstage ( $n = 8$ )	0.702	0.720	0.890	0.724	0.690	0.743
No T downstage ( $n = 7$ )	0.803	0.824	0.877	0.875	0.851	0.947
<i>P</i> value	0.339	0.463	0.909	0.202	0.108	0.114
Good regression ( $n = 9$ )	0.658	0.670	0.851	0.618	0.673	0.713
Poor regression ( $n = 6$ )	0.885	0.907	0.933	0.938	0.903	1.027
<i>P</i> value	0.021	0.081	0.452	0.032	0.016	0.010

correlated with tumor necrosis and reduced cell density<sup>[15]</sup>, and most studies have found an increase in ADC after CRT<sup>[12,16,17]</sup>. For example, Kim *et al.*<sup>[12]</sup> recently showed that neoadjuvant CRT caused a significant increase in the ADC values of 76 rectal cancer patients. In contrast, Hein *et al.*<sup>[18]</sup> reported a decrease in the ADC after CRT in all nine of their patients, and they attributed this result to intratumoral radiation-induced fibrosis and cytotoxic edema as well as to the method employed (ROI excluding apparent necrotic areas).

Our results indicate that the mean pre-CRT ADC was negatively correlated with tumor regression ( $P = 0.021$ ) but not with T downstaging ( $P = 0.339$ ). T downstaging and TRG criteria were used because these are common factors used for the evaluation of treatment responses<sup>[19,20]</sup>. The TRG was not completely concordant with T downstaging, and some studies have shown that the pre-treatment ADC value is negatively correlated with treatment response in rectal cancer and other tumors<sup>[10,21-25]</sup>. Dzik-Jurasz *et al.*<sup>[10]</sup> found a strong negative correlation between the mean pretreatment tumor water ADC and the percent change in the size of the tumor after chemotherapy and chemoradiation in 2002. ADC values are generally higher for necrotic tumors than for solid or viable tumors<sup>[26]</sup>. Because necrotic areas in tumors are resistant to radiation, it may be hypothesized that tumors with necrotic areas, and thus high pretreatment ADC values, would have less favorable treatment responses. However, other studies have obtained different results; for example, several studies of rectal and other tumors found no correlation between the pretreatment ADC value and treatment response<sup>[11,27,28]</sup>, whereas another study found a positive correlation<sup>[12]</sup>. Several factors may explain these different correlations, such as small sample sizes, the use of different methods for calculating the ADC, and the use of different indicators for the evaluation of treatment response.

A substantial change in the mean ADC value at the 2<sup>nd</sup> week of CRT predicted the tumor response of LARC in our study. Most studies have assumed that CRT decreases tumor cellularity and results in a substantial change in the ADC value<sup>[18,26]</sup>. Although decreasing tumor cellularity will lead to a reduction in tumor size, this reduction is typically observed 3 wk or more after the start of CRT<sup>[29,30]</sup>. Thus, a more rapid evaluation or prediction of treatment response would be clinically useful. We

found a significant increase in the mean ADC at the 2<sup>nd</sup> week in the T downstage ( $P = 0.011$ ) and good regression ( $P = 0.004$ ) groups but not in the groups of patients without T downstaging and with poor regression. We believe that the significant increase in the mean ADC at the 2<sup>nd</sup> week of treatment was correlated with tumor necrosis and apoptosis, which reduce cell density, after the start of therapy. Similar results have been obtained in several other studies. For example, one study examined the ADC data of nine patients with LARC, and a significant change in the mean ADC starting at week 2 of CRT was observed<sup>[18]</sup>. In another study focused on the early detection of responses to CRT in cervical cancer, the changes in the ADC value after 2 wk of therapy were also significantly correlated with the treatment response<sup>[27]</sup>.

There are several limitations of our study. First, the study sample size was small. Second, the sample slice with the largest tumor extension was selected to determine the ADC value, and the use of this slice may not have adequately captured the heterogeneity of the tumor. Third, the ROIs were drawn manually, and this process may have influenced the ADC value and introduced subjectivity. The reason the ROIs were drawn manually by a single experienced radiologist was to obtain more uniform and stable ADC values.

Our study and several previous studies highlight the value of DW-MRI as a predictive tool for the response of rectal cancer to chemoradiation. However, there are some difficulties associated with incorporating DW-MRI into routine clinical practice. The reproducibility of DWI has been insufficiently investigated, and the cut-off values used to determine treatment response vary between treatments and ADC measurement techniques. Thus, a standardized guideline to predict or assess treatment response is needed before DWI can be implemented in clinical practice.

In this study, the tumor ADC values changed during the course of neoadjuvant chemoradiation. The pretreatment tumor ADC value was negatively correlated with tumor regression after chemoradiation for the treatment of LARC, and the ADC value at the 2<sup>nd</sup> week of therapy was significantly correlated with the tumor response. Our results indicate that DW-MRI may be a valuable clinical tool to help predict or assess the responses of rectal tumors to neoadjuvant concurrent chemoradiation at an early timepoint.



## COMMENTS

### Background

Neoadjuvant (chemo)radiation followed by surgery has become the standard treatment for locally advanced rectal cancer (LARC). However, approximately 20%-30% of patients do not benefit from this neoadjuvant treatment due to radioresistance of the tumor. Functional non-invasive diffusion-weighted magnetic resonance imaging (DW-MRI) studies are increasingly used to predict response to cancer therapy, but definitive evidence is limited, especially for patients with rectal cancer treated with neoadjuvant chemoradiation therapy (CRT).

### Research frontiers

DW-MRI is a non-invasive functional MRI technique. To date, published data on the value of DW-MRI as a predictive tool for assessing responses to anti-cancer treatment in patients with rectal cancer are scarce and conflicting.

### Innovations and breakthroughs

The authors found that CRT induced a significant increase in the mean apparent diffusion coefficient (ADC) value of LARC. The pretreatment tumor ADC was negatively correlated with tumor regression after CRT for the treatment of LARC, and the ADC value at the 2<sup>nd</sup> week of therapy was significantly correlated with the tumor response.

### Applications

The results of this study suggest that DW-MRI may be a valuable clinical tool to help predict or assess the responses of rectal tumors to neoadjuvant concurrent CRT at an early timepoint.

### Terminology

DW-MRI is a non-invasive functional MRI technique that provides information by measuring water proton mobility in tissues. ADC values can be calculated from DWI measurements according to the impediment to free diffusion of water molecules in a single voxel due to restricting barriers such as membranes, macromolecules, and fibers inside different tissue compartments.

### Peer review

This is an interesting study that investigates the use of DW-MRI as a predictor of the tumor response in 15 patients with rectal cancer undergoing CRT therapy by measuring the tumor ADC. This is an emerging field in which new knowledge is needed, and this study, despite its limits, provides novel information that may help to settle the current debate about the utility of DW-MRI as a predictive tool for the response to anti-cancer treatment.

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## Dual-sided composite mesh repair of hiatal hernia: Our experience and a review of the Chinese literature

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### Abstract

**AIM:** To summarize our experience in the application of Crurasoft® for antireflux surgery and hiatal hernia (HH) repair and to introduce the work of Chinese doctors on this topic.

**METHODS:** Twenty-one patients underwent HH repair with Crurasoft® reinforcement. Gastroesophageal reflux disease (GERD) and HH-related symptoms including heartburn, regurgitation, chest pain, dysphagia, and abdominal pain were evaluated preoperatively and 6 mo postoperatively. A patient survey was conducted by phone by one of the authors. Patients were asked about "recurrent reflux or heartburn" and "dysphagia". An internet-based Chinese literature search in this field was also performed. Data extracted from each study included: number of patients treated, hernia size, hiatorrhaphy, antireflux surgery, follow-up period, recurrence rate, and complications (especially dysphagia).

**RESULTS:** There were 8 type I, 10 type II and 3 type III HHs in this group. Mean operative time was 119.29 min (range 80-175 min). Intraoperatively, length and width of the hiatal orifice were measured, ( $4.33 \pm 0.84$  and  $2.85 \pm 0.85$  cm, respectively). Thirteen and eight Nissen and Toupet funduplications were performed, respectively. The intraoperative complication rate was 9.52%. Despite dysphagia, GERD-related symptoms improved significantly compared with those before surgery. The recurrence rate was 0% during the 6-mo follow-up period, and long-term follow-up disclosed a recurrence rate of 4.76% with a mean period of 16.28 mo. Eight patients developed new-onset dysphagia. The Chinese literature review identified 12 papers with 213 patients. The overall recurrence rate was 1.88%. There was no esophageal erosion and the rate of dysphagia ranged from 0% to 24%.

**CONCLUSION:** The use of Crurasoft® mesh for HH repair results in satisfactory symptom control with a low recurrence rate. Postoperative dysphagia continues to be an issue, and requires more research to reduce its incidence.

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**Key words:** Hiatal hernia; Gastroesophageal reflux disease; Anti-reflux surgery; Mesh; Prosthetic

**Core tip:** With a focus on the mesh fixation technique, the application of Crurasoft® for antireflux surgery and hiatal hernia repair achieved satisfactory outcome. The recurrence rate was 0% during the 6-mo follow-up period, and long-term follow-up disclosed a recurrence rate of 4.76% with a mean period of 16.28 mo. Eight patients developed new-onset dysphagia and this gradually resolved without difficulty in swallowing solid food in 6 patients. The Chinese literature review identified 12 papers with 213 patients. The overall recurrence rate was 1.88%. There was no esophageal erosion and



the rate of dysphagia ranged from 0% to 24%.

Zhang W, Tang W, Shan CX, Liu S, Jiang ZG, Jiang DZ, Zheng XM, Qiu M. Dual-sided composite mesh repair of hiatal hernia: Our experience and a review of the Chinese literature. *World J Gastroenterol* 2013; 19(33): 5528-5533 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i33/5528.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i33.5528>

## INTRODUCTION

Laparoscopic fundoplication is a safe and effective alternative to long-term medical treatment for patients with gastroesophageal reflux disease (GERD) and hiatal hernia (HH)<sup>[1]</sup>. Cryoplasty is considered to be an essential part of antireflux surgery<sup>[2]</sup>. Possible reasons for failed laparoscopic antireflux surgery and disruption of HH repair are lateral tension following simple hiatal closure, or poor character of the crural musculature. The use of a mesh, either by reducing tension or reinforcing the crural musculature, is associated with a significantly lower recurrence rate<sup>[1-3]</sup>.

Despite this, most concerns are focused on mesh-related complications (including intraluminal erosion, fibrosis, and esophageal stenosis)<sup>[3,4]</sup>. Although few mesh-related complications at the hiatus have been reported, anecdotal observations suggest that this complication may be more common<sup>[7]</sup>. Moreover, surgery to manage these complications is complex and may require esophagectomy or gastrectomy<sup>[3]</sup>. For these reasons, many surgeons avoid the use of synthetic mesh in HH repair<sup>[8]</sup>.

The ideal mesh generates adhesion to the diaphragmatic surface and not the visceral side<sup>[4]</sup>. "V" shaped composite polytetrafluoroethylene (PTFE) and expanded polytetrafluoroethylene (ePTFE) prostheses (dual-sided composite mesh, Crurasoft®) have some of these features. PTFE encourages ingrowth of host tissue from the underlying crura, producing local fibrosis and a more uniform mesh-tissue complex. ePTFE was thought to have a benign behavior as opposed to hollow viscera<sup>[9]</sup>, with encapsulation of the material and neomesothelialization of the exposed abdominal surface, thus becoming isolated from the esophagus and stomach<sup>[3]</sup>. That is, dual-sided mesh has the merits of prosthetic mesh and may avoid possible major complications. Chilintseva *et al*<sup>[10]</sup> reported the preliminary results of the use of this dual-sided prosthesis for large HH repairs, demonstrating satisfactory results. Although there was no erosion of the esophagus or stomach, severe periprosthetic fibrosis resulted in postoperative dysphagia in two patients, requiring reoperation. The authors proposed that positioning the mesh with care should be emphasized<sup>[10]</sup>.

To reduce postoperative dysphagia, some propose that space should be allowed between the esophagus and the mesh<sup>[11]</sup>. We summarize our experience in the ap-

plication of Crurasoft® for antireflux surgery and HH repair, focusing on whether a reduction in postoperative complications, especially erosion and dysphagia, can be achieved if technical attention to mesh fixation is applied. Moreover, Crurasoft® is the most commonly used prosthetic mesh in China. We also analyzed and introduced the work of Chinese doctors on this topic, as the Chinese language is still an obstacle for academic communication.

## MATERIALS AND METHODS

### Patients

From May 2010 to July 2012, 48 patients underwent surgery for pH-proven symptomatic GERD with HH in our institution. Of these, 21 patients (14 male, 7 female) underwent hiatal repair with an onlay Crurasoft® mesh reinforcement and were enrolled in this retrospective analysis. The indication for mesh implantation included a HH length longer than 3 cm, obesity, and weak hiatus tissue.

### Symptom evaluation

GERD and HH related symptoms including heartburn, regurgitation, chest pain, dysphagia, and abdominal pain were evaluated preoperatively and 6 mo postoperatively. The severity of symptoms was evaluated using a scaled 0-10 visual analog score, as previously described in the literature<sup>[12]</sup>.

### Preoperative work-up

Preoperative barium contrast swallowing or a computed tomography scan was used to evaluate the type and size of the HH. The presence and severity of esophagitis was confirmed by upper endoscopy. pH monitoring (24-h) and esophageal manometry were performed in all patients to evaluate lower esophageal sphincter function and esophageal motility.

### Surgical technique

Five trocars were used during laparoscopic surgery. The stomach was first reduced into the abdomen, followed by mobilization of the distal esophagus with at least 3 cm of intraabdominal esophagus restored to the abdominal cavity. All patients underwent primary closure of the hiatus with between 2 and 5 nonabsorbable sutures for posterior Cryoplasty, depending on the size of the hiatus defect (Figure 1). Additional anterior Cryoplasty was also performed if the defect was wide. A V-shaped dual-sided composite mesh (Crurasoft®, Composix mesh, CR Bard, Cranston, United States) was used to reinforce the primary repair, with the PTFE side facing the diaphragm (Figure 2). The lower of the two arms was positioned about 2-3 mm below the first stitch, and fixed with staples (EMS, Johnson and Johnson). Additional staples were applied to secure the mesh to the right and left crura and flatten it. The small ePTFE "tongue" was placed to protect the posterior esophageal wall from contacting the PTFE margin. After closing the hiatus, a fundoplication (Nissen/Toupet) was performed.

**Table 1** Classification of hiatal hernia

Type	Description
I	Sliding hernia with the GEJ above the diaphragm
II	Paraesophageal hiatus hernia. A part of the stomach herniates through the hiatus and lies beside the esophagus, without movement of the GEJ
III	Combined hernia. The combination of type I and II
IV	A large defect in the hiatus, allowing other organs to enter the hernia sac

GEJ: Gastroesophageal junction.

**Table 2** Baseline characteristics of the patients who underwent laparoscopic antireflux surgery

Item	Value
Age (yr)	53.81 ± 13.76 (21-75)
Body mass index (kg/m <sup>2</sup> )	28.95 ± 3.11 (21-35)
Hiatal hernia length (cm)	4.33 ± 0.84 (3.1-6.3)
Hiatal hernia width (cm)	2.85 ± 0.85 (1.8-5.4)
DeMeester score	50.30 ± 27.73 (12.7-112.7)
Surgery duration (min)	119.29 ± 23.84 (80-175)
Postoperative stay (d)	4.71 ± 0.85 (4-7)

Data are expressed as absolute mean ± SD (range).

### Intraoperative data

Operative duration and size of HH (length and width), were recorded.

### Phone questionnaire

In January 2013, a patient survey was conducted by phone by one of the authors. Patients were asked about “recurrent reflux or heartburn” and “dysphagia.” Dysphagia was defined as new-onset difficulty in swallowing; severity (mild/severe) and duration of symptoms (temporary/permanent, duration shorter than 6 mo was defined as temporary) were also surveyed. Patients were also asked about “whether you are satisfied with the outcome of surgery”.

### Data sources and study selection

An internet-based Chinese literature search was performed using the Chinese Medical Literature database (Chongqing VIP) between January 2000 and December 2012. The key words “hiatal hernia”, “GERD”, and “mesh” were used in all possible combinations to identify relevant articles. If data appeared appropriate for analysis, the abstract and full article were retrieved for in-depth review. All reference lists in the papers were manually searched for relevant articles. Inclusion criteria were: (1) antireflux surgery with HH repair using Crurasoft® composite mesh; (2) reports described surgical technique details; and (3) reports documented outcome of recurrence and follow-up data. The literature search, study selection, and data extraction were performed by two independent authors. Data extracted from each study included: number of patients treated, hernia size, hiatorrhaphy, antireflux surgery, follow-up period, recurrence rate, and

**Table 3** Symptom evaluation before and after surgery

	Before surgery	After surgery	P value
Heartburn	5.33 ± 1.65	2.14 ± 1.74	0
Regurgitation	5.00 ± 1.64	1.95 ± 1.16	0
Chest pain	3.62 ± 1.99	1.29 ± 1.15	0
Dysphagia	2.57 ± 1.66	1.62 ± 1.86	0.087
Abdominal pain	2.33 ± 1.28	1.38 ± 1.12	0.014

complications (especially dysphagia).

### Ethical approval of the study protocol

All patients were informed about the study protocol. Written consent for the investigation in accordance with the ethical guidelines of Changzheng Hospital was obtained.

### Statistical analysis

The student's *t* test and Pearson  $\chi^2$  test were used to compare means and categorical variables, respectively. A *P* value of less than 0.05 was considered statistically significant.

## RESULTS

### Perioperative data and 30-d complications

Classification of HH is listed in Table 1. There were 8 type I, 10 type II, and 3 type III HHs in this group. Mean operative time was 119.29 min (range 80-175 min). Intraoperatively, both the length and width of the hiatal orifice were measured, (4.33 ± 0.84 and 2.85 ± 0.85 cm, respectively; Table 2). Thirteen and 8 Nissen and Toupet funduplications were performed, respectively.

There was no mortality and no conversion to open surgery. The intraoperative complication rate was 9.52% (one spleen capsular laceration and 1 pneumothorax, all repaired laparoscopically without sequelae). Eight patients complained of new-onset dysphagia with difficulty eating solid food. No early reoperation or intervention (*e.g.*, endoscopic dilatation) was required. The median length of postoperative hospital stay was 5 (range 4-7) d.

### Symptomatic improvement

Preoperative and postoperative symptoms of GERD and HH were compared. As listed in Table 3, all relevant symptoms (except for dysphagia) improved significantly. 10 patients agreed to have a barium meal; no recurrence was demonstrated.

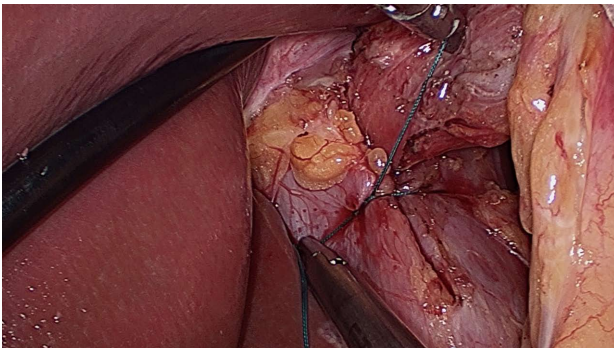
### Long-term complications

One patient was lost to follow-up. The mean follow-up period was 16.28 (6-32) mo. The overall satisfaction rate was 85.71% (18/21). One patient had a recurrence confirmed at the 8 mo postoperative visit, with the major complaint being dysphagia, different from her preoperative symptoms of heartburn. Barium meal examination showed a type II paraesophageal hernia. In a review of her history, she developed dysphagia at postoperative month 3, following an episode of severe vomiting. This

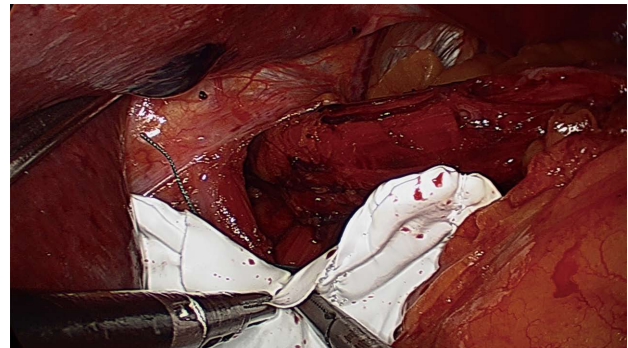
**Table 4** Chinese literature on the use of dual-sided mesh for hiatal hernia repair

Author	Patients	Hernia size	Hiatorrhaphy	Antireflux surgery	Follow-up (mo)	Recurrence rate	Complications
Chu <i>et al</i> <sup>[13]</sup>	12	III (8), IV (4)	Yes	Nissen	12-60	0/12	-
Wang <i>et al</i> <sup>[14]</sup>	15	I (6), II (7), III (2)	Yes	Toupet	Median 18	0/15	1 dysphagia, 2 PPI treatment
Tai <i>et al</i> <sup>[15]</sup>	21	I (9), II (4), III (6), IV (2)	Yes	Toupet	1-16	0/21	3 dysphagia
Ji <i>et al</i> <sup>[16]</sup>	7	-	Yes	Nissen	6-24	0/7	-
Ma <i>et al</i> <sup>[17]</sup>	40	I 1 (3), II (4), III (15), IV (8)	Yes	Toupet/Dor	3-25	0/40	6 dysphagia
Zhao <i>et al</i> <sup>[18]</sup>	25	All > 6 cm	Yes	Nissen/Toupet/Dor	3-35	1 (1)/25 <sup>1</sup>	6 dysphagia, 1 PPI treatment
Xu <i>et al</i> <sup>[19]</sup>	3	13-18 cm	Yes	Toupet	6-12	0/3	-
Zhang <i>et al</i> <sup>[20]</sup>	21	I 1 (4), II (5), III (2)	Yes	Toupet	6-36	0/21	-
Zou <i>et al</i> <sup>[21]</sup>	20	All > 6 cm	Yes	Dor	> 12	2 (5)/20 <sup>1</sup>	-
Yao <i>et al</i> <sup>[22]</sup>	33	I (5), II (23), III (5)	Yes	Nissen	> 12	1 (10)/33 <sup>1</sup>	3 dysphagia, 1 gastric retention
Li <i>et al</i> <sup>[23]</sup>	4	-	Yes	Nissen/Toupet	1-36	0/4	-
Fei <i>et al</i> <sup>[24]</sup>	12	< 5 cm (10), > 5 cm (2)	Yes	Nissen	12	0/12	1 dysphagia

<sup>1</sup>Anatomic recurrence (symptomatic recurrence)/total patient number. PPI: Proton pump inhibitor.



**Figure 1** Primary closure of the hiatus with nonabsorbable stitches for posterior Cryoplasty.



**Figure 2** Lower margin of the two arms was positioned about 2-3 mm below the first stitch.

patient scored the outcome of surgery as dissatisfactory and was reluctant to undergo reoperation. The other two patients presented with recurrence of heartburn or regurgitation 4 and 7 mo following surgery, respectively. In these cases, barium swallowing failed to detect HH recurrence. Both patients underwent Nissen fundoplication without symptoms of dysphagia.

Of the 8 patients who complained of postoperative dysphagia, this gradually resolved without difficulty in swallowing solid food in 6 patients. The average period to resolution of dysphagia was 5.2 (range 4-7) mo. The remaining 2 patients complained of mild to moderate dysphagia, unabated even at the final phone call contact (11 and 19 mo postoperatively, respectively). One patient was confirmed to have a slight stricture at the level of the hiatus, for which dilatation achieved slight resolution. The other patient refused further workup and intervention.

### Chinese literature review

Our literature search identified 24 articles for review. Twelve papers fulfilled the inclusion criteria, with a total of 213 patients included in the final analysis. Reasons for exclusion were: no follow-up data ( $n = 8$ ), pediatric surgery ( $n = 1$ ), review ( $n = 1$ ), and overlapping study populations ( $n = 2$ ) (Table 4)<sup>[13-24]</sup>. All surgery involved hiatoplasty and fundoplication other than gastropexy. There were only 3 randomized controlled trials. Fei *et*

*al*<sup>[24]</sup> concluded that reinforcement of HH repair with Crurasoft® significantly improved HH-related symptoms. Zou *et al*<sup>[21]</sup> confirmed that the use of Crurasoft® significantly reduced recurrence from 36.4% with simple closure to 10%, with a follow-up period greater than 1 year. Yao *et al*<sup>[22]</sup> reported that there was 1 hernia recurrence following mesh placement in the 1-year follow-up period, compared with 3 cases in the simple Cryoplasty group. However, this difference was not statistically significant ( $P = 0.300$ ). Recurrence rates varied from 0% to 25%. This disparity might be due to differences in the definition of recurrence. Some authors define recurrence as symptoms, without radiological confirmation. The highest anatomic recurrence was reported by Zou *et al*<sup>[21]</sup> (10%), where all the HH in that cohort were larger than 6 cm. The overall anatomic recurrence rate was 1.88% (4/213). There were no cases of esophageal or stomach erosion. Postoperative dysphagia varied from 0% to 24% (median 6.67%).

### DISCUSSION

A survey on the use of mesh for HH repair by members of the Society of American Gastrointestinal and Endoscopic Surgeons (SAGES) showed that 33% preferred nonabsorbable to absorbable mesh<sup>[25]</sup>. This reflects the fact that prosthetic mesh has the advantage of reducing HH recurrence; biomaterial tends to be associated with



failure<sup>[9]</sup>. On the other hand, concerns still exist regarding mesh-related complications, including erosion, stricture, and fibrosis. Thus, there may be a trade-off in the choice of mesh repair for HH: permanent mesh risks erosion, while biologic mesh risks recurrence<sup>[9]</sup>.

Crurasoft® has the advantages of permanent mesh, while reducing mesh-related complications. Chilintseva *et al*<sup>[10]</sup> reported 38 cases who underwent HH repair using Crurasoft®, with no recurrences. Priego *et al*<sup>[26]</sup> concluded that Crurasoft®-reinforced hiatoplasty reduced HH recurrence in patients with large hiatal defects (larger than 5 cm), similar to that in patients with smaller hiatal defects (2% *vs* 2.1%). Granderath *et al*<sup>[27]</sup> selected a tailoring strategy for HH repair according to hiatal surface area (HAS). Those with HAS larger than 8 cm<sup>2</sup> underwent Crurasoft® placement in a tension-free, posterior onlay fashion. During a mean follow-up period of 6.3 mo, only 1 patient (1.8%) developed postoperative partial intrathoracic wrap migration. In the Chinese literature review, recurrence was between 0% and 10% (Table 3). The highest recurrence rate (2/20, 10%) was reported by Zou *et al*<sup>[21]</sup>, in whose series all HH were large, with orifices larger than 6 cm or herniation of more than half of the stomach. In our study cohort, we found a type II HH anatomic recurrence. Paraesophageal herniation is a complication that occurs in the immediate postoperative period following laparoscopic antireflux surgery, with an incidence of up to 7%. Vomiting in the early postoperative period, which occurred in this patient, has been identified as a risk factor for recurrence<sup>[3]</sup>. Violent diaphragmatic movements might also dislodge the mesh if fixation is inadequate<sup>[7]</sup>. Sufficient fixation of the mesh and avoidance of lifting or straining have been advocated to reduce this complication.

Two patients suffered symptomatic recurrence without any proof of anatomic recurrence. Both patients underwent Nissen fundoplication and the barium meal examination showed an intact wrap. A possible explanation for this could be poor correlation between postoperative symptoms and actual reflux<sup>[28]</sup>. Both patients presented with heartburn and acid regurgitation, the cardinal symptoms of GERD. However, these symptoms have a low specificity and sensitivity for the actual diagnosis of GERD. One patient agreed to resume manometry and pH monitoring, and all data indicated an improvement compared with that before surgery. As postoperative GERD symptoms actually indicate acid reflux in only 30% of patients and are not even accurate to rule out acid reflux in patients who are completely free of symptoms after surgery, Khajanchee *et al*<sup>[28]</sup> insisted that surgeons should explain the presence of symptomatic recurrence cautiously and that objective testing should be introduced to determine the actual cause.

In a collection of case reports pertaining to mesh complications after prosthetic hiatoplasty with special emphasis on mesh erosion, Stadlhuber *et al*<sup>[7]</sup> identified 17 cases of intraluminal erosion, involving not only different mesh material (polypropylene, PTFE, and biomaterial), but also different mesh configurations (keyhole, horse-

shoe and heart shaped). No apparent relationship between these parameters and mesh erosion was observed, thus the technique for mesh fixation was questioned. Fixation techniques such as the proximity of placement of the mesh at the esophagus are important factors in the development of postoperative complications. The edge of the mesh may “cheese wire” its way into the esophagus if it touches the esophagus or if shrinkage occurs. It is also possible that the mesh can migrate if fixation is insufficient, or traumatic events such as vomiting or repeated coughing may dislodge the mesh, causing it to be apposed to the esophageal wall, leading to erosion or stricture<sup>[7]</sup>. Use of Crurasoft® cannot completely eliminate this complication. In one case report, total migration of Crurasoft® into the stomach was detected by endoscopy 2 years after repeat fundoplication<sup>[29]</sup>. Both our series and a review of the Chinese literature failed to disclose any cases of this complication. However, a case of erosion was discussed at a conference without confirmation of its exact source and details (personal communication). Thus, the exact incidence of this complication may be underestimated<sup>[7,11]</sup>.

As opposed to erosion, esophageal stricture due to fibrosis associated with the prosthesis, may be a more common complication. Although ePTFE is less fibrogenic and is designed to prevent contact between the mesh and viscera, severe fibrosis enveloping the mesh can develop, leading to stricture refractory even to endoscopic dilatation<sup>[10]</sup>. Even though Wassenaar’s recommendations to maintain a 2-3 mm distance between the mesh and esophagus were followed, postoperative dysphagia cannot be completely eliminated (38.10% in our cohort). Only 2 patients had permanent symptoms, due to stricture at the hiatus and not the fundoplication itself (confirmed radiographically). Fortunately, most of these patients presented with mild dysphagia, which resolved within the first postoperative year, and did not require reoperation.

In conclusion, the use of Crurasoft® mesh for HH repair results in satisfactory symptom control with a low recurrence rate. Postoperative dysphagia continues to be an issue, and requires more research to reduce its incidence.

## COMMENTS

### Background

Cryoplasty is considered to be an essential part of antireflux surgery and use of a mesh, either by reducing tension or reinforcing the crural musculature, is associated with a significantly lower recurrence rate. Despite this, most concerns are focused on mesh-related complications.

### Research frontiers

Prosthetic mesh-related complications, including intraluminal erosion, fibrosis, and esophageal stenosis may be more common than reported. Moreover, surgery to manage these complications is complex and may require esophagectomy or gastrectomy. The ideal mesh generates adhesion to the diaphragmatic surface and not the visceral side.

### Innovations and breakthroughs

“V” shaped composite polytetrafluoroethylene (PTFE) and expanded polytetrafluoroethylene (ePTFE) prostheses (dual-sided composite mesh, Crurasoft®) might be an ideal mesh. PTFE encourages ingrowth of host tissue from the underlying crura, producing local fibrosis and a more uniform mesh-tissue complex. ePTFE was thought to have a benign behavior as opposed to hollow viscera,



with encapsulation of the material and neomesothelialization of the abdominal exposed surface, thus becoming isolated from the esophagus and stomach.

### Applications

Crurasoft® is the most commonly used prosthetic mesh in China. The authors summarize their experience in the application of Crurasoft® for antireflux surgery, focusing on whether reduction of postoperative complications, especially erosion and dysphagia, can be achieved if technical attention to mesh fixation is applied.

### Peer review

Clinical study and review by the authors demonstrate the benefit of the mesh Crurasoft in surgical therapy of hiatal hernia. The study is of clinical interest.

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## Proposal of new classification for postoperative patients with hepatocellular carcinoma based on tumor growth characteristics

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patients with vascular involvement and patients with regional lymph node metastasis ( $21.667 \pm 4.773$  and  $14.619 \pm 2.456$  mo, respectively,  $P = 0.801$ ). The OS of patients with distant metastasis ( $6.417 \pm 1.395$  mo) was shorter than that of the other groups ( $P < 0.001$ ). No significant difference in survival was observed between patients with expansive tumor growth and vascular and/or regional lymph node involvement and patients with invasive tumor growth and no vascular and/or lymph node involvement ( $25.762 \pm 7.024$ ,  $21.200 \pm 7.794$  and  $39.533 \pm 5.840$  mo, respectively;  $P = 0.871$ ,  $0.307$  and  $0.563$ , respectively).

**CONCLUSION:** These data led to the proposal of a new staging system: the Expansive-Invasive-Disseminative growth staging classification.

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### Abstract

**AIM:** To propose an appropriate staging system for hepatocellular carcinoma (HCC) classification.

**METHODS:** Here, 288 in-patients with HCC were studied and divided into three groups: those with expansive growth, invasive growth (including satellite nodules, nodule fusions and direct tumor invasion of adjacent organs), or disseminative growth (including vascular involvement, regional lymph node metastasis and distant metastasis). A survival analysis was performed using a Kaplan-Meier analysis, and prognostic factors for overall survival were determined by the Cox proportional hazards regression model.

