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# A systematic review of the efficacy and safety of herbal medicines used in the treatment of obesity

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caffeine and Bofutsushosan. In conclusion, compounds containing ephedra, CQ, ginseng, bitter melon, and zingiber were found to be effective in the management of obesity. Attention to these natural compounds would open a new approach for novel therapeutic and more effective agents.

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**Key words:** Animal; Herbal medicine; Human; Obesity

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## Abstract

This review focuses on the efficacy and safety of effective herbal medicines in the management of obesity in humans and animals. PubMed, Scopus, Google Scholar, Web of Science, and IranMedex databases were searched up to December 30, 2008. The search terms were "obesity" and ("herbal medicine" or "plant", "plant medicinal" or "medicine traditional") without narrowing or limiting search elements. All of the human and animal studies on the effects of herbs with the key outcome of change in anthropometric measures such as body weight and waist-hip circumference, body fat, amount of food intake, and appetite were included. *In vitro* studies, reviews, and letters to editors were excluded. Of the publications identified in the initial database, 915 results were identified and reviewed, and a total of 77 studies were included (19 human and 58 animal studies). Studies with *Cissus quadrangularis* (CQ), *Sambucus nigra*, *Asparagus officinalis*, *Garcinia atroviridis*, ephedra and caffeine, Slimax (extract of several plants including *Zingiber officinale* and Bofutsushosan) showed a significant decrease in body weight. In 41 animal studies, significant weight loss or inhibition of weight gain was found. No significant adverse effects or mortality were observed except in studies with supplements containing ephedra,

## INTRODUCTION

The prevalence of obesity is increasing worldwide<sup>[1]</sup> resulting in an association with major health problems such as type 2 diabetes, ischemic heart disease, stroke, and cancer. It is necessary to treat obese individuals by both lifestyle interventions and/or pharmacological therapy. Pharmacologic treatment and surgical interventions used in some circumstances are not always appropriate<sup>[2]</sup>. Unfortunately, drug treatment of obesity despite short-term benefits, is often associated with rebound weight gain after the cessation of drug use, side effects from the medication, and the potential for drug abuse<sup>[3]</sup>. Pharmacologic options include sibutramine, orlistat, phentermine, diethylpropion, and fluoxetine or bupropion. Phentermine and diethylpropion have potential for abuse and are only approved for short-term use. Approved medications for long term use in the treatment of obesity are sibutramine and orlistat, however, these agents should be used with caution in patients with a history of cardiovascular disorders<sup>[4]</sup>. The general public uses many other methods for weight

loss including herbs, vitamins, nutritional supplements, and meal replacement preparations. Rigorous scientific studies have not been carried out on these products, and in many cases safety and efficacy take a back seat to marketing.

Complementary and alternative therapies have long been used in the Eastern world but recently these therapies are being used increasingly worldwide<sup>[5]</sup>. When conventional medicine fails to treat chronic diseases and conditions such as obesity efficaciously and without adverse events, many people seek unconventional therapies including herbal medicine<sup>[6]</sup>. Although the number of randomized trials on complementary therapies has doubled every 5 years and the Cochrane library included 100 systematic reviews of unconventional interventions<sup>[7]</sup>, none of these studies specifically mentioned herbal therapy in obesity.

This review aimed to evaluate the current science on the efficacy and safety of herbal medicines in the management of obesity.

## DATA SOURCES AND STUDY SELECTIONS

PubMed, Scopus, Google Scholar, Web of Science, and IranMedex databases were searched up to December 30, 2008 for all human and animal studies investigating the effects (both harmful and beneficial) of treating obesity with herbal medicines. The search terms were "obesity" and ("herbal medicine" or "plant", "plant medicinal" or "medicine traditional") without narrowing or limiting search elements. Only publications with available abstracts were reviewed. The main outcome measures sought at the end of treatments as anti-obesity effects, were body weight, body fat including fat mass/fat weight or fat percentage/visceral adipose tissue weight, triceps skin fold thickness, waist or hip circumference, and appetite or amount of food intake.

Herbal medicines are defined in this review as raw or refined products derived from plants or parts of plants (e.g. leaves, stems, buds, flowers, roots, or tubers) used for the treatment of diseases. The synonyms of herbal medicines are herbal remedies, herbal medications, herbal products, herbal preparations, medicinal herbs, and phytopharmaceuticals, etc.

All of the abstracts from human and animal studies with the main outcome of change in anthropometric measures such as body weight and waist-hip circumference, body fat (weight or mass of visceral adipose tissue, fat mass or percent), amount of food intake, and appetite in participants were included. Even studies on other relevant diseases such as diabetes were also reviewed and included if the appropriate outcomes were shown. *In vitro* studies, review articles, and letters to the editor were excluded. Unpublished data such as theses were also excluded. Two reviewers independently examined the title, abstract and references of each article meeting the inclusion criteria and eliminated duplications and those showing exclusion criteria.

## FINDINGS

Of the publications identified from the initial database search, 915 results were identified and reviewed for inclusion or exclusion. A total of 77 studies were included (19 human and 58 animal studies). Human studies included 17 randomized clinical trials (RCTs) and two before-after clinical trials<sup>[8-26]</sup>. RCTs reported random allocation of humans to herbal medicines *vs* (placebo/another plant/combination of plants) with or without specific dietary and exercise programs outlined in Tables 1 and 2 as weight loss programs. Human subjects were healthy overweight, obese or with impaired glucose tolerance test volunteers. Animal studies included healthy, genetically or experimentally obese or diabetic mice, rats and other rodents. The route of administration of herbs in almost all studies was oral intake with the exception of some animal studies as indicated in Table 2.

## HUMAN STUDIES

### **Change in human body weight**

All studies showed loss of body weight except one<sup>[21]</sup> which seemed to have problems with the study design, and one other study<sup>[10]</sup> which showed a significant decrease only in body fat. Studies with *Cissus quadrangularis* (CQ)<sup>[26]</sup> or combined with *Irrwingia gabonensis* (IG)<sup>[15]</sup>, a combination of *Sambucus nigra* and *Asparagus officinalis*<sup>[16]</sup>, calcium hydroxycitrate in *Garcinia atroviridis*<sup>[18]</sup>, supplements containing ephedra and caffeine<sup>[9,13,20]</sup>, and Slimax as an extract of several plants including *Zingiber officinale*<sup>[8]</sup> and Bofutsushosan<sup>[14]</sup> showed significant decreases in body weight.

### **Body fat**

A significant decrease in body fat was shown with CQ<sup>[26]</sup>, supplements containing ephedra and caffeine<sup>[9,13]</sup>, a natural compound containing capsicum and some lipotropic nutrients<sup>[10]</sup>, Bofutsushosan<sup>[14]</sup>, and calcium hydroxycitrate in *Garcinia atroviridis*<sup>[18]</sup>. These phytopharmaceuticals showed a significant decrease in triceps skin fold thickness indicating significant loss of fat.

### **Waist and hip circumference**

Efficient decreases in both waist and hip circumferences in trials with a supplement containing ephedra and caffeine<sup>[9]</sup> and Slimax (extract of several plants including *Zingiber officinale*<sup>[8]</sup>) were shown whereas *Caralluma fimbriata*<sup>[19]</sup> and CQ with or without IG<sup>[15]</sup> significantly decreased waist size.

### **Food intake**

Decreases in appetite or amount of food or energy intake with a supplement containing ephedra and caffeine<sup>[20]</sup> and *Caralluma fimbriata*<sup>[19]</sup> were shown (not significant) but hydroxycitric acid (HCA-SX) with or without *Gymnema sylvestre*<sup>[23]</sup> decreased the amount of food intake efficiently. A natural compound containing capsicum and other lipotropic nutrients<sup>[10]</sup> did not significantly change energy intake.