**RESULTS:** The overall survival (OS) of patients with invasive tumor growth was shorter than that of patients with expansive tumor growth ( $27.796 \pm 3.730$  and  $57.398 \pm 4.873$  mo, respectively,  $P < 0.001$ ). No significant difference in survival was observed between

**Key words:** Hepatocellular carcinoma; Lymph node; Metastasis; Invasive growth; Staging system classification

**Core tip:** A number of staging systems were designed for all of hepatocellular carcinoma (HCC) patients based on some character of tumor, such as tumor size, vascular invasion, regional lymph node metastasis and extra-hepatic spread. But those systems failed to adequately stratify HCC patients with respect to prognosis. In our study, we explore an appropriate staging system for resectable patients with HCC based on tumor's growth characteristics, the Expansive-Invasive-Disseminative growth staging classification, which is a simple and efficacious prognostic model for postoperative patients with HCC.

Zhu CH, Liu XH, Cao R, Wu XZ. Proposal of new classification for postoperative patients with hepatocellular carcinoma based on tumor growth characteristics. *World J Gastroenterol* 2013; 19(33): 5534-5541 Available from: URL: <http://www.wjgnet.com>

## INTRODUCTION

A staging system has been widely used for malignant diseases to stratify patients into comparable groups to predict patients' long-term outcomes. The American Joint Committee on Cancer (AJCC) uses the tumor-node-metastasis (TNM) system as a staging system for many malignant diseases to predict prognosis. Nevertheless, the TNM system fails to adequately stratify hepatocellular carcinoma (HCC) patients with respect to prognosis. In fact, prognosis of patients with cirrhosis and HCC depends on both residual liver function and tumor characteristics. Staging systems that include liver function status were first proposed by Okuda *et al*<sup>[1]</sup>. In 1998, investigators from the Cancer of the Liver Italian Program (CLIP) proposed the CLIP score that is based on Child-Pugh stage, tumor morphology and extension, alpha-fetoprotein (AFP) level, and portal vein thrombosis<sup>[2,3]</sup>. Although the CLIP score has good prognostic value in HCC patients, this score has some limitations when applied to patients with resectable HCC<sup>[4]</sup>. The Chinese University Prognostic Index for HCC was identified in 2002<sup>[5]</sup>. It combines the conventional TNM system with liver function and AFP. In 2003, the Japan Integrated Staging score was proposed by Kudo *et al*<sup>[4]</sup>. It is based on new adapted TNM system and Child-Pugh grading.

Some of the tumor characteristics of HCC include the tumor size, tumor number, invasive growth, vascular invasion, regional lymph node metastasis, and extrahepatic spread<sup>[6-17]</sup>. The TNM staging system is based on tumor characteristics, such as tumor size, vascular invasion, regional lymph node metastasis and extrahepatic spread. In 1999, the Barcelona Clinic Liver Cancer (BCLC) staging classification for HCC was proposed by Llovet *et al*<sup>[18]</sup> based on certain tumor characteristics. Cammà *et al*<sup>[19]</sup> reported that the overall predictive ability of BCLC, CLIP and French classification staging systems was unsatisfactory for patients with both cirrhosis and HCC and did not have uniform predictive results for treated patients and untreated patients. None of the scoring systems provided confident prediction of survival in individual patients. However, because the liver function of the most resectable patients is either an A or B score by Child-Pugh analysis, the TNM staging system provides an effective means of assessing the prognosis of patients following curative resection of HCC<sup>[11]</sup>. Unfortunately, the TNM system fails to include comprehensive characteristics of the tumor, especially the tumor's growth pattern. Thus, it is crucial to design a system to evaluate the effects of tumor characteristics on the clinical outcome of resectable patients with HCC.

In this study, 288 postoperative patients with HCC were studied and followed until August 2012. Patients with a C score from the Child-Pugh analysis were ex-

cluded to eliminate the effect of poor liver function on the long-term outcome. The purpose of the study was to explore an appropriate staging system for resectable patients with HCC based on the patient's tumor growth characteristics.

## MATERIALS AND METHODS

### Patients

Two hundred and eighty-eight in-patients who were diagnosed with HCC and underwent curative resection of HCC at Tianjin Medical University Cancer Institute and Hospital from March 1999 to July 2007 were included in this study and were followed until August 2012. Pathological testing for all patients was performed to confirm HCC. Contrast-enhanced computed tomography (CT), magnetic resonance imaging or positron emission tomography-CT was performed to confirm patients without metastatic disease. The patients' medical records were reviewed, and demographic, clinical and histological variables were derived from the medical records. The pT and pN status were identified based on the 7<sup>th</sup> edition of AJCC TNM classification. This study was approved by our institutional research review board.

### Statistical analysis

Overall survival (OS) curves were plotted by the Kaplan-Meier method and compared using the Log-rank test. The prognostic factors which showed the potential associations with OS were analyzed using a univariate analysis. They Cox proportional hazard model was used to find independent characteristic factors for survival time for the multivariate analysis from the univariate analyses. Statistical calculations were performed using SPSS (Version: 16.0, Chicago, United States).

## RESULTS

### Characteristics of patients

All of the patients with HCC had an A and B score by Child-Pugh analysis. The patients and tumor characteristics are summarized in Table 1. The median age of patients in this study was 54 years. In total, 119 patients had stage I, 22 patients had stage II, 24 patients had stage IIIA, 25 patients had stage IIIB, 66 patients had stage IIIC, and 21 patients had stage IVA HCC. Although most patients with stage IVB HCC were excluded from tumor resection, 11 patients at Tianjin Medical University Cancer Institute and Hospital in stage IVB had a resected primary tumor from March 1999 to July 2007. The OS and median survival time were  $45.704 \pm 3.380$  and  $20.000 \pm 2.314$  mo, respectively, for postoperative patients with HCC. Tumor size, tumor status, regional lymph node metastasis, distant metastasis, Child-Pugh score, AFP and tumor growth pattern were the factors affecting survival (Table 2).

### Survival analysis of invasive growth

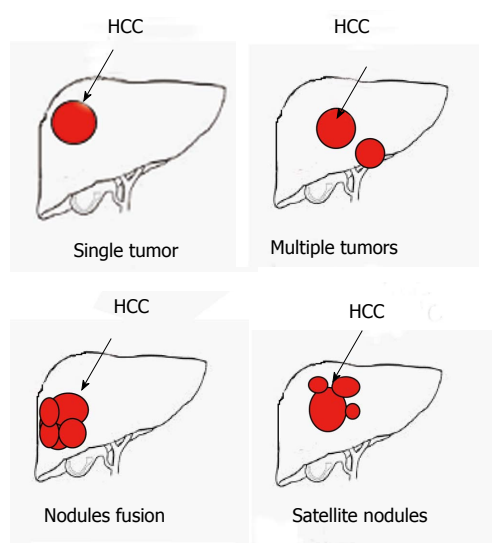
Patients were divided into five groups based on tumor



**Table 1** Patients and tumor characteristics *n* (%)

Characteristics	<i>n</i> = 288
Age (yr)	
≤ 60	208 (72.2)
> 60	80 (27.8)
Gender	
Female	49 (17.0)
Male	239 (83.0)
HBV infection	211 (75.36)
TNM stage	
I	119 (41.3)
II	22 (7.6)
III A	24 (8.3)
III B	25 (8.7)
III C	66 (22.9)
IV A	21 (7.3)
IV B	11 (3.8)
Tumor size (cm)	
≤ 5	105 (36.5)
5 < size ≤ 10	125 (43.4)
> 10	58 (20.1)
Child-Pugh score	
A	260 (90.3)
B	28 (9.7)
AFP (ng/mL)	
≤ 200	161 (57.7)
> 200	118 (42.3)

HBV: Hepatitis B virus; TNM: Tumor-node-metastasis; AFP: Alpha-fetoprotein.



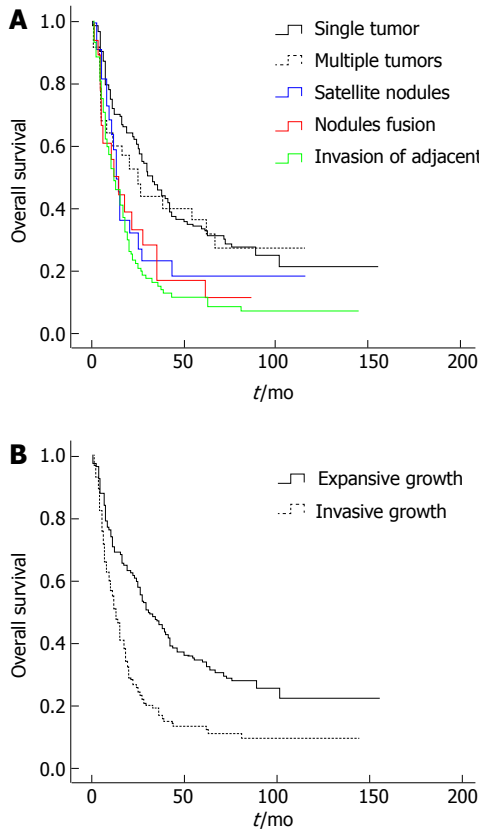
**Figure 1** Growth characteristics of hepatocellular carcinoma. HCC: Hepatocellular carcinoma.

number and invasive growth characteristics as follows: single tumor, multiple tumors, satellite nodules (including perforation the tumor encapsulation), nodule fusion (including diffuse growth lack tumor encapsulation) and invasion of adjacent organs (tumor with direct invasion of adjacent organs other than the gallbladder or with perforation of visceral peritoneum) (Figure 1). No significant difference in survival was observed between patients with a single tumor and patients with multiple tumors (Figure 2A). Moreover, no significant difference in survival was

**Table 2** Analysis of factors affecting survival

	Death/ all cases	Median survival time (95%CI)	<i>P</i> value
Age (yr)			0.612
≤ 60	165/208	19 (13.470-24.530)	
> 60	63/80	23 (15.988-30.012)	
Gender			0.605
Male	36/49	20 (6.283-33.717)	
Female	193/239	20 (15.131-24.869)	
Tumor size (cm)			< 0.001
≤ 5	67/105	34 (17.568-50.432)	
5 < size ≤ 10	107/125	17 (8.965-25.035)	
> 10	55/58	8 (4.890-11.110)	
Tumor status			< 0.001
T1	90/126	33 (24.933-41.067)	
T2	14/22	25 (0.000-73.264)	
T3a	26/31	15 (5.184-24.816)	
T3b	24/27	8 (5.470-10.530)	
T4	75/82	12 (7.905-16.095)	
Regional lymph node metastasis			< 0.001
No	205/264	22 (16.882-27.118)	
Yes	24/24	8 (3.999-12.001)	
Distant metastasis			< 0.001
No	217/276	22 (17.691-26.309)	
Yes	12/12	4 (2.303-5.697)	
Child-Pugh score			< 0.001
A	203/260	23 (18.485-27.515)	
B	26/28	7 (2.851-11.149)	
AFP (ng/mL)			< 0.001
≤ 200	118/161	28 (21.162-34.838)	
> 200	102/118	12 (8.198-15.802)	
Expansive growth and invasive growth			< 0.001
Single tumor	103/143	33 (24.408-41.592)	
Multiple tumors	18/25	25 (8.680-41.320)	
Satellite nodules (including perforation tumor encapsulation)	18/22	13 (10.242-15.758)	
Nodules fusion (including diffuse growth which lack tumor encapsulation)	16/18	12 (3.684-20.316)	
Direct invasion of adjacent organs other than the gallbladder or with perforation of visceral peritoneum	74/80	12 (7.956-16.044)	
Disseminative growth			< 0.001
Without vascular or regional lymph node involvement	155/210	28 (21.928-34.072)	
Vascular involvement	42/46	8 (4.979-11.021)	
Regional lymph node involvement	21/21	10 (4.019-15.981)	
Distant metastasis	12/12	4 (2.303-5.697)	
EID stage			< 0.001
I	96/140	36 (27.626-44.374)	
II	84/99	16 (11.450-20.550)	
III	38/38	8 (4.375-11.625)	
IV	11/11	5 (3.436-6.564)	
TNM stage			< 0.001
I	84/119	35 (26.448-43.552)	
II	14/22	25 (0.000-73.264)	
III A	19/24	16 (2.557-29.443)	
III B	22/25	8 (1.880-14.120)	
III C	58/66	15 (10.033-19.967)	
IV A	21/21	10 (4.019-15.981)	
IV B	11/11	5 (3.436-6.564)	

AFP: Alpha-fetoprotein; EID: Expansive-Invasive-Disseminative growth; TNM: Tumor-node-metastasis.

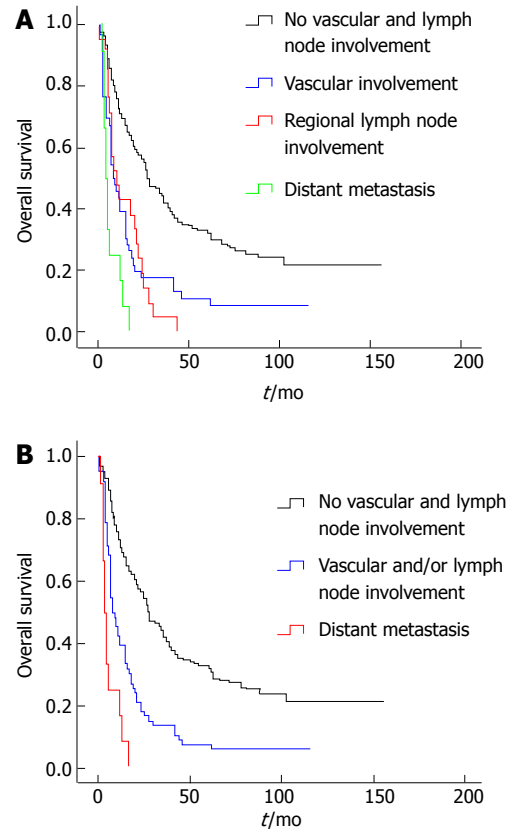


**Figure 2 Overall survival curves for patients with expansive tumor growth and invasive tumor growth.** A: No significant difference in survival was observed between patients with a single tumor and patients with multiple tumors. Moreover, no significant difference in survival was observed among patients with satellite nodules, patients with nodule fusion and patients with tumor invasion of adjacent organs; B: Based on the data, patients were divided to two groups: expansive tumor growth (single tumor and multiple tumors) and invasive tumor growth (satellite nodules, nodules fusion, and tumors with direct invasion of adjacent organs). The overall survival was  $57.398 \pm 4.873$  mo for patients with expansive tumor growth, while it was  $27.796 \pm 3.730$  mo for patients with invasive tumor growth ( $P < 0.001$ ).

observed among patients with satellite nodules, patients with nodule fusion or patients with tumor invasion of adjacent organs (Figure 2A). Based on the data, patients were divided into two groups: expansive tumor growth (single tumor and multiple tumors) and invasive tumor growth (satellite nodules, nodules fusion, and tumor with direct invasion of adjacent organs). The OS of patients with invasive tumor growth was shorter than that of patients with expansive tumor growth ( $P < 0.001$ , Figure 2B). The OS was  $57.398 \pm 4.873$  mo for patients with expansive tumor growth, while it was  $27.796 \pm 3.730$  mo for patients with invasive tumor growth. The median survival time of patients with expansive tumor growth and patients with invasive tumor growth were 30 and 13 mo, respectively.

### Survival analysis of disseminative growth

Based on the extrahepatic metastatic tendency of tumor, the patients were divided to four groups: tumor without vascular and regional lymph node involvement; tumor

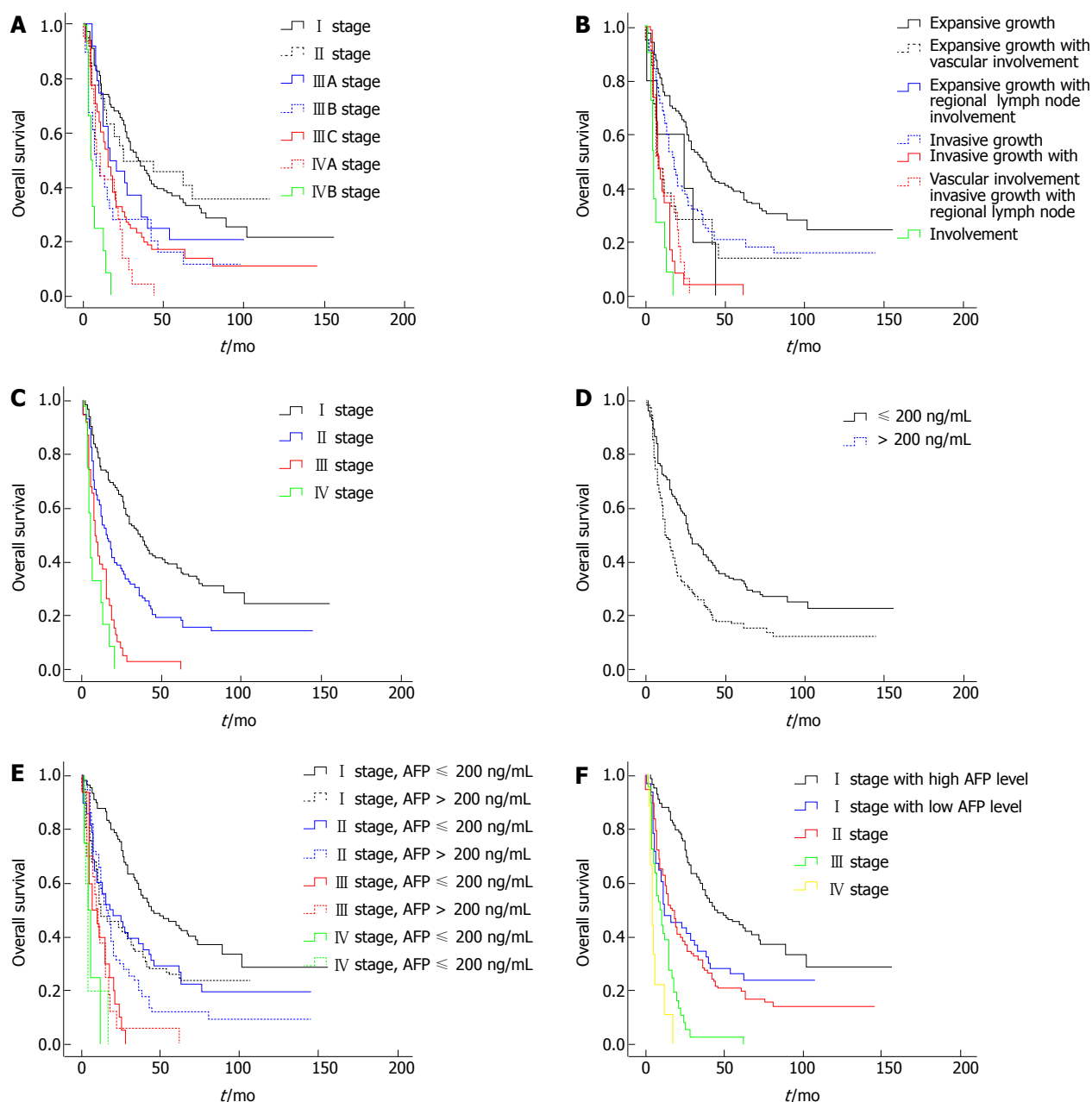


**Figure 3 Overall survival curves for patients with disseminative growth.** A: No significant difference in survival was observed between patients with vascular involvement of tumor and patients with regional lymph node metastasis. The overall survival (OS) of patients with tumors that lacked vascular and regional lymph node involvement was longer than those of other groups, while the OS of patients with distant metastasis was shorter than those of other groups; B: Based on these data, patients were divided into three groups: tumors without vascular and regional lymph node involvement, tumors with vascular and/or regional lymph node involvement and distant metastasis.

with vascular involvement; regional lymph node metastasis; and distant metastasis. No significant differences in survival were observed between patients with vascular involvement of the tumor and patients with regional lymph node metastasis (Figure 3A). The OS of patients who lacked vascular and regional lymph node involvement was better than other groups, while the OS of patients with distant metastasis was shorter than those of other groups (Figure 3A). The OS of patients who lacked vascular and regional lymph node involvement was  $55.532 \pm 4.237$  mo, while the OS of patients with distant metastasis was  $6.417 \pm 1.395$  mo. The OS of patients with vascular and regional lymph node tumor involvement was  $21.667 \pm 4.773$  and  $14.619 \pm 2.456$  mo, respectively. The median survival times for each group were 28, 8, 10 and 4 mo, respectively. Based on these data, the patients were divided into three groups: tumor without vascular and regional lymph node involvement; tumor with vascular and/or regional lymph node involvement; and distant metastasis (Figure 3B).

### New classification of HCC

According to the 7<sup>th</sup> edition TNM classification, no sig-



**Figure 4** New classification for hepatocellular carcinoma. A: Overall survival (OS) curves for patients based on tumor-node-metastasis system classification; B: OS curves for patients with expansive growth, invasive growth and disseminative growth; C: OS curves for patients based on Expansive-Invasive-Disseminative growth (EID) staging classification; D: OS curves for patients with different levels of alpha-fetoprotein (AFP); E: OS curves for patients based on EID staging classification and AFP; F: Overall survival curves for patients combined EID staging classification and AFP.

nificant difference in survival was observed between the groups with stage I or II HCC (Figure 4A). The OS of patients with stage I was  $59.460 \pm 5.806$  and  $55.585 \pm 10.289$  mo for patients with stage II. Moreover, no significant differences in survival were observed among the different stage III groups (Figure 4A). The OS of patients with stage IIIA, IIIB and IIIC were  $35.917 \pm 7.138$ ,  $24.760 \pm 6.250$  and  $31.996 \pm 5.457$  mo, respectively.

Patients were divided to seven groups based on the growth characteristics of the tumor: expansive growth, expansive growth with vascular involvement, expansive growth with regional lymph node involvement, invasive growth, invasive growth with vascular involvement, inva-

sive growth with regional lymph node involvement, and distant metastasis. The OS of each group was  $62.632 \pm 5.415$ ,  $25.762 \pm 7.024$ ,  $21.200 \pm 7.794$ ,  $39.533 \pm 5.840$ ,  $11.478 \pm 2.635$ ,  $12.653 \pm 2.076$  and  $6.727 \pm 1.490$  mo, respectively. There were significant differences in survival among patients with expansive tumor growth, invasive tumor growth and disseminative tumor growth. No significant difference in survival was observed between patients with expansive tumor growth along with vascular and/or regional lymph node involvement and patients with invasive tumor growth who lacked vascular and regional lymph node involvement (Figure 4B).

These data enable the proposal of a new staging sys-



**Table 3** Expansive-Invasive-Disseminative growth staging classification

Phase	Growth characteristics
I	Expansive tumor growth (single tumor and multiple tumors)
II	Expansive tumor growth along with vascular and/or regional lymph node involvement
III	Invasive tumor growth (satellite nodules, nodule fusion and tumor direct invasion of adjacent organs)
IV	Invasive tumor growth along with vascular/regional lymph node involvement
IV	Distant metastasis of tumor

tem: the Expansive-Invasive-Disseminative growth (EID) staging classification, which comprises four stages that select the best candidates for the best therapies currently available. Stage I includes patients with expansive tumor growth. Stage II has two subgroups: the first group consists of patients with expansive tumor growth along with vascular and/or regional lymph node involvement, and the second group includes patients with invasive tumor growth. Stage III includes patients with invasive tumor growth along with vascular/regional lymph node involvement. Stage IV includes patients with distant metastasis (Figure 4C). The OS values of patients at each stage were  $62.637 \pm 5.453$ ,  $36.880 \pm 7.779$ ,  $12.053 \pm 1.796$  and  $6.727 \pm 1.490$  mo, respectively. The overall median survival times of patients at each stage were 36, 16, 8 and 5 mo, respectively.

Table 2 showed the univariate analysis of the factors measured association with survival. The tumor characteristics that were statistically significant were tumor size, AFP, Child Pugh score, growth pattern, TNM stage and EID stage. Multivariate analyses identified EID stage and AFP as independent factors associated with OS ( $P < 0.001$ ,  $P = 0.008$ , respectively). There was no significant difference between the TNM stage and Child Pugh score. The OS of patients with high levels of AFP (AFP > 200 ng/mL;  $56.229 \pm 4.849$  mo) was shorter than that of patients with low levels of AFP ( $33.208 \pm 4.212$  mo;  $P < 0.001$ , Figure 4D). Moreover, the OS of stage I patients with high levels of AFP was shorter than that of patients with low levels of AFP ( $72.240 \pm 6.793$  mo,  $37.804 \pm 6.054$  mo, respectively,  $P < 0.001$ ). No significant difference in survival was observed between I stage patients with high levels of AFP and II stage patients. Dramatically, for stage II, III and IV patients, no significant difference in survival was observed between patients with high levels of AFP and patients with low levels of AFP (Figure 4E). Thus, AFP levels only affect the OS of stage I patients (Figure 4F).

## DISCUSSION

There are four main factors affecting the prognosis of HCC: (1) the stage, aggressiveness and growth rate of the tumor; (2) the general health of the patient; (3) the liver function of the patient; and (4) the specific intervention<sup>[20]</sup>. A number of staging systems have been devised for patients with HCC. Each staging system includes variables which evaluate one or more of the first 3 factors listed above. For example, the TNM staging system eval-

uates only the tumor characteristics, whereas the Child Pugh score provides information regarding liver function. Some of the characteristics of HCC tumors include the tumor size, tumor number, aggressiveness of growth, vascular involvement, regional lymph node metastasis, and extrahepatic spread. Although the TNM and BCLC staging classification for HCC were proposed based on certain tumor characteristics, no staging system systematically evaluates the effect of tumor growth patterns on the clinical outcome of patients with HCC.

In this study, 288 postoperative patients with HCC were studied, and the tumor growth patterns were divided into three types: (1) expansive growth (single tumor and multiple tumors without invasive and disseminative growth); (2) invasive growth (satellite nodules including perforation of the tumor encapsulation, nodule fusion including diffuse growth that lacks tumor encapsulation, and tumors with direct invasion of adjacent organs); and (3) disseminative growth (vascular involvement, regional lymph node metastasis and distant metastasis). Cheng *et al*<sup>[6]</sup> reported that the lack of tumor encapsulation was an independent factor for HCC. Other research showed that the presence of satellite nodules was an independent factor for the long-term survival of patients with HCC after curative resection<sup>[9,21]</sup>. The OS of patients with invasive tumor growth was shorter than that of patients with expansive tumor growth. No significant difference in survival was observed between patients with vascular involvement and patients with regional lymph node metastasis. The OS of patients with expansive tumor growth was longer than those of other groups, while the OS of patients with distant metastasis was shorter than those of other groups. No significant differences in survival were observed between patients with expansive tumor growth with vascular and/or regional lymph node involvement and patients with invasive tumor growth that lacked vascular and regional lymph node involvement.

These data enable the proposal of a new staging system to select the best candidates for the best therapies currently available: the four-stage EID staging classification (Table 3). Stage I includes patients with expansive tumor growths. Stage II has two subgroups: the first group contains patients with expansive tumor growth along with vascular and/or regional lymph node involvement, and the second group consists of patients with invasive tumor growth. Stage III includes patients with invasive tumor growth along with vascular/regional lymph node involvement. Stage IV comprises patients with distant tumor metastasis. The OS values of each stage

**Table 4** Characteristics of patients as defined by Expansive-Invasive-Disseminative growth stage *n* (%)

Characteristics	I	II	III	IV
Age (yr)				
≤ 60	96 (68.6)	78 (78.8)	26 (68.4)	8 (72.9)
> 60	44 (31.4)	21 (21.2)	12 (31.6)	3 (27.3)
Gender				
Female	27 (19.3)	15 (15.2)	5 (13.2)	2 (18.2)
Male	113 (80.7)	84 (84.8)	33 (86.8)	9 (81.8)
HBV infection	105 (75.0)	75 (75.8)	23 (60.5)	8 (72.7)
TNM stage				
I	117 (83.6)	2 (2.0)	0	0
II	12 (8.6)	10 (10.1)	0	0
III A	9 (6.4)	15 (15.2)	0	0
III B	0	20 (20.2)	5 (13.2)	0
III C	2 (1.4)	46 (44.5)	18 (47.4)	0
IV A	0	6 (6.1)	15 (39.5)	0
IV B	0	0	0	11 (100)
Tumor size (cm)				
≤ 5	71 (50.7)	27 (27.3)	5 (13.2)	2 (18.2)
5 < size ≤ 10	52 (37.1)	50 (50.5)	18 (47.4)	5 (45.5)
> 10	17 (12.1)	22 (22.2)	15 (39.5)	4 (36.4)
Child-Pugh score				
A	128 (91.4)	92 (92.9)	30 (78.9)	10 (90.9)
B	12 (8.6)	7 (7.1)	8 (21.1)	1 (9.1)
AFP (ng/mL)				
≤ 200	91 (66.4)	46 (47.4)	20 (55.6)	4 (44.4)
> 200	46 (33.6)	51 (52.6)	16 (44.4)	5 (55.6)

HBV: Hepatitis B virus; TNM: Tumor-node-metastasis; AFP: Alpha-fetoprotein.

were  $62.637 \pm 5.453$ ,  $36.880 \pm 7.779$ ,  $12.053 \pm 1.796$  and  $6.727 \pm 1.490$  mo, respectively. The median survival times of patients at each stage were 36, 16, 8 and 5 mo, respectively.

Univariate analysis showed that the statistically significant factors were EID staging classification and AFP (Table 4). There were no statistically significant correlative differences between OS and the Child-Pugh score. There is a substantial amount of research showing that the Child-Pugh score and AFP values > 200 ng/mL are independent factors for HCC<sup>[6-8,15,22]</sup>. In fact, patients with a C Child-Pugh score were excluded for this study to eliminate the effects of poor liver function on long-term outcome. The OS of stage I patients with high levels of AFP was shorter than that of patients with low levels of AFP. There is research showing that the Japan Integrated Staging Score and BCLC staging system combined with AFP levels may serve as a better staging system for early-stage HCC patients<sup>[22,23]</sup>. Dramatically, for patients in stages II, III and IV, no significant difference in survival was observed between patients with high levels of AFP and patients with low levels of AFP. Thus, AFP levels likely only affect the OS of stage I patients.

In conclusion, the EID staging classification is a simple and efficacious prognostic model for postoperative patients with HCC. Because the EID staging classification is easily obtained and objective, we propose it for widespread use in clinical practice as a staging system for postoperative patients with HCC.

## COMMENTS

### Background

The tumor-node-metastasis (TNM) system fails to include comprehensive characteristics of the tumor, especially the tumor's growth pattern. Thus, it is crucial to design a system to evaluate the effects of tumor characteristics on the clinical outcome of resectable patients with hepatocellular carcinoma (HCC).

### Research frontiers

The characteristics of HCC tumors include the tumor size, tumor number, aggressiveness of growth, vascular involvement, regional lymph node metastasis, and extrahepatic spread. Although the TNM and Barcelona Clinic Liver Cancer staging classification for HCC were proposed based on certain tumor characteristics, no staging system systematically evaluates the effect of tumor growth patterns on the clinical outcome of patients with HCC.

### Innovations and breakthroughs

These data enable the proposal of a new staging system to select the best candidates for the best therapies currently available: the four-stage Expansive-Invasive-Disseminative growth (EID) staging classification. Stage I includes patients with expansive tumor growths. Stage II has two subgroups: the first group contains patients with expansive tumor growth along with vascular and/or regional lymph node involvement, and the second group consists of patients with invasive tumor growth. Stage III includes patients with invasive tumor growth along with vascular/regional lymph node involvement.

### Applications

The EID staging classification is a simple and efficacious prognostic model for postoperative patients with HCC. Because the EID staging classification is obtained easily, authors propose it for postoperative patients with HCC.

### Terminology

Expansive growth includes single tumor and multiple tumors without invasive and disseminative growth; invasive growth consists of satellite nodules including perforation of the tumor encapsulation, nodule fusion including diffuse growth that lacks tumor encapsulation and tumors with direct invasion of adjacent organs; disseminative growth contains vascular involvement, regional lymph node metastasis and distant metastasis

### Peer review

The prognostic significance of the new staging method was confirmed by the detailed statistical analysis of 288 patients treated in a single facility, being compared with other staging systems previously proposed. This clinical study is interesting and novel. The author found a simply and efficacy prognostic model for postoperative patients with HCC.

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## Impact of intraoperative blood loss on survival after curative resection for gastric cancer

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### Abstract

**AIM:** To elucidate the potential impact of intraoperative blood loss (IBL) on long-term survival of gastric cancer patients after curative surgery.

**METHODS:** A total of 845 stage I-III gastric cancer patients who underwent curative gastrectomy between January 2003 and December 2007 in our center were enrolled in this study. Patients were divided into 3 groups according to the amount of IBL: group 1 (< 200 mL), group 2 (200-400 mL) and group 3 (> 400 mL). Clinicopathological features were compared among the three groups and potential prognostic factors were analyzed. The Log-rank test was used to assess statistical differences between the groups. Independent prognostic factors were identified by the Cox proportional haz-

ards regression model. Stratified analysis was used to investigate the impact of IBL on survival in each stage. Cancer-specific survival was also compared among the three groups by excluding deaths due to reasons other than gastric cancer. Finally, we explored the possible factors associated with IBL and identified the independent risk factors for IBL  $\geq$  200 mL.