**Table 1** Human studies considering the anti-obesity effects of herbal medicines

Authors	Target	Herbs (scientific name)	Study	Dose/duration	Groups	Main outcome	Other relevant effects & complications
Ignjatovic et al <sup>[8]</sup> 2000	Healthy volunteers	Slimax: extract of several plants: <i>Hordeum vulgare</i> , <i>Polygonatum multiflorum</i> , <i>Dimocarpus longan</i> , <i>Ligusticum sinense</i> , <i>Lilium brownie</i> , and <i>Zingiber officinale</i>	RCT	6 wk	C: Placebo I: Compound	Sig. decrease in body wt. & waist & hip Cir. & BMI	Modification of lipid metabolism with sig. effect on the accumulation & the release of lipid from adipose tissue
Boozer et al <sup>[9]</sup> 2001	Over wt. (n = 35)	An herbal supplement: ( <i>Ma Huang</i> & <i>Guarana</i> )	RCT (double-blind)	72 mg (ephedra) 240 mg (caffeine)/8 wk	C: Placebo (n = 24) I: Compound (n = 24)	Sig. decrease in body wt. & total body fat & sig. greater reduction in hip & waist Cir.	Greater reduction in serum TG, potentially treatment-related dropouts (23%) in the active group and none in the placebo group. Dry mouth, insomnia & headache were reported
Hoeger et al <sup>[10]</sup> 1998	Healthy	A natural dietary compound of chromium picolinate, inulin, capsicum, L-phenylalanine, and other lipotropic nutrients	RCT (double-blind)	4 wk	C: wt. loss program (n = 67) I: wt. loss program + compound (n = 56)	Sig. decrease in body fat percent, fat mass & FFM, but no sig. difference in body wt. BMI and energy intake	
Ziauddin et al <sup>[11]</sup> 2004	Hyperlipidemic (n = 30)	<i>Terminalia arjuna Roxb</i>	Before-after CT			Sig. improvement in obesity. Reduction in body wt. in some cases	Sig. decrease in serum total lipid levels. Sig. relief of palpitation, dyspnea, chest & joint pain. Reduction in BP in some cases
Abidov et al <sup>[12]</sup> 2006	Obese non-diabetic women (n = 32)	A compound of <i>Aralia mandshurica</i> (A) and <i>Engelhardtia chrysolepis</i> (E) extracts named ARALOX	RCT	450 mg (A) & 450 mg (E)/d	C: Diet + placebo I: Diet + compound	Decrease in total body wt. & fat wt.	Reduction in perilipin content in adipocytes and plasma TG. Stimulate activity of hormone sensitive lipase
Greenway et al <sup>[13]</sup> 2004	Human (obese & over wt.) healthy	Herbal supplement containing caffeine and ephedra	RCT (double-blind)	210 mg (e) & 72 mg (c)/12 wk	C: Placebo I: Compound	Sig. decrease in body wt. & the percentage of fat	No differences in lipid levels, or BP were shown. No serious adverse effect
Hioki et al <sup>[14]</sup> 2004	Obese women with IGT (n = 80)	Bofu-tsusho-san containing ( <i>Ephedrae Herba</i> , <i>Glycyrrhizae Radix</i> , <i>Forsythiae Fructus</i> , <i>Schizonepetae Spica</i> &...)	RCT (double-blind)	Equivalent of (24 mg/ephedrine & 280 mg caffeine/24 wk)	C: wt. loss program I: wt. loss program + compound	Compared to baseline the I group lost significantly more body wt. & abdominal visceral fat & the placebo group lost sig. body wt. & had no sig. change in abdominal visceral fat	No decrease in RMR. Sig. improvement in insulin resistance compared to week 0. Loose bowel movements resulted in three withdrawals
Oben et al <sup>[15]</sup> 2008	Human (obese & over wt.)	A combination of <i>Cissus quadrangularis</i> (CQ) & <i>Irvingia gabonensis</i> (IG)	RCT (double-blind)	300 mg (CQ) & 500 mg (IG) per day/10 wk	C: Placebo I: CQ CQ + IG	Sig. decrease in body wt. & body fat percent & waist size in both I groups but the combination group (CQ + IG) resulted in larger reductions	Sig. decrease in Chol & LDL of plasma and fasting blood glucose levels
Chrubasik et al <sup>[16]</sup> 2008	Healthy (n = 80)	A combination of <i>Sambucus nigra</i> (S) and <i>Asparagus officinalis</i> (A)	Before-after CT	(S): 1 mg anthocyanin, 370 mg flavonol, 150 mg hydroxycinnamate (A): 19 mg saponin per day	-	Sig. decrease in mean of the wt.	Sig. improvement of BP, physical and emotional well-being and quality of life

Udani <i>et al</i> <sup>[17]</sup> 2007	Healthy ( <i>n</i> = 25)	Proprietary fractionated white bean extract	RCT (double-blind)	2000 mg/14 wk	C: Placebo + wt. loss program I: Extract + wt. loss program	In both groups, decrease in body wt. & waist size from baseline was sig. but no sig value between groups	There were no adverse effect
Roongpisuthipong <i>et al</i> <sup>[18]</sup> 2007	Obese women	Calcium hydroxycitrate in <i>Garcinia atroviridis</i>	RCT	2 mo	C: Diet I: Diet + extract	Sig. decrease in body wt. & greater reduction in BMI. Sig. decrease in the triceps skin fold thickness	
Kuriyan <i>et al</i> <sup>[19]</sup> 2007	Over wt. ( <i>n</i> = 50)	Caralluma fimbriata	RCT	1 g/60 d	C: wt. loss program I: wt. loss program + extract	Sig. decrease in waist Cir. & hunger levels. Greater decrease in body wt., BMI, hip Cir., body fat & energy intake but not sig.	
Hackman <i>et al</i> <sup>[20]</sup> 2007	Obese & over wt. women ( <i>n</i> = 41)	Multinutrient supplement containing ephedra (e) and caffeine (c)	RCT (double-blind)	40 mg (e) and 100 mg (c)/9 mo	C: Control supplement I: Multinutrient supplement	Sig. decrease in body wt. decrease in appetite	Sig. decline in serum chol, TG, glucose, fasting insulin & leptin levels & minor adverse effects like dry mouth, insomnia, nervousness and palpitation were reported
Garrison <i>et al</i> <sup>[21]</sup> 2006	Over wt. women	Proprietary extracts of Magnolia officinalis and phellodendron amurense	RCT	750 mg/6 wk	C: Placebo I: Extract	No sig. wt. gain for the I group but sig. wt. gain in C. groups	The I groups tended to have lower levels of cortisol in the evening
Coffey <i>et al</i> <sup>[22]</sup> 2004	Human (over wt. & obese) ( <i>n</i> = 102)	Product containing ephedrine, caffeine & other ingredients.	RCT (double-blind)	12 wk	C: Placebo I: Compound	Additional wt. loss (1/5 kg) & greater reduction in BMI & waist Cir. No difference in body fat & fat mass percent was shown	No difference in pulse, diastolic & systolic BP & adverse events
Preuss <i>et al</i> <sup>[23]</sup> 2004	Obese ( <i>n</i> = 60)	Hydroxycitric acid (HCA-SX) and a combination of HCA-SX and niacin-bound chromium (NBC) and Gymnema sylvestre extract (GSE)	RCT (double-blind)	HCA-SX: 4667 mg GSE: 400 mg NBC: 4 mg/8 wk	C: Placebo I1 = HCA-SX I2 = GSE + NBC + HCA-SX All groups had wt. loss program	5%-6% decrease in body wt. & BMI & sig. decrease in food intake in both I groups	Sig. decrease in serum lipids & leptin & increase in HDL & excretion of urinary fat metabolites in both I groups. There were mild adverse effects but not significant between groups
Udani <i>et al</i> <sup>[24]</sup> 2004	Obese ( <i>n</i> = 24)	A proprietary fractionated white bean ( <i>Phaseolus vulgaris</i> )	RCT (double-blind)	3000 mg/8 wk	C: Placebo I: Extract	Decrease of body wt. with 129% difference	Reduction of TG three times greater than C. group. No adverse effect was shown
Bhatt <i>et al</i> <sup>[25]</sup> 1995	Healthy ( <i>n</i> = 58)	Guggulu (Medohar)	RCT	1/5, 3 g/30 d	C: wt. loss program I: wt. loss program + extract	Higher mean wt. reduction in I group. In I group, all patients > 90 kg lost wt. but 3 in C group did not lose wt.	
Oben <i>et al</i> <sup>[26]</sup> 2007	Over wt. & obese	<i>Cissus quadrangularis</i>	RCT (double-blind)	300, 1028 mg	C: Placebo I: Two extract formulation: CQR-300, CORE	Sig. decrease in body wt & body fat	Sig. decrease in serum lipids and glucose. Sig. increase in HDL-C plasma 5-HT and creatinine levels

Cir: Circumference; BP: Blood pressure; BMI: Body mass index; sig.: Significant; C: Control; I: Intervention; RCT: Randomized control trial; CT: Clinical trial; TG: Triglyceride; HDL: High density lipoprotein; LDL: Low density lipoprotein; Chol: Cholesterol; IGT: Impaired glucose tolerance.

### Other effects

Anti-hyperlipidemic, antihyperglycemic, and other relevant anti-obesity effects of medicinal plants in human studies are summarized in Table 1.

### Adverse effects

No significant adverse effects compared to controls were mentioned and no mortality was reported, except in studies with supplements containing ephedra and

**Table 2 Animal studies on the anti-obesity effects of herbal medicines**

<b>Authors</b>	<b>Target</b>	<b>Herbs (scientific name)</b>	<b>Dose/duration</b>	<b>Groups</b>	<b>Main outcome</b>	<b>Other relevant effects &amp; complications</b>
Wang et al <sup>[27]</sup> 2000	Rat (obese)	Haidonghua powder: Laminaria japonica Aresch & Benincasa hispida (Thunb.) Cogn. <i>etc</i>	(2.5 g/kg)	-	Sig. decrease in Lee's index & size of fat cells	Did not influence the function of thyroid gland & metabolism of water & salt
Jeon et al <sup>[28]</sup> 2003	Mouse	Rhus vemiciflua Stokes	8 wk	C: HFD I: HFD + extract	Sig. suppression of body wt. gain and lower wt. of subcutaneous adipose tissue	Lowered plasma TG
Alarcon-Aguilar et al <sup>[29]</sup> 2007	Mouse	Hibiscus sabdariffa	120 mg/kg 60 d	C: Healthy & obese (by MSG) + placebo I: Same groups + extract	Sig. decrease in body wt. gain in obese mice & increased liquid intake in both groups	No sig. change in TG & Chol levels. Increase in ALT levels was shown but was not sig.
Urías-Silvas et al <sup>[30]</sup> 2008	Mouse	Fructans extracted from Agave tequilana (TEQ) and Dasylirion spp (DAS)	10% supplement	C: STD I: STD + Raftilose/DAS/TEQ	Sig. decrease in body wt. gain & food intake. The (TEQ) group had the lowest value	Lower serum glucose & Chol level but Sig. decrease in TG levels was shown in Raftilose group. Higher concentration of GLP-1 & its precursor & proglucagon mRNA in I groups
Park et al <sup>[31]</sup> 2007	Rat	Platycodon grandiflorum	150 mg/kg 7 wk	C: NLD/HFD I: Same groups + extract	Sig. decrease in body wt & subcutaneous adipose tissue wt. & adipocytes size in I group	Sig. decrease in plasma TG & Chol concentrations, up-regulation of FABP mRNA expression induced by HFD
Jongwon et al <sup>[32]</sup> 2005	Rat (obese by HFD)	Allium victorialis var. platyphyllum leaves	100 mg/kg 2 wk	-	Considerable reduction of retroperitoneal, epididymal and total abdominal fat pad wt.	Sig. decrease in hyperlipidemia and increased lipid content in feces
Kobayashi et al <sup>[33]</sup> 2001	Rat	Evodiamine an alkaloid of a fruit: Evodia rutaecarpa	0/02%, 0/03% of the diet 12 wk	C: Control I: Extract	Sig. decrease in perirenal fat wt. & decrease of epididymal fat mass	Sig. decrease of lipid in liver & serum FFAs. Sig. increase of lipolytic activity in perirenal fat tissue & specific GDP binding in brown adipose tissue mitochondria as the biological index of heat production
Jin et al <sup>[34]</sup> 1994	Rat	Jiang-zhi jian-fei yao: the refined Rhubarb	Injected intragastrically		No sig. increase in body wt. but reduction of food intake. Decreased size of abdominal adipose cells	Prolongation of stomach evacuation time and acceleration of intestinal movements
Kim et al <sup>[35]</sup> 2008	Rat	Juniperus chinensis	1% supplement /79 d	C: NLD/HFD I: HFD + extract	Sig. decrease in body wt gain & visceral fat pad wt.	Sig. decrease in blood lipid, leptin & insulin levels. Sig. reversal of the down-regulation of genes implicated in adipogenesis & increased gene expressions & phosphorylations related to FABO
Shih et al <sup>[36]</sup> 2008	Mouse (obese by HFD)	Momordica charantia (bitter melon)	4 wk	C: Control I: Rosiglitazone/extract	Sig. decrease in epididymal white adipose tissue wt. & visceral fat wt.	Sig. improvement in blood glucose, leptin, and FFA. Influenced PPAR $\alpha$ /PPAR $\gamma$ expression
Pang et al <sup>[37]</sup> 2008	Rat (obese by HFD)	Ilex paraguariensis			Sig. decrease in body wt. of visceral fat-pad wt.	Sig. decrease in blood and hepatic lipid, glucose, insulin and leptin levels. Reversed the down-regulation of genes implicated adipogenesis, thermogenesis & enhanced expression of uncoupling proteins in adipose tissue
Bruno et al <sup>[38]</sup> 2008	Mouse	Green tea	0%, 1%, 2% (wt:wt.)/6 wk	C: Obese/lean I: Same groups + extract	Sig. decrease in body wt. of both I groups	In obese I group, sig. decrease in hepatic steatosis was observed dose dependently. Liver enzymes decreased. 30%-41% and 22%-33% lower serum ALT and AST activities were shown, respectively