**RESULTS:** Overall survival was significantly influenced by the amount of IBL. The 5-year overall survival rates were 51.2%, 39.4% and 23.4% for IBL less than 200 mL, 200 to 400 mL and more than 400 mL, respectively (< 200 mL vs 200-400 mL,  $P < 0.001$ ; 200-400 mL vs > 400 mL,  $P = 0.003$ ). Age, tumor size, Borrmann type, extranodal metastasis, tumour-node-metastasis (TNM) stage, chemotherapy, extent of lymphadenectomy, IBL and postoperative complications were found to be independent prognostic factors in multivariable analysis. Following stratified analysis, patients staged TNM I-II and those with IBL less than 200 mL tended to have better survival than those with IBL not less than 200 mL, while patients staged TNM III, whose IBL was less than 400 mL had better survival. Tumor location, tumor size, TNM stage, type of gastrectomy, combined organ resection, extent of lymphadenectomy and year of surgery were found to be factors associated with the amount of IBL, while tumor location, type of gastrectomy, combined organ resection and year of surgery were independently associated with IBL  $\geq$  200 mL.

**CONCLUSION:** IBL is an independent prognostic factor for gastric cancer after curative resection. Reducing IBL can improve the long-term outcome of gastric cancer patients following curative gastrectomy.

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**Key words:** Gastric carcinoma; Intraoperative blood loss; Blood transfusion; Postoperative complication; Prognosis



**Core tip:** Intraoperative blood loss (IBL) has been shown to be associated with poor outcome in various types of malignancy. In this study, we found that the overall survival of gastric cancer patients was significantly affected by the amount of IBL, and IBL was an independent prognostic factor in multivariate analysis. We suggest that meticulous surgery and new surgical methods such as the application of an ultrasonic scalpel in lymph node dissection should be used to decrease the amount of IBL and improve the long-term outcome of gastric cancer patients following curative gastrectomy.

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## INTRODUCTION

Radical gastrectomy with regional lymph node dissection is the only possible curative treatment for gastric cancer<sup>[1]</sup>. Even after R0 resection, a significant number of patients suffer from recurrence, especially those with advanced gastric cancer<sup>[2-4]</sup>. Tumor depth and lymph node status are well-known prognostic factors, and patient age and performance status have also been reported to have an impact on the long-term outcome of patients<sup>[5-7]</sup>. Besides these factors, a number of potential prognostic factors have been reported in recent years, such as perioperative blood transfusion and intraoperative blood loss (IBL)<sup>[8-11]</sup>.

The impact of IBL on long-term outcome has previously been reported in patients with colorectal cancer, prostate cancer and pancreas cancer<sup>[12-14]</sup>. However, there are few reports assessing the relationship between IBL and long-term outcome in gastric cancer patients. Dhar *et al*<sup>[10]</sup> reported that more than 500 mL blood loss during surgery was an independent predictor of survival in gastric cancer patients with transmural depth invasion. Kamei *et al*<sup>[11]</sup> demonstrated that IBL was a crucial risk factor for peritoneal recurrence after curative resection for advanced gastric cancer. Unfortunately, the numbers of patients included in these aforementioned studies were small, and no further meticulous analysis was performed to explore the correlation between the prognosis of gastric cancer patients and the accurate amount of IBL.

The aim of the present study is to elucidate the potential impact of IBL on the long-term survival of gastric cancer patients after curative surgery in a single high-volume center in China.

## MATERIALS AND METHODS

### Patients

The surgical and pathological data of 845 patients with

gastric cancer who had undergone curative gastrectomy (R0 resection) with lymph node dissection and had been followed up between January 2003 and December 2007 at Tianjin Medical University Cancer Institute and Hospital were reviewed in this study. All the patients had been histologically diagnosed with adenocarcinoma of the stomach. Patients who previously underwent gastric surgery or received preoperative chemotherapy were excluded. Patients with distant metastasis were also excluded. The study population consisted of 845 patients, 607 males (71.8%) and 238 females (28.2%) with a median age of 62 years (range, 23-89 years).

### Surgical treatment and perioperative management

All the patients underwent gastrectomy with D1 or D2 lymph node dissection. The choice of surgical procedure for reconstruction was made by the surgeon. Resection margin was pathologically confirmed as negative. Postoperative adjuvant chemotherapy was administered according to tumor stage, physical condition and the patient's willingness. Chemotherapeutics consisted of 5-fluorouracil, leucovorin and oxaliplatin. Radiotherapy was not administered in the present study.

IBL was visually estimated according to the weight or volume of blood absorbed by gauze and suction pump by anesthesiologists immediately after surgery. We obtained this information from anesthesia records. IBL ranged from 50 to 1500 mL and the median IBL was 200 mL for the whole group. The patients were divided into 3 groups according to the amount of IBL: group 1 (< 200 mL), group 2 (200-400 mL) and group 3 (> 400 mL). The entire transfusion history during hospital stay for surgery was recorded. Patients whose perioperative hemoglobin was less than 70 g/L or who lost a lot of blood during surgery were routinely given a red blood cell transfusion. Of the 845 patients, 211 had a perioperative red blood cell transfusion, and the remaining 634 did not receive a transfusion. Postoperative complications during hospitalization only included those directly associated with surgery, such as hemorrhage, wound dehiscence, anastomotic leak, pancreatic fistula, lymphatic fistula and abdominal or wound infection.

### Evaluation of clinicopathological variables and survival

The clinicopathological features studied included gender, age, tumor location, tumor size, Borrmann type, histology, extranodal metastasis (EM), type of gastrectomy, combined organ resection, postoperative chemotherapy, tumour-node-metastasis (TNM) stage, extent of lymphadenectomy, postoperative complications, perioperative transfusion, and IBL. Clinicopathological features were first compared among the three groups and the impact of each factor on survival was evaluated to identify independent prognostic factors. We next determined whether IBL influenced cancer-specific survival by comparing overall survival among the three groups by excluding deaths due to reasons other than gastric cancer. Finally, we explored the possible factors associated with IBL and identified

**Table 1 Case characteristics *n* (%)**

Characteristics	IBL (mL)			$\chi^2$	<i>P</i> value
	< 200	200-400	> 400		
IBL (mean $\pm$ SD)	99.3 $\pm$ 25.0	223.2 $\pm$ 41.6	484.4 $\pm$ 179.9		
Gender				4.307	0.116
Male	269 (70.2)	285 (71.6)	53 (71.8)		
Female	114 (29.8)	113 (28.4)	11 (28.2)		
Age (yr)				2.488	0.288
$\leq$ 65	230 (60.1)	227 (57.0)	32 (50.0)		
> 65	153 (39.9)	171 (43.0)	32 (50.0)		
Tumor location				40.555	< 0.001
Lower 1/3	205 (53.5)	148 (37.2)	14 (21.9)		
Middle 1/3	36 (9.4)	41 (10.3)	6 (9.4)		
Upper 1/3	98 (25.6)	164 (41.2)	34 (53.1)		
2/3 or more	44 (11.5)	45 (11.3)	10 (15.6)		
Tumor size				17.677	< 0.001
< 5 cm	180 (47.0)	155 (38.9)	13 (20.3)		
$\geq$ 5 cm	203 (53.0)	243 (61.1)	51 (79.7)		
Borrmann type				5.180	0.075
I / II	169 (44.1)	153 (38.4)	33 (51.6)		
III / IV	214 (55.9)	245 (61.6)	31 (48.4)		
Histology				0.982	0.612
Differentiated	121 (31.6)	139 (34.9)	21 (32.8)		
Undifferentiated	262 (68.4)	259 (65.1)	43 (67.2)		
Extranodal metastasis				1.963	0.375
Positive	59 (15.4)	71 (17.8)	14 (21.9)		
Negative	324 (84.6)	327 (82.2)	50 (78.1)		
Depth of invasion				14.719	0.023
pT1	14 (3.7)	11 (2.8)	0 (0.0)		
pT2	53 (13.8)	44 (11.1)	0 (0.0)		
pT3	21 (5.5)	28 (7.0)	6 (9.4)		
pT4	295 (77.0)	315 (79.1)	58 (90.6)		
Lymph node metastasis				15.793	0.015
pN0	173 (45.2)	146 (36.7)	19 (29.7)		
pN1	56 (14.6)	82 (20.6)	9 (14.1)		
pN2	85 (22.2)	87 (21.9)	15 (23.4)		
pN3	69 (18.0)	83 (20.9)	21 (32.8)		
TNM stage				15.313	0.004
I	53 (13.8)	43 (10.8)	0 (0.0)		
II	132 (34.5)	118 (29.6)	19 (29.7)		
III	198 (51.7)	237 (59.5)	45 (70.3)		
Chemotherapy				2.036	0.361
Yes	104 (27.2)	119 (29.9)	14 (21.9)		
No	279 (72.8)	279 (70.1)	50 (78.1)		
Type of gastrectomy				37.357	< 0.001
Total	51 (13.3)	117 (29.4)	24 (37.5)		
Subtotal	332 (86.7)	281 (70.6)	40 (62.5)		
Combined organ resection				22.256	< 0.001
Yes	16 (4.2)	38 (9.5)	13 (20.3)		
No	367 (95.8)	360 (90.5)	51 (79.7)		
Extent of lymphadenectomy				7.230	0.027
D2 and D2+	189 (49.3)	188 (47.2)	20 (31.3)		
D1	194 (50.7)	210 (52.8)	44 (68.8)		
Postoperative complications				7.500	0.024
Present	20 (5.2)	34 (8.5)	9 (14.1)		
Absent	363 (94.8)	364 (91.5)	55 (85.9)		

IBL: Intraoperative blood loss; TNM: Tumour-node-metastasis.

the independent risk factors for IBL  $\geq$  200 mL. The tumors were staged according to the 7<sup>th</sup> edition Union for International Cancer Control TNM classification system, whereas lymphadenectomy and lymph node stations were defined according to the 3<sup>rd</sup> English Edition of the Japanese Classification of Gastric Carcinoma. Tumors were

classified into two groups based on histology: differentiated type including papillary, well or moderately differentiated adenocarcinoma; and undifferentiated type including poorly differentiated or undifferentiated adenocarcinoma, signet ring cell carcinoma and mucinous carcinoma.

### Follow-up

The patients were followed up every 3 mo up to 2 years after surgery, then every 6 mo up to 5 years, and then every year or until death. Physical examination, laboratory tests, imaging and endoscopy were performed at each visit. The median follow-up was 39 mo (range 1-103 mo), and the last follow-up date was December 20, 2012. The overall survival rate was calculated from the day of surgical resection until time of death or final follow-up.

### Statistical analysis

Categorical variables were analyzed by means of the  $\chi^2$  or Fisher's exact test. Overall survival curves were calculated using the Kaplan-Meier method based on the length of time between primary surgical treatment and final follow-up or death; the Log-rank test was used to assess statistical differences between the groups. Independent prognostic factors were identified by the Cox proportional hazards regression model. One-way analysis of variance (ANOVA) analysis or *t* test was used in univariate analysis to identify possible factors associated with IBL. Independent risk factors for IBL  $\geq$  200 mL were determined by logistic regression. *P* < 0.05 was considered statistically significant. The statistical analysis was performed using the statistical program SPSS 17.0 (SPSS, Chicago, IL, United States).

## RESULTS

### Clinicopathological features

Of the 845 patients, 397 (47.0%) patients underwent D2 or greater lymph node dissection, and the remaining 448 (53.0%) patients underwent D1 lymph node dissection. Sixty-seven patients underwent gastrectomy combined with other organ resections and 237 patients received postoperative adjuvant chemotherapy.

The patients were divided into three groups according to IBL (Table 1). The mean IBL was 99.4 mL in group 1, 223.2 mL in group 2 and 484.4 mL in group 3. There were no statistical differences in gender, age, Borrmann type, histology, EM and postoperative chemotherapy among the three groups. Tumors located in the upper one-third were more frequent in group 2 and group 3, while in group 1, 53.5% of tumors were located in the lower one-third. The incidence of postoperative complications and the ratios of tumors with a diameter  $\geq$  5 cm increased when the amount of IBL was high. Total gastrectomy and combined organ resection were more frequently performed in group 3 than in group 1 and group 2. Patients in group 2 and group 3 were more likely to have advanced tumor (T), node (N), and TNM stage than

**Table 2** Survival analysis of all patients with gastric cancer

Characteristics	n (%)	5-yr OS	Univariate analysis		Multivariate analysis	
			$\chi^2$	P value	HR (95%CI)	P value
Gender			1.609	0.205		
Male	607 (71.8)	42.20%				
Female	238 (28.2)	47.10%				
Age (yr)			21.037	< 0.001		
≤ 65	489 (57.9)	50.10%			1 (ref)	
> 65	356 (42.1)	34.60%			1.372 (1.140-1.652)	0.001
Tumor location			26.417	< 0.001		
Lower 1/3	367 (43.4)	50.10%			1 (ref)	
Middle 1/3	83 (9.8)	45.80%			0.978 (0.680-1.407)	0.905
Upper 1/3	296 (35.0)	39.50%			0.931 (0.741-1.169)	0.538
2/3 or more	99 (11.7)	29.30%			1.149 (0.832-1.586)	0.398
Tumor size			58.693	< 0.001		
< 5 cm	348 (41.2)	57.80%			1 (ref)	
≥ 5 cm	497 (58.8)	33.60%			1.411 (1.152-1.730)	0.001
Borrmann type			13.517	< 0.001		
I / II	355 (42.0)	50.40%			1 (ref)	
III / IV	490 (58.0)	38.60%			1.285 (1.062-1.556)	0.010
Histology			6.783	0.009		
Differentiated	281 (33.3)	49.80%			1 (ref)	
Undifferentiated	564 (66.7)	40.40%			1.151 (0.939-1.412)	0.176
Extranodal metastasis			52.773	< 0.001		
Negative	701 (83.0)	47.50%			1 (ref)	
Positive	144 (17.0)	24.30%			1.543 (1.236-1.925)	< 0.001
TNM stage			147.103	< 0.001		
I	96 (11.4)	82.30%			1 (ref)	
II	269 (31.8)	58.40%			2.253 (1.362-3.727)	0.002
III	480 (56.8)	27.50%			4.736 (2.898-7.740)	< 0.001
Chemotherapy			10.999	0.001		
Yes	237 (28.0)	50.60%			1 (ref)	
No	608 (72.0)	40.80%			1.357 (1.093-1.684)	0.006
Extent of lymphadenectomy			6.668	0.010		
D2 and D2+	397 (47.0)	48.40%			1 (ref)	
D1	448 (53.0)	39.30%			1.372 (1.126-1.671)	0.002
Type of gastrectomy			21.400	< 0.001		
Subtotal	653 (77.3)	47.00%			1 (ref)	
Total	192 (22.7)	31.80%			1.102 (0.849-1.430)	0.466
Combined organ resection			10.310	0.001		
No	778 (92.1)	44.60%			1 (ref)	
Yes	67 (7.9)	31.30%			1.116 (0.811-1.536)	0.501
Intraoperative blood loss			29.175	< 0.001		
< 200 mL	383 (45.3)	51.20%			1 (ref)	
200-400 mL	398 (47.1)	39.40%			1.242 (1.017-1.516)	0.033
> 400 mL	64 (7.6)	23.40%			1.590 (1.140-2.217)	0.006
Perioperative transfusion			6.145	0.013		
No	634 (75.0)	45.70%			1 (ref)	
Yes	211 (25.0)	37.00%			0.962 (0.748-1.180)	0.708
Postoperative complications			28.320	< 0.001		
Absent	782 (92.5)	44.90%			1 (ref)	
Present	63 (7.5)	27.00%			2.096 (1.525-2.881)	< 0.001

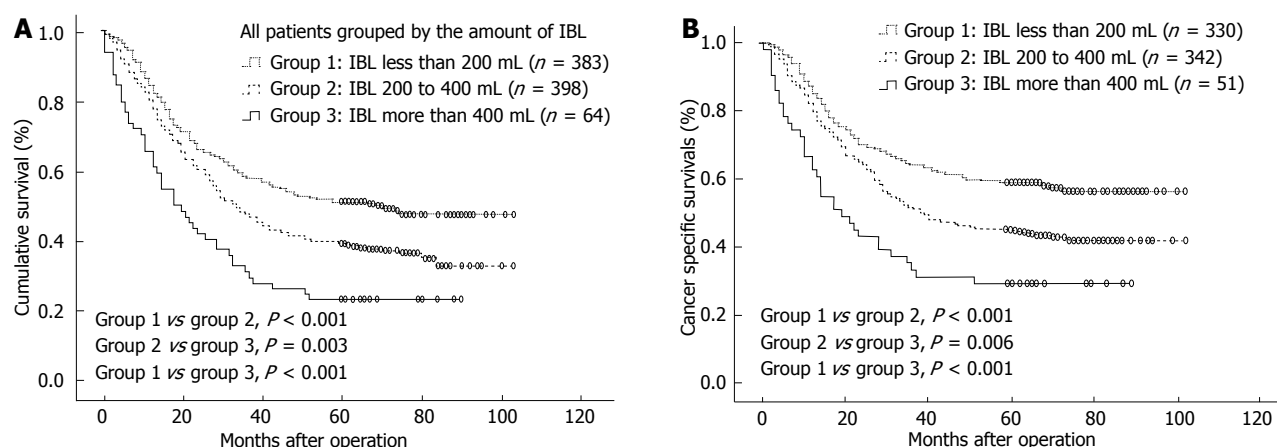
OS: Overall survival; TNM: Tumour-node-metastasis.

patients in group 1.

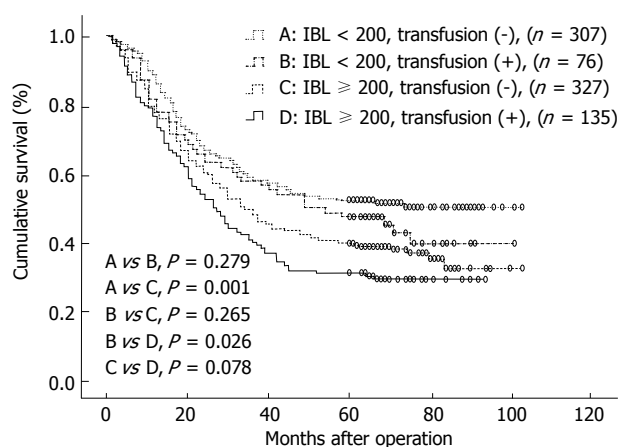
**Prognostic value of IBL in gastric cancer**

Data from univariate and multivariate survival analyses are shown in Table 2. A total of 14 factors evaluated in the univariate analysis had a significant effect on survival: age (≤ 65 years *vs* > 65 years), tumor location, tumor size, Borrmann type (types I and II *vs* types III and IV), histology, EM, TNM stage, postoperative chemotherapy, type of gastrectomy, combined organ resection, extent of lymphadenectomy, IBL, perioperative transfusion and

postoperative complications. Gender did not influence survival. In multivariate analysis, age, tumor size, Borrmann type, EM, TNM stage, postoperative chemotherapy, extent of lymphadenectomy, postoperative complications and IBL were found to be independent prognostic factors for overall survival (OS). The 5-year OS rates were 51.2%, 39.4% and 23.4% for IBL < 200, 200-400, and > 400 mL, respectively, (< 200 mL *vs* 200-400 mL, *P* < 0.001; 200-400 mL *vs* > 400 mL, *P* = 0.001) (Figure 1A). When deaths due to factors other than gastric cancer were excluded, cancer-specific survival was still sig-



**Figure 1** Overall survival and cancer-specific curves for all patients grouped by intraoperative blood loss. A: Overall survival curve; B: Cancer-specific survival curve. IBL: Intraoperative blood loss.



**Figure 2** Overall survival curves for all patients classified according to intraoperative blood loss and red blood cell transfusion. IBL: Intraoperative blood loss.

nificantly influenced by IBL (Figure 1B). The 5-year OS rates for patients with red blood cell transfusion *vs* those without were 37.0% and 45.7% ( $P = 0.013$ ), respectively.

To assess the association between IBL and red blood cell transfusion, patients were categorized into 4 groups [IBL < 200 mL and transfusion (-); IBL < 200 mL, transfusion (+); IBL ≥ 200 mL, transfusion (-); IBL ≥ 200 mL, transfusion (+)], and OS was compared among these groups (Figure 2). As a blood loss of 200 mL was the median for the whole group, it was used for dichotomization in the statistical analysis. As a result, a IBL of 200 mL or more was a significant factor when excluding the influence of red blood cell transfusion ( $P = 0.001$ ;  $P = 0.026$ ). However, there was no significant difference in OS between patients with and without transfusion when the influence of IBL was excluded ( $P = 0.279$ ;  $P = 0.078$ ).

The results of the stratified analysis are shown in Table 3. In patients with TNM stage I, those with IBL less than 200 mL had significantly better survival than those with IBL 200-400 mL (Figure 3A). In the patients

**Table 3** Tumour-node-metastasis-stratified analysis of the overall survival

	Group 1 <sup>1</sup>		Group 2 <sup>1</sup>		Group 3 <sup>1</sup>		$\chi^2$	<i>P</i> value
	<i>n</i>	5-yr OS	<i>n</i>	5-yr OS	<i>n</i>	5-yr OS		
TNM								
I	53	88.7	43	74.4			4.538	0.037
II	132	68.2	118	50.0	19	42.1	10.763	0.005
III	198	29.8	237	27.8	45	15.6	8.035	0.018

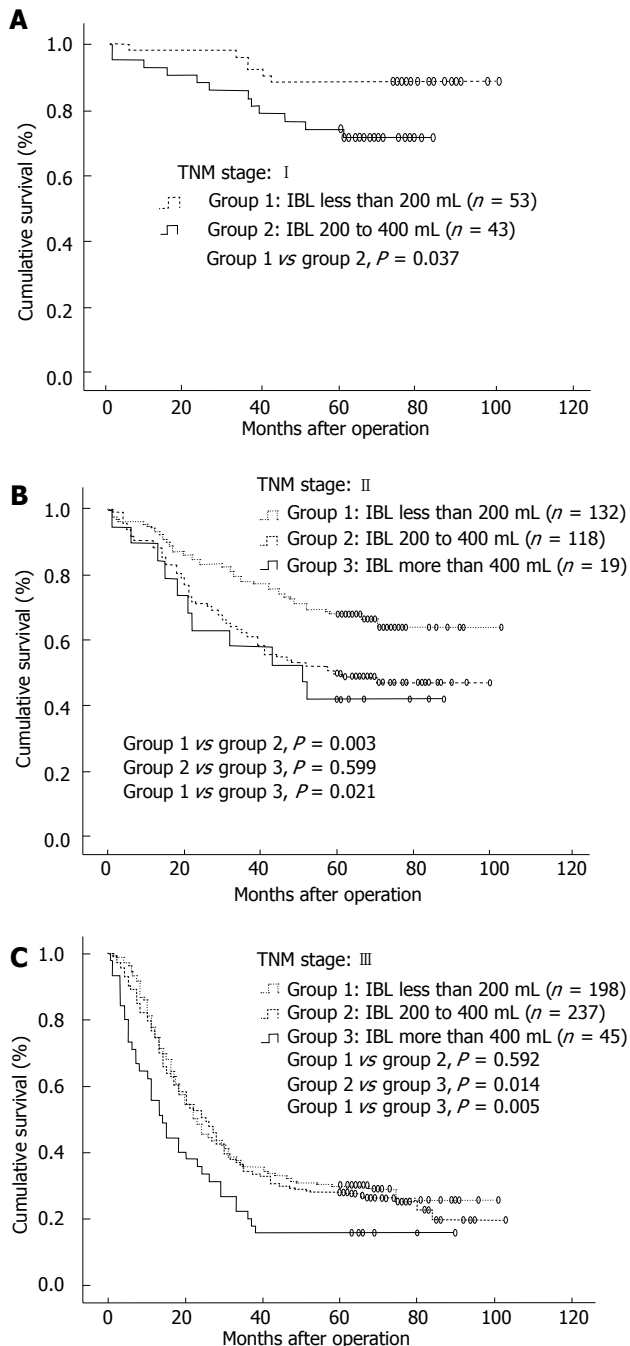
<sup>1</sup>Group 1: IBL < 200 mL; Group 2: IBL 200-400 mL; Group 3: IBL > 400 mL. OS: Overall survival; TNM: Tumour-node-metastasis; IBL: Intraoperative blood loss.

staged with TNM II, those with IBL less than 200 mL had a significantly higher 5-year OS than those with IBL 200-400 mL or more than 400 mL, while there were no statistical differences in OS between those with IBL 200-400 mL and more than 400 mL (Figure 3B). For patients staged TNM III, OS did not differ significantly between those with IBL less than 200 mL and 200-400 mL, however, these patients had significantly higher 5-year OS than those with IBL more than 400 mL (Figure 3C).

### Risk factors associated with IBL

Univariate analysis of factors associated with the amount of IBL is shown in Table 4. Following one-way ANOVA analysis or *t* test, tumor location, tumor size, TNM stage, type of gastrectomy, combined organ resection, extent of lymphadenectomy and year of surgery were found to be significant factors associated with the amount of IBL. Factors which had no influence on IBL were gender, age, Borrmann type, histology, and EM. As patients with IBL less than 200 mL had the best survival, we further identified the independent risk factors for IBL ≥ 200 mL. Factors significant in the univariate analysis were included in the multivariate analysis. Tumor location, type of gastrectomy, combined organ resection and year of surgery were found to be independent risk factors for IBL ≥ 200 mL in the multivariate analysis (Table 5).





**Figure 3 Overall survival curves.** A: 96 patients staged tumour-node-metastasis (TNM) I; B: 269 patients staged TNM II; C: 480 patients staged TNM III. IBL: Intraoperative blood loss.

## DISCUSSION

The prognosis of gastric cancer is mainly associated with tumor depth and lymph node status<sup>[5,6]</sup>. To improve the outcome of gastric cancer, standard surgery with D2 lymph node dissection is recommended<sup>[15,16]</sup>. However, even after curative gastrectomy with D2 dissection, the prognosis remains poor. In the present study, we evaluated the potential prognostic factors and found that IBL was significantly associated with the survival of patients

**Table 4 Association between clinicopathologic factors and the amount of intraoperative blood loss: univariate analysis**

Characteristics	<i>n</i> (%)	Amount of IBL (mL) (mean $\pm$ SD)	<i>t</i> / <i>F</i>	<i>P</i> value
Gender			1.770	0.077
Male	607 (71.8)	191.4 $\pm$ 128.6		
Female	238 (28.2)	175.2 $\pm$ 92.5		
Age (yr)			-1.128	0.260
$\leq 65$	489 (57.9)	182.9 $\pm$ 121.8		
$> 65$	356 (42.1)	192.3 $\pm$ 116.7		
Tumor location			12.455	$< 0.001$
Lower 1/3	367 (43.4)	160.9 $\pm$ 87.8		
Middle 1/3	83 (9.8)	179.5 $\pm$ 103.0		
Upper 1/3	296 (35.0)	213.2 $\pm$ 127.5		
2/3 or more	99 (11.7)	210.6 $\pm$ 177.5		
Tumor size			-4.129	$< 0.001$
$< 5$ cm	348 (41.2)	166.7 $\pm$ 92.8		
$\geq 5$ cm	497 (58.8)	200.9 $\pm$ 133.7		
Borrmann type				
I / II	355 (42.0)	187.5 $\pm$ 127.0	0.128	0.899
III / IV	490 (58.0)	186.4 $\pm$ 114.3		
Histology			-0.160	0.873
Differentiated	281 (33.3)	185.9 $\pm$ 107.7		
Undifferentiated	564 (66.7)	187.3 $\pm$ 125.3		
Extranodal metastasis			-1.040	0.299
Negative	701 (83.0)	184.9 $\pm$ 119.7		
Positive	144 (17.0)	196.3 $\pm$ 119.6		
TNM stage			4.974	0.007
I	96 (11.4)	154.2 $\pm$ 67.1		
II	269 (31.8)	183.3 $\pm$ 135.9		
III	480 (56.8)	195.4 $\pm$ 117.1		
Type of gastrectomy			-5.963	$< 0.001$
Subtotal	653 (77.3)	173.8 $\pm$ 102.3		
Total	192 (22.7)	231.2 $\pm$ 158.1		
Combined organ resection			-5.329	$< 0.001$
Absent	778 (92.1)	180.5 $\pm$ 110.9		
Present	67 (7.9)	260.4 $\pm$ 180.0		
Extent of lymphadenectomy			-2.676	0.008
D2 and D2+	397 (47.0)	175.2 $\pm$ 95.4		
D1	448 (53.0)	197.2 $\pm$ 136.9		
Year of surgery			-2.494	0.013
2003-2005	489 (57.9)	195.1 $\pm$ 133.6		
2006-2007	356 (42.1)	174.3 $\pm$ 97.6		

TNM: Tumour, node, metastasis; IBL: Intraoperative blood loss.

**Table 5 Multivariate analysis of risk factors for intraoperative blood loss  $\geq 200$  mL**

Feature	HR	95%CI	<i>P</i> value
Tumor location Upper 1/3 and 2/3 or more vs lower and middle 1/3	1.717	1.272-2.317	$< 0.001$
Tumor size $\geq 5$ cm vs $< 5$ cm	1.129	0.833-1.513	0.434
TNM stage III vs I, II	1.174	0.872-1.580	0.290
Extent of gastrectomy D1 vs D2 and D2+	1.161	0.860-1.566	0.330
Type of gastrectomy Total vs subtotal	2.501	1.707-3.663	$< 0.001$
Combined organ resection Present vs absent	1.996	1.089-3.659	0.025
Year of surgery 2003-2005 vs 2006-2007	1.452	1.080-1.954	0.014

TNM: Tumour, node, metastasis.

with gastric cancer after curative resection.

IBL has been reported to be associated with the prognosis of many malignant tumors<sup>[12-14]</sup>. Mörner *et al*<sup>[12]</sup> reported that the degree of IBL in colon cancer influenced long-term survival. In their study, blood loss of 250 mL or more during surgery was a risk factor for overall mortality in both univariate and multivariate analyses. Nagai *et al*<sup>[13]</sup> demonstrated that IBL greater than 2000 mL was related to poor prognosis in patients with pancreatic cancer. These authors suggested that successful curative resection with limited blood loss can contribute to improved survival. With regard to gastric cancer, few studies have focused on IBL. Dhar *et al*<sup>[10]</sup> reported that IBL more than 500 mL was an independent prognostic factor. Kamei *et al*<sup>[11]</sup> demonstrated that the cumulative survival rate was significantly lower in patients with IBL  $\geq 475$  mL than in patients with IBL  $< 475$  mL ( $P = 0.0038$ ), and IBL was a critical risk factor for peritoneal recurrence after curative resection of advanced gastric cancer. Our data are consistent with those results and strongly suggest that IBL, rather than transfusion, was an independent prognostic factor for gastric cancer after curative resection.

In previous studies, blood loss of 475 or 500 mL was proposed as a threshold for prognostic significance<sup>[10,11]</sup>. To date, no study has conducted a detailed statistical analysis by classifying patients into groups based on the level of IBL during resection for gastric cancer. When the thresholds were set at 200 and 400 mL, the OS was significantly affected based on a comparison between these 3 groups. The 5-year OS rates were 51.2%, 39.4% and 23.4% for IBL  $< 200$  mL, 200-400 mL and  $> 400$  mL, respectively ( $< 200$  mL *vs* 200-400 mL,  $P < 0.001$ ; 200-400 mL *vs*  $> 400$  mL,  $P = 0.003$ ;  $< 200$  mL *vs*  $> 400$  mL,  $P < 0.001$ ). Even when deaths due to factors other than gastric cancer were excluded, the differences in cancer-specific survival among the three groups were still significant. This clearly demonstrated the negative influence of IBL on survival after curative gastrectomy. Pathological stage is assumed to be the most important prognostic factor for gastric cancer following curative gastrectomy. Therefore, we stratified patients by TNM stage. Even after stratification, the same trend, *i.e.*, better outcomes in patients with a small amount of IBL, was still observed in each stage. Thus, reducing IBL in resectable gastric cancer may provide further improvements in survival. According to the results of the present study, for patients staged TNM I and II, IBL should be controlled within 200 mL to achieve a better outcome. In patients staged TNM III, IBL should be no more than 400 mL.

Blood transfusion is needed when performing complex surgery with a large amount of IBL. Although many studies<sup>[17-21]</sup> have confirmed that perioperative blood transfusion leads to poor outcome in gastric cancer, some studies<sup>[22-26]</sup> do not support this. In the present study, perioperative transfusion was a prognostic factor, but not an independent prognostic factor in the multivariate analysis. When the influence of IBL was excluded, OS did not dif-

fer significantly between patients with and without transfusion, although 5-year OS was higher in patients without transfusion than in patients with transfusion if the IBL was similar. However, when excluding the influence of transfusion, patients whose IBL was less than 200 mL had significantly better survival than those with IBL of 200 mL or more. The effect of IBL on survival was more pronounced than that of red blood transfusion.

It is still unclear why IBL affects the long-term outcome of patients. It is thought that excessive IBL reduces the body's immunity and thus its ability to fight cancer cells<sup>[10]</sup>. In a study conducted by Bruns *et al*<sup>[27]</sup>, IBL more than 700 mL following gastrointestinal surgery was associated with a significant decrease in natural killer cell activity, producing an unfavorable effect on patient survival. However, the degree of immune suppression was not assessed in this study. This should be examined in a future trial to clarify whether patients with excessive IBL have severe immune suppression resulting in a poor overall survival rate. Another possible explanation is that IBL is associated with peritoneal recurrence which leads to poor survival. It has been reported that operative blood loss is an independent risk factor for peritoneal recurrence of curatively resectable advanced gastric cancer<sup>[11]</sup>. In open abdominal surgery, most operative blood loss accumulates in the abdominal cavity, and thus, the peritoneal surface is considered to have direct contact with blood components. As extravascular blood cells, such as leukocytes and platelets, are activated, they may produce a number of soluble factors that may produce a favorable microenvironment for malignant cells. In fact, activated neutrophils, macrophages, and platelets are capable of producing a large amount of angiogenic factors, such as vascular endothelial growth factor, on the peritoneal surface, which is critical for the survival of isolated cancer cells<sup>[28,29]</sup>. Unfortunately, recurrence data was not obtained in our study.