Lee <i>et al</i> <sup>[39]</sup> 2008	Mouse	A combination of <i>Morus alba</i> , <i>Melissa officinalis</i> and <i>Artemisia capillaries</i>	12 wk	-	Sig. decrease in body wt. gain & adipose mass	Decreased serum levels of TG, Chol & inhibited hepatic lipid accumulation, and increased hepatic mRNA levels of enzymes responsible for FABO
Choi <i>et al</i> <sup>[40]</sup> 2008	Mouse (obese by HFD)	Cucurbita moschata	500 mg/kg 8 wk	-	Sig. suppression of body wt. & fat storage increase but amount of food intake was not affected	
Huang <i>et al</i> 2008 <sup>[41]</sup>	Rat	<i>Momordira charantia</i> L. (Bitter melon)	5%	C: HFD I1: HFD + plant I2: HFD + thiazolidinedione	Sig. decrease in the number of large adipocytes in both I groups. Sig. decrease in adipose tissue mass in I group compared to L group	Sig. decrease in enzymes of adipose tissue implicating reduction of insulin resistance in I group as compared to C group
Lemaire <i>et al</i> <sup>[42]</sup> 2007	Rat (obese)	<i>Cyperus rotundus</i> <td>45, 220 mg/kg 60 d</td> <td>-</td> <td>Sig. decrease in wt. gain without affecting food consumption</td> <td></td>	45, 220 mg/kg 60 d	-	Sig. decrease in wt. gain without affecting food consumption	
Lei <i>et al</i> <sup>[43]</sup> 2007	Mouse	Pomegranate leaf	400/800 mg per kilogram 5 wk	C: HFD/NLD I: Same groups + extract	Sig. decrease in body wt. & energy intake and adipose pad wt. percents in I. group. Sig. decrease in appetite of obese mice on NLD was shown	Sig. decrease in serum TG, Chol, glucose levels & Chol/HDL ratio, inhibition of intestinal fat absorption
Aoki <i>et al</i> <sup>[44]</sup> 2007	Mouse (obese by HFD)	Licorice flavonoids oil (LFO)	0/5%, 1%, 2% 8 wk	C: Placebo I: Extract	Sig. decrease of abdominal white adipose tissue & body wt. gain with 1% & 2% LFO groups, decrease of adipocyte size	Improvement of fatty degeneration of hepatocytes and changes in genes implicating regulation of lipid metabolism with 2% concentration
Oluyemi <i>et al</i> <sup>[45]</sup> 2007	Rat	<i>Garcinia cambogia</i> seed (bitter cola)	200, 400 mg/kg 5 wk	C: Placebo I: Extract	Sig. decrease in body wt.	Sig. decrease in TG pool of adipose tissue & liver but sig. increase of HDL & decreased LDL
Han <i>et al</i> <sup>[46]</sup> 2006	Mouse (obese by HFD)	<i>Kochia scoparia</i>	1%, 3%/3 d	-	Prevented the increases in body & parametrial adipose tissue wt.	Sig. increase the fecal content & fecal TG levels in day 3
Goyal <i>et al</i> <sup>[47]</sup> 2006	Mouse (obese gold thioglucose)	<i>Zingiber officinale</i>	250 mg/kg 8 wk	C: Placebo I: Extract	Sig. decrease in body wt.	Sig. decrease in serum Chol, TG, glucose, and insulin
Kishino <i>et al</i> <sup>[48]</sup> 2006	Rat and mouse	<i>Salacia reticulata</i>	0/5% 8 wk in mice 0/2% 35 d in rats	C: HFD I: HFD + plant	Sig. decrease in the body wt. and visceral fat mass increase	Sig. decrease in plasma TG, 4 h after ingestion; Sig. decrease in energy efficiency, plasma leptin and adiponectine levels
Jayaprakasam <i>et al</i> <sup>[49]</sup> 2006	Mouse	Cornelian cherry (cornus mas) (Purified anthocyanins (A) & ursolic acid (u))	1 g/kg (A), 500 mg/kg (u) 8 wk	C: HFD I: HFD + A/A + u	24% decrease in wt. gain in (A) group	Elevated insulin levels; Sig. decrease of liver TG in A + u group
Moreno <i>et al</i> <sup>[50]</sup> 2006	Rat	<i>Arachis hypogaea</i> nutshell	1% (wt:wt) /12 wk	C: HFD I: HFD + extract	Sig. decrease in body wt. gain and liver size	Increased fecal lipid excretion. Reduced TG content of liver and serum glucose and insulin
Galisteo <i>et al</i> <sup>[51]</sup> 2005	Rat (obese)	<i>Plantago Ovata</i>	3/5% 25 wk	C: STD I: STD + extract	Sig. decrease in body wt. gain	Sig. improvement of lipid profile, FFA & insulin & TNF- $\alpha$ & hypoaldinectinemia
Zhao <i>et al</i> <sup>[52]</sup> 2005	Mouse (obese by hyperalimentation)	Phillyrin (Fructose forsythia)			Sig. decrease in wet wt. of fat & fat index & diameter of fat cells & lee index	Decrease in jejunum microvillus area, and serum levels of TG & Chol
Chen <i>et al</i> <sup>[53]</sup> 2005	Rat	Bitter melon ( <i>Momordica charantia</i> )	0/75% or 7/5 g per kilogram 7 wk	C: LFD/HFD I: LFD/HFD + extract	Lower energy efficiency and visceral fat mass after 4 wk in I group	Reduced plasma glucose and hepatic TG but higher serum FFA after 4 wk; Higher plasma catecholamine after 7 wk in I group; Sig. decrease in hepatic TG & steatosis and sig. increase of serum epinephrine & FFA in HFD group of I

Han et al <sup>[54]</sup> 2005	Rat	Coleus forskohlii	50 g/kg	C: Sham operated/ ovariectomized + control diet I: Same groups + extract	Reduced body wt.& food intake & fat accumulation
Han et al <sup>[55]</sup> 2005	Mouse	Chikusetsu saponins isolated from Panax japonicus rhizomes	1%, 3%/9 wk	C: HFD I: HFD + extract	Prevented body wt. gain & increase of parametrial adipose tissue wt.
Han et al <sup>[56]</sup> 2005	Mouse	Zingiber officinale Roscoe	1%, 3%/8 wk	C: HFD I: HFD + plant	Sig. decrease in body wt. gain at 2-8 wk with 3% & in final parametrial adipose tissue wt. with 1% concentration
Cha et al <sup>[57]</sup> 2004	Mouse	Acanthopanax senticosus	0/5 g per kilogram 12 wk	C: NLD/HFD I: NLD/HFD + extract	HFD group of I had lower wt. gain but no difference in food consumption was shown
Kim et al <sup>[58]</sup> 2005	Rat	Crude saponin of Korean red ginseng	200 mg/kg 3 wk, ip	C: NLD/HFD I: NLD/HFD + extract	Reduced body wt., food intake & fat content in HFD group of I similar to those fed with NLD
Yun et al <sup>[59]</sup> 2004	Mouse	Wild Ginseng	250, 500 mg/kg	C: HFD I: HFD + extract	Sig. inhibition of body wt. gain dose dependently. Decrease of white & brown adipocytes diameters
Junbao et al <sup>[60]</sup> 2004	Rat (obese)	Semen cassiae	6%	-	Sig. decrease in body wt. & lee index
Kim et al <sup>[61]</sup> 2004	Rat	Adlay seed (CoixLachrymajobi var. mayuen)	50 mg/100 g of body wt.	C1: NLD C2: HFD + saline (sham group) I: HFD + plant	Sig. decrease in body wt. & food intake & epididymal and peritoneal fat & white adipose tissue size as compared to sham group
Kwon et al <sup>[62]</sup> 2003	Rodent	Dioscorea nipponica Makino	5%/8 wk	C: HFD I: HFD + plant	Sig. decrease in body wt. & adipose gain
Lu et al <sup>[63]</sup> 1999	Rat (obese by hyperalimentation)	Inspissation tea (Guangdong kudingcha)		C: Control II: Extract I2: Fenfluramine	Stronger modulation on lymphocytes hypertrophy and quantity was shown in II group
Yoshikawa et al <sup>[64]</sup> 2002	Rat (obese)	Salacia reticulata	125 mg/kg 27 d		Suppression of body wt. and periuterine fat storage increase in female rats but no effect on male rats
Xie et al <sup>[65]</sup> 2002	Mouse	Ginseng berry	150 mg/kg 12 d, ip	C: Diabetic/lean diabetic + placebo I: Same groups + extract	Sig. decrease in body wt. as compared to day 0 in diabetic group of I. wt. loss in lean mice was shown
Yamamoto et al <sup>[66]</sup> 2000	Rat	CT-II, an extract from Nomame Herba	8 wk, 12 wk, 6 mo	C: Lean/obese + HFD I: Same groups + HFD + plant	Sig. inhibition of body wt. gain dose dependently without affecting food intake in lean rats after 12 wk. Sig. decrease in body wt. gain in obese mice after 24 wk
Han et al <sup>[67]</sup> 1999	Mouse	Oolong tea	10 wk	C: HFD I: HFD + extract	No sig. difference in food intake but prevented obesity & liver induced by a HFD
					Enhancement of noradrenalin induced lipolysis & inhibition of pancreatic lipase activity