IBL has been shown to be correlated with postoperative complications<sup>[30]</sup>. In the present study, the incidence of postoperative complications increased when the amount of IBL was high. Previous studies have affirmed the negative influence of postoperative complications on survival for many malignancies<sup>[31-35]</sup>. Sierzega *et al*<sup>[7]</sup> reported that anastomotic leakage was an independent prognostic factor for gastric adenocarcinoma following total gastrectomy. Tokunaga *et al*<sup>[35]</sup> found that postoperative intra-abdominal infectious complications had an adverse effect on 5-year OS and relapse-free survival rate. Our results were in accordance with those reports and showed that the presence of postoperative complications was an independent prognostic factor for OS. As a higher rate of complications was associated with a larger amount of IBL, we consider that the difference in the incidence of postoperative complications among the three groups was a possible contributing factor to the survival difference among the three groups.

As IBL is an independent prognostic factor and patients with IBL less than 200 mL had the best outcome,

it is necessary to explore the potential factors influencing IBL and to develop new surgical methods to reduce IBL. It is obvious that IBL could be affected by the type of gastrectomy and combined organ resection. Patients with tumors located in the upper 1/3 or more than 2/3 the area usually undergo a total gastrectomy or combined spleen resection, which may result in a larger amount of IBL. Lymph node dissection is considered to be a complex procedure and can easily lead to bleeding, especially dissection of the lymph nodes around the celiac trunk. We have used an ultrasonic scalpel for lymph node dissection of gastric cancer since 2006. Ultrasonic surgical devices have been reported to provide advantages in terms of operative time and blood loss<sup>[36,37]</sup>. A study conducted by Inoue K and colleagues showed that blood loss was significantly lower in patients using ultrasonic scalpel than in those not using the ultrasonic scalpel (median 351.0 mL *vs* 569.5 mL;  $P = 0.016$ )<sup>[38]</sup>. From this point of view, it is actually the application of the ultrasonic scalpel that leads to reduced IBL rather than the year, although year of surgery was found to be an independent risk factor for IBL in the present study.

In conclusion, IBL was found to be an independent prognostic factor for gastric cancer after curative resection. It can be used to stratify the risk for gastric cancer prognosis. Meticulous surgery is needed and new methods should be considered to decrease the amount of IBL and improve the long-term outcome of patients following curative gastrectomy.

## COMMENTS

### Background

Intraoperative blood loss (IBL) has been shown to be associated with poor outcome in various types of malignancy. However, the relationship between the amount of IBL and outcome of gastric cancer is still unclear.

### Research frontiers

IBL can not be avoided in surgery. Excessive blood loss may result in more postoperative complications and poorer prognosis. Research has shown the negative association between IBL and prognosis of many malignancies. Few researchers have focused on IBL during resection of gastric cancer. In this study, the authors demonstrated that IBL was an independent prognostic factor for gastric cancer after curative resection.

### Innovations and breakthroughs

Many studies have affirmed that perioperative blood transfusion leads to poor outcome in gastric cancer. However, when performing complex surgery, blood transfusion is required due to a large amount of IBL, which was also reported to have an adverse effect on survival. The impact of IBL on survival may be confused by blood transfusion. This study evaluated the prognostic value of both factors on survival in gastric cancer patients after curative resection and found that IBL influenced the prognosis of gastric cancer rather than blood transfusion.

### Applications

By understanding the negative association between the amount of IBL and prognosis of gastric cancer, this study may stimulate surgeons to pay attention to decreasing the amount of IBL during curative gastrectomy.

### Terminology

IBL is the amount of blood loss during surgery which is visually estimated by anesthesiologists immediately after surgery. Extranodal metastasis was defined as the presence of tumor cells in extramural soft tissue that was discontinuous with either the primary lesion or locoregional lymph nodes.

### Peer review

The IBL and perioperative transfusion have been the topics concerned by sur-

geons. And IBL has been shown to be associated with poor outcome in various types of malignancy. This study shows that IBL is an independent prognostic factor for gastric cancer patients after curative resection. This conclusion has some significance for guiding clinical work.

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## Effects of extended lymphadenectomy and postoperative chemotherapy on node-negative gastric cancer

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### Abstract

**AIM:** To investigate the effects of extended lymphadenectomy and postoperative chemotherapy on gastric cancer without lymph node metastasis.

**METHODS:** Clinical data of 311 node-negative gastric cancer patients who underwent potentially curative gastrectomy with more than 15 lymph nodes resected, from January 2002 to December 2006, were analyzed retrospectively. Patients with pT4 stage or distant metastasis were excluded. We analyzed the relationship between the D2 lymphadenectomy and the 5-year survival rate among different subgroups stratified by clinical features, such as age, tumor size, tumor location and depth of invasion. At the same time, the relationship between postoperative chemotherapy and the 5-year survival rate among different subgroups were also analyzed.

**RESULTS:** The overall 5-year survival rate of the entire

cohort was 63.7%. The 5-year survival rate was poor in those patients who were: (1) more than 65 years old; (2) with tumor size larger than 4 cm; (3) with tumor located in the upper portion of the stomach; and (4) with pT3 tumor. The survival rate was improved significantly by extended lymphadenectomy only in patients with pT3 tumor ( $P = 0.019$ ), but not in other subgroups. Moreover, there was no significant difference in survival rate between patients with and without postoperative chemotherapy among all of the subgroups ( $P > 0.05$ ).

**CONCLUSION:** For gastric cancer patients without lymph node metastasis, extended lymphadenectomy could improve the survival rate of those who have pT3-stage tumor. However, there was no evidence of a survival benefit from postoperative chemotherapy alone.

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**Key words:** Gastric cancer; Lymph node negative metastasis; Extended lymphadenectomy; D2 lymphadenectomy; Chemotherapy

**Core tip:** Little information is available regarding the effects of D2 lymphadenectomy and postoperative chemotherapy in patients with node-negative early gastric cancer. Data of 311 gastric cancer patients without lymph node metastasis were analyzed retrospectively. Results showed that D2 lymphadenectomy could improve the survival rate of patients with pT3-stage tumor. However, there was no evidence of a survival benefit from postoperative chemotherapy. In conclusion, it is recommended that D2 lymphadenectomy with gastrectomy be applied for node-negative patients with pT3 gastric cancer whereas the effects of postoperative chemotherapy in patients with node-negative early gastric cancer need to be further studied.

Xue Q, Wang XN, Deng JY, Zhang RP, Liang H. Effects of extended lymphadenectomy and postoperative chemotherapy

on node-negative gastric cancer. *World J Gastroenterol* 2013; 19(33): 5551-5556 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i33/5551.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i33.5551>

## INTRODUCTION

Gastric cancer is one of the most common malignancies worldwide, with a high mortality rate<sup>[1]</sup>. Many studies indicate that, in gastric cancer, the presence or absence of lymph node metastasis is an important prognostic factor that could influence the prognosis of patients following curative gastrectomy<sup>[2-5]</sup>. It has been shown that an extended (D2) lymphadenectomy could bring benefits to the long-term survival rate of patients with node-positive gastric cancer<sup>[6,7]</sup>, and D2 lymphadenectomy has become a standard surgical procedure for curative treatment in South Korea and Japan<sup>[8]</sup>. However, recurrence and metastasis are also noted in node-negative gastric cancer after curative resection, and there are few studies on the effects of D2 lymphadenectomy in patients with node-negative gastric cancer. At the same time, postoperative chemotherapy is considered an effective treatment option for patients with advanced gastric cancer<sup>[9-11]</sup>, nevertheless, whether it could bring benefit to node-negative gastric cancer patients who received curative gastrectomy still needs to be further elucidated. Hence, the aim of this study was to investigate whether extended lymphadenectomy and postoperative chemotherapy could bring a survival benefit to patients with node-negative gastric cancer.

## MATERIALS AND METHODS

Between January 2002 and December 2006, 867 patients diagnosed with gastric adenocarcinoma were treated with curative gastrectomy (R0 resection) and with more than 15 lymph nodes resected at the Department of Gastric Cancer Surgery, Tianjin Medical University Cancer Hospital and City Key Laboratory of Cancer Prevention and Therapy, Tianjin, China. Of these patients, 311 had lymph node-negative metastasis. There were 230 males and 81 females with ages ranging from 21 to 82 years ( $60.0 \pm 11.2$  years). Patients with pT4 stage or distant metastasis were excluded. D2 lymphadenectomy was performed according to the guidelines of lymph node stations defined by the Japanese Gastric Cancer Association<sup>[12]</sup>.

Patients were stratified according to clinical features including age, sex, tumor size, location, Borrmann type, depth of invasion, and pathologic examination. Furthermore, patients with poor prognosis were stratified into subgroups according to the number of resected lymph nodes (LNs) and whether they received postoperative chemotherapy. According to the number of resected LNs, patients were divided into a 15-24 subgroup and a  $\geq 25$  subgroup. Patients were also divided into groups according to whether or not they received postoperative chemotherapy.

**Table 1 Clinicopathologic factors of patients with node-negative gastric cancer**

Characteristics	n	5-yr survival rate	$\chi^2$	P value
Gender			1.416	0.234
Male	230	67.40%		
Female	81	72.80%		
Age (yr)			4.979	0.026
< 65	156	75.20%		
$\geq 65$	155	62.40%		
Tumor size (cm)			5.930	0.015
$\leq 4$	166	73.80%		
> 4	145	63.00%		
Tumor location			8.721	0.033
Upper	103	58.70%		
Middle	45	67.90%		
Lower	150	76.50%		
Total	13	68.40%		
Borrmann type			3.834	0.280
I	60	71.60%		
II	129	74.20%		
III	108	62.80%		
IV	14	57.10%		
Depth of invasion			13.676	0.001
T1	22	100.00%		
T2	69	78.40%		
T3	220	62.20%		
Pathology			2.689	0.101
Differentiated	124	73.90%		
Undifferentiated	187	65.30%		

Patients received postoperative chemotherapy (FOLF-  
OX6): oxaliplatin ( $100 \text{ mg/m}^2$ ) and leucovorin ( $400 \text{ mg/m}^2$ ), followed by 5-FU ( $400 \text{ mg/m}^2$ ) bolus, then a 46 h continuous infusion of 5-FU ( $3000 \text{ mg/m}^2$ ). The regimen was repeated every 2 wk for 6-8 cycles and follow-up was conducted until November 2011 or until death. Data collection was based on review of clinical charts and on telephone interviews with discharged patients.

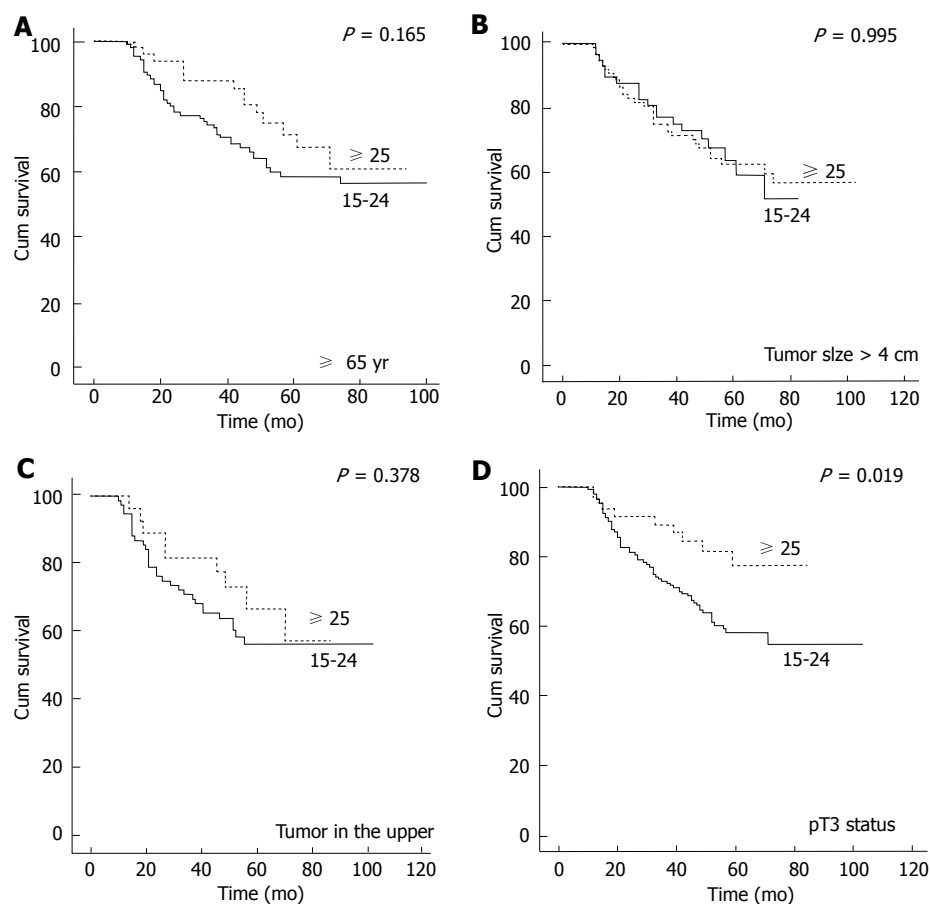
## Statistical analysis

The analysis was performed using the Statistical Package for Social Science (SPSS), version 13.0 for Windows. Actuarial survival rate was determined *via* the Kaplan-Meier method, and univariate comparisons of survival between different groups were performed using the log rank test. Significance of differences was accepted at  $P$  value  $< 0.05$ .

## RESULTS

The overall 5-year survival rate (5-YSR) of the entire cohort was 63.7%. Factors influencing the 5-YSR were as follows: age ( $P = 0.026$ ), tumor size ( $P = 0.015$ ), tumor location ( $P = 0.033$ ) and depth of invasion ( $P < 0.001$ ). The survival rate was lower in patients who were more than 65 years old, with tumor size larger than 4 cm, with tumor located in the upper portion of the stomach, or with pT3 status. Gender ( $P = 0.234$ ), Borrmann type ( $P = 0.280$ ) and pathological types ( $P = 0.101$ ) had no significant influence on the survival rate. The clinicopathological variables tested in the univariate analysis are shown in Table 1.

The survival rate of different groups divided by the



**Figure 1** Five-year survival curve for patients with N0 gastric cancer according to the number of resected lymph nodes. A: In  $\geq 65$  years group, survival curve for 155 patients with N0 gastric cancer according to the number of resected lymph nodes (15-24 and  $\geq 25$ ); B: In tumor size  $> 4$  cm group, survival curve for 145 patients with N0 gastric cancer according to the number of resected lymph nodes (15-24 and  $\geq 25$ ); C: In the upper location group, survival curve for 103 patients with N0 gastric cancer according to the number of resected lymph nodes (15-24 and  $\geq 25$ ); D: In pT3 group, survival curve for 220 patients with N0 gastric cancer according to the number of resected lymph nodes (15-24 and  $\geq 25$ ).

**Table 2** Major postoperative complications observed in the study

Type of complications	15-24 LNs removed ( <i>n</i> = 189)	Above 25 LNs removed ( <i>n</i> = 122)	$\chi^2$	<i>P</i> value
Pulmonary	16	13		
Abdominal abscess	15	9		
Pancreatic fistula	5	3		
Anastomotic leak	2	2		
Lymphorrhea	4	3		
Paralytic ileus	2	2		
Others	3	2		
Total	47	34	0.347	0.556

LN: Lymph nodes.

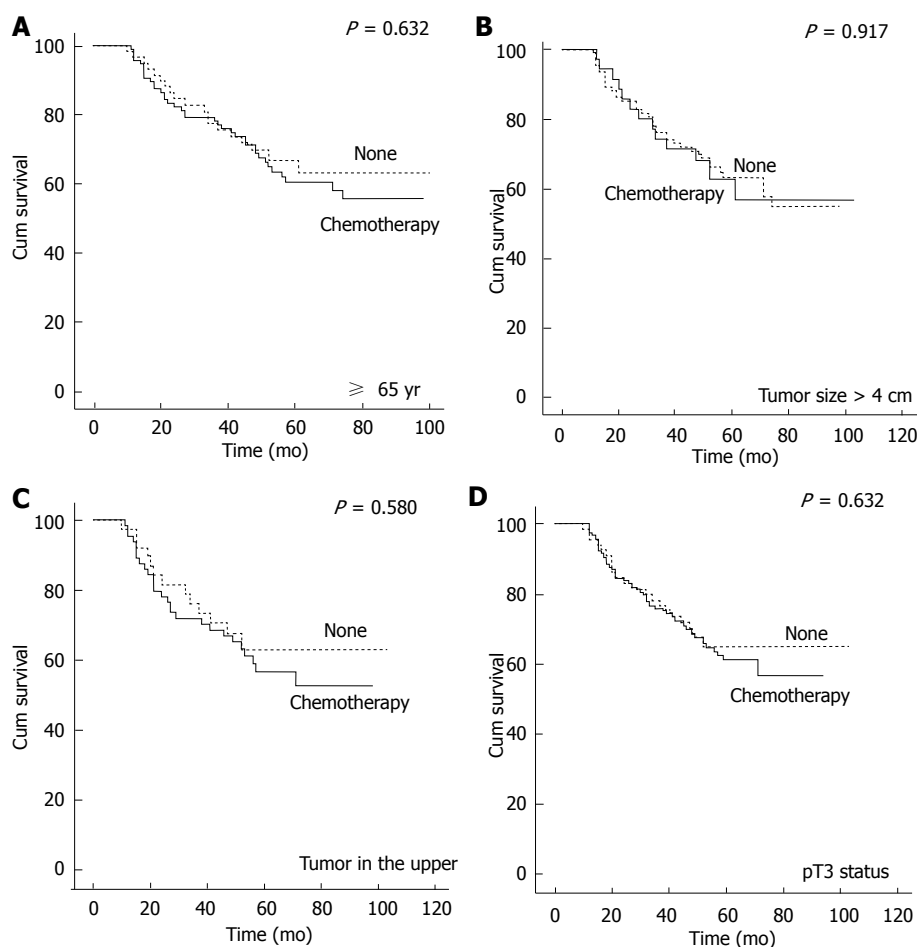
number of resected LNs and whether patients received post-operative chemotherapy were compared between groups stratified by age, tumor size, tumor location and pT status. In patients who were more than 65 years old, with tumor size larger than 4 cm, with tumor located in the upper portion of the stomach, the survival rate was not significantly different between the two subgroups of patients with 15-24 and  $\geq 25$  LNs dissected ( $P = 0.165, 0.995, 0.378$ , respectively). However, for patients with pT3 cancer, the survival rate in patients with  $\geq 25$  LNs dissected was significantly higher than that of patients with 15-24 LNs dissected ( $P = 0.019$ ). The survival curves are presented in Figure 1.

There was no significant difference in survival rates between patients with or without postoperative chemotherapy in all 4 groups, divided according to whether patients were more than 65 years old, with tumor size larger than 4 cm, with tumor located in the upper portion of the stomach or in pT3 status ( $P = 0.632, 0.917, 0.580, 0.632$ , respectively). The survival curves are shown in Figure 2.

Eighty-one of the 311 patients developed postoperative general and surgical complications (morbidity: 26.0%), such as pulmonary affections, abdominal abscess, pancreatic fistula, anastomotic leak, lymphorrhea, paralytic ileus, and no patients died during the perioperative period. Forty-seven patients with complications were in the patient group with 15-24 LNs dissected, and thirty-four were in the group with  $\geq 25$  LNs dissected. There was no significant difference in the post-operative complication rate between these two groups ( $P = 0.556$ ). Table 2 lists the type of complications and their frequency.

## DISCUSSION

Nowadays, due to the significant improvements in diagnosing techniques as well as the popularization of health screening, gastric cancers tend to be detected in their early stages. Of all the patients with gastric cancer treated in our hospital, 35.9% were in the early period. It is commonly considered that lymph node metastases is one of the most important prognostic factors for patients with gastric cancer after curative surgery<sup>[13]</sup>. What's more, re-



**Figure 2** Five-year survival curve for patients with N0 gastric cancer according to whether patients received postoperative chemotherapy. A: In  $\geq 65$  years group, survival curve for 155 patients with N0 gastric cancer according to whether patients received postoperative chemotherapy; B: In tumor size  $> 4$  cm group, survival curve for 145 patients with N0 gastric cancer according to whether patients received postoperative chemotherapy; C: In the upper location group, survival curve for 103 patients with N0 gastric cancer according to whether patients received postoperative chemotherapy; D: In pT3 group, survival curve for 220 patients with N0 gastric cancer according to whether patients received postoperative chemotherapy.

currence and metastasis were also noted in gastric cancer without lymph node metastasis after curative resection. The recurrence rate of early gastric cancer (EGC) was reported as 1.7%-3.4%<sup>[14-17]</sup>. In previous studies<sup>[18-20]</sup>, it was reported that some variables such as pT status, tumor size, tumor location, Lauren type and the number of resected LNs were associated with survival in pN0 gastric cancer. According to our study, the survival rate was lower in patients whose age was more than 65 years old, tumor size was larger than 4 cm, tumor location was in the upper portion of the stomach, or tumor stage was pT3.

Studies have shown that D2 lymphadenectomy could improve the overall survival of patients with advanced node-positive gastric cancer<sup>[21,22]</sup>. D2 lymphadenectomy for pN0 gastric cancer patients who received gastrectomy has been a topic of much discussion. Some recent studies reported that D2 lymphadenectomy with gastrectomy could prolong the survival rate of patients with node-negative advanced gastric cancer<sup>[23-25]</sup>. Consistently, in this study we found that the survival rate of node-negative patients with pT3 gastric cancer could be improved by D2 lymphadenectomy ( $P = 0.019$ ). One possible reason is that the node and tissue with micrometastasis were removed by D2 lymphadenectomy. In one recent study<sup>[26]</sup> it is reported that lymph node micro-metastasis was detectable in 10% of node-negative EGC patients, and occurred more frequently in cases with larger tumor,

lymphatic invasion, or venous invasion. Based on these results, it is recommended that, for node-negative patients diagnosed with pT3 gastric cancer by endoscopic ultrasound preoperatively or at operation, the D2 lymphadenectomy should be performed even without clinically detectable node metastases. However, for other patients with poor survival rate, the effect of D2 lymphadenectomy is inconspicuous.

Previously, it was claimed that the postoperative morbidity and mortality may be increased by D2 lymphadenectomy<sup>[27,28]</sup>. However, with the improvement of surgical techniques, this situation has been changed. As reported in one study<sup>[29]</sup>, there was no difference in the incidence of four major complications (anastomotic leak, pancreatic fistula, abdominal abscess, pneumonia) between the D2 group and D2 plus group. In this study, we also found that the mortality of postoperative general complications was not significantly different between two groups with and without D2 lymphadenectomy (24.9% *vs* 27.9%,  $P = 0.556$ ).

To date, it has been recommended that postoperative chemotherapy should be used in advanced gastric cancer<sup>[9-11,30,31]</sup>. The efficacy and safety of FOXFOL6 regimen for advanced gastric cancer has been demonstrated by a phase II study<sup>[32]</sup>. However, the therapeutic value of chemotherapy for pN0 gastric cancer is still unclear and scarcely reported. Inconsistent with results



from advanced gastric cancer, we found that the survival rate of pN0 gastric cancer patients with postoperative chemotherapy was not significantly different from that of patients without chemotherapy, regardless of whether patients were more than 65 years old ( $P = 0.632$ ), with tumor size larger than 4 cm ( $P = 0.917$ ), with tumor located in the upper portion of the stomach ( $P = 0.580$ ) or in pT3 status ( $P = 0.632$ ).

There were several limitations to the current study. First, in this study, the overall survival is evaluated as an endpoint, while disease-free or recurrence-free survival was not investigated, which are also important for patients with gastric cancer. Second, the extent of lymphadenectomy was variable according to the decisions made by different surgeons, which may affect the results of this study. Finally, as this is a retrospective study, the regimen and dose of chemotherapy might be multifarious, which may affect the accuracy of the comparison of groups.

In conclusion, it is recommended that D2 lymphadenectomy with gastrectomy be applied for node-negative patients with pT3 gastric cancer. However, the effect of postoperative chemotherapy in pN0 gastric cancer patients still need to be further studied.

## COMMENTS

### Background

Many studies have shown that D2 lymphadenectomy could bring benefits to the long-term survival rate of patients with node-positive gastric cancer, however, little information is available regarding its effects in patients with node-negative gastric cancer. At the same time, although the efficacy and safety of FOXFOL6 regimen for advanced gastric cancer has been validated by many studies, the effects of postoperative chemotherapy for pN0 gastric cancer are still unclear and scarcely reported.

### Research frontiers

Some recent studies reported that D2 lymphadenectomy with gastrectomy could prolong the survival rate of patients with node-negative gastric cancer, whereas the impact of postoperative chemotherapy on the survival is scarcely reported.

### Innovations and breakthroughs

The authors retrospectively reviewed 311 patients with node-negative gastric cancer, who were treated with curative gastrectomy and with more than 15 lymph nodes resected at a hospital in Tianjin between 2002 and 2006, to assess whether D2 lymphadenectomy and postoperative chemotherapy may affect their survival rate.

### Applications

The authors suggest that, for node-negative patients diagnosed with pT3 gastric cancer, D2 lymphadenectomy be performed even without clinically detectable node metastases. However, for other patients with poor survival rate, the effect of D2 lymphadenectomy is less obvious.

### Peer review

This article demonstrated the necessity of extended lymphadenectomy for gastric cancer patients without lymph node metastasis. In this study, the authors found that the survival rate of node-negative patients with pT3 gastric cancer could be improved by D2 lymphadenectomy.

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## Hypermethylation of *TGF-β1* gene promoter in gastric cancer

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**METHODS:** We examined the frequency and extent of *TGF-β1* promoter methylation using methylation-specific PCR in the gastric tissues from 47 gastric cancer patients and 39 non-gastric cancer subjects. *H. pylori* infection was confirmed by a positive result from either a serological test, histological analysis or C<sup>13</sup> urea breath test. GES-1 and MKN-45 cells co-cultured with *H. pylori* or treated with IL-1β for 12, 24 and 48 h *in vitro* tested the effects of *H. pylori* or IL-1β on *TGF-β1*.

**RESULTS:** Twenty-four/forty-seven (51%) cases of gastric cancer (GC) tissues showed *TGF-β1* promoter methylation, 15/47 (31.9%) cases of matched non-cancerous gastric mucosa tissues from the GC patients, and 11/39 (28%) case of the normal gastric mucosa tissues from non-GC subjects showed *TGF-β1* promoter methylation (51% vs 28%, *P* < 0.05). Significantly higher levels of methylation of *TGF-β1* were found in the tumor tissues than in non-tumor tissues from GC patients (0.24 ± 0.06 vs 0.17 ± 0.04, *P* < 0.05) and normal gastric tissues from non-GC subjects (0.24 ± 0.06 vs 0.15 ± 0.03, *P* < 0.05). *TGF-β1* methylation was found in 48.3% of *H. pylori*-positive gastric mucosal tissues whereas only 23.1% of *H. pylori*-negative gastric mucosal tissues showed *TGF-β1* methylation (48.3% vs 23.1%, *P* < 0.05). IL-1β appeared to induce a dose-dependent methylation of *TGF-β1* and the strongest methylation was observed in GES-1 cells treated with 2.5 ng/mL of IL-1β for 48 h. Further studies showed that pre-treatment of GES-1 cells with 20 ng/mL IL-1RA for 1 h could partially abolish the effect of IL-1β on *TGF-β1* methylation. Infection of GES-1 cells by *H. pylori* was not found to induce significant *TGF-β1* promoter methylation.

**CONCLUSION:** Our data revealed that *TGF-β1* promoter is methylated in GC patients. IL-1β may be an important mediator for *H. pylori* induced gene methylation during GC development.

### Abstract

**AIM:** To examine transforming growth factor-β1 (*TGF-β1*) promoter methylation in gastric cancer and to determine if *Helicobacter pylori* (*H. pylori*) or interleukin (IL)-1β could induce *TGF-β1* hypermethylation *in vitro*.

**Key words:** Transforming growth factor- $\beta$ 1; Interleukin-1 $\beta$ ; Methylation; *Helicobacter pylori*; Gastric cancer

**Core tip:** *In vitro* studies showed that GES-1 cells exposed to *Helicobacter pylori* (*H. pylori*) did not show significant transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) methylation. However, treatment of the GES-1 cells with interleukin (IL)-1 $\beta$  led to a dose-dependent methylation of TGF- $\beta$ 1, which was partially abolished by IL-1RA. The high levels of TGF- $\beta$ 1 promoter methylation in *H. pylori* positive patients was likely the result of *H. pylori*-induced inflammation rather than *H. pylori* itself. IL-1 $\beta$  may be an important mediator for *H. pylori*-induced gene methylation during gastric cancer development.

Wang YQ, Li YM, Li X, Liu T, Liu XK, Zhang JQ, Guo JW, Guo LY, Qiao L. Hypermethylation of TGF- $\beta$ 1 gene promoter in gastric cancer. *World J Gastroenterol* 2013; 19(33): 5557-5564 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i33/5557.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i33.5557>

## INTRODUCTION

Genetic and epigenetic alterations in tumor suppressor genes or oncogenes are implicated in cancer formation. DNA methylation is the major form of epigenetic change in eukaryotic genomes. It involves the addition of a methyl group to the carbon 5 position of the cytosine ring within the CpG dinucleotide. CpG islands (CGIs) are regions of the genome that contain a large number of CpG dinucleotide repeats. In mammalian genomes, CGIs usually extend for 300-3000 base pairs. They are located within, and close to, sites of about 40% of gene promoters. It is estimated that in mammalian genomes, about 80% of CpG dinucleotides are methylated. However, CpG dinucleotides in regions abundant in GC pairs, such as CGIs, are normally protected from DNA methylation, and this is an important controlling mechanism for gene promoters and gene expression<sup>[1]</sup>. Although most CGIs linked to promoters are non-methylated, recent studies have revealed that promoter CpG hypermethylation, associated with transcriptional inactivation, may play a pivotal role in tumorigenesis<sup>[2]</sup>.

Gastric cancer (GC) remains a major health threat because of its high incidence, poor prognosis and limited treatment options. Multiple epigenetic and genetic alterations have been identified in GC patients. High levels of aberrant CpG island methylation and DNA methylation in the gastric mucosae correlate with increased GC risk, and as such, they are increasingly recognized as candidate markers for GC<sup>[3]</sup>.

Clinical and epidemiological studies have demonstrated that *Helicobacter pylori* (*H. pylori*) infection is correlates strongly with aberrant methylation in GC, whereas eradication of *H. pylori* significantly reduces gene meth-

ylation<sup>[4,5]</sup>. Clearly, *H. Pylori*-induced aberrant methylation plays a role in GC formation. However, the precise molecular mechanisms of how *H. Pylori* might induce aberrant CpG island methylation remain elusive.

Chronic inflammation is a well-known promoting factor for many cancers. Approximately 15%-20% of all human cancers are related to chronic inflammation<sup>[6]</sup>. GC is a typical inflammation-related malignancy, being closely linked to *H. Pylori*-induced chronic inflammation in gastric mucosa. Chronic inflammation in the esophagus and colon may precipitate aberrant methylation, but whether *H. pylori* itself or the chronic inflammation caused by *H. pylori* infection induces methylation in CGIs remains controversial<sup>[7,8]</sup>.

*H. pylori* infection is characterized by infiltration of inflammatory cells, such as neutrophils and lymphocytes, into the gastric mucosa, as well as increased production of inflammatory cytokines<sup>[9,10]</sup>. Interleukin (IL)-1 $\beta$  is a pro-inflammatory cytokine primarily secreted by activated monocytes/macrophages in response to bacterial infection. IL-1 $\beta$  mediates many pathophysiological events during host-environment interactions. Recent studies have demonstrated that the levels of several inflammatory cytokines including IL-1 $\beta$ , IL-6, IL-8 and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) are significantly higher in the gastric mucosal tissues from *H. pylori*-positive patients than those from *H. pylori*-negative patients<sup>[11-13]</sup>. It was further demonstrated that IL-1 $\beta$  could directly induce promoter methylation of *E-cadherin*, an important extracellular matrix component involved in the maintenance of epithelial stability: *H. pylori*-induced methylation of *E-cadherin* promoter was mediated through IL-1 $\beta$ <sup>[14]</sup>.

Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) is an anti-inflammatory cytokine with multiple, and perhaps even opposite, biological effects in many tissues. TGF- $\beta$ 1 was shown to inhibit the growth of epithelial cells, but stimulates the proliferation of mesenchymal cells<sup>[15,16]</sup>. Many studies have shown that TGF- $\beta$ 1 is overexpressed in epithelial cancers and exerts its transforming potential through several mechanisms, such as stimulating the progression of stromal cells, promoting angiogenesis and suppressing immune surveillance<sup>[17]</sup>. However, TGF- $\beta$ 1 was reported to function as a tumor suppressor, because it could inhibit potently the proliferation of many types of cancer cells derived from breast, prostate, lung, colon and liver<sup>[18,19]</sup>. Furthermore, methylation-induced silencing of TGF- $\beta$ 1 has been implicated in the development of several solid tumors, and silencing of TGF- $\beta$ 1 signaling through methylation of the gene encoding its receptor have been reported<sup>[20,21]</sup>.