Pusztai <i>et al</i> <sup>[68]</sup> 1998	Rat	Kidney bean (Phaseolus vulgaris)	130, 150, 280 g/kg 10-70 d	C: Lean/obese + LFD/HFD I: Same groups + extract	The growth was retarded dose- dependently lower body fat	Sig. decrease of body protein in lean I group. Sig. decrease in plasma insulin levels in obese I group. Sig. pancreatic growth after long term feeding in all I groups
Nagasawa <i>et al</i> <sup>[69]</sup> 1991	Mouse (obese)	Tree peony root (Paenia suffruticosa)	0/5% 30 wk	C: Control I: Extract	Sig. decrease in food intake and Lee index	Improvement in glucose tolerance. No sig. difference in serum FFA levels
Wang <i>et al</i> <sup>[70]</sup> 2008	Mouse	Parasitic loranthus from Loranthaceae or Viscaceae	20 d	-	Sig. decrease in body wt. & food intake	High inhibitory ability on FAS- Loran thaea was nearly 400 fold stronger than that from the viscaceae
Hu <i>et al</i> <sup>[71]</sup> 2008	Mouse (female)	Escins extracted from Aesculus turbinata Blume (Hippocastanaceae)	2%/11 wk	I: HFD C: HFD + extract	Suppressed the increase in body & parametrial adipose tissue wt.	Suppressed the increase of liver TG content; increased TG in feces after fat ingestion
Ohkoshi <i>et al</i> <sup>[72]</sup> 2007	Mouse	Nelumbo nucifera Gaertn leaves (Nymphaeaceae)	50%	C: STD/HFD I: Same groups + extract	Sig. suppression of body wt. gain	Exhibition of lipolytic activity especially in visceral adipose tissue; $\beta$ adrenergic receptor pathway was partly involved
Kang <i>et al</i> <sup>[73]</sup> 2004	Rat	PM-F2-OB composed of <i>Lycii Fructus</i> , <i>Rehmanniae Radix</i> , <i>Coicis Semen</i> , <i>Carthami Flos</i> , <i>Hoelen</i> , <i>Angelicae Radix</i> , <i>Nelumbinis Semen</i> , <i>Radix Dioscorea</i> and <i>Aurantii Fructus</i>	6 wk	C: STD/HFD I: Same groups + plant	No sig. difference in wt. change if STD was used but in HFD group of I resulted in sig. decrease in body wt. gain but showed no sig. difference in amount of food intake	Sig. decrease in serum Chol/ LDL and total lipids; reduction of kidney fat wt./FFA/PL & TG to levels equal or below the normal diet
Mary <i>et al</i> <sup>[74]</sup> 2003	Rabbit	Caps HT2 A herbal formulation	5 mg/kg (iv) 30 d 100/200/300/ 400/mg per kilogram orally	-	Sig. decrease in body wt.	Sig. increase in HDL after oral administration and decrease in atherogenic index in oral administration; Sig. increase of the release of LPL enzyme and sig. hypolipidemic effect in IV groups
Wu <i>et al</i> <sup>[75]</sup> 2005	Rat (diabetic by STZ )	Astragalus polysaccharide (APS) a component of Astragalus membranaceus roots	400 mg/kg (APS) 5 wk	-	Sig. decrease in body wt.	Sig. decrease in plasma glucose; improved insulin sensitivity
Xie <i>et al</i> <sup>[76]</sup> 2005	Mouse (Genetically obese)	Total, Ginsenosides in Chinese ginseng (TG CG), from leaves and the stem of Panax ginseng	100, 200 mg/kg (ip) 12 wk & 150, 300 mg/kg (oral)/12 wk	C: Placebo I: Extract	Sig. decrease in body wt.	Sig. decrease in FBG in 200 mg/kg dose after injection Sig. decrease in FBG in 300 mg/kg dose
Palit <i>et al</i> <sup>[77]</sup> 1999	Mouse	Galega officinalis	10% (w/w) of the diet 28 d	C: Diabetic/NL I: Same groups + plant	Sig. decrease in body wt. in both I groups, sig. wt. loss in normal mice independent of a reduction in food intake but in diabetic mice wt. loss was with reduced food intake	Striking loss of body fat in both groups; Sig. decrease in serum glucose in both groups but Sig. decrease in serum insulin in diabetic mice
Oi <i>et al</i> <sup>[78]</sup> 1999	Rat	Garlic	8 g/kg of diet 28 d	C: HFD I: HFD + extract	Sig. decrease in body wt. & perirenal adipose tissue wt. & epididymal fat pad	Sig. decrease in plasma TG levels; sig. decrease in mitochondrial protein and (UCP) in brown adipose tissue, and in urinary noradrenaline and adrenaline excretion
Yoshida <i>et al</i> <sup>[79]</sup> 1995	Mouse (obese and lean)	Bofu-tsusho-san	1/4%, 4/7% of wt. of food 8 wk	-	Sig. decrease in body wt. & retroperitoneal white adipose tissue wt. and no change in food intake	Sig. increase in GDP binding dose dependently
He <i>et al</i> <sup>[80]</sup> 2008	Rat (obese by STZ & HFD)	Yi-Qi-Yang-Yin-Ye	2, 4, 8 g/kg 4 wk	-	Body wt. decreased	Decrease in TG/Chol/ LDL/FFA/FBG/insulin; improvement of glucose tolerance

Jeong et al <sup>[81]</sup> 2008	Rat (fatty)	Gyeongshang angjeehwan: Liriope platyphylla F.T./Wang & T. Tang (Liliaceae), Platycodongrandiflorum A. DC. (Campanulaceae). Schisandrachinensis K. Koch (Magnoliaceae). Ephedra sinica Stapf (Ephedraceae)	8 wk	C: Placebo I: Compound	Sig. decrease in food intake & body wt. gain & abdominal fat	Sig. decrease in plasma leptin levels; decrease in circulating TG and inhibition of lipid accumulation in liver; increase of mRNA of genes responsible for FABO
Park et al <sup>[82]</sup> 2005	Rat (obese by diet)	Platycodon grandiflorum	150 mg/kg 7 wk	C: Convert to NLD/HFD I: Same groups + extract	Sig. decrease in wt. of body & adipose tissues in rats converted to NLD as compared to those remained on HFD	Sig. decrease in fat cell number & size in both I groups as compared to their state before intervention; decrease of FABP expression in HFD group of I
Akagiri et al <sup>[83]</sup> 2008	Mouse (obese by HFD)	Bofutsushosan (BOF)	1%/4 wk	C: Placebo I: Compound	The wt. of WAT and increase in size of adipocytes inhibited	Expression of UCP1 mRNA in WAT was found but not sig.
Kim et al <sup>[84]</sup> 2005	Mouse (diabetic)	Pine extract (bark and needle)	21 d	C: Control I: Extract	Sig. decrease in body wt.	Effectively suppressed the increase of postprandial blood glucose level by delaying absorption of diet
Attele et al <sup>[85]</sup> 2002	Mouse (obese diabetic)	Panax ginseng berry	150 mg/kg (ip) 12 d	C: Control I: Extract	Sig. loss of wt. with a sig. reduction in food intake & a very sig. increase in energy expenditure & body temperature	Sig. improvement in glucose tolerance & sig. reduction in serum insulin levels & plasma chol levels

MSG: Monosodium glutamate; FABO: Fatty acid  $\beta$  oxidation; STD: Standard diet; LFD: Low fat diet; NLD: Normal diet; HFD: High fat diet; FABP: Fatty acid binding protein; FFM: Fat free mass; sig.: Significant; AST: Aspartate transaminase; ALT: Alanine transaminase; C: Control; I: Intervention; FAS: Fatty acid synthetase; UCP: Uncoupling protein; GDP: Guanosine 5' diphosphate; FAS: Fatty acid synthetase; TG: Triglyceride; HDL: High density lipoprotein; LDL: Low density lipoprotein; FBC: Fasting blood glucose; ip: Intraperitoneal; iv: Intravenous. Caps HT2 is a herbal formulation containing methanolic extract of selected parts of plants: commiphora mukul; Allium Sativum; Plumbago indica/some carpus anacardium/Hemidesmus indicus/Terminalia arjuna/Tinospora cordifolia/Withania somnifera ocimum sanctum.

caffeine<sup>[9,20]</sup> which caused minor adverse effects such as dry mouth, insomnia, nervousness, palpitation and headache. Bofutsushosan<sup>[14]</sup> caused loose bowel movements.

## ANIMAL STUDIES

### Change in body weight and body fat

The majority of animal studies (41 out of 58) showed significant weight loss or inhibition of weight gain when supplemented with high fat diets containing extracts of plants, with or without an efficient decrease in fat mass<sup>[27-85]</sup> (Table 2).

### Food intake

Clinical trials with *Agave tequilana* (TEQ) and *Dasylirion* spp (DAS)<sup>[50]</sup>, Pomegranate leaf<sup>[43]</sup>, Korean red ginseng<sup>[58]</sup>, Tree peony<sup>[69]</sup>, Gyeongshang angjeehwan containing a variety of plants including platycodon grandiflorum, Magnoliaceae and Ephedra<sup>[81]</sup>, Parasitic loranthus<sup>[70]</sup>, and Panax ginseng berry<sup>[85]</sup> showed significant reductions in food intake or appetite. In studies with *Cucurbita moschata*<sup>[40]</sup>, *Cyperus rotundus*<sup>[42]</sup>, Nomame Herba<sup>[66]</sup>, *Acanthopanax senticosus*<sup>[57]</sup> PM-F2-OB (a traditional herbal medicine used for the treatment of obesity in Korea composed of *Lycii Fructus*), and several other plants<sup>[73]</sup>, bofu-tsusho-san<sup>[75]</sup>, *Galega officinalis*<sup>[77]</sup>, and Oolong tea<sup>[67]</sup>, no change in the amount of food intake or appetite was observed.

## DISCUSSION

In many studies<sup>[8-10,12-16,20-23,27,39,73,74,79-81,83]</sup>, a combination of plants or compounds containing minerals and/or chemical extracts of plants were investigated and the scientific names are summarized in Tables 1 and 2. Most of these studies showed anti-obesity effects such as decreasing body weight in humans or body weight gain in animals with or without changes in body fat.

Currently available anti-obesity medications attack the body fat dilemma in three different ways. They can stimulate metabolism, suppress appetite, affect serotonin, or they can impede digestion of fat. In this review, we can categorize the target effects of herbal medicines in the same way.

*Arachis hypogaea*<sup>[50]</sup> decreased body weight gain, liver triglyceride content and liver size in association with increased fecal lipid excretion, suggesting an inhibitory mechanism on lipid absorption. *Phillyrin*<sup>[52]</sup>, *Allium victorialis*<sup>[32]</sup>, Pomegranate leaf<sup>[43]</sup>, *Kochia scoparia*<sup>[46]</sup>, *Panax japonicus*<sup>[55]</sup>, Oolong tea<sup>[67]</sup>, and *Aesculus turbinata* Blume<sup>[71]</sup> also had the same effect.

A decrease in food intake as a result of a decrease in appetite and an influence on hormonal status was observed with TEQ and DAS<sup>[50]</sup>, Pomegranate leaf<sup>[43]</sup>, Korean red ginseng<sup>[58]</sup>, Tree peony<sup>[69]</sup>, Gyeongshang angjeehwan containing a variety of plants including platycodon grandiflorum and Magnoliaceae and

ephedra<sup>[81]</sup>, and Parasitic loranthus<sup>[70]</sup>, refined Rhubarb<sup>[34]</sup>, *Caralluma fimbriata*<sup>[19]</sup> and Panax ginseng berry<sup>[85]</sup>. Possible stimulation of metabolism has been reported as a mechanism of action for compounds such as Slimax<sup>[8]</sup>, supplements containing ephedra<sup>[9,13,14,20]</sup> and *Terminalia arjuna Roxb*<sup>[11]</sup> which showed modification of lipid metabolism and a reduction in serum lipid levels.

Ephedra known as *Ma Huang* is a well known natural product with amphetamine-like stimulation effects. Although its efficacy in weight loss need more investigations, its adverse effects are well established in the literature. In this review, nine studies investigated the effects of ephedra as one of the major components in the combinations with caffeine<sup>[9,13,22]</sup> or with several other plants<sup>[14,20,79,81,83]</sup> 5 of which were human studies<sup>[9,13,14,20,22]</sup>.

In one study<sup>[13]</sup>, efficient decreases in body weight and fat were observed with the administration of 210 mg caffeine and 72 mg ephedra per day for 12 wk with an improvement in lipid metabolism and blood pressure without serious adverse effects. In this study, the weight loss at 12-wk was  $-3.5 \pm 0.6$  kg with the test compound which was significantly ( $P < 0.02$ ) higher than that of the placebo. The percentage fat loss shown by DXA was  $-7.9\% \pm 2.9\%$  and  $-1.9\% \pm 1.1\%$ , respectively ( $P < 0.05$ ). In another study<sup>[20]</sup>, ephedra at a dose of 40 mg/d and caffeine at a dose of 100 mg/d for a longer time (9 mo) was found to be more efficient than the previous study in lowering body fat and weight, improving lipid metabolism and blood pressure and had no serious adverse effects. The treatment group lost significantly more body weight (-7.18 kg) and body fat (-5.33 kg) than the control group (-2.25 and -0.99 kg, respectively). The difference in data from these two studies possibly resulted from the different dosages and duration of interventions.

In a human study<sup>[9]</sup>, a significantly greater weight loss was observed ( $-4.0 \pm 3.4$  kg or 3.5% of baseline) in the test group vs ( $-0.8 \pm 2.4$  kg or 0.09% of baseline) in the placebo group. Changes were significantly greater for body fat and percentage of body fat in the active group ( $-3.5 \pm 3.3$  kg and  $-2.1\% \pm 3.0\%$ ) in comparison to the placebo group ( $-0.7 \pm 2.9$  kg and  $-0.2\% \pm 2.3\%$ ). The tested product also produced several untoward side effects, leading to some actively treated subjects withdrawing from the study. Additional long-term studies are needed to elucidate the effects of chronic treatment. Thus further examinations in healthy individuals using scientific combinations and dose/duration adjustments are required.

Four studies<sup>[58,59,65,70]</sup> investigated different doses and types of ginseng which is a very popular Chinese herbal medicine. Ginseng significantly decreased weight gain and efficiently improved glucose tolerance<sup>[59,76]</sup>.