It has been reported that the expression level of host TGF- $\beta$ 1 in gastric mucosa was an important determinant for the pathogenesis of *H. pylori*-associated gastric diseases<sup>[22-24]</sup>. The gastric mucosa of TGF- $\beta$ 1 null mice exhibit similar changes to those observed in *H. pylori*-associated gastritis, and these mice were found to progressively develop inflammatory diseases and die within 3-4 wk of birth<sup>[25]</sup>. However, the role of TGF- $\beta$ 1 methylation in the development of *H. pylori*-related GC remains largely unknown.



In this study, we aimed investigate if *H. pylori* could induce promoter methylation of TGF- $\beta$ 1 in GC and whether IL-1 $\beta$  plays a role in this process.

## MATERIALS AND METHODS

### Patients and specimens

This study involved 47 consecutive GC patients (35 males and 12 females, mean age 56.2 years) who underwent gastrectomy and 39 consecutive non-GC subjects (28 males and 11 females, mean age 52.1 years) who underwent upper gastroduodenoscopy. *H. pylori* infection was confirmed by a positive result from either one of the following diagnostic approaches: serological test, histological analysis or C<sup>13</sup> urea breath test. No patients received prior *H. pylori* eradication therapy. Ninety-four gastric specimens (two from each patient, from tumor and non-tumor gastric mucosa) from GC patients and 39 gastric mucosa tissues from non-GC subjects were collected. The tissues were snap-frozen in liquid nitrogen and subsequently stored at -80 °C for the studies described below. Patients who received eradication therapy for *H. pylori* before the study and those with severe systemic diseases (such as major organ failure, server infection, autoimmune disease, organ transplantation and immunosuppressive therapy) were excluded.

### Cell culture

Human gastric epithelium cell line GES-1 was kindly provided by Professor Bingdong Zhu (School of Basic Medical Sciences, Lanzhou University). Cells were cultured in DMEM medium containing 10% FBS supplemented with 100 IU/mL penicillin, 100 IU/mL streptomycin and maintained at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>.

### Bacterial strain and conditions

NCTC11637, a *CagA*-positive strain of *H. pylori*, was purchased from the American Type Cell Culture (ATCC) (Rockville, MI, United States). *H. pylori* were cultured on 4.2% Columbia Blood A gar (Youkang Foundation of Biological Science and Technology Beijing Co. Ltd. Beijing, China) containing 7.5% normal sheep blood and 0.5% antibiotics (vancomycin, 10 mg/mL; polymyxin, 0.025 mg/mL; and amphotericin B, 10 mg/mL) under micro-aerophilic conditions for 72 h. Bacteria were harvested and re-suspended in sterile phosphate buffered saline (PBS) and counted by absorbance at 660 nm (1 OD<sub>660</sub> = 1 × 10<sup>8</sup> colony forming units/mL).

### Infection of gastric epithelial cells by *H. pylori*

To establish an *in vitro* model of *H. pylori* infected gastric mucosa, GES-1 cells were grown to 80% confluence under the above-mentioned conditions. Cells were infected with live *H. pylori* at *H. pylori*/cell ratios of 5:1, 10:1, 50:1, and 100:1. To determine the involvement of IL-1 $\beta$  in *H. pylori*-induced pathology, GES-1 cells were pre-treated with human interleukin-1 receptor antagonist (IL-1RA) (Peprotech, Rocky Hill, NJ, United States) for 1 h before

*H. pylori* infection. IL-1RA was used at various concentrations (10, 20, 50 and 100 ng/mL) to select the concentration at which it can effectively block the IL-1 $\beta$  signaling. 20 ng/mL for 48 h was found to be an effective dose. All cells were cultured in 6-well plates at 37 °C in a humidified atmosphere for 12, 24 and 48 h.

### Treatment of GES-1 cells with IL-1 $\beta$

GES-1 cells were pre-treated with or without various concentrations of IL-1RA (10, 20, 50 and 100 ng/mL) for 1 h, followed by treatment with different concentrations IL-1 $\beta$  (Peprotech, Rocky Hill, NJ, United States) (0.1, 0.25, 1.0 and 2.5 ng/mL) for 12, 24, and 48 h in fresh serum-free DMEM medium.

### Methylation-specific polymerase chain reaction

Genomic DNA was extracted from each sample using the TIANamp Genomic DNA Kit (Tiangen Biotech Beijing Co. Ltd, Beijing, China), according to the manufacturer's instructions. The extracted DNA was treated with sodium bisulfite using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Los Angeles, CA, United States), according to the manufacturer's instructions. After bisulfite treatment, DNA was purified using a Zymo-Spin™ IC Column (Zymo Research, Los Angeles, CA, United States) and resuspended in 10  $\mu$ L of dilution buffer (M-Elution Buffer, Zymo Research, Los Angeles, CA, United States). DNA methylation of the TGF- $\beta$ 1 promoter was analyzed by methylation-specific polymerase chain reaction (MSPCR), using Zymo Taq™ PreMix (Zymo Research), according to the manufacturer's instructions. Briefly, the bisulfite-modified DNA (2  $\mu$ L) was amplified using specific primers for methylated and unmethylated sequences of TGF- $\beta$ 1. The primer sequences used in the study were as follows. For methylated TGF- $\beta$ 1, forward: TATATCGTTCGTAAAGTTATAGCGT, reverse: AACATAAAAAAACTAAACCACCGTC. For unmethylated TGF- $\beta$ 1, forward: ATTTATATTGTTTGTA-AAGTTATAGTGT, and reverse: AACATAAAAAAACTAAACCACCATC. PCR was performed in a 50- $\mu$ L reaction system, which contains Zymo Taq™ PreMix (25  $\mu$ L), forward primer (10  $\mu$ mol/L) 4  $\mu$ L, reverse primer (10  $\mu$ mol/L) 4  $\mu$ L, DNA template (2  $\mu$ L), and double distilled water (15  $\mu$ L). The reactions were hot-started at 97 °C for 10 min, followed by 40 cycles of reactions (15 s at 95 °C, 35 s for annealing, and 30 s at 72 °C) and a final 7-min extension in a Thermal Cycler (Veriti, ABI Co., Foster, CA, United States). For positive controls, we used CpGenome Universal Methylated DNA (Intergen, New York, NY, United States). Five microliters of PCR products were separated by electrophoresis on 2% agarose gel stained with ethidium bromide, and imaged using a VersaDoc Imaging System (Bio-Rad Laboratories Co., Ltd. Hercules, CA, United States).

### Densitometric analysis of TGF- $\beta$ 1 methylation Levels

Quantity One software v4.62 (Bio-Rad Laboratories Co., Ltd. Hercules, CA, United States) was used to perform

**Table 1** Methylation frequency for transforming growth facto-β1 in gastric tissues

Case ID	GC patients (n = 47)		Non-GC subjects (n = 39)	
	<i>H. pylori</i>	<i>TGF-β1</i> methylation	<i>H. pylori</i>	<i>TGF-β1</i> methylation
		Tumor	Non-tumor	
1	+	Methylated	Methylated	+
2	+			
3	+	Methylated	Methylated	+
4	+	Methylated		
5	+	Methylated		
6	+			
7	+			
8	+			
9	+	Methylated		
10	+	Methylated	Methylated	+
11	+			
12	+			
13	+	Methylated		
14	+			
15	+	Methylated	Methylated	+
16	+			
17	+			
18	+	Methylated		
19	+	Methylated	Methylated	+
20	+			
21	+	Methylated	Methylated	+
22	+			
23	+			
24	+			
25	+	Methylated		
26	+	Methylated	Methylated	-
27	+			
28	+			
29	+	Methylated	Methylated	-
30	+	Methylated	Methylated	-
31	+	Methylated		
32	+			
33	+			
34	+	Methylated	Methylated	-
35	+			
36	+	Methylated		
37	+	Methylated	Methylated	-
38	+	Methylated		
39	-			
40	-	Methylated	Methylated	
41	-	Methylated	Methylated	
42	-			
43	-			
44	-			
45	-			
46	-	Methylated	Methylated	
47	-			

GC: Gastric cancer; TGF-β1: Transforming growth factor-β1; *H. pylori*: *Helicobacter pylori*.

densitometric analyses of methylated and un-methylated bands of *TGF-β1*, and the results were presented as the mean of three independent experiments. The methylation levels were calculated as the ratio of the value of methylated band to methylated plus unmethylated bands.

Statistical analysis

The frequencies of promoter methylation were compared between the two groups by two-sided Fisher’s exact test or Pearson  $\chi^2$  test. Differences in methylation levels of *TGF-β1*

between two groups were examined by Student’s *t* test. A *P* value of < 0.05 was considered statistically significant.

RESULTS

Methylation frequency and levels of TGF-β1 in GC and non-GC subjects

As shown in Table 1, 24/47 (51%) cases of GC tissues showed *TGF-β1* promoter methylation, 15/47 (31.9%) cases of matched non-cancerous gastric mucosa tissues from the GC patients, and 11/39 (28%) cases of the normal gastric mucosa tissues from non-GC subjects showed *TGF-β1* promoter methylation (51% *vs* 28%, *P* = 0.032).

To evaluate the levels of *TGF-β1* methylation, we analyzed quantitatively the MSPCR product bands by their fluorescence intensities. Typical MSPCR bands are shown in Figure 1A, and the quantitative data for the methylation levels of *TGF-β1* in gastric tissues are shown in Figure 1B. Significantly higher levels of methylation of *TGF-β1* were found in the tumor tissues (0.24 ± 0.06) than in non-tumor tissues from GC patients (0.17 ± 0.04) (*P* = 0.001) and normal gastric tissues from non-GC subjects (0.15 ± 0.03) (*P* = 0.001).

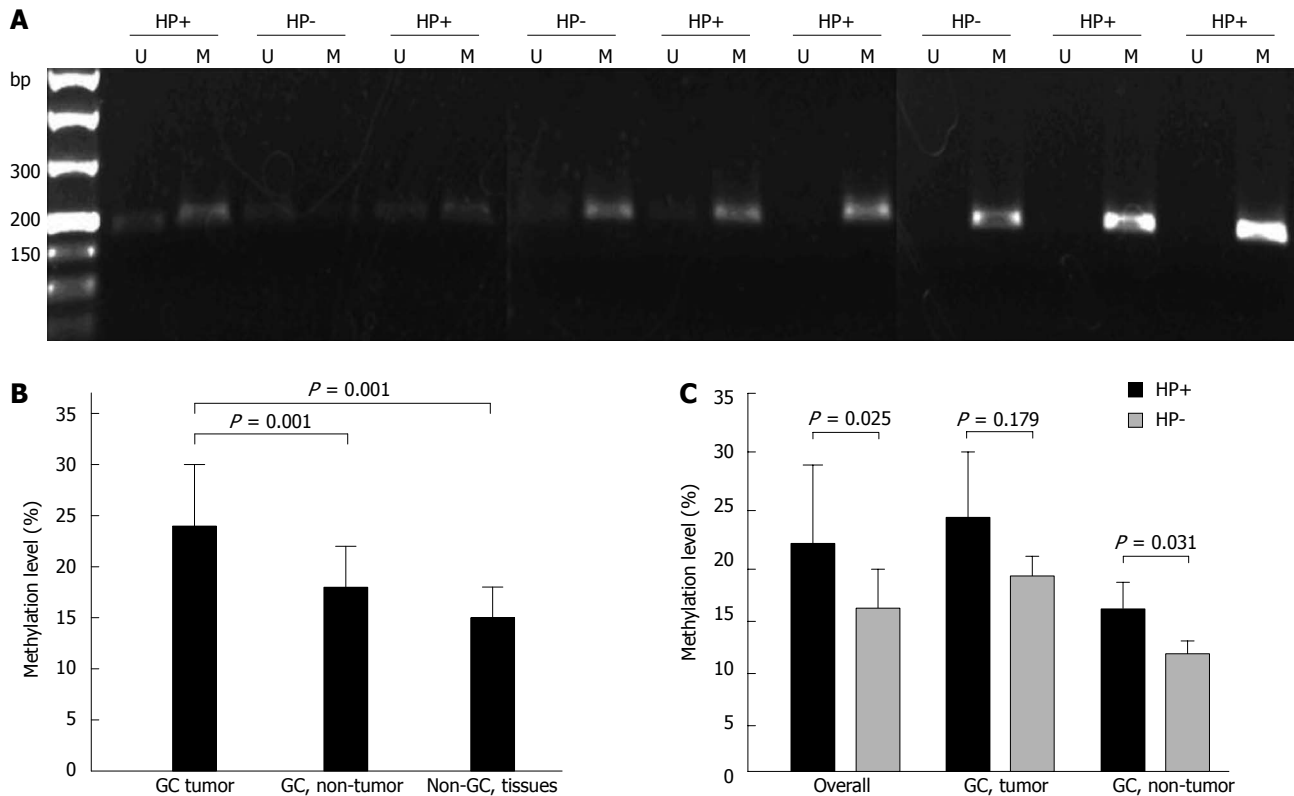
Methylation frequency and levels of TGF-β1 in H. pylori-positive and H. pylori-negative subjects

To examine the impact of *H. pylori* on the methylation of *TGF-β1*, we compared the methylation frequency of *TGF-β1* in *H. pylori*-positive and *H. pylori*-negative GC tissues and non-cancerous gastric tissues. As shown in Table 1 and best shown in Table 2, 21/38 (55.3%) cases of *H. pylori*-positive GC tissues showed *TGF-β1* methylation, whereas only 3/9 (33.3%) cases of *H. pylori*-negative GC tissues showed *TGF-β1* methylation. In non-cancerous gastric tissues obtained from GC patients, the frequency of *TGF-β1* methylation appeared to be similar between *H. pylori*-positive and *H. pylori*-negative gastric tissues (31.6% *vs* 33%, *P* = 0.919). In normal gastric mucosa from non-GC patients, *H. pylori*-positive tissues exhibited more frequent *TGF-β1* methylation than the *H. pylori*-negative gastric tissues (36.4% *vs* 17.6%, *P* = 0.288). Overall, *TGF-β1* methylation was found in 48.3% of *H. pylori*-positive gastric mucosal tissues, whereas only 23.1% of *H. pylori*-negative gastric mucosal tissues showed *TGF-β1* methylation (48.3% *vs* 23.1%, *P* = 0.029).

Further densitometric analysis showed that *H. pylori*-positive GC tumor tissues exhibited much higher levels of *TGF-β1* methylation than *H. pylori*-negative GC tumor tissues (Figure 1A and C). Furthermore, higher levels of *TGF-β1* methylation were also found in *H. pylori* positive non-GC mucosal tissues (Figure 1C). Overall, more *TGF-β1* methylation was present in *H. pylori* positive gastric mucosa than in *H. pylori*-negative gastric tissues (0.23 ± 0.06 *vs* 0.16 ± 0.03, *P* = 0.025) (Figure 1C).

Effect of IL-1β signaling on TGF-β1 promoter methylation

To examine the mechanisms of *TGF-β1* methylation, GES-1 cells were incubated in the presence or absence



**Figure 1** Detection of transforming growth factor- $\beta$ 1 promoter methylation by methylation-specific polymerase chain reaction. Genomic DNA was extracted from human gastric tissues. A: Representative methylation-specific polymerase chain reaction results from gastric cancer (GC) tissues with or without *Helicobacter pylori* (*H. pylori*) infection are shown; B: Levels of transforming growth factor- $\beta$ 1 (*TGF- $\beta$ 1*) promoter methylation in GC tissues, non-cancerous gastric mucosa from the GC patients (GC, non-tumor) and normal gastric mucosa from non-GC subjects (Non-GC tissues); C: Impact of *H. pylori* status on the levels of *TGF- $\beta$ 1* promoter methylation in GC tissues (GC, tumor), non-cancerous gastric mucosa from the GC patients (GC, non-tumor) and combined samples (overall). HP+: *H. pylori*-positive; HP-: *H. pylori*-negative; U: Unmethylated; M: Methylated.

**Table 2** Impact of *Helicobacter pylori* on the frequency of transforming growth factor- $\beta$ 1 methylation in gastric tissues

	<i>TGF-<math>\beta</math>1</i> methylation in GC patients		<i>TGF-<math>\beta</math>1</i> methylation in Non-GC patients	Total <sup>1</sup>
	Tumor	Non-tumor	Normal gastric mucosa	
<i>H. pylori</i> (+)	21/38 (55.3%)	12/38 (31.6%)	8/22 (36.4%)	41/98 (41.8%)
<i>H. pylori</i> (-)	3/9 (33%)	3/9 (33%)	3/17 (17.6%)	9/35 (25.7%)

<sup>1</sup>Combined tumor and non-tumor tissue. GC: Gastric cancer; *TGF- $\beta$ 1*: Transforming growth factor- $\beta$ 1; *H. pylori*: *Helicobacter pylori*.

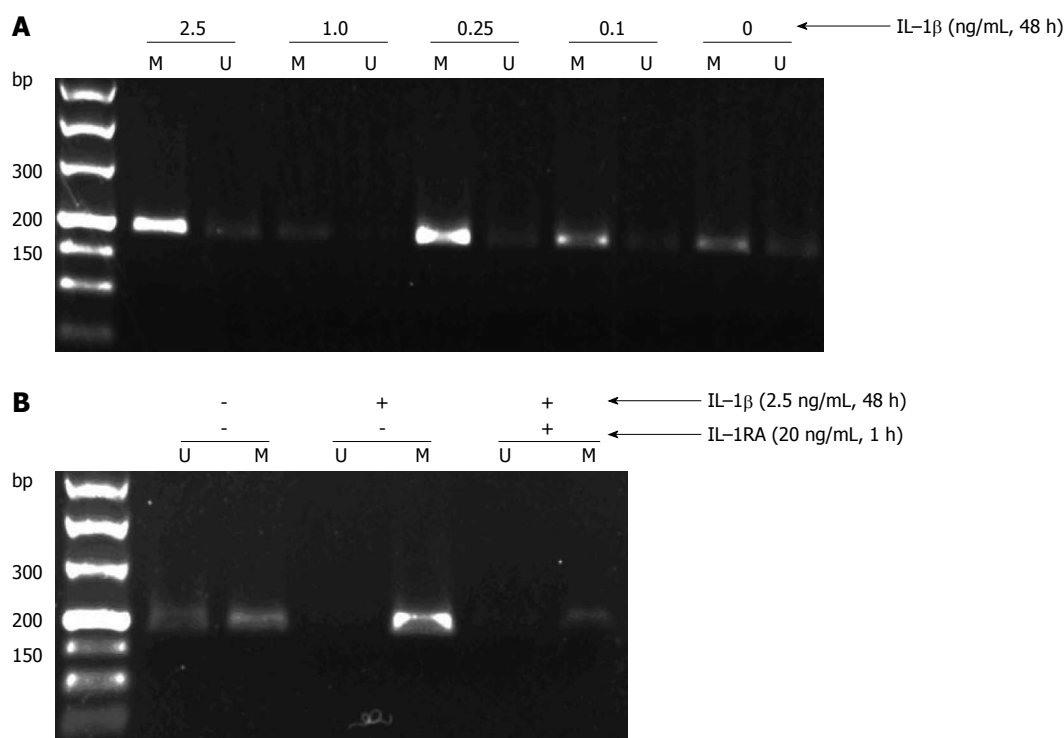
of different concentrations of IL-1 $\beta$  (0.1, 0.25, 1.0 and 2.5 ng/mL) for 12, 24 and 48 h, and *TGF- $\beta$ 1* promoter methylation was then measured by MSPCR. As shown in Figure 2A, IL-1 $\beta$  appeared to induce a dose-dependent methylation of *TGF- $\beta$ 1*, with the strongest methylation being observed in GES-1 cells treated with 2.5 ng/mL of IL-1 $\beta$  for 48 h. Further studies showed that pre-treatment of GES-1 cells with 20 ng/mL of IL-1RA for 1 h could partially abolish the effect of IL-1 $\beta$  on *TGF- $\beta$ 1* methylation (Figure 2B).

#### *H. pylori* alone dose not induce *TGF- $\beta$ 1* methylation in vitro

Infection of GES-1 cells by *H. pylori* did not induce significant *TGF- $\beta$ 1* promoter methylation (data not shown).

## DISCUSSION

Promoter hypermethylation leading to epigenetic inactivation of tumor suppressor genes plays a pivotal role in tumorigenesis. Aging, chronic inflammation, and viral and bacterial infections promote methylation of promoter CpG islands and may represent the “environmental” triggers of carcinogenesis. The stomach is one of the organs that constantly undergoes DNA methylation of CpG islands in its epithelial cells. The oncogenic role of *H. pylori* for gastric malignancies, mainly gastric carcinoma and MALT lymphoma, has been well documented, and as such, *H. pylori* has been designated a Class I carcinogen. However, the mechanism by which *H. pylori* induces GC remained poorly defined.



**Figure 2** Induction of transforming growth factor- $\beta$ 1 methylation by interleukin-1 $\beta$  in GES-1 cells. A: Treatment of GES-1 cells by interleukin (IL)-1 $\beta$  led to a dose-dependent methylation of transforming growth factor (TGF)- $\beta$ 1; B: IL-1 $\beta$ -induced TGF- $\beta$ 1 methylation in GES-1 cells was partially abolished by IL-1RA. U: Unmethylated; M: Methylated; *H. pylori*: *Helicobacter pylori*.

In the present study, we have revealed, for the first time, that significantly more frequent and higher levels of TGF- $\beta$ 1 promoter methylation are present in GC patients than in non-cancerous controls. Although aging is a recognized risk factor for DNA methylation, from the current study, the impact of age (and sex) on TGF- $\beta$ 1 DNA methylation could be excluded, because our study populations in each group were well-balanced in their distribution of age and sex.

TGF- $\beta$ 1 shows biphasic effects in tumorigenesis<sup>[26]</sup>. In the initial stage, it may function as a tumor suppressor by inhibiting cell growth. This was demonstrated in breast cancer in which constitutive activation of the TGF- $\beta$ 1 pathway prolonged the latency of tumorigenesis or resulted in smaller tumor formation in mice<sup>[27,28]</sup>. However, in the later stage of tumorigenesis (*i.e.*, when the tumors are well established), activation of the TGF- $\beta$ 1 signaling can strongly promote tumor progression<sup>[29]</sup>.

Chronic inflammation-induced methylation of promoter CGIs has been closely linked to the development of human cancers<sup>[30,31]</sup>. In gastric cancer, whether the gene hypermethylation observed in *H. pylori*-infected individuals is caused directly by *H. pylori*, or the gene hypermethylation results from *H. pylori*-induced gastric inflammation remains a topic of debate. In our study, we first examined if *H. pylori* could directly cause methylation of TGF- $\beta$ 1. We made use of a gastric epithelial cell line GES-1, and co-cultured this cell line with *H. pylori*. Contrary to our initial hypothesis, *H. pylori* did not induce apparent promoter methylation of TGF- $\beta$ 1. However, treatment of GES-1 cells with IL-1 $\beta$  led to a marked

methylation of the TGF- $\beta$ 1 promoter, and this was partially reversed by antagonizing IL-1 $\beta$  signaling using its receptor blocker IL-1RA. IL-1 $\beta$  is an important pro-inflammatory cytokine that initiates and amplifies the inflammatory responses to *H. pylori* infection. IL-1 $\beta$  is closely linked to DNA methylation of gastric epithelial cells, particularly in *H. pylori* infected individuals. We think that IL-1 $\beta$  may be an important mediator in *H. pylori*-induced TGF- $\beta$ 1 methylation<sup>[32,33]</sup>.

Our *in vitro* data did not correlate with the *in vivo* data, which showed that *H. pylori* infected individuals, particularly *H. pylori*-positive GC tissues, showed more frequent and higher levels of TGF- $\beta$ 1 methylation. This inconsistency may be explained in several ways. Firstly, the acute infection of GES-1 cells by *H. pylori* may not be a valid model for studying the impact of *H. pylori* on gastric mucosa. It was reported that chronic, rather than acute, *H. pylori* infection was responsible for methylation induction. Secondly, *H. pylori* may not directly induce hypermethylation, but rather it induces inflammation and the production of pro-inflammatory cytokines, such as IL-1 $\beta$ , is likely a contributing factor for TGF- $\beta$ 1 methylation. Our data support this view. Lastly, inflammatory cytokines or molecules may also be involved in *H. pylori*-induced methylation. For example, nitric oxide may be involved in *H. pylori* infection-related DNA methylation<sup>[34]</sup>.

## COMMENTS

### Background

Hypermethylation of promoter CpG islands (CGIs) leads to functional silenc-



ing of some tumor suppressor genes and is thus involved in carcinogenesis. Chronic inflammation is closely associated with cancer formation. Inflammation-induced gene methylation is an important mechanism for inflammation-associated cancers, of which gastric cancer is a classical example. During gastric cancer formation, frequent aberrant CGI methylation has been reported and the role of *Helicobacter pylori* (*H. pylori*) infection during this process is controversial. Inactivation of transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) by promoter methylation has been implicated as an important mechanism for the development of several malignancies, such lung and prostate cancers; however, the role of TGF- $\beta$ 1 methylation in gastric cancer remains unknown.

### Research frontiers

Multiple epigenetic and genetic alterations have been identified in gastric cancer (GC) patients. High level of aberrant CpG island methylation in the gastric mucosae is correlated with increased GC risk, and as such, they are increasingly recognized as candidate markers for GC. Clinical and epidemiological studies have demonstrated that *H. pylori* infection is strongly correlated with aberrant methylation in GC. However, the precise molecular mechanism by which *H. pylori* might induce aberrant CpG island methylation remain elusive. GC is a typical inflammation-related malignancy, being closely linked to *H. pylori* induced chronic inflammation in the gastric mucosa. Whether *H. pylori* itself or the chronic inflammation caused by *H. pylori* infection can induce methylation in CGIs remains controversial.

### Innovations and breakthroughs

TGF- $\beta$ 1 is an anti-inflammatory cytokine with multiple effects in many tissues. Many studies have shown that TGF- $\beta$ 1 is overexpressed in epithelial cancers and exerts its transforming potential through several mechanisms, such as stimulating the progression of stromal cells, promoting angiogenesis and suppressing immune surveillance. In the present study, the authors have revealed, for the first time, that significantly more frequent and higher levels of TGF- $\beta$ 1 promoter methylation is present in GC patients with *H. pylori* infection than in non-cancerous controls. *In vitro* studies showed that normal gastric epithelial cell line GES-1 cells exposed to *H. pylori* did not show significant TGF- $\beta$ 1 methylation. However, treatment of the GES-1 cells with IL-1 $\beta$  led to a dose-dependent methylation of TGF- $\beta$ 1. The data show that *H. pylori* may not directly induce hypermethylation, but rather this bacteria induced inflammation and the production of pro-inflammatory cytokines, such IL-1 $\beta$ , is likely a contributing factor for TGF- $\beta$ 1 methylation.

### Applications

High levels of TGF- $\beta$ 1 methylation in the gastric cancer tissue suggest that TGF- $\beta$ 1 functions as a tumor suppressor in *H. pylori* related gastric cancer.

### Terminology

CpG island: CpG islands are DNA segments, at least 0.5 kb in size, that are rich in G:C and CpG content, and are often located in the promoter or 50-exon sequences of genes. Promoter CpG islands have traditionally been thought to be unmethylated in normal cells, with the exception of those on the inactive X chromosome and those associated with imprinted genes. DNA methylation: DNA methylation is the major epigenetic phenomenon of eukaryotic genomes, and involves the addition of a methyl group to the carbon 5 position of the cytosine ring within the CpG dinucleotide. DNA methylation is required for the normal development of cells, whereas aberrant methylation of CpG islands confers a selective growth advantage that results in cancerous growth.

### Peer review

The manuscript by Wang *et al* demonstrates that high levels of TGF- $\beta$ 1 promoter methylation in *H. pylori* positive patients was the result of *H. pylori*-induced inflammation rather than by *H. pylori* itself, and that IL-1 $\beta$  may be an important mediator for *H. pylori*-induced gene methylation during gastric cancer development. The overall goal of the paper is relevant. The data presented are solid and credible. The results are interesting, clinically important and worthy of publication in World Journal of Gastroenterology.

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## Palliative treatment for incurable malignant colorectal obstructions: A meta-analysis

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### Abstract

**AIM:** To perform a meta-analysis of palliative stent placement *vs* palliative surgical decompression for management of incurable malignant colorectal obstructions.

**METHODS:** The databases of Medline, Web of Science, Embase, and the Cochrane Central Register of Controlled Trials were searched from their inception to July 2012 for studies (prospective, retrospective, randomized controlled trials, and case-control trials) designed as comparative analyses of patients with incurable malignant colorectal obstructions treated by self-expanding metallic stents (SEMS) or palliative surgery. No language restrictions were imposed. The main outcome measures were hospital stay, intensive care unit admission, clinical success rate, 30-d mortality, stoma formation, complications, and overall survival time. The data extraction was conducted by two investigators

working independently and using a standardized form. The Mantel-Haenszel  $\chi^2$  method was used to estimate the pooled risk ratios with 95%CI under a fixed-effects model; when statistical heterogeneity existed in the pooled data (as evaluated by  $Q$  test and  $I^2$  statistics, where  $P < 0.10$  and  $I^2 < 25\%$  indicated heterogeneity), a random-effects model was used.

**RESULTS:** Thirteen relevant articles, representing 837 patients (SEMS group,  $n = 404$ ; surgery group,  $n = 433$ ), were selected for analysis. Compared to the surgery group, the SEMS group showed lower clinical success (99.8% *vs* 93.1%,  $P = 0.0009$ ) but shorter durations of hospital stay (18.84 d *vs* 9.55 d,  $P < 0.00001$ ) and time to initiation of chemotherapy (33.36 d *vs* 15.53 d,  $P < 0.00001$ ), and lower rate of stoma formation (54.0% *vs* 12.7%,  $P < 0.00001$ ). Additionally, the SEMS group experienced a significantly lower rate of 30-d mortality (4.2% *vs* 10.5%,  $P = 0.01$ ). Stent-related complications were not uncommon and included perforation (10.1%), migration (9.2%), and occlusion (18.3%). Surgery-related complications were slightly less common and included wound infection (5.0%) and anastomotic leak (4.7%). The rate of total complications was similar between these two groups (SEMS: 34.0% *vs* surgery: 38.1%,  $P = 0.60$ ), but the surgery-related complications occurred earlier than stent-related complications (rate of early complications: 33.7% *vs* 13.7%,  $P = 0.03$ ; rate of late complications: 32.3% *vs* 12.7%,  $P < 0.0001$ ). The overall survival time of SEMS- and surgery-treated patients was not significantly different (7.64 mo *vs* 7.88 mo).

**CONCLUSION:** SEMS is less effective than surgery for palliation of incurable malignant colorectal obstructions, but is associated with a shorter time to chemotherapy and lower 30-d mortality.

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**Key words:** Self-expandable metal stents; Palliative sur-

gery; Incurable malignant colorectal obstruction; Large-bowel obstruction; Treatment outcomes

**Core tip:** This meta-analysis demonstrates the advantages of self-expandable metal stent (SEMS) placement as palliative therapy for incurable malignant colorectal obstructions. Specifically, when compared to the outcomes of surgical treatment, the SEMS treatment is associated with shorter hospital stay and interval to chemotherapy initiation, as well as lower early morbidity and 30-d mortality rates. These advantageous features may surmount the overall lower rate of palliative efficacy when considering treatment options for cases with extensive metastatic disease or severe comorbid medical illness that disqualify a patient from operative candidacy; regardless, SEMS application should be performed as an alternative to surgery with caution.

Zhao XD, Cai BB, Cao RS, Shi RH. Palliative treatment for incurable malignant colorectal obstructions: A meta-analysis. *World J Gastroenterol* 2013; 19(33): 5565-5574 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i33/5565.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i33.5565>

## INTRODUCTION

Malignant colorectal obstruction, a type of large bowel obstruction (LBO), is a frequent and serious complication of advanced cancers, including colorectal cancer or those with near organ (*e.g.*, ovary, vagina, and prostate) or distant metastases<sup>[1]</sup>. LBO initially manifests non-specific gastrointestinal symptoms, such as vomiting, abdominal distention and abdominal pain; however, if left untreated, the condition may progress to a life-threatening status, as the weak necrotic areas of the bowel become more susceptible to tears and a risk for rapid onset infection and septicemia.

The traditional therapeutic approach for LBO is surgical, and the Hartmann's pouch procedure and loop colostomy are the most widely applied surgical methods used for treating obstruction of incurable advanced cancer. Unfortunately, these procedures are associated with substantial drawbacks, including high mortality and morbidity<sup>[2-4]</sup>, as well as detrimental impacts on a patient's quality of life when irreversible ostomies necessitate a colostomy bag<sup>[1,5,6]</sup>. The alternative method of colonic stent insertion was introduced by Dohmoto<sup>[7]</sup> to overcome the risks associated with open surgery. Since then, self-expanding metallic stents (SEMS) have been widely applied to patients with incurable malignant obstructions as palliative treatment or as a bridge to elective primary resection and anastomosis.

SEMS placement is achieved by feeding the metal tube in a collapsed state to the site of obstruction by using a guidewire and visualization by fluoroscopy and/or endoscopy. The inserted stents then undergo passive expansion to create a strong, passable space and relieve

the obstruction. Numerous stents of various lengths and maximal expanded diameter have been designed specifically for treating lower gastrointestinal obstructions, so that the appropriate stent can be chosen for each patient based on location and length of the lesion and severity of the obstruction.

Despite the widespread availability and application of SEMS, its efficacy and safety for treating incurable malignant colorectal obstruction, as compared to that of the traditional surgical approach, has been addressed in relatively few studies with small populations. Thus, this meta-analysis was designed to provide stronger evidence of the outcomes, benefits, and risks of these two palliative treatments through the increased statistical power afforded by pooling data of the previously studied patient populations.

## MATERIALS AND METHODS

### Literature search strategy and data extraction

Two investigators (Zhao XD and Cai BB) performed independent searches of the Medline, Web of Science, Embase, and Cochran Central Register of Controlled Trials databases. These literature collections were queried from inception to July 2012 using the following keywords and medical subject heading terms: stents, colonic stent, colorectal stent, Hartmann's procedures, Hartmann's, colostomy, palliative surgery, intestinal obstruction, large bowel obstruction, colorectal obstruction, comparative study, treatment outcomes, and human. The search strategy was widened or narrowed by applying Boolean operators (NOT, AND, and OR), and no language restriction was applied. All potentially relevant abstracts, studies, and citations were retrieved for review, and the references cited in each were further searched to identify any additional potentially relevant publications.

The two investigators also performed the data extraction (inclusion and exclusion criteria described below), working independently and using pre-determined forms to record first author, year of publication, study design including inclusion and exclusion criteria, and study population characteristics. The extracted datasets were compared and any disagreements were resolved by discussion and consensus.

### Inclusion criteria

Potentially relevant studies were selected for inclusion in the meta-analysis according to the following criteria: (1) comparative analysis of palliative SEMS and palliative surgery for treating malignant colorectal obstructions that were unresectable and had negative margins; (2) patients lacked signs of peritonitis and perforation; (3) reporting of at least one of the outcomes measures listed below; (4) designed as randomized controlled trials (RCTs) or other case-control study; and (5) performed with human patients.

### Exclusion criteria

Studies were excluded from the meta-analysis according to the following criteria: (1) evaluation of SEMS as



**Table 1 Study characteristics of included nonrandomized controlled studies *n* (%)**

Ref.	Design	Diagnosis	Palliative SEMS ( <i>n</i> )	Palliative surgery ( <i>n</i> )	Matching	Female	Study quality (NOS score)
Law <i>et al</i> <sup>[10]</sup>	P	a	30	31	1, 2, 3	21 (34.4)	8
Carne <i>et al</i> <sup>[11]</sup>	R	a	25	19	3	19 (43.2)	4
Johnson <i>et al</i> <sup>[12]</sup>	M	a	20	18	2, 3	17 (47.2)	6
Tomiki <i>et al</i> <sup>[13]</sup>	P	a, b, c	18	17	4	15 (42.9)	4
Ptok <i>et al</i> <sup>[14]</sup>	P	a	40	38	2, 3, 4	34 (44.7)	7
Faragher <i>et al</i> <sup>[15]</sup>	R	a	29	26	1, 2, 4	22 (40.0)	6
Vemulapalli <i>et al</i> <sup>[16]</sup>	R	a	53	70	1, 2, 4	49 (41.2)	5
Suárez <i>et al</i> <sup>[17]</sup>	P	a	45	53	1, 4, 6	31 (31.6)	7
Lee <i>et al</i> <sup>[18]</sup>	P	a	71	73	1, 2, 6	50 (34.7)	7
Lee <i>et al</i> <sup>[19]</sup>	R	a	36	52	1, 2, 4	39 (44.3)	6

Study design is prospective (P), retrospective (R), or case-matched (M); diagnosis is colorectal cancer (a), ovarian cancer (b), or disseminated upper gastrointestinal malignancy (c); matching for age (1), sex (2), diagnosis (3), tumor site (4), or American Society of Anesthesiologists score (6); NOS scores of 5 or more indicate high-quality. SEMS: Self-expanding metallic stents.

**Table 2 Study characteristics of included randomized controlled studies *n* (%)**

Ref.	Design	Diagnosis	Palliative SEMS ( <i>n</i> )	Palliative surgery ( <i>n</i> )	Matching	Female	Study quality (modified Jadad score)
Xinopoulos <i>et al</i> <sup>[20]</sup>	RCT	a,b	15	15	NC	14 (46.7)	High
Fiori <i>et al</i> <sup>[21]</sup>	RCT	a	11	11	1, 2, 4	9 (40.9)	High
van Hooft <i>et al</i> <sup>[22]</sup>	RCT	a	11	10	1, 2, 4, 5	10 (47.6)	High

Study design is randomized controlled trial (RCT); diagnosis is colorectal cancer (a) or ovarian cancer (b); matching for age (1), sex (2), tumor site (4), tumor stage (5), or the publication made no comment (NC) on the matching status; all studies were classified as high-quality according to the modified Jadad score between 4 and 7. SEMS: Self-expanding metallic stents.

a bridge to surgery (SBTS) or as a treatment for benign strictures, or comprehensive studies in which the data could not be clearly separated for exclusion; and (2) missing or unclear data for the outcomes of interest.

### Assessment of methodology quality

The Newcastle-Ottawa scale<sup>[8]</sup> was employed to assess the quality of non-randomized studies, with scores of  $\geq 5$  indicating high quality. The modified Jadad score<sup>[9]</sup> was employed to assess the quality of randomized studies, with the cumulative scores of 4 to 7 indicating high quality.

### Statistical analysis

The meta-analysis was performed by the RevMan 5.0.25 software (The Cochran Collaboration, Oxford, England) and the statistical analysis was carried out by the Stata 12.0 software (StataCorp, College Station, TX, United States). The risk ratios (RRs, with 95%CI) of dichotomous data were estimated by the Mantel-Haenszel  $\chi^2$  method; *P* values of  $< 0.05$  were considered to indicate statistically significant differences between groups. Between-study heterogeneity was evaluated by the *Q* test and *I*<sup>2</sup> statistic, for which *P* values  $> 0.10$  and *I*<sup>2</sup>  $< 25\%$  indicated a lack of heterogeneity, respectively. In order to broaden the effect estimate in the presence of heterogeneity, the random-effects model was applied for evaluation of the pooled data. Finally, publication bias was estimated by Egger's and Begg's funnel plots, for which *P* values  $> 0.05$  indicated a lack of publication bias.

## RESULTS

### Characteristics of selected studies

Thirteen studies, including 10 nonrandomized controlled studies<sup>[10-19]</sup> and three RCTs<sup>[20-22]</sup>, met the criteria for inclusion in the meta-analysis. The studies' characteristics and quality assessment scores are presented in Tables 1 and 2, respectively. Eleven (84.62%) of the studies were categorized as high-quality. The total number of included patients was 837, of which 404 were treated by SEMS (48.3%) and 433 (51.7%) by palliative surgery. Eleven of the studies<sup>[10-12,14-19,21,22]</sup> focused solely on cases with colorectal cancer etiology, and the remaining two studies<sup>[13,20]</sup> also included etiologies of ovarian cancer and disseminated upper gastrointestinal malignancy. The studies also used different definitions of palliative surgery, with four of the studies<sup>[12,13,20,21]</sup> specifically reporting the colostomy procedure and the others reporting primary resection with anastomosis, primary resection without anastomosis, bypass, or Hartmann's procedure, as well. Complications reported for the total case population were categorized as early (occurring  $\leq 30$  d post-treatment) or late (occurring  $> 30$  d post-treatment).

### Features of clinical management

**Length of hospital stay:** The mean length of hospital stay for the pooled SEMS group was significantly lower than that of the pooled surgery group (9.6 d *vs* 18.8 d, *P*  $< 0.00001$ ).

**Intensive care unit admission:** Three studies<sup>[10,12,18]</sup> reported cases requiring intensive care unit (ICU) admission after treatment. Analysis of the 241 patients, including 119 treated with SEMS and 122 treated with surgery, indicated that the rate of ICU usage was significantly lower in the SEMS group than in the surgery group (0.8% *vs* 18.0%,  $P = 0.001$ ; Figure 1A).

**Time to chemotherapy initiation:** Three studies<sup>[17-19]</sup> reported cases receiving chemotherapy after treatment. Analysis of the 330 patients, including 152 treated with SEMS and 178 treated with surgery, indicated that the mean time to chemotherapy initiation following treatment was significantly lower in the SEMS group than in the surgery group (15.5 d *vs* 33.4 d).

### Short-term outcomes and complications

**Clinical relief of obstructions:** Data of treatment efficacy were available for all cases from all 13 studies. The surgery-treated patients showed a significantly higher rate of clinical relief of obstructions than the SEMS-treated patients (93.1% *vs* 99.8%,  $P = 0.0009$ ; Figure 1B).

**30-d mortality or in-hospital mortality:** Two studies<sup>[19,21]</sup> reported zero mortalities during both the in-hospital stay period and the 30-d follow-up. Meta-analysis of the 688 patients in the remaining ten studies, including 334 treated with SEMS and 354 treated with surgery, indicated that the SEMS group experienced fewer overall deaths than the surgery group (4.2% *vs* 10.5%,  $P = 0.01$ ; Figure 1C).

**Overall, early- and late-onset complications:** Data of treatment-related complications were available for all cases from all 13 studies. Although a slightly lower percentage of the SEMS-treated patients experienced complications, the amount was not significantly different from that in the surgery-treated patients (34.0% *vs* 38.1%,  $P = 0.60$ ; Figure 1D). Nine of the studies<sup>[10,14-18,20-22]</sup> reported data sub-categorized as early complications; while five studies<sup>[15-18,22]</sup> reported data as late complications. Compared to the surgery group ( $n = 326$ ), the SEMS-treated patients ( $n = 300$ ) experienced significantly less early complications (13.7% *vs* 33.7%,  $P = 0.03$ ; Figure 1E) but significantly more late complications (32.3% *vs* 12.7%,  $P < 0.0001$ ; Figure 1F).

### Stent-related complications

Eleven studies<sup>[10-19,22]</sup> reported stent-related complications. The overall rate of perforation was 10.1% (for 367 patients), of stent migration was 9.2% (for 361 patients), of stent obstruction was 18.3% (for 331 patients).

**Surgery-related complications:** Seven studies<sup>[10,11,15-19]</sup> reported surgery-related complications. Six of those studies<sup>[10,15-19]</sup> reported wound infection, and the rate was 5.0% (for 15 patients). Three of those studies<sup>[11,17,19]</sup> reported anastomotic leak, and the rate was 4.7% (for 95 patients).

### Long-term outcomes

**Overall survive time:** Data of survival time were available for all cases from all 13 studies. The overall survival time was similar between the SEMS-treated and surgery-treated patients (7.6 mo *vs* 7.9 mo;  $P > 0.05$ ).

**Stoma formation:** Ten studies<sup>[10-13,15,17-21]</sup> reported stoma formation. Among the 299 patients for whom colonic stent insertion was attempted, 12.7% ( $n = 38$ ) ultimately required a stoma. Among the 315 surgery-treated patients, 54.0% ( $n = 170$ ) required stoma formation. The amount of patients with stoma formation was significantly lower in the SEMS group (*vs* surgery group,  $P < 0.00001$ ; Figure 1G).

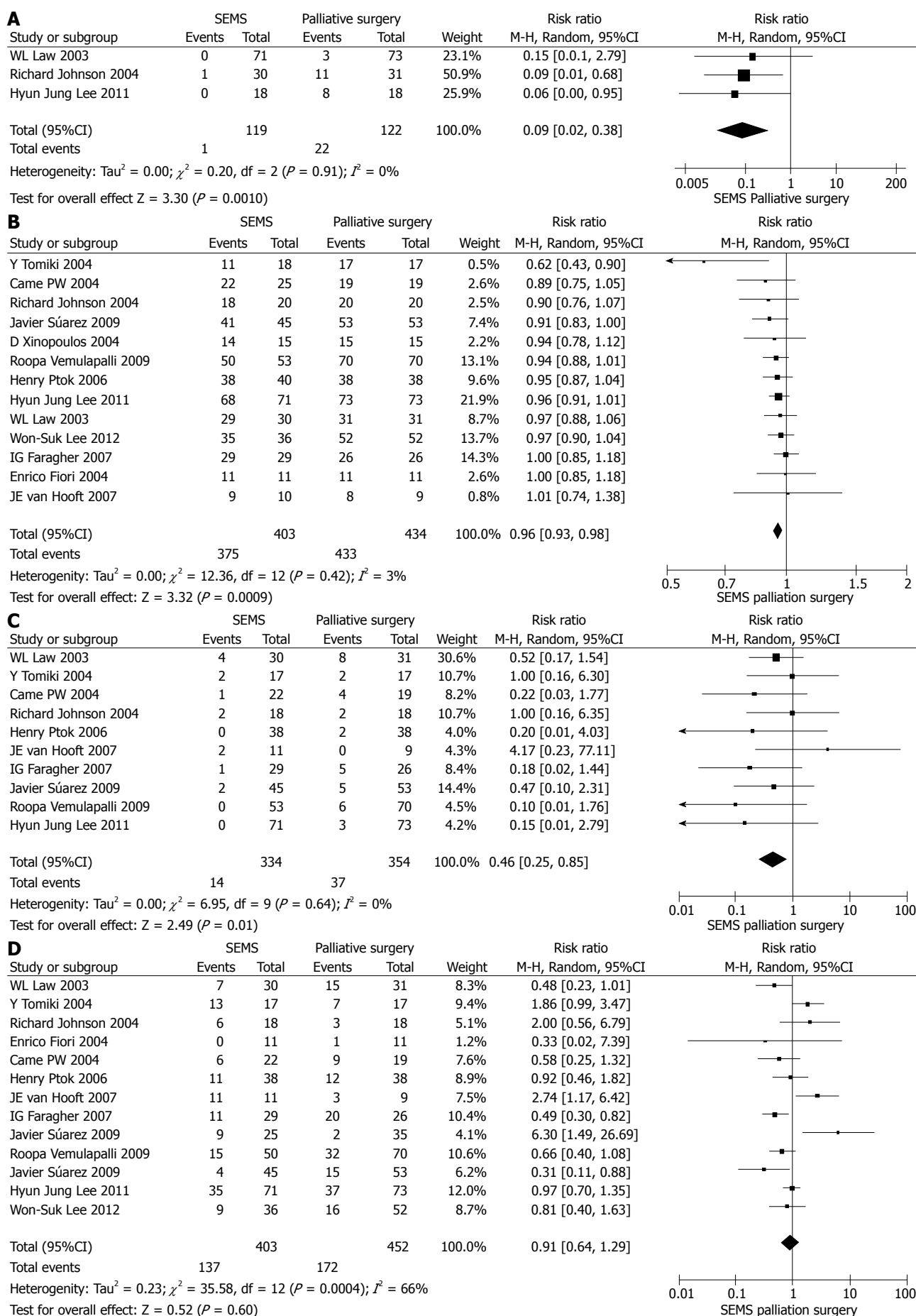
### Sensitivity analysis

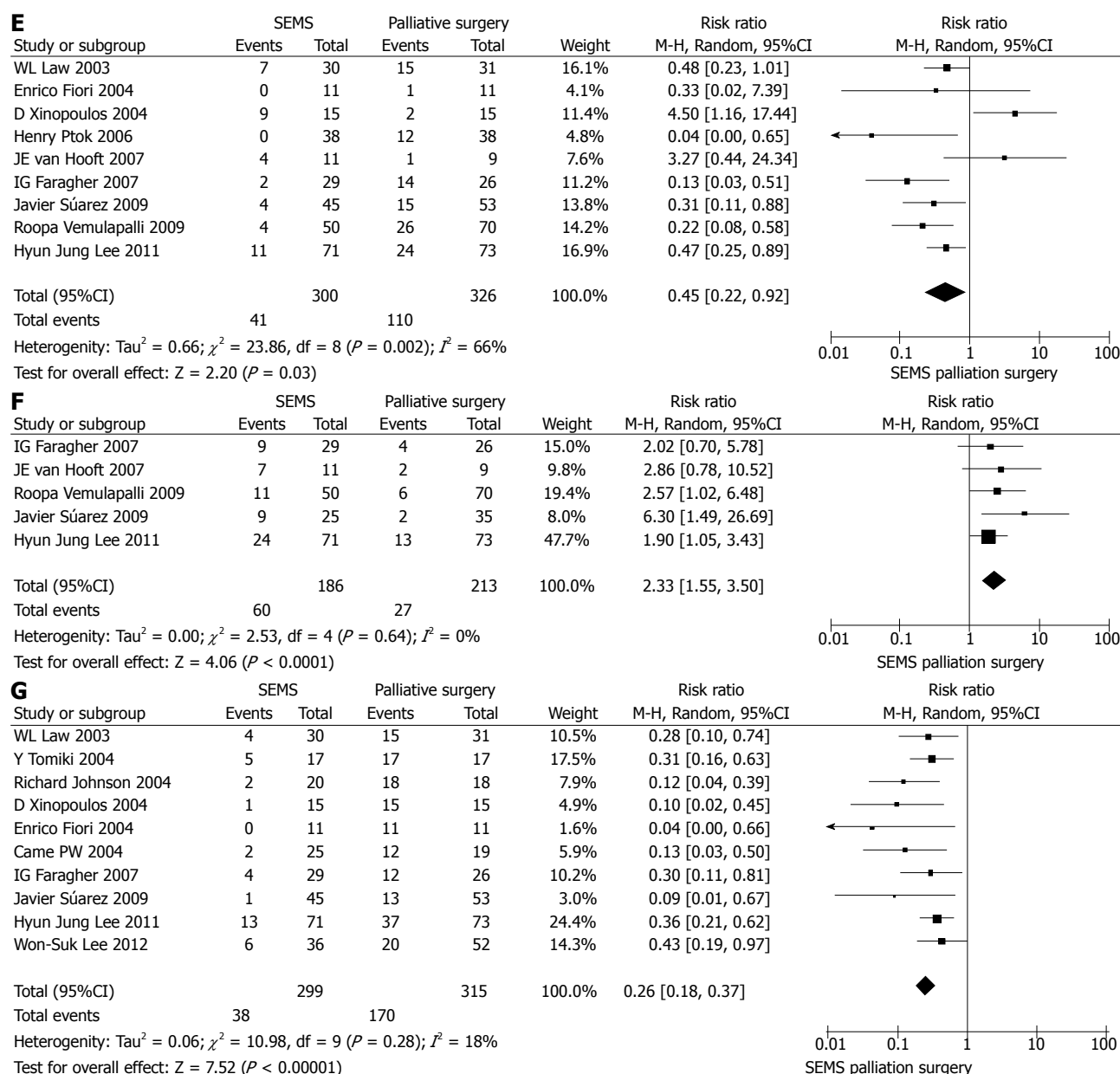
**Therapeutic efficacy and outcomes of SEMS and surgery for colorectal cancer-related obstructions:** Comparative analysis of the therapeutic efficacies of SEMS and surgery for resolving colorectal cancer-related obstructions<sup>[10-12,14-19,21]</sup> and obstructions caused by other advanced cancers<sup>[13,20]</sup> revealed no differences between the two treatment approaches. However, among the subset of patients with colorectal cancer-related obstructions ( $n = 772$ ), the SEMS-treated patients ( $n = 370$ ) showed significantly lower rates of 30-d mortality (3.79% *vs* surgery-treated patients: 10.4%,  $P = 0.008$ ), early complications (11.2% *vs* 34.7%,  $P = 0.0002$ ), and stoma formation (12.0% *vs* 48.8%,  $P < 0.00001$ ). Unfortunately, these SEMS-treated patients also showed a significantly lower rate of clinical relief of the colorectal cancer-related obstructions (94.6% *vs* 99.8%,  $P = 0.002$ ). No significant difference was observed between the two treatments for total complications (SEMS: 32.1% *vs* surgery: 37.9%,  $P = 0.34$ ) (Table 3).

**Therapeutic efficacy and outcomes of SEMS *vs* the colostomy surgical treatment:** Four studies<sup>[12,13,20,21]</sup> compared outcomes of SEMS against the colostomy surgical approach. In contrast to the results of SEMS compared to all types of surgeries for treating incurable malignant colorectal obstructions, there was no significant difference found between clinical relief attained by SEMS and colostomy (84.4% *vs* 100%,  $P = 0.18$ ). The SEMS-treated patients, however, did require significantly less stoma formation than the colostomy-treated patients (12.7% *vs* 100%,  $P < 0.00001$ ), and experienced significantly less total complications (23.9% *vs* 41.3%,  $P = 0.04$ ). The rates of 30-d mortality and early complications were not significantly different between the SEMS-treated group and the colostomy-treated group ( $P = 1.00$  and  $P = 0.64$ , respectively) (Table 3).

### Publication bias

As shown in Figure 2, three comparisons showed potential bias: clinical relief of incurable malignant LBO (Egger's test  $P = 0.04$  and Begg's test,  $P = 0.12$ ) and stoma formation (incurable malignant LBO: Egger's test,  $P = 0.001$





**Figure 1 Forest plot.** A: Intensive care unit admissions; B: Overall clinical success rates; C: Overall 30-d mortality rates; D: Total complications; E: Complications with early-onset ( $\leq 30$  d post-treatment); F: Complications with late-onset ( $> 30$  d post-treatment); G: Stoma formation. SEMS: Self-expanding metallic stents.

and Begg's test,  $P = 0.03$ ; incurable malignant colorectal cancer-related obstructions: Egger's test,  $P = 0.005$  and Begg's test,  $P = 0.04$ ). However, the statistical analysis revealed no evidence of publication bias among any of these comparisons.

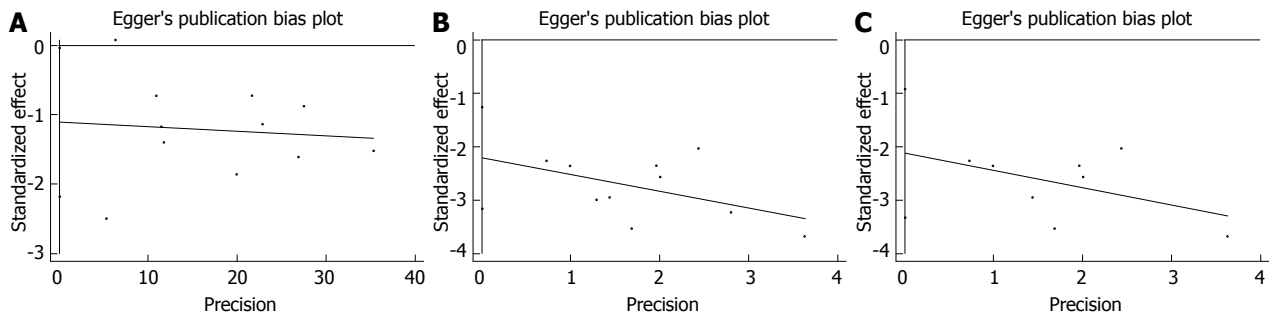
## DISCUSSION

Previous studies have demonstrated the risks associated with the traditional surgical approach for treating malignant LBO, namely high rates of morbidity, mortality, and stoma formation<sup>[23,24]</sup>. The less invasive alternative approach of colonic stent insertion, particularly of SEMS, promised to overcome the high hospitalization costs and poor quality of life related to these outcomes. While subsequent meta-analyses have been conducted to investigate

the benefit and risk of endoscopic SBTS<sup>[25-28]</sup>, no study to date had performed a focused comparison of palliative SEMS and palliative surgery for treating incurable malignant LBO-as is described herein.

In the current meta-analysis, palliative surgery was found to be superior to SEMS for decompressing incurable malignant LBO; while this finding is contrary to the majority of individual studies of this subject<sup>[29-32]</sup>, it is consistent with the investigations by Cirocchi *et al*<sup>[28]</sup> and Sagar<sup>[33]</sup>. An important distinguishing feature among these collective studies is the variable definitions of palliative surgery that were used as the basis of analysis; in addition, these studies have yet to address whether and to what extent primary tumor resection affects the mean survival time of those patients suffering from advanced cancer<sup>[34,35]</sup>. In our meta-analysis of eleven studies, the





**Figure 2** Egger's and Begg's plots of publication bias among the included trials. A: Clinical relief of incurable malignant large bowel obstruction (LBO); B: Stoma formation of incurable malignant LBO; C: Stoma formation of incurable colorectal cancer obstruction.

**Table 3** Sensitivity analysis of the included studies

	Studies (n)	Patients (n)	RR (95%CI)	P value
Studies including colorectal cancer only				
Clinical success rate	11	772	0.96 (0.93, 0.98)	0.002
30-d mortality	9	654	0.42 (0.22, 0.80)	0.008
Total complications	12	821	0.84 (0.59, 1.20)	0.340
Early complications	8	596	0.35 (0.20, 0.60)	0.0002
Stoma formation	8	550	0.26 (0.17, 0.39)	< 0.00001
Studies including colostomy only				
Clinical success rate	4	127	0.89 (0.76, 1.05)	0.18
30-d mortality	2	70	1.00 (0.27, 3.68)	1.00
Total complications	3	92	1.79 (1.03, 3.09)	0.04
Early complications	2	52	1.80 (0.16, 20.79)	0.64
Stoma formation	4	124	0.16 (0.07, 0.38)	< 0.00001

overall clinical success rate of SEMS treatment ranged from 70%-95%. A previous multicenter study<sup>[36]</sup> of SEMS with long-term follow-up revealed that the clinical success rate increased gradually over time (87.8% at 30 d, 89.7% at 3 mo, 92.8% at 6 mo, and 96% at 12 mo). The follow-up period in our included studies are different but all within 12 mo and the clinical success rate was approximately similar. In addition, our meta-analysis revealed that obstructions caused by colorectal cancer benefited more from the surgical approach. Fernández-Esparrach *et al.*<sup>[37]</sup> have reported a similar finding and hypothesized that the severe complications associated with the SEMS procedure, such as migration, obstruction and perforation, limited its long-term clinical efficacy. Moreover, the authors advised that adjunct palliative chemotherapy may help to promote the life expectancy of SEMS-treated patients. A retrospective study conducted in Korean patients advanced gastric cancer<sup>[38]</sup> also indicated that SEMS insertion was less effective than emergency surgery for the palliative treatment for colorectal obstructions. In light of these previous findings, and in agreement with the opinions expressed by other interested groups in this field<sup>[39,40]</sup>, it is possible that the clinical stent success rate observed in our current meta-analysis had nothing to do with the stent placement or the etiology of the obstructions. Indeed, Sebastian *et al.*<sup>[31]</sup> suggested that the clinical success rate of stenting is mainly associated with the site and extent of the obstruction.

Our meta-analysis also indicated that SEMS treatment is associated with shorter lengths of hospital stay,

reduced ICU admissions, fewer stoma formation, and shorter time to initiation of adjunct chemotherapy; These findings are consistent with results from other relevant studies<sup>[30,32,33,41]</sup> and suggest that the less trauma endured produced by the SEMS approach eliminates delay of post-procedure chemotherapy, thereby promoting beneficial patient outcome. It was unfortunate that the current meta-analysis was limited by a lack of comparative data concerning quality of life outcome and cost-effectiveness between these two palliative treatments; analysis of such data will be necessary for comprehensively assessing the feasibility of these palliative management approaches for advanced disease. Only one of the studies included in the meta-analysis, a RCT<sup>[20]</sup> comprised of 30 patients, attempted to address the monetary expense of stent placement, as compared to colostomy treatment; however, the analysis was abandoned due to the high rate of colonic perforation that occurred in the nonsurgical arm. However, some studies<sup>[32,42]</sup> that did not meet the criteria for inclusion in our meta-analysis have suggested that SEMS may be less costly than the conventional surgical approach for treating colonic cancer obstructions; but, we cannot comment on the quality or appropriateness of these data or the implications related to our findings.

The safety of stent placement was also evaluated in the current meta-analysis. Although SEMS insertion is considered a less invasive method than surgery, and advanced procedure-related devices, such as hydrophilic elastic guidewires and stent delivery systems, have improved the ease and successful application of this method, complications still occur. Fortunately, the majority of complications are minor, such as low fever and abdominal discomfort, and resolved easily by medication. While less frequent, the major complications of the stent procedure, such as bleeding, colonic perforation, stent migration and stent occlusion, can be life-threatening<sup>[43]</sup>. In a systematic review<sup>[30]</sup> of 88 articles reporting on stent-related complications in cases of LBO, the median rates of stent migration, perforation, and reconstruction were reported as 11%, 4.5% and 12%, respectively. In the current meta-analysis, the rates of perforation and reconstruction were slightly higher; we believe this finding reflects the fact that data on perforations caused by tumor infiltration were included in the analysis and that the data on reconstructions included not only the etiologies of tu-

mor ingrowth/overgrowth and stent migration, but also of fecal implant.

The contributing factors to complications of stent insertion have been extensively studied. Factors related to stent type have been particularly well studied, and it is believed that covered stents provide the optimal resistance to tumor ingrowth, thereby helping to reduce reconstruction events, while uncovered stents are believed to minimize stent migration<sup>[30,39,44]</sup>. The type of stent, however, does not appear to be related to perforation events<sup>[30]</sup>, nor to have a significant effect on the safety of stent placement<sup>[45]</sup>. Furthermore, a retrospective analysis of uncovered SEMS for treating primary colorectal cancer vs non-colorectal extrinsic cancer found no significant difference in migration or occlusion events<sup>[46]</sup>. That study also suggested that insufficient stent expansion (< 70%) at 48 h after insertion may be a predictor of subsequent stent occlusion. Another retrospective analysis of 168 SEMS-treated LBO patients<sup>[47]</sup> identified five risk factors of therapeutic inefficacy, including male sex, complete obstruction, stent diameter  $\leq 22$  cm, premature dilation of the stent, and operators' experience. In addition, subsequent chemotherapy, especially Bevacizumab therapy, was demonstrated to nearly triple the risk of perforation. This latter finding was not supported by the study by Kim *et al*<sup>[39]</sup>, who demonstrated that chemotherapy had no affect on migration or reconstruction and that stent length had no relationship with complications, but showed that stent diameter < 24 cm had negative impact on palliative SEMS migration. In another study, stent migration was shown to occur more frequently in the distal colon<sup>[51]</sup>.

Despite significant improvements in the surgical procedures used for managing incurable malignant colorectal obstructions, the perioperative morbidity and mortality rates have remained high. Similarly, the patients treated with surgery in the current meta-analysis experienced appreciable levels of anastomotic dehiscence, wound infection, and death. The former two complications may have a negative influence on tumor recurrence, metastasis, and long-term survival. In the current meta-analysis, a greater number of surgery-treated patients died within 30 d after treatment, as compared to those treated with SEMS. While this result is contrary to those obtained with other similar patient series<sup>[30,33]</sup> and meta-analyses comparing SBTS<sup>[25-28]</sup>, it may be explained by the lower amount of total complications that were experienced by the overall SEMS-treated group. Another study also found significantly lower complications in a stent-treated group, but we cannot comment on the related implications for our findings as the previous data had significant heterogeneity<sup>[25]</sup>. In an attempt to address this issue, we performed sub-group analysis of the complications, independently assessing the early- and late-onset complications; the results indicated that surgery had a higher risk of early complications, while SEMS insertion had a higher risk of late complications. Future studies should further investigate the roles of early and late complications in therapeutic efficacy and overall survival.

Two of the studies<sup>[13,20]</sup> included in the overall meta-analysis were excluded from the focused comparison of SEMS and surgery outcomes for incurable colorectal-related obstructions. The results were not impacted by their removal and were in accordance with the findings reported by Kim *et al*<sup>[40]</sup>. Then, we investigated the comparison between SEMS and colostomy for incurable malignant LBO (using four studies). Unlike the previous results, these results suggested that, compared to colostomy, SEMS could be an effective palliative treatment for incurable malignant LBO; no significant difference was found for the clinical success rates between groups with fewer stoma, but the 30-d mortality and the complications should be taken into account. Unfortunately, the current meta-analysis was underpowered to investigate the differences in overall survival time between these two groups.

Other limitations of our meta-analysis design may have impacted our results and their interpretation. First, only three of the 13 included studies are RCTs. Second, the pooled sample size was still relatively small and the data from the included studies was not uniform for the outcome measures. Third, publication bias existed among four of the studies; indeed, a general limitation of all meta-analyses is publication bias introduced by the fact that positive results are more likely to be published. To overcome these limitations, long-term RCTs should be conducted with large numbers of patients to achieve a sufficient level of statistical power for accurately estimating the optimal palliative treatment for incurable malignant LBO.

In summary, palliative SEMS does not appear to have a significant advantage over palliative surgery for decompressing incurable malignant colorectal obstructions, regardless of etiology; however, the use of colonic stents is safe. The shorter interval to chemotherapy and significantly lower rates of 30-d mortality and short-term complications suggest that SEMS may be a reasonable alternative for treating patients with extensive metastatic disease or who are poor operative candidates due to severe comorbid medical illnesses.

## COMMENTS

### Background

Malignant colorectal obstruction is a common and serious complication of advanced cancer. The traditional treatment approach, surgery, is associated with high risks of morbidity and mortality. The more recently developed approach of stent insertion is less invasive and has been widely applied, especially using self-expanding metallic stents (SEMS), but its risks and benefits in patients with incurable malignant large bowel obstruction (LBO) remain to be definitively established.