It has been reported<sup>[58]</sup> that hormonal influences can reduce food intake and decrease serum leptin and neuropeptide Y in the brain hypothalamus although not significantly. Thus the anti-obesity effect of this plant requires further investigation.

CQ, a succulent vine native to West Africa and Southeast Asia, has been used in traditional African and Ayurvedic medicine for more than a century. Although some studies have examined other uses for CQ, its role in fighting against obesity and for symptoms of

metabolic syndrome has recently attracted interest in other parts of the world, because of its milder adverse effects comparing to ephedra. In this review, two studies focused on this herb<sup>[15,26]</sup>. CQ in combination with IG<sup>[15]</sup> induced marked reductions in body weight and fat. In addition, a reduction in waist size of 1.0 cm in the placebo group vs 21.9 cm in the CQ-IG group was observed.

As we focused on herbal medicines, all dietary interventions such as the consumption of fruits and vegetables, whole grains, different types of fibers, functional food components including omega three fatty acids or phytochemicals such as flavonoids were omitted. Lifestyle modification is still the safest and efficacious method of inducing a persistent weight loss. In this review, some of the studies were carried out on subjects who simultaneously received diet and exercise programs (mentioned as weight loss programs in Tables). These results demonstrated that specific phytochemical supplements increase the effectiveness of weight loss programs and additional significant anti-obesity effects are observed.

Although few studies mentioned adverse effects, it should be noted that many serious adverse events which would have stopped a trial of a pharmaceutical agent would likely not have been identified by the authors' search methods. Moreover, important safety issues including significant adverse events or supplement-drug interactions relevant to many clinical populations may not be fully addressed by the trials available for review.

## CONCLUSION

Compliance with conventional weight-management programs, which often include increasing energy expenditure via physical activity, is low. It is not surprising to see the marketing of many new dietary slimming aids aimed at satisfying the need for palatable (as well as safe, effective, and therapeutic) options. In accord with this approach there are numerous investigations on the effectiveness of medicinal plants as natural supplements to reduce body weight. In this paper a variety of herbal supplements had beneficial effects on obesity especially compounds containing ephedra, CQ, ginseng, bitter melon (*Momordica charantia*), and zingiber. Most of the introduced herbals (Tables 1 and 2) have also been shown to have antioxidant effects, and with regard to the role of oxidative stress in the pathophysiology of some diseases and conditions, their further positive effects may be very promising<sup>[86-95]</sup>. Attention to these natural compounds and further work on the isolation and characterization of their constituents is highly recommended.

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REVIEW

# An overview of animal models for investigating the pathogenesis and therapeutic strategies in acute hepatic failure

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of the inoculation of rabbits with the virus of rabbit hemorrhagic disease. This model displays biochemical and histological characteristics, and clinical features that resemble those in human AHF. In the present article an overview is given of the most widely used animal models of AHF, and their main advantages and disadvantages are reviewed.

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**Key words:** Acute hepatic failure; Surgical models; Chemical models; Viral models

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## Abstract

Acute hepatic failure (AHF) is a severe liver injury accompanied by hepatic encephalopathy which causes multiorgan failure with an extremely high mortality rate, even if intensive care is provided. Management of severe AHF continues to be one of the most challenging problems in clinical medicine. Liver transplantation has been shown to be the most effective therapy, but the procedure is limited by shortage of donor organs. Although a number of clinical trials testing different liver assist devices are under way, these systems alone have no significant effect on patient survival and are only regarded as a useful approach to bridge patients with AHF to liver transplantation. As a result, reproducible experimental animal models resembling the clinical conditions are still needed. The three main approaches used to create an animal model for AHF are: surgical procedures, toxic liver injury and infective procedures. Most common models are based on surgical techniques (total/partial hepatectomy, complete/transient devascularization) or the use of hepatotoxic drugs (acetaminophen, galactosamine, thioacetamide, and others), and very few satisfactory viral models are available. We have recently developed a viral model of AHF by means

## INTRODUCTION

Acute or fulminant hepatic failure (AHF) is a severe liver injury accompanied by hepatic encephalopathy which causes multiorgan failure with an extremely high mortality rate, even if intensive care is provided. Management of severe AHF continues to be one of the most challenging problems in clinical medicine<sup>[1]</sup>. Liver transplantation has been shown to be the most effective therapy, but the procedure is limited by shortage of donor organs combined with the disadvantage of needing immunosuppressant treatment<sup>[2,3]</sup>. Survival rates are substantially improved today compared with the mortality rate that approximated 100% when the

syndrome was first described nearly five decades ago. Nonetheless, survival has plateaued in recent years, prompting us to consider whether major new advances in disease understanding are needed to further improve the overall outcome<sup>[4]</sup>.

Since 1970, when Trey and Davidson<sup>[5]</sup> introduced the term fulminant hepatic failure, various authors have suggested different classifications aimed to establish prognosis and adequate therapeutic strategies. These classifications are fundamentally based on time elapsed from onset of clinical symptoms or jaundice to the development of encephalopathy<sup>[6-9]</sup>. The causes of AHF are varied and in many patients remain unknown. A 6-year study (1992-1998) carried out in Spain into the causes of AHF indicated that the basic etiopathogenic agents of AHF were viral hepatitis (39%), unknown cause (30%), toxins or drugs (21%) and others (10%)<sup>[10]</sup>. Acute viral hepatitis constitutes a frequent cause of AHF<sup>[7,11,12]</sup>. The viruses causing hepatitis A, B and E are capable of producing AHF, which is rarely seen with hepatitis C virus. The hepatitis B virus is the main causal agent worldwide, responsible for 70% of all cases of viral origin<sup>[13]</sup>. The hepatitis E virus causes AHF principally in women in their third term of pregnancy. Other viruses involved comprise the herpes virus, varicela-zoster, cytomegalovirus, Epstein-Barr virus, human herpes type 6, adenovirus and paramyxovirus, mainly in the setting of immunosuppression<sup>[14]</sup>. Non-steroidal anti-inflammatory analgesic and anti-bacterial drugs are among the pharmaceuticals which most frequently trigger AHF<sup>[7,15-17]</sup>. Other less common causes of AHF include pregnancy, veno-occlusive disease, Budd-Chiari syndrome, Wilson's disease, hemochromatosis, tumoral metastases, sepsis, ischemia and hepatic transplant failure<sup>[18,19]</sup>.

Etiologies also vary worldwide with considerable differences apparent between Western countries and the developing world. In Europe and North America a large proportion of cases are due to acetaminophen and to idiosyncratic drug reactions<sup>[20]</sup>, whereas reports from emerging countries in Asia and Africa feature viral illnesses, particularly hepatitis B and E<sup>[17]</sup>. The resulting clinical picture is remarkably similar across the different etiologies, reflecting common patterns of response of the innate immune system and the resulting inflammatory response<sup>[21]</sup>. Determining etiology is important for two reasons: specific antidotes or therapies may be indicated once the diagnosis is known, and knowing the cause provides a reasonably valid guide to predicting outcome.

The AHF syndrome occurs as a result of the functional failure of a large part of the hepatic parenchyma, and severity is proportional to the level of hepatic damage. AHF provokes profound physiological alterations characterized by encephalopathy, hemodynamic changes and coagulopathy, with frequent development of cerebral edema and renal failure<sup>[14,22,23]</sup>. Diagnosis is based on biochemical and hematological data indicating hepatic cell hypofunction. Although prolonged prothrombin time not corrected by vitamin K and impairment of factor V are

widely used, research into prognostic indexes remains an open field of investigation<sup>[24]</sup>.