### Research frontiers

The current meta-analysis was carried out to comparatively assess the outcomes of palliative surgery and palliative SEMS insertion in patients with incurable malignant LBO; the main outcome measure included length of hospital stay, intensive care unit admission, clinical success rate, 30-d mortality, complications, stoma formation, and overall survival time.

### Innovations and breakthroughs

The current meta-analysis demonstrated the advantages of SEMS as a pal-

liative therapy for incurable malignant LBO, in terms of shortened durations of hospital stay and time to chemotherapy initiation, and decreased rates of 30-d mortality and early-onset complications. However, SEMs failed to show a greater efficacy than palliative surgery for resolving obstructions.

### Applications

The results from this meta-analysis suggests that colonic stent insertion may be a safe and feasible alternative palliative treatment for patients who are otherwise poor candidates for the traditional surgical treatment, such as those with extensive metastatic disease or severe comorbid medical illnesses. SEMs is not absolutely recommended, however, since it is associated with significant late-onset complications and mortality. Until further randomized controlled trials, with large patient populations, are carried out, application should be considered on a case-by-case basis.

### Terminology

SEMS: expandable metal tubes that are placed in the collapsed state at the site of obstruction by means of a guidewire and fluoroscopy and/or endoscopy visualization; gradual, automatic expansion to the maximum diameter of the stent serves to relieve the obstruction and create a strong and passable space. Meta-analysis: the collection, combination, and analysis of data from multiple previously completed studies on a particular topic of interest that is carried out with the aim of increasing statistical power to draw stronger conclusions about a controversial subject.

### Peer review

The current meta-analysis was designed to evaluate the risks and benefits of SEMs treatment for incurable malignant colorectal obstructions, as compared to surgical treatment. The analysis included a total of 13 studies, nine of which scored high upon established quality assessment systems. The research design is solid, and its results have clinical relevancy as they demonstrate that, in patients with incurable malignant colorectal obstruction, stent placement improves treatment outcome, specifically by shortening the time to chemotherapy initiation and lowering the 30-d mortality rate.

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## Lactic acidosis during telbivudine treatment for HBV: A case report and literature review

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**Author contributions:** Jin JL diagnosed and treated the patient and wrote the manuscript; Hu P followed up the patient; Lu JH and Luo SS performed the muscle biopsy and provided the histopathologic pictures; Huang XY was involved in the preparation of the manuscript; Weng XH and Zhang JM helped diagnose and manage the patients and prepare the manuscript.

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modialysis treatment for 16 times and usage of glucocorticosteroid. The patient fully recovered after 16 wk of treatment. This is the first documented case with severe LA caused by telbivudine monotherapy. Besides serum creatine phosphokinase, blood lactate level should also be closely monitored in patients receiving telbivudine.

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**Key words:** Lactic acidosis/hyperlactatemia; Telbivudine; Hepatitis B virus; Nucleoside analogue; Adverse effects

**Core tip:** Myopathy is the most common side effect resulting from telbivudine. Lactic acidosis (LA) is rare but fatal, and LA caused by telbivudine has never been reported. Here, we report the first case of chronic hepatitis B developing severe refractory LA during telbivudine monotherapy. This case shows that telbivudine may cause muscle damage and even lead to fatal LA in chronic hepatitis B patients. Patients under telbivudine treatment should be closely monitored for muscular, blood lactate and other mitochondrial toxicity associated side effects.

### Abstract

All oral nucleoside analogues against hepatitis B virus, with an exception of telbivudine, have been reported causing lactic acidosis (LA). Here we report the first case of chronic hepatitis B developing severe refractory LA during telbivudine monotherapy. A 36-year-old man of Chinese origin received telbivudine antiviral treatment for chronic hepatitis B. After 11 mo of therapy, he developed anorexia, nausea, and vomiting with mild muscle weakness. The patient was found with elevated serum creatine phosphokinase up to 3683 U/L (upper limit of normal 170 U/L) and marked LA. LA did not resolve immediately following discontinuation of telbivudine. His condition began to improve after he-

Jin JL, Hu P, Lu JH, Luo SS, Huang XY, Weng XH, Zhang JM. Lactic acidosis during telbivudine treatment for HBV: A case report and literature review. *World J Gastroenterol* 2013; 19(33): 5575-5580 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i33/5575.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i33.5575>

### INTRODUCTION

Suppression of hepatitis B virus (HBV) DNA is a principal goal in treating chronic hepatitis B because this was shown to significantly improve liver histology as well as

to decrease rates of hepatic complications and hepatocellular carcinoma. Current treatment options are pegylated interferon alpha and nucleoside analogues including lamivudine, telbivudine, entecavir, adefovir dipivoxil and tenofovir disoproxil. These agents have relatively fewer side effects than interferon alpha, and generally well tolerated<sup>[1]</sup>. Infrequent but serious adverse events have been reported in clinical trials and post-marketing surveillance in individual cases. Lactic acidosis (LA) is one of the severe adverse events and has been reported in the patients treated by all the other four nucleoside analogues except for telbivudine.

All of the five approved oral antiviral agents for HBV treatment can inhibit the polymerase activity of HBV, leading to a reduction in viral replication and serum HBV DNA levels. At the same time, some of these agents have a low level of activity against the human mitochondrial DNA (mtDNA) polymerase gamma and can lead to impaired mitochondrial replication with mitochondrial loss or dysfunction<sup>[1]</sup>. Clinical manifestations of mitochondrial toxicity vary based on the affected tissues, but may include myopathy, neuropathy, hepatic steatosis, pancreatitis, macrocytosis, nephrotoxicity, hyperlactatemia and LA. All nucleoside analogues have a “black box” warning regarding potential mitochondrial toxicity in their product labeling.

Telbivudine is a potent oral nucleoside analogue approved for the treatment of chronic hepatitis B in 2006 at a dose of 600 mg/d. A significantly higher incidence of grade 3-4 serum creatine phosphokinase (CPK) elevation (*i.e.*, > 7 times upper limit of normal) was reported in a large, multinational registration clinical trial<sup>[2]</sup>. However, to date, there has been no published report of LA caused by telbivudine monotherapy. Here, we report a case of LA during telbivudine treatment, discuss the pathophysiology, clinical features and potential treatment of LA.

## CASE REPORT

The patient is a 36-year-old, HIV-negative young male farmer. He was admitted to our hospital because of nausea and vomiting repeatedly for 40 d.

He had suffered from chronic hepatitis B for 13 years. His liver function test (LFT) revealed an intermittent elevation of alanine aminotransferase (ALT) levels between 1999 and 2011, and recovered to normal level after some symptomatic treatment. In September 2011, his LFT became abnormal again, the ALT was 704 U/L and HBV DNA was  $7.0 \times 10^7$  copies/mL, HBV markers showed HBsAg, HBeAg and HBcAb were positive. Subsequently, he began to take telbivudine 600 mg/d regularly (Figure 1). In early September 2012 (47 d before admission), he began to develop anorexia, nausea and vomiting without apparent causes. There were no other concurrent symptoms, such as fever, headache, abdominal pain and altered level of consciousness. But he had mild muscle pain and weakness. The diagnostic workup including gastroscopy, cranial CT and abdominal plain

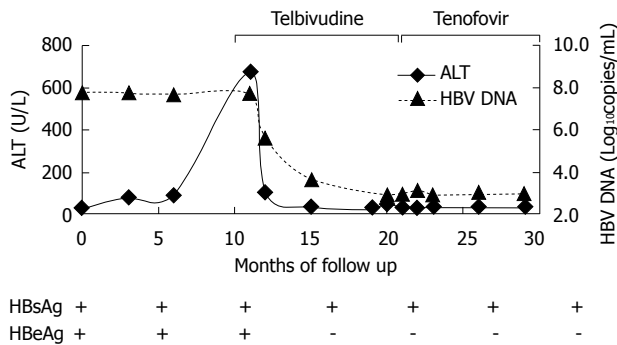
film revealed bilateral multiple renal calculi. CPK was significantly elevated at 3683 U/L (normal range: 25-170 U/L) 20 d before admission (Figure 2). The arterial blood gas analysis at that time showed pH 7.41, carbon dioxide partial pressure 37.2 mmHg, oxygen partial pressure 87.1 mmHg, actual bicarbonate 23.2 mmol/L, standard bicarbonate 23.6 mmol/L, base excess -1.4 mmol/L, and blood lactate level 4.4 mmol/L (upper limit of normal 2.5 mmol/L). It was considered that hyperlactatemia was caused by telbivudine at a local clinic. Subsequently telbivudine was discontinued.

However, the patient's condition continued to deteriorate despite alkalization treatment. Two weeks before admission, his CPK level decreased to 1183 U/L, but the arterial blood gas analysis demonstrated a worsening of metabolic acidosis: pH 7.2, actual bicarbonate 10.6 mmol/L, base excess 15.8 mmol/L, and blood lactate level elevated to 10.7 mmol/L (Figure 3). The clinical symptoms included persisting nausea and vomiting. The blood lactate level rose further to more than 12 mmol/L (the upper limit can be detected in the laboratory) (Figure 3). A week before admission, the patient received eight times of hemodialysis treatment at a local clinic. His blood lactate returned to a normal level each time after hemodialysis, however, it would rebound the next day. The patient was eventually transferred to our hospital because of refractory LA. On the day of admission, the blood lactate was 7.93 mmol/L, ALT was 42 U/L, aspartate aminotransferase was 66 U/L, LDH was 349 U/L and CPK was 632 U/L. Physical examination on admission revealed waddling gait and proximal muscular weakness in both lower limbs, quantitative value was 4 grade.

The patient was noticed to have a history of hypokalemic periodic paralysis for more than 10 years after a serious inquiry. His first attack was the most severe one, with paralysis affecting both of his legs but recovered after potassium supplement. There was no further event in the recent years.

The examination after admission also revealed hypothyroidism: TSH 12.39 mIU/L, T4 110.1 nmol/L, T3 1.31 nmol/L, and FT4 14.42 pmol/L. B-mode ultrasonography showed diffuse enlargement of thyroid. Endocrinologist consultation considered a subclinical hypothyroidism, and 25 µg euthyrox was prescribed daily.

Electromyography revealed mild myopathic changes. Prolonged exercise test was normal. Muscle biopsy on left biceps revealed moderate variation in fiber size as well as increased muscle nucleus (Figure 4). A substantial number of degenerative muscle fibers occurred. Regeneration of muscle fiber could be seen, with no inflammatory cells infiltration. Mitochondrial damage was identified by modified Gomori trichrome stain and other histopathological studies. Modified Gomori trichrome staining revealed many ragged red fibers (RRF); reduced form of nicotinamide-adenine dinucleotide (NADH) and succinic dehydrogenase (SDH) staining showed disorganized enzyme activity in the fibers with RRF. ATP staining showed mosaic arrangement of type I and type



**Figure 1** Progression of serum hepatitis B virus DNA and aminotransferase. Telbivudine was introduced when alanine aminotransferase (ALT) and hepatitis B virus (HBV) DNA level was both high. The indication was clear and sufficient, and lactic acidosis happened after 11 mo of antiviral treatment when liver function was controlled well. HBV DNA continued to be normal after telbivudine was stopped and changed to tenofovir soon after.

II fibers. Oil Red O staining showed that several muscle fibers were filled with increased lipid droplets. Histo-Immunohistochemical tests were Rod-Dystrophin (+), C-Dystrophin (+), N-Dystrophin (+), Dysferlin (+), Merosin (+),  $\alpha$ -Sarcoglycan (+),  $\beta$ -Sarcoglycan (+), and  $\gamma$ -Sarcoglycan (+). The patient was diagnosed with LA (type B2), HBeAg negative chronic hepatitis B and drug-induced myopathy.

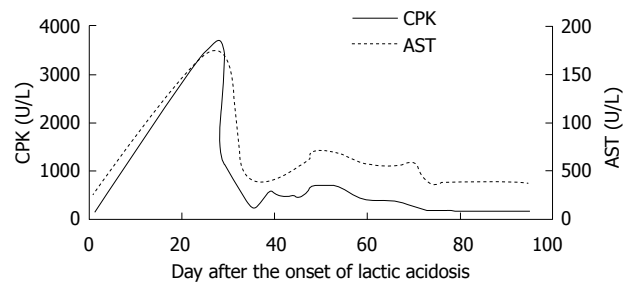
He was given hemodialysis for more than eight times after admission. The blood lactate level reduced to normal range (less than 2.5 mmol/L) after hemodialysis but slightly elevated the following day. The symptoms of nausea and vomiting completely recovered, so the hemodialysis was discontinued. He was given hydration, alkalization and supplementation with Coenzyme Q 10 and Levocarnitine. Two weeks after hemodialysis, the blood lactate level still fluctuated between 5 and 7 mmol/L. As a result, methylprednisolone tablets (24 mg/d) was given. Meanwhile, HBV DNA was rechecked and showed a slight rebound at  $1.59 \times 10^3$  copies/mL, consequently tenofovir (300 mg/d) was given to suppress the HBV.

In the following two weeks, his blood lactate level returned to a normal range, and the HBV DNA was undetectable (less than 1000 copies/mL), so methylprednisolone was tapered off within a ten-week period. The patient has remained very well and followed up regularly to date.

## DISCUSSION

Our patient had marked LA without evidence of infection or organ hypoperfusion. It is very likely that his acidosis was secondary to the nucleoside analogue, telbivudine, during treatment of HBV.

In basic terms, lactic acid is the normal endpoint of the anaerobic breakdown of glucose in the tissues. In the setting of decreased tissue oxygenation, lactic acid is produced as the anaerobic cycle is utilized for energy production. The normal blood lactate concentration in unstressed patients is 0.5-1 mmol/L. Lactate concentration



**Figure 2** Progression of serum creatine kinase level. Creatine kinase (CPK) elevated at the very beginning of lactic acidosis and returned to normal range quickly. AST: Aspartate aminotransferase.

of less than 2 mmol/L can be considered to be normal in patients with critical illness. Hyperlactatemia is defined as a persistent, mild to moderate (2-4 mmol/L) increase in blood lactate concentration without metabolic acidosis; whereas LA is characterized by constant increased in blood lactate levels (usually  $> 5$  mmol/L) in association with metabolic acidosis (usually present as pH  $< 7.3$  and serum bicarbonate  $< 10$  mmol/L)<sup>[1]</sup>.

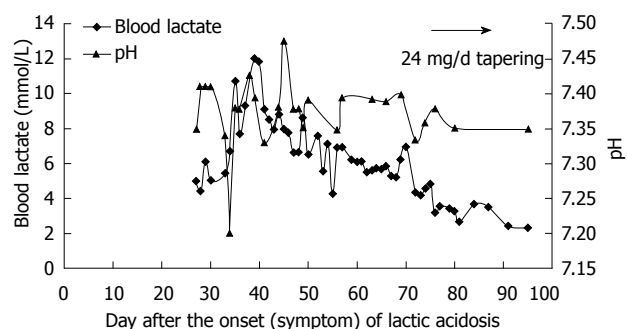
The LA syndrome linked to nucleoside analogue is associated with steatosis, abnormal mitochondrial appearance and function, pancreatitis, neuropathy, and myopathy. The onset may be abrupt or insidious, it generally begins with nausea, vomiting, and abdominal pain. It will progress to tachypnea, shortness of breath, and hypoxia. Patients with severe LA may subsequently develop renal failure, liver failure, coagulopathy, seizures, arrhythmias, and even death. The patient reported here was a severe LA case with a lactate level of more than 12 mmol/L and pH value of 7.2. His blood lactate level did not recover to normal even after hemodialysis treatment for 16 times.

The Food and Drug Administration approved oral nucleoside analogues for HBV treatment, including lamivudine, adefovir, telbivudine, entecavir, and tenofovir, are well tolerated. However, these still carry the “black box” warning for the potential development of mitochondrial damage with resultant LA based on the data from the human immunodeficiency virus (HIV) treatment literature<sup>[3-7]</sup> and the experience using fialuridine (FIAU) in HBV treatment<sup>[8]</sup>.

Lamivudine<sup>[4,5]</sup> and tenofovir<sup>[3,7]</sup> associated LA was reported only in HIV patients treated with combination regimens (Table 1), while their mitochondrial toxicity is far less than those antiretroviral nucleoside analogues. The risk of LA with entecavir treatment in chronic hepatitis B patients remain controversial. However, it was reported to occur more often in patients with impaired liver function<sup>[1,9,10]</sup>, especially in those with high MELD (model for end stage liver diseases) scores and multi-organ failure (Table 1). Report of LA caused by adefovir is rare, and all reported cases were present in a combination regimens<sup>[9]</sup>.

Telbivudine, as with all the other approved nucleoside analogues, has a potential of mitochondrial toxicity which will lead to LA in theory. However, no single case has been reported to date. This will be the first documented case of type B LA in a chronic hepatitis B patient who





**Figure 3** A refractory lactic acidosis case and the fluctuation of blood lactate level. Symptoms lasted more than 3 mo and recovered slowly after 16 times of hemodialysis and small dosage of glucocorticoid helped to resolve the persistent serum lactate elevation.

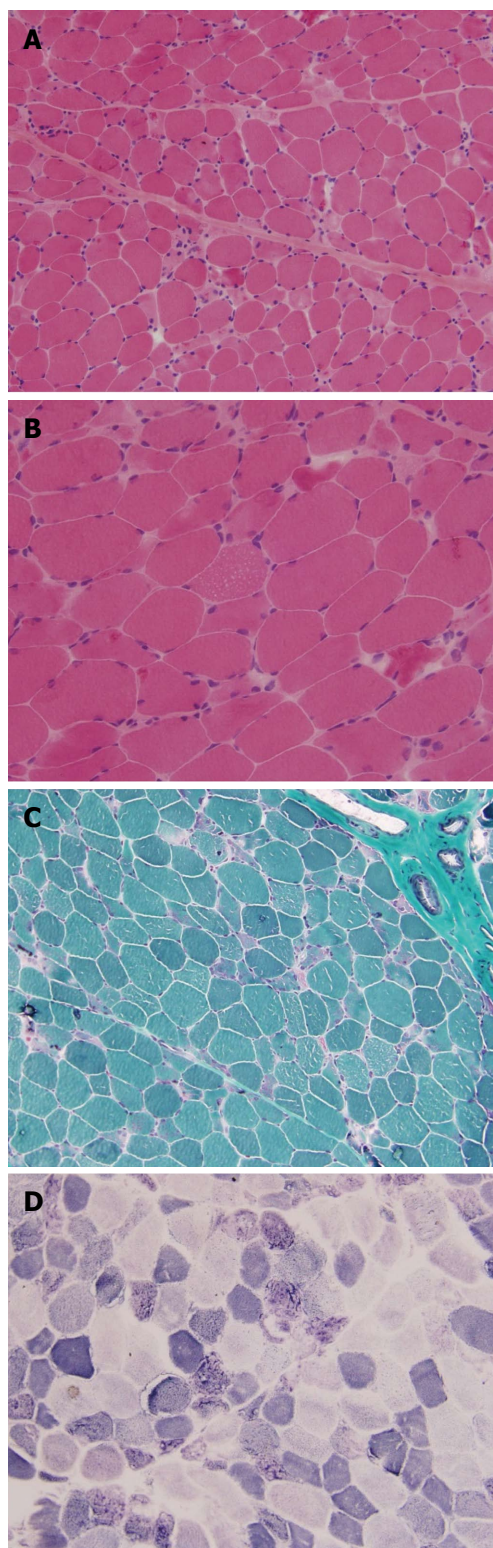
received telbivudine monotherapy.

Among the five nucleoside analogues approved for the use in hepatitis B, the inhibitory strength of mtDNA polymerase gamma in an *in vitro* test system is actually far less than that seen in antiretroviral agents. In the registration trial of telbivudine for HBV, the side-effect profile of telbivudine was generally favorable<sup>[2]</sup> and similar to comparator arm of lamivudine throughout 2 years of treatment. There was no LA case reported, however, a significantly higher incidence of grade 3 to 4 serum CPK elevations (*i.e.*, 7 times upper limit of normal) was noted in telbivudine-treated compared to lamivudine-treated patients at 2 years (12.9% *vs* 4.1%).

We noticed that our patient had a history of hypokalemic periodic paralysis. Hypokalemic periodic paralysis is an autosomal-dominant disorder characterized by episodic attacks of muscle weakness with hypokalemia. Whether there was pre-existence of myopathy in our patient prior to telbivudine treatment is uncertain, only transient CPK elevation was observed and most of time the CPK value was normal before LA occurred. The reason that LA and CPK elevation does not co-exist in most cases during monotherapy of nucleoside analogues in chronic hepatitis B patients is unclear. Interestingly, our case is a rare incident where CPK elevation and LA occurred simultaneously (Table 1). This case has suggested that besides CPK, serum lactate level should also be monitored closely during the treatment of telbivudine.

LA can be divided into 2 categories, type A and type B. Type A is LA occurring in association with clinical evidence of poor tissue perfusion or oxygenation of blood (*e.g.*, hypotension, cyanosis, cool and mottled extremities). Type B is LA occurring when no clinical evidence of poor tissue perfusion or oxygenation exists. Type B can be divided into 3 subtypes based on underlying etiology. Type B1 occurs in relation to systemic disease, such as renal and hepatic failure, diabetes and malignancy. Type B3 is due to inborn errors of metabolism. Type B2 is caused by several classes of drugs and toxins, including biguanides, alcohols, iron, isoniazid, zidovudine, and salicylates.

Our patient had marked LA without evidence of in-



**Figure 4** Histopathology of muscle biopsy specimens showed mitochondrial toxicity. A: Many regenerating and necrotic muscle fibers, mild nuclear proliferation and necrosis around muscle fibers (HE, magnification  $\times 200$ ); B: Part of muscle fibers filled with fatty droplets (HE, magnification  $\times 400$ ); C: Ragged red fibers under envelope of shrinking muscle cells (modified Gomori trichrome stain, magnification  $\times 200$ ); D: The figure revealed the structural disorders of mitochondria. The myocytes different in size; Type I and Type II muscle fibers showed mosaic arrangement (nicotinamide-adenine dinucleotide, magnification  $\times 200$ ).



**Table 1** Characteristics of patients with lactic acidosis treated with nucleoside analogues

Patient ID	Age (yr)	Liver condition	Underlying disease	Child-Pugh	MELD score	Drug	LA therapy	Peak lactate (mmol/L)	Nadir pH	BE (mmol/L)	Peak CPK (U/L)	Prognosis	Ref.
1	35	CHB	HOKPP	A	7	LDT	11 mo	> 12	7.2	-15.8	3683	Resolved	This paper
2	36	OLT, ITBL	-	C	38	ETV	9 mo	5.20	7.2	-18	Normal	Resolved	[7]
3	79	ALF	-	-	29	ETV	6 d	20.82	7.1	-17	Normal	Death	[7]
4	60	OLT, re-cirrhosis	-	C	28	ETV	1 mo	3.86	7.4	-5	Normal	Resolved	[7]
5	60	Cirrhosis HCC	-	B	25	ETV	10 d	6.77	7.3	-12	Normal	Resolved	[7]
6	61	Cirrhosis HCC	-	B	22	ETV	4 d	2.70	7.4	-6	Normal	Resolved	[7]
7	63	CHB, HCC	massive bilobar pneumonia	C	30	ETV	10 d	9.20	7.24	-	Normal	Resolved	[8]
8	54	CHB, cirrhosis	CML	C	24	ETV + ADV	10 d	9.50	6.95	-	Normal	Resolved	[9]
9	42	HIV	-	A	7	HARRT (stavudine + LAM)	9 mo	5.48	7.15	-	Normal	Resolved	[6]
10	51	HIV	DM	A	7	HARRT (tenofovir)	12 mo	6.40	7.21	-	Normal	Resolved	[7]

MELD: Model for end stage liver diseases; LA: Lactic acidosis; BE: Base excess; CPK: Creatine phosphokinase; CHB: Chronic hepatitis B; OLT: Orthotopic liver transplantation; ITBL: Ischemic-type biliary lesions; ALF: Acute liver failure; HCC: Hepatocellular carcinoma; HIV: Human immunodeficiency virus; HOKPP: Hypokalemia periodic paralysis; CML: Chronic myelogenous leukemia; DM: Diabetes mellitus; LAM: Lamivudine; ETV: Entecavir; ADV: Adefovir; LDT: Telbivudine; HARRT: Highly active antiretroviral treatment; Lactate mmol/L  $\times$  9.608 = mg/dL.

fection or organ hypoperfusion. In view of the fact that no other underlying causes were identified, his acidosis may be due to telbivudine (Type B2 LA). The patient also had mild muscle pain and proximal muscle weakness consistent with a myopathy, as shown on the electromyography. It is likely LA and myopathy arise from the same pathological origin, *i.e.*, mitochondrial dysfunction. Indeed, subsequent muscle biopsy showed RRF, lipid storage and mitochondrial dysfunction, which indicated the mitochondrial toxicity.

Management options for type B LA may include treatment for primary diseases, renal replacement therapy, bicarbonate alkalization and supplementation with thiamine, L-acetylcarnitine as well as Coenzyme Q 10<sup>[10]</sup>. In term of nucleoside analogues, discontinuation should be instantaneously. Most of the LA cases can resolve rapidly after discontinuation of the causative drug. Majority of the patients who developed LA secondary to nucleoside analogues had a good outcome. The recovery progression for our patient was slow with a total period of more than three months. The symptoms improved after hemodialysis therapy for 16 times, and blood lactate level normalized to the upper limit of normal, but halted for a period of time. No plausible reasons can be found for this phenomenon, but small dosage of glucocorticoid seems to be effective. The use of low-dose glucocorticoid for a short period of time may have an unusual effect. However, a larger controlled clinical trial is required for further clarification. It should be applied cautiously by an experienced clinical hepatologist.

This case shows that telbivudine may cause muscle damage and even lead to fatal LA in telbivudine-treated chronic hepatitis B patients. Thus patients receiving tel-

bivudine should be closely monitored for muscular abnormalities, blood lactate level and other mitochondrial toxicity associated side effects.

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**E-Editor** Ma S



## Selective endoscopic ligation for treatment of upper gastrointestinal protuberant lesions

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children.

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**Key words:** Endoscopy; Ligation; Endoscopic ultrasonography; Protuberant lesion; Children

**Core tip:** Endoscopic ligation is an effective method in the management of protuberant lesions. It is less invasive and less expensive than surgical interventions. However, there are few studies of this technique in the treatment of upper gastrointestinal (GI) lesions in children. This paper reports selective endoscopic ligation for the treatment of different upper GI protuberant lesions in children. Endoscopic ultrasonography was used to determine the depth of invasion and provided a preliminary characterization of the lesions.

### Abstract

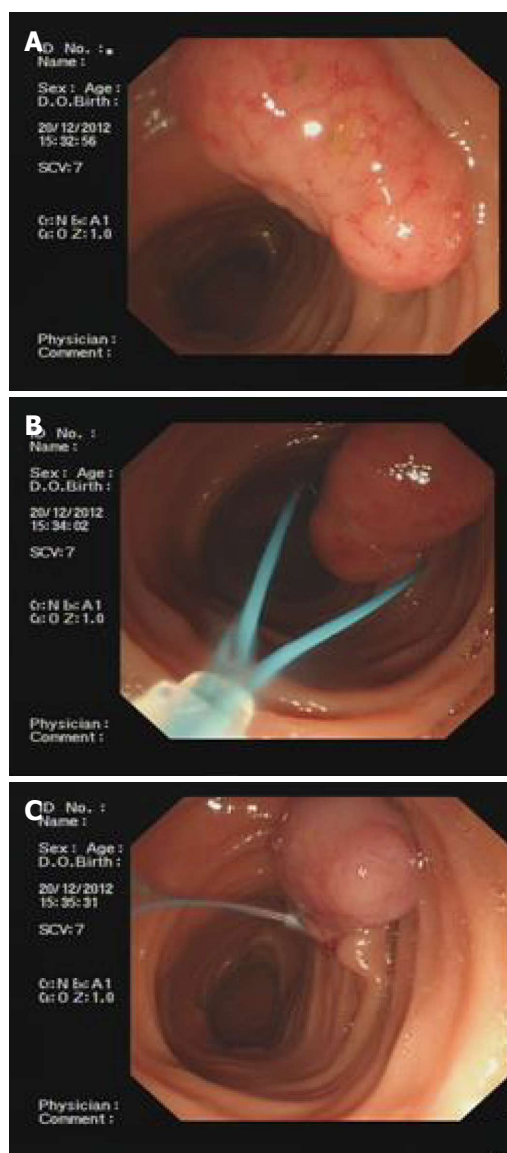
This study explored the clinical value of endoscopic ligation for the treatment of upper gastrointestinal (GI) protuberant lesions in children. According to the appearance and size of lesions, we used different ligation techniques for the treatment of the lesions. Endoscopic ultrasonography was used for preliminary characterization of the lesions. One case diagnosed with Peutz-Jeghers syndrome was successfully treated by a detachable snare. Two cases with semi-pedunculated or broad-base lesions originating from the submucosal layer of the upper GI were treated with endoscopic variceal ligation; endoscopic examination showed that one case had complete healing 11 wk after ligation, while an ulcer scar was observed at the ligation site after 6 wk in the other case. All lesions were successfully ligated at the first attempt. No significant complications occurred either during or after the procedure. Selective endoscopic ligation of upper GI lesions is an effective and safe treatment for upper GI protuberant lesions in

Wang L, Chen SY, Huang Y, Wu J, Leung YK. Selective endoscopic ligation for treatment of upper gastrointestinal protuberant lesions. *World J Gastroenterol* 2013; 19(33): 5581-5585 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i33/5581.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i33.5581>

### INTRODUCTION

Protuberant lesions in the gastrointestinal (GI) tract may cause clinical symptoms (e.g., abdominal pain, bleeding, intussusception, obstruction) and have malignant potential. In addition, the presence of the lesion is a source of psychological distress. With the development of endoscopic techniques and devices, endoscopic treatment has become an effective method for protuberant lesions in the gastrointestinal tract. It is less invasive than surgical interventions.

Endoscopic ligation has been widely used in the management of post polypectomy bleeding, bleeding of esophageal and gastric varices, and angiodysplasias<sup>[1]</sup>. In



**Figure 1** Imaging in case 1. A: Endoscopic view of the large pedunculated polyp in the descending part of duodenum; B: Endoscopic ligation with a detachable snare; C: Endoscopic view of the ligated polyp, with the endoloop placed around the stalk.

2004, Sun *et al.*<sup>[2]</sup> first reported endoscopic band ligation without electrosurgery was an effective and safe treatment for resection of small upper GI leiomyoma. They found most leiomyomas could slough spontaneously within 3.6 to 4.5 wk. Complications related to use of electrosurgery were avoided. There was a case report about colonoscopic polypectomy with a detachable snare to remove a large juvenile polyp in 1-year-old girl<sup>[3]</sup>. However, there are few published reports regarding endoscopic ligation to treat upper GI protuberant lesions in children. Here, we report three patients with upper GI protuberant lesions who received endoscopic treatment with ligation.

## CASE REPORT

### Case 1

A 4-year-old girl presented with abdominal pain for 6 mo.

She was diagnosed with Peutz-Jeghers syndrome. Under general anesthesia, conventional upper GI endoscopic examination (GIF-XQ260, Olympus, Japan) revealed multiple polyps. A large, 25 mm × 15 mm, pedunculated polyp with a hyperemic and edematous surface was found in the descending part of the duodenum (Figure 1). Another polyp with a thick stalk, 16 mm × 10 mm in size, also appeared in the opposite side of the duodenal papilla. To remove the two large polyps safely, we performed endoscopic ligation with detachable snares (MAJ339, Olympus, Japan). The device was composed of an elliptically shaped nylon loop and a silicone-rubber stopper which maintained the tightness of the loop. The nylon loop was placed at the base of the stalk, tightened around the stalk, then the stopper was detached from the device. We observed the color change in the target lesion to ensure proper tightening, when the ligated lesion changed to dark red because of congestion. A smaller lesion, 3 mm × 3 mm, was found in the gastric body. It was removed using the electrocoagulation technique. No complications occurred during or after the procedure using the detachable snare.