The pathophysiology of AHF is an area of great interest. It is evident that a relationship must exist between different pathogenic factors, such as bacteria toxins, cytokines, free radicals and other components of the inflammatory system which cause local lesions<sup>[25]</sup>. It seems that the endothelium is the first to release vasoactive agents which affect local and distal blood flow in the critical phase of the disease, with nitric oxide, prostacyclins and endothelins being essential components of the response<sup>[26]</sup>. Hyperbilirubinemia is generally conjugated and jaundice is an early indicator which progresses rapidly. Severe coagulation problems arise as a result of a variety of mechanisms. Consumption of factor V indicates hepatic damage regardless of vitamin K levels. Renal failure occurs in 30%-75% of cases, and is associated with a poor prognosis. Thrombocytopenia is also common<sup>[27]</sup>. An increase in the plasma concentration of aromatic amino acids (AAA) and normal or slightly elevated values for branched amino acids (ACR) are typical findings in patients with AHF. In fact, a fundamental clinical parameter for AHF is the Fischer index, that is, the ACR/AAA molar ratio, which decreases as the severity of hepatic symptoms develops<sup>[28]</sup>. An increase in the amino acids phenylalanine and tyrosine, and a decrease in the Fischer index have been reported in both surgical models<sup>[29]</sup> and models using galactosamine<sup>[30]</sup>.

Intracranial hypertension is a major cause of morbidity and mortality of patients suffering from fulminant hepatic failure. The etiology of this intracranial hypertension is not fully determined, and is probably multifactorial, combining a cytotoxic brain edema due to the astrocytic accumulation of glutamine, and an increase in cerebral blood volume and cerebral blood flow; in part due to inflammation, to glutamine and to toxic products of the diseased liver<sup>[31]</sup>. Cerebral edema is a potential life-threatening complication in patients with AHF who progress to grade III/IV encephalopathy<sup>[26]</sup>. The current view on the pathogenesis of cerebral edema is that hyperammonemia plays a main role. High arterial ammonia concentrations have been proposed as a predictor of brain herniation and mortality in patients with AFL<sup>[32-34]</sup>. Moreover, arterial ammonia concentration, ammonia delivery to the brain, and its metabolic rate are higher in patients with high intracranial pressure, and increased arterial ammonia correlates with increased cerebral flow<sup>[35]</sup>. Recent work has also suggested free radical formation occurring at a mitochondrial level as being the potential mediator of cellular dysfunction as opposed to ammonia *per se*<sup>[36]</sup>.

Research into the molecular mechanisms of hepatic regeneration has aroused wide-spread interest<sup>[37,38]</sup>. Although little is still known about the hepatic regenerative process, it is clear that cellular loss and damage in the liver are accompanied by a lack of regenerative activity<sup>[39]</sup>. Plasma levels of hepatocyte growth factor (HGF) and transforming growth factor (TGF)- $\beta$  rise<sup>[40]</sup>. An increase in the activity of the

fibrinolytic system, responsible for the activation of both HGF and TGF- $\beta$ , is also observed<sup>[41]</sup>. It has been reported that the serum of people affected by AHF has a negative effect upon culture cell growth when compared with a control serum, due to cell proliferation inhibition rather than to an increase of apoptosis<sup>[42]</sup>. Recent studies using various animal models have shown that the over-expression of calpastatin, an endogenous inhibitor of calpain, helps prevent further liver damage when hepatic regeneration is compromised<sup>[43]</sup>.

Knowledge concerning the pathophysiological basis of the AHF hemodynamic alterations, immunological dysfunction, and multiorgan failure is still very rudimentary. It is therefore crucial to investigate the molecular basis of AHF in more depth<sup>[44]</sup>. Furthermore, although many AHF treatment options have been proposed and applied in recent years, only hepatic transplantation is widely accepted among clinical specialists. However, the lack of donors combined with the high costs, technical difficulties, viability issues and the disadvantage of needing life-long pharmacological immunosuppressant treatment following surgical intervention (with the added complication that the immunosuppressant agents used themselves produce side effects in the kidneys, liver and other organs), mean that liver transplantation is not always an option. For these reasons, other therapeutic options to bridge patients to recovery or stabilization have to be considered. Artificial liver support, intended to remove protein-bound toxins and water-soluble toxins without providing synthetic function, and bioartificial liver support systems, using hepatocytes in an extracorporeal device connected to the patient's circulation, are being tested, and molecular adsorbent recirculating systems (MARS)<sup>[45]</sup> or cell-based therapies are increasingly the focus of attention<sup>[46,47]</sup>. These systems improve clinical and biochemical parameters and can be applied safely to patients<sup>[48]</sup>, but their effectiveness and viability have not yet been conclusively demonstrated<sup>[38]</sup>. In terms of clinical applications, functional studies using animal models are absolutely crucial.

## ANIMAL MODELS OF AHF

Knowledge of the pathophysiology and treatment of AHF are limited by the lack of satisfactory animal models. Many attempts have been made to develop a suitable model which can be replicated, using a wide variety of species and approaches, from surgical models to the use of hepatotoxic drugs (Table 1). However, to date a simple model which accurately reproduces the pattern of human AHF has not been reported, and the models currently in use present significant limitations<sup>[49,50]</sup>.

An ideal model would present well-defined clinical and biochemical criteria, and, as in the case of the King's College AHF prognostic criteria<sup>[11]</sup>, be capable of providing an accurate prognosis. However, none of the models which have been developed until now meet these requirements. Furthermore, the clinical and biochemical criteria used to indicate the existence of AHF in animal models often have very little in common with those used in clinical practice. However, given the current state of

knowledge concerning AHF and the difficulties involved in carrying out research on patients, animal models have a fundamental role to play in future studies despite their limitations. Therefore, although progress is being made, research in this field must continue, with the aim of developing a reliable and suitable animal model, capable of accurately reflecting the human clinical syndrome and presenting a minimum of disadvantages<sup>[51]</sup>.

Ideal AHF models, according to criteria widely accepted (Table 2)<sup>[49-54]</sup>, would benefit from complying with a series of requirements including that the model should be reversible, ie that some animals would survive the process if a suitable treatment were administered, and that the results obtained can be replicated, i.e. that death occurs at recognised intervals and that the extent of hepatic damage can be measured and standardised. Furthermore, death would need to be a result of hepatic damage, i.e. the complications produced following damage would need to accurately reflect the typical human clinical picture and death should be the direct result of the liver damage produced. Therefore, the untreated animals should die with signs of progressive hepatic failure within a recognised period of time. In addition, the animal used would need to be of a size permitting sufficient samples of blood and tissue to be taken during treatment. Finally, all the methods used should represent the lowest possible health risk for personnel participating in the research. An additional criterion could be the use of a conscious animal model to evaluate the development of hepatic encephalopathy, since this is an essential part of the pathology of AHF<sup>[55]</sup>.

Numerous studies have been carried out in an attempt to develop a suitable AHF model. The majority of animal models are based on surgical techniques or hepatotoxic drugs. Surgical models include the use of hepatic ischemia and partial/total hepatectomy, whilst chemical models are based on the use of drugs and toxins such as acetaminophen, azoxymethane, concanavalin A, galactosamine, halothane, thioacetamide, amatoxin-endotoxin, etc. Nevertheless, to date, no model accurately reflects human AHF, and most demonstrate significant limitations.

### Surgical models

Surgical models of AHF can be divided into three categories: hepatectomy (total or partial), devascularization (total or partial) and models which are a combination of the previous two.

**Total and partial hepatectomy:** Surgical models employing total or partial hepatectomy have been successfully developed in various animal species following the first attempt carried out by Mann on dogs in 1921<sup>[56]</sup>. It has been demonstrated that 95% liver