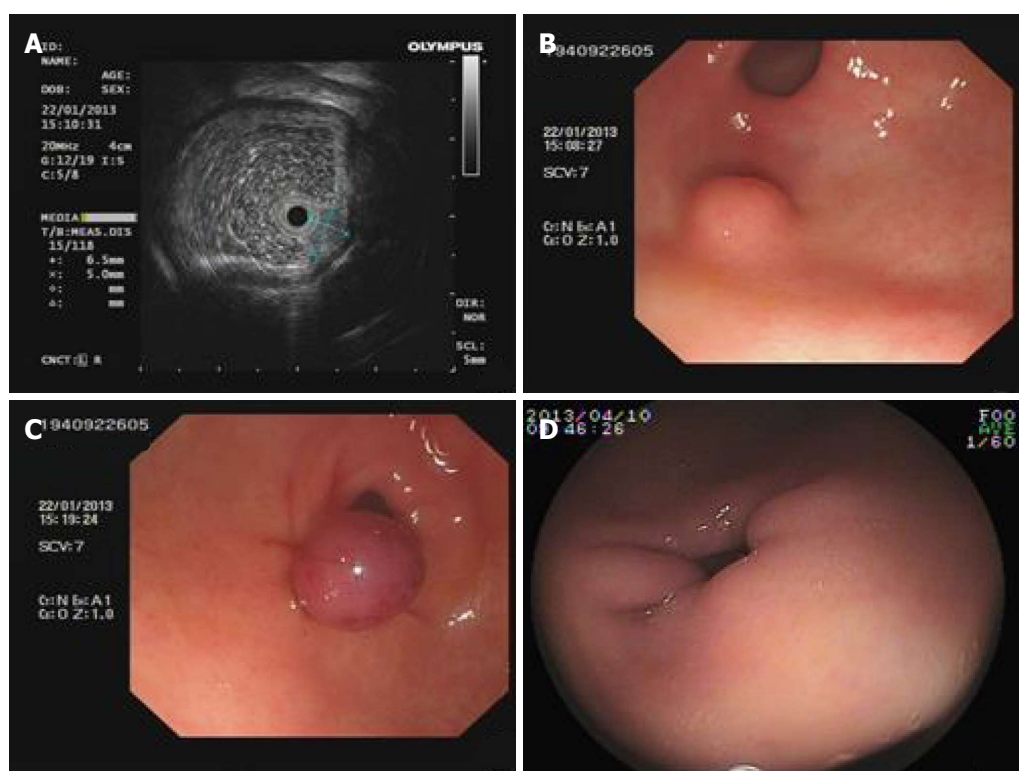
### Case 2

A 10-year-old boy with nausea and belching for 2 mo underwent upper gastrointestinal endoscopy (GIF-XQ260, Olympus, Japan) in our hospital. The result revealed a protuberant lesion located in the gastric antrum. Endoscopic ultrasonography (EUS) was performed using a radial echoendoscope with a 20 MHz catheter probe (UM-DP12-25R, Olympus, Japan), and showed a 6.5 mm × 5.0 mm hypoechoic, homogeneous lesion originating from the submucosal layer. The lesion did not involve the muscularis propria. Endoscopic ligation was performed using an attached endoscopic variceal ligation (EVL) device (Six Shooter Saeed Multiband Ligator, Wilson-Cook Medical, Winston-Salem, NC, United States), which aspirated the lesion into the ligator cap, and then an elastic rubber band was released then tightened around the base of the lesion (Figure 2). The goal of ligation was to create a polypoid form with a pseudo stalk. For complete ligation, suction should be maintained for at least 1 min before releasing the rubber band. There were no significant procedure-related complications. The lesion completely healed within 11 wk after the ligation.

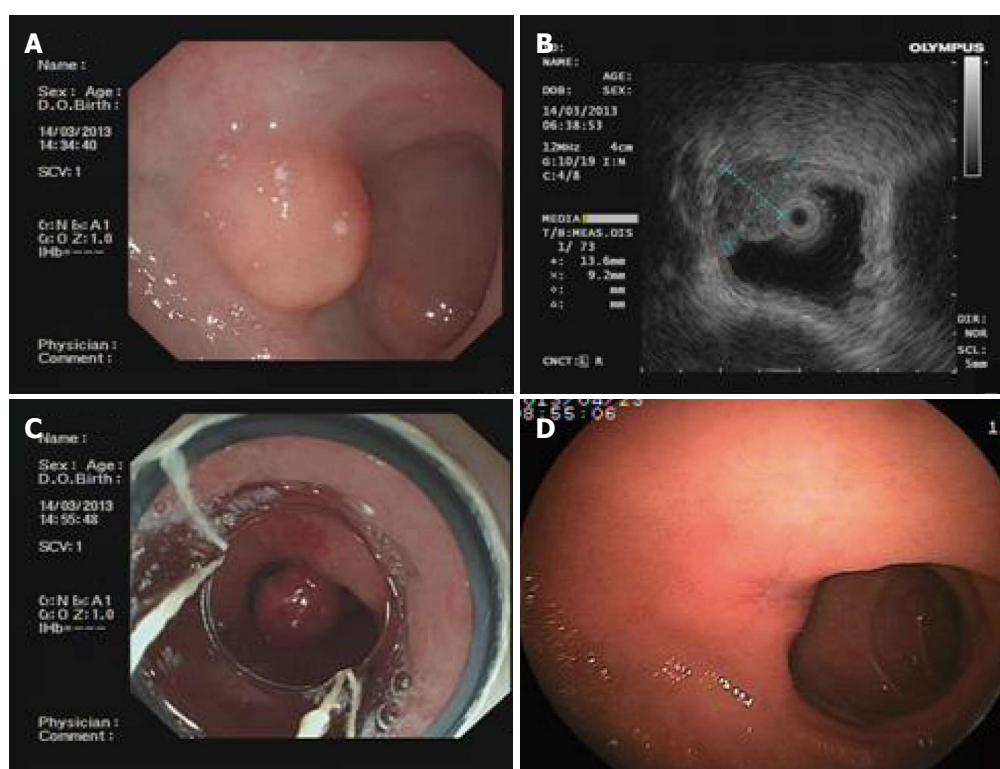
### Case 3

A 10-year-old boy with an episode of recurrent abdominal pain was referred to our hospital for gastrointestinal endoscopy (GIF-XQ260, Olympus, Japan). Under general anesthesia, EUS examination showed a hypoechoic homogeneous mass, 13.6 mm × 9.2 mm in size, originating from the submucosal layer of the duodenal bulb (Figure 3). It did not extend to the muscularis propria. The ligation of this lesion was carried out with the EVL device. The lesion was sucked sufficiently into the ligator cap and the band was tightened to ligate the base of the tissue. No complications were reported during the proce-





**Figure 2** Imaging in case 2. A: Endoscopic ultrasonography image of the submucosal lesion; B: Endoscopic view of the lesion in gastric antrum; C: Endoscopic view showing the ligated lesion; D: Endoscopic image at 11 wk after ligation, showing healing at the ligation site.



**Figure 3** Imaging in case 3. A: Endoscopic view of the protuberant lesion in the duodenal bulb; B: Endoscopic ultrasonography image of the size of the lesion; C: Endoscopic view of the ligated lesion using the endoscopic variceal ligation device; D: Endoscopic view of an ulcer scar at the ligation site after 6 wk.

dure. Four days later, GI endoscopy showed sloughing of the raised lesion, and an ulcer could be observed. A scar

was seen at the ligation site on a follow-up examination of 6 wk later.

## DISCUSSION

There has been remarkable progress in the use of endoscopic treatment for gastrointestinal diseases. A detachable snare for endoscopic use was first developed by Hachisu<sup>[4]</sup>. Large polyps or other raised lesions have been successfully removed by detachable snares. In a randomized trial, Iishi *et al.*<sup>[5]</sup> used endoscopic ligation with a detachable snare for the stalk of a large pedunculated polyp and evaluated the safety and effectiveness of the procedure in comparison with conventional endoscopic snare polypectomy. Results showed that no bleeding occurred in 47 patients assigned to colonoscopic polypectomy with a detachable snare, but bleeding occurred in five of 42 patients who received conventional colonoscopic polypectomy. Moreover, the use of a detachable snare reduced the duration of hospitalization after polypectomy. In 2005, Raju *et al.*<sup>[6]</sup> first described a new technique for successful removal of a large pedunculated, 4 cm wide, broad-based colonic lipoma using endoloops without the need for cautery. Lee *et al.*<sup>[7]</sup> reported that nine cases diagnosed with large pedunculated GI submucosal tumors were successfully treated by endoloop ligation in 2008, and the tumors were removed within 4 wk. Recently, a trial was published to evaluate the clinical impact of selective ligation using a detachable snare for small intestinal polyps in three adult patients with Peutz-Jeghers syndrome<sup>[8]</sup>. The technique of endoscopic ligation is safer than conventional snare polypectomy or endoscopic mucosal resection. It could reduce the risk of bleeding and injury of the deeper tissue layers. However, to our knowledge, the effect of endoscopic ligation for upper GI protuberant lesions in children has not been reported.

According to our experience, there are two aspects to be considered in endoscopic treatment. One is the appearance of elevated lesions. A study described some instances where the use of a detachable snare was ineffective for colonoscopic polypectomy of large polyps with thin stalks or for lesions that were semi-pedunculated<sup>[9]</sup>. It is difficult to tighten the lesion sufficiently in semi-pedunculated or broad-based lesions, as the loop is more likely to slip off. A target lesion positioned at the 5 o'clock to 7 o'clock position is easier to remove by an endoloop<sup>[1]</sup>. Huang *et al.*<sup>[10]</sup> reported the methodology for different lesions using an EVL device or endoloop. Small GI stromal tumors ( $\leq 12$  mm) were treated by endoscopic band ligation with an EVL device. Large pedunculated tumors ( $> 12$  mm) could be managed by endoscopic ligation with a detachable endoloop, while ligation of large sessile tumors was carried out with a large-sized transparent cap plus an endoloop. In our cases, we used a detachable snare for large pedunculated lesions. If the lesion was semi-pedunculated or sessile, endoscopic ligation was carried out with an attached band ligator device. In one of the two cases using an EVL device, the lesion was larger than 12 mm in diameter. For smaller lesions (3 mm  $\times$  3 mm), conventional electrocoagulation was performed. Thus, selective ligation is essential to avoid complications.

The second consideration is that endoscopic band li-

gation is associated with a risk of perforation. It is necessary to be careful to avoid aspirating excessive tissue into the cap. Perforations were reported in two studies after endoscopic band ligation<sup>[11,12]</sup>; two cases were GI stromal tumors in the gastric fundus, and one was a gastric submucosal tumor partly connecting with the muscularis propria. The reason might be that all the layers of the gastric wall were ligated. It seems that the risk of perforation is greater with deeper layer tumors. Determination of the origin of the lesion and appropriate suction force are essential factors that should be considered.

Endoscopic ligation allows the lesion to slough spontaneously. The main limitation of this technique is the difficulty in retrieving the tissue specimen. Histopathologic diagnoses could not be made. However, protuberant lesions in children are mostly considered as benign and to have very low potential for malignant transformation. Sun *et al.*<sup>[13,14]</sup> reported that EUS-assisted band ligation with systematic follow-up by EUS was an effective treatment for small upper GI stromal tumors. EUS was used to determine the histologic layer of origin and evaluate whether the mass was confined completely by the band. It has significant value in the diagnosis of submucosal lesions. In the present study, EUS examination was used to identify the depth of invasion. It provided preliminary identification of the property of the lesion. We recommend that our patients should have close follow-up in order to detect any recurrence early.

In conclusion, endoscopic ligation appears to be a feasible method for removal of upper GI lesions in children. According to the characteristics and volume of the lesions, selecting the correct application of ligation and controlling the suction force can reduce the risk of related complications, such as hemorrhage and perforation. In addition, combining endoscopic ligation with EUS examination may be a useful technique for submucosal tumors in children. Pediatric experience with this endoscopic technique remains limited. Thus, studies involving more subjects and longer follow-up are needed to further define the clinical role of endoscopic ligation in children.

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**P- Reviewer** Ozkan OV **S- Editor** Wen LL  
**L- Editor** Cant MR **E- Editor** Li JY



## Gallstone ileus: Case report and literature review

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**Author contributions:** Dai XZ and Li GQ contributed equally to this work; Dai XZ and Zhang CY designed the report; Dai XZ and Li GQ were attending doctors for the patients; Dai XZ, Li GQ and Zhang CY performed the surgical operation; Zhang F and Wang XH performed imaging diagnosis; Zhang CY organized the report; Dai XZ wrote the paper.

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population with a female predominance. The advent of computed tomography and magnetic resonance imaging has made it easier to diagnose GI. Enterotomy with stone extraction alone remains the most common surgical method because of its low incidence of complications.

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**Key words:** Gallstone ileus; Fistula closure; Intestinal obstruction; Bowel obstruction; Enterolithotomy

**Core tip:** We present the case of a 56-year-old female who presented at our institution with symptoms of bowel obstruction. Abdominal computed tomography (CT) and exploratory laparotomy revealed a large gallstone in the terminal ileus. She underwent enterolithotomy and had an uneventful postoperative course. The literature suggests that gallstone ileus (GI) is a rare condition affecting mainly the older population and has a female predominance. CT and magnetic resonance imaging have made it easier to diagnose GI. Enterotomy with stone extraction alone remains the most common surgical method because of its low incidence of complications.

### Abstract

Gallstone ileus (GI) is characterized by occlusion of the intestinal lumen as a result of one or more gallstones. GI is a rare complication of gallstones that occurs in 1%-4% of all cases of bowel obstruction. The mortality associated with GI ranges between 12% and 27%. Classical findings on plain abdominal radiography include: (1) pneumobilia; (2) intestinal obstruction; (3) an aberrantly located gallstone; and (4) change of location of a previously observed stone. The optimal management of acute GI is controversial and can be: (1) enterotomy with stone extraction alone; (2) enterotomy, stone extraction, cholecystectomy and fistula closure; (3) bowel resection alone; and (4) bowel resection with fistula closure. We describe a case to highlight some of the pertinent issues involved in GI management, and propose a scheme to minimize recurrent disease and postoperative complications. We conclude that GI is a rare condition affecting mainly the older

Dai XZ, Li GQ, Zhang F, Wang XH, Zhang CY. Gallstone ileus: Case report and literature review. *World J Gastroenterol* 2013; 19(33): 5586-5589 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i33/5586.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i33.5586>

### INTRODUCTION

Gallstone ileus (GI) is characterized by occlusion of the intestinal lumen as a result of one or more gallstones<sup>[1,2]</sup>. According to reports from the 1990s, GI is a rare complication of gallstones that occurs in 1%-4% of all cases of bowel obstruction and in  $\leq 25\%$  of cases of non-strangulated small-bowel obstruction in patients aged  $> 65$  years. The mortality associated with GI ranges between





Figure 1 Abdominal radiographs were normal.

12% and 27%<sup>[3]</sup>. GI accounts for only 0.095% of cases of mechanical bowel obstruction in the United States<sup>[4]</sup>. The optimal management of acute GI is controversial<sup>[3,4]</sup>. We describe a case here to highlight some of the pertinent issues involved in GI management, and propose a scheme to minimize recurrent disease and postoperative complications.

## CASE REPORT

A 56-year-old female presented with intermittent vomiting and abdominal pain for 7 d. This patient had cholecystolithiasis for 10 years and had been treated with antibiotics. She had been treated in another hospital with intravenous fluids and antibiotics. She had undergone gastrointestinal decompression and regulation of water and electrolytes for approximately 1 wk, but the symptoms had not abated.

One week after the latest attack, she was referred to our hospital for nausea, vomiting, constipation and abdominal pain. Upon examination, her abdomen was moderately distended, and tympanic and high-pitched bowel sounds were audible. Rectal examination was normal. Blood tests revealed an elevated total leukocyte count ( $14.4 \times 10^9$  cells/L) and unremarkable liver function test values. Plain radiographs of the abdomen were normal (Figure 1). She was treated with intravenous fluids and antibiotics. However, 2 d after hospital admission, clinical deterioration was investigated with computed tomography (CT). CT demonstrated a small-bowel obstruction due to a 50-mm calculus within an ileal loop. CT showed air in the gallbladder and adhesions between the thickened gallbladder wall and duodenal wall. A severe air-fluid level was also seen (Figure 2). We therefore made a diagnosis of GI.

The patient underwent an exploratory laparotomy, during which a wide fistula from the gallbladder to the second part of the duodenum was found. Abdominal adhesions around the gallbladder were very severe. Exploration revealed massively dilated loops of the small bowel proximal to the distal ileum. An obstruction was seen 100 cm from the terminal ileum, where an enterotomy was made to reveal a large gallstone (5 cm  $\times$  3 cm  $\times$  4 cm).

The gallstone was removed and the enterotomy repaired in two layers (Figure 3). The patient had an uneventful postoperative course and was discharged home on postoperative day 10.

## DISCUSSION

GI is more common in women, and the ratio of females to males is 3.5 to 1<sup>[4]</sup>. The gallstone may enter the intestine through a fistula and it can impact anywhere in the gastrointestinal tract<sup>[5]</sup>. The gallstone must be  $\geq 2$ -2.5 cm in diameter to cause obstruction<sup>[6-9]</sup>. As shown by Reisner and Cohen, impaction of the stone can occur in any part of the bowel, *i.e.*, the ileum (60.5% of cases), jejunum (16.1%), stomach (14.2%), colon (4.1%), and duodenum (3.5%). It can also be passed spontaneously (1.3%)<sup>[3,10]</sup>. It occurs most frequently in the terminal ileum and the ileocecal valve because of their narrow lumen and potentially less active peristalsis<sup>[10]</sup>.

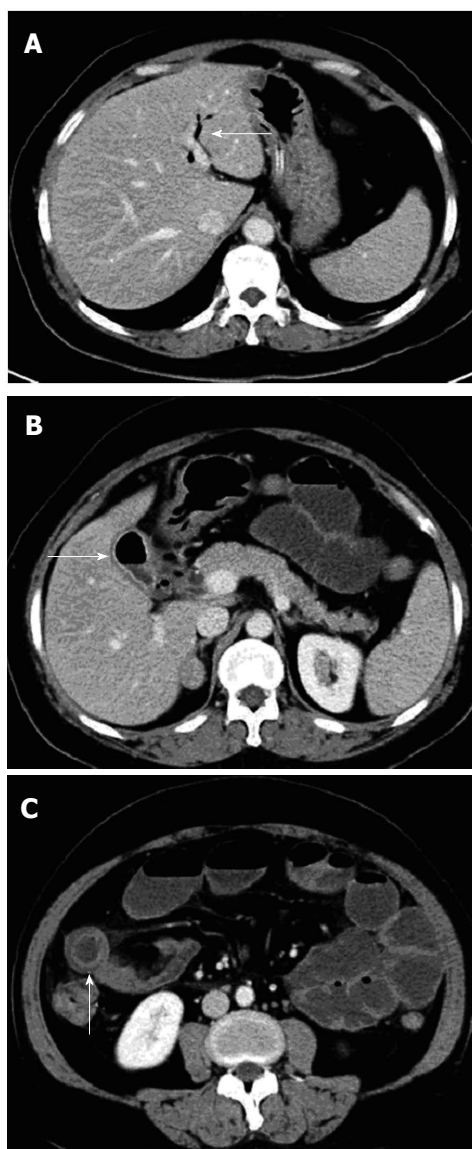
If GI occurs in elderly patients with comorbidities, the often vague, intermittent symptoms may delay the diagnosis by days<sup>[3]</sup>. Presentation is typically non-specific, and often with intermittent symptoms of nausea, vomiting, abdominal distension and pain. We should pay more attention to those patients who have the history of cholecystolithiasis and with symptoms such as nausea, vomiting, abdominal distension and pain. In the past, confirming the diagnosis was difficult, but the advent of CT and magnetic resonance imaging (MRI) has made it easier to diagnose GI<sup>[10,11]</sup>.

Classical findings on plain abdominal radiography include: (1) pneumobilia; (2) intestinal obstruction; (3) an aberrantly located gallstone; and (4) a change in location of a previously observed stone<sup>[9,11-14]</sup>. The widespread use of CT with an overall sensitivity, specificity, and diagnostic accuracy of 93%, 100% and 99%, respectively, has aided diagnosis<sup>[14]</sup>. Interestingly, no aerobilia which can be easily detected by transabdominal ultrasound may be one reason for the delayed diagnosis. Additionally, the absence of significant calcification of the stone reduces the chance of an early diagnosis. In 50% of cases, the diagnosis is often only made at laparotomy<sup>[3]</sup>.

GI is a mechanical intestinal obstruction caused by impaction of gallstones within the lumen of the bowel. Most reports indicate that stones smaller than 2.5 cm usually pass through spontaneously, so conservative treatment (decompression by nasogastric drainage) is conducted before a decision is made to remove the impacted stone by surgical means<sup>[6,8]</sup>.

Management of GI is controversial and includes: (1) enterotomy with stone extraction alone; (2) enterotomy, stone extraction, cholecystectomy and fistula closure; (3) bowel resection alone; and (4) bowel resection with fistula closure<sup>[4,6,15]</sup>.

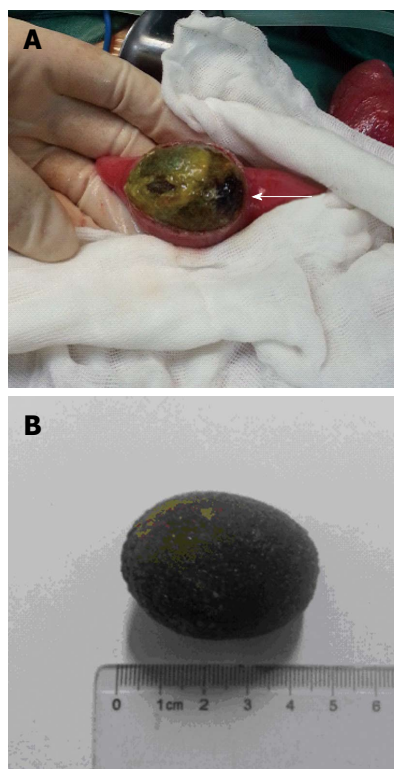
Enterotomy with stone extraction alone remains the most common surgical method because of its low incidence of complications<sup>[4]</sup>. Spontaneous closure of the fistulous tract is observed in  $> 50\%$  of cases<sup>[12]</sup>. Small-



**Figure 2 Severe air-fluid level.** A: Axial view of the upper abdomen demonstrates air in an intrahepatic bile duct (arrow); B: Axial view of the upper abdomen demonstrates an air in the gallbladder (arrow); C: Computed tomography demonstrating gallstones of approximately 5 cm in diameter (arrow) within the small bowel.

bowel obstruction requires enterolithotomy with a longitudinal incision placed on the anti-mesenteric border proximal to the site of impaction. Careful closure of the enterolithotomy is needed to avoid narrowing of the intestinal lumen, and we usually employ a transverse closure for this reason. The choice of surgical procedure is determined largely by clinical status. GI patients are usually elderly and have comorbidities so enterotomy with stone extraction alone appears to more suitable than more invasive techniques<sup>[4,16]</sup>.

However, 5% of patients who undergo enterolithotomy alone go on to develop biliary symptoms, and 10% require an unplanned reoperation. In the presence of residual stones, the estimated prevalence of recurrence ranged from 5% to 17%, and more than half of these recurrences occur within 6 mo of the index presentation.



**Figure 3 The gallstone was removed and the enterotomy repaired in two layers.** A: Impacted stone (arrow) removed from the intestine; B: Gallstone measuring 3 cm × 4 cm × 5 cm.

Retrospective cohort and literature reviews of GI reveal a prevalence of biliary malignancy of 2%-6%<sup>[4,17]</sup>.

We noted that fistula closure, if conducted urgently or as an emergency during the initial procedure, was independently associated with a higher prevalence of mortality than enterotomy and stone extraction alone. The reason may be that elderly patients have multiple comorbidities and an edematous surrounding area. Bowel resection is sometimes necessary, particularly in the presence of a perforation.

Laparoscopy-assisted methods have been reported by Sarli *et al.*<sup>[18]</sup>, who successfully treated three women with GI. Their patients made uneventful recoveries. However, laparoscopy is somewhat more challenging in cases of dilated and an edematous bowel<sup>[18,19]</sup>.

Some special types of GI, such as Bouveret's syndrome (stones impacting in the duodenum causing gastric outlet obstruction), and stones in the stomach or the colon are suitable for non-surgical therapeutic options in around 20% of the patients. For example laserlithotripsy in Bouveret's syndrome<sup>[20]</sup> or extracorporeal shock wave lithotripsy<sup>[21]</sup> or even only endoscopic extraction<sup>[22]</sup> may be a promising and fast therapeutic alternative.

Historically, wound infections and dehiscence have been cited as being the most common complications after surgery in 25% to 50% of GI cases. In contrast to what has been published so far, the most common post-operative complication is acute renal failure followed by urinary tract infection and wound infections. Gastrointes-

tinal complications related to anastomotic leaks and intra-abdominal abscesses are highest in patients undergoing enterotomy with fistula closure<sup>[3,4,12]</sup>.

If the gallbladder is preserved at the initial procedure, delayed cholecystectomy must be addressed. This is because 5% of patients who have undergone enterolithotomy alone go on to develop biliary symptoms, and the risk of patent fistula reflux and resulting biliary malignancy<sup>[3,4,12]</sup>. In conclusion, GI is a rare condition affecting mainly the older population with a female predominance. If GI occurs in elderly patients with comorbidities, the often vague, intermittent symptoms may delay the diagnosis by days. The advent of CT and MRI has made it easier to diagnose GI. Enterotomy with stone extraction alone remains the most common surgical method because of its low incidence of complications.

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## Photodynamic therapy for high-grade dysplasia of bile duct *via* a choledochoscope

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High-grade dysplasia; Choledochoscope

**Core tip:** Due to significant surgical trauma and a low risk of canceration, surgeons face a dilemma regarding the decision to perform pancreaticoduodenectomy for high-grade dysplasia of the distal bile duct. This report is the first to describe the successful treatment of high-grade dysplasia of the distal bile duct using photodynamic therapy *via* a choledochoscope. This clinical case demonstrated that photodynamic therapy *via* a trans-T-tube choledochoscope may be an effective and promising protocol for carcinoma *in situ* or high-grade dysplasia of the distal common bile duct.

Zhou JJ, Xiong L, Li QL, Gu Y, Wen Y, Deng XF, Miao XY. Photodynamic therapy for high-grade dysplasia of bile duct *via* a choledochoscope. *World J Gastroenterol* 2013; 19(33): 5590-5592 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i33/5590.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i33.5590>

### Abstract

When a distal common bile duct neoplasm is at the stage of carcinoma *in situ* or high-grade dysplasia, it is difficult for the surgeon to decide whether to perform pancreaticoduodenectomy. Here we describe a patient with a progressive dysplastic lesion in the common bile duct, which developed from moderate-high to high-grade dysplasia in approximately 2 mo. The patient refused major surgery. Therefore, endoscopic-assisted photodynamic therapy was performed. The result at follow-up using a trans-T-tube choledochoscope showed that the lesion was completely necrotic. This report is the first to describe the successful treatment of high-grade dysplasia of the distal bile duct using photodynamic therapy *via* a choledochoscope.

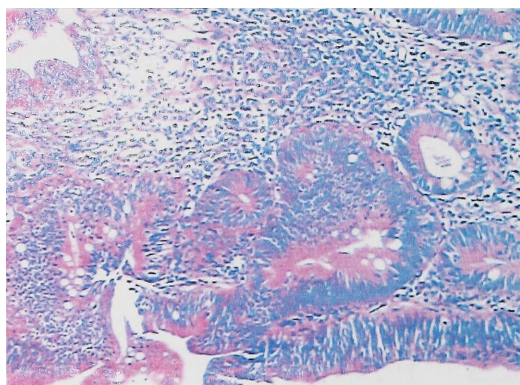
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**Key words:** Photodynamic therapy; Common bile duct;

### INTRODUCTION

Endoscopic treatment is an alternative treatment option with low morbidity. Premalignant lesions such as high-grade dysplasia are being treated increasingly *via* endoscopy. Furthermore, as a promising clinical protocol, endoscopic-assisted photodynamic therapy may result in a more precise effect due to its selective damage to tumor cells. Saleem *et al*<sup>[1]</sup> reported successful photodynamic treatment (PDT) *via* endoscopic retrograde cholangiopancreatography (ERCP) for a villous adenoma with high-grade dysplasia. Here we report a unique case of progressive dysplasia in the distal common bile duct, which developed from moderate-high to high-grade dysplasia in approximately 2 mo, and was successfully treated by PDT *via* a choledochoscope.





**Figure 1** Pathological findings showed moderate-high grade dysplasia of the distal common bile duct lesion (hematoxylin/eosin staining,  $\times 100$ ).

## CASE REPORT

A 47-year-old man with previous common bile duct exploration and T-tube drainage for obstructive jaundice and poor general condition was admitted for follow-up examination. The patient had undergone surgery approximately 2 mo previously, which revealed an easily bleeding lesion of 3 cm in diameter occupying the distal common bile duct, and the pathological findings showed moderate-high grade dysplasia (Figure 1).

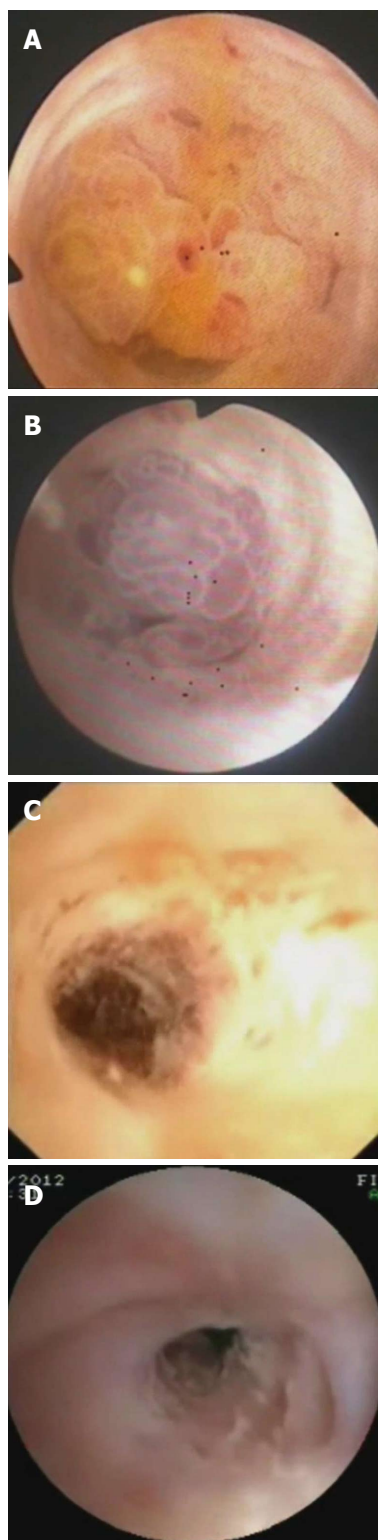
Choledochoscopy showed the same easily bleeding lesion as before (Figure 2A), and a second endoscopic biopsy revealed villous adenoma-like epithelia with moderate-high dysplasia and focal high-grade dysplasia. A trans-T-tube cholangiogram was performed, which demonstrated a dilated biliary tree with an irregular filling defect in the distal common bile duct (Figure 3).

The patient refused major surgery. Therefore, endoscopic-assisted PDT was performed. Informed consent was obtained from the patient prior to PDT. Hematoporphyrin (5 mg/kg) was administered intravenously 48 h before PDT. According to the surgical record and a previous computer tomography (CT) scan, we performed PDT through the T-tube sinus tract using a 4-cm long, cylindrical light diffuser at a power output of 250 mW/cm<sup>2</sup> in one application for a total dose of 70 J/cm<sup>2</sup>. This covered the whole distal common bile duct area. We noticed that the lesion became purple soon after light irradiation (Figure 2B). The patient felt well after the procedure and was discharged the next day.

Two weeks later, trans-T-tube choledochoscopy showed that the lesion was completely necrotic (Figure 2C). The patient remained symptom-free, and at repeat choledochoscopy 3 mo later, no intraductal lesion was seen (Figure 2D). Furthermore, the video showed a functional duodenal papilla. The latest follow-up by choledochoscopy showed that the distal common bile duct was patent and the patient was asymptomatic and in good condition in March 2013.

## DISCUSSION

Tumors of the distal common bile duct present a serious



2012/8/1 Choledochoscopy finding: easily bleeding tumor in the lower extreme of common bile duct.

2012/8/13 Choledochoscopy view: the tumor 10 min after PDT.

2012/8/27 Two weeks after PDT: necrosis of the tumor.

A new bile duct with function observed 3 mo after PDT.

**Figure 2** Images taken *via* the trans-T-tube choledochoscope. A: Before photodynamic treatment (PDT); B: Ten minutes after PDT; C: Two weeks after PDT; D: Three months after PDT.

surgical problem because they necessitate a complicated and extensive resection. Pancreaticoduodenectomy (the Whipple procedure) is one of the most difficult procedures in general surgery, but should only be considered if the tumor is locally contained when identified. However, these small lesions tend to present late in the disease



**Figure 3 Trans-T-tube cholangiogram.** This image shows the dilated common bile duct and intrahepatic bile duct with an irregular filling defect in the distal common bile duct.

course, and many are unresectable<sup>[2]</sup>. Unfortunately, these lesions are not sensitive to chemotherapy or radiotherapy. In addition, studies have shown that intrahepatic bile duct hyperplasia is a potent precursor of cholangiocellular carcinoma. Livers with cholangiocellular carcinoma are closely associated with atypical hyperplasia and carcinoma *in situ*<sup>[3]</sup>. Therefore, when they are at the stage of carcinoma *in situ* or high-grade dysplasia, a new set of management dilemmas occur with regard to major surgery.

With more attention being paid to earlier detection and more careful pathological assessment, these previously overlooked histological features are attracting more interest not only in the diagnosis but also in the treatment.

Endoscopic surgery is an alternative treatment option with low morbidity in many cases<sup>[4]</sup>. Endoscopic-assisted photodynamic therapy is a promising method for lesions in the biliary tree due to the thickness of the bile duct wall being within the penetration depth of the light. PDT can be regarded as a standard palliative therapy for

unresectable cholangiocarcinoma as reported in previous studies<sup>[5]</sup>. In our case, it demonstrated high selectivity for the dysplastic lesion, and did not damage the duodenal papilla. This indicates the potential for endoscope-assisted PDT to provide precise ablation of intraductal lesions with progressive dysplasia. This protocol may serve as a standard modality for patients who are not candidates for surgery, and the T-tube sinus provides a good tract for both treatment and follow-up.

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## GENERAL INFORMATION

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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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/index.htm>

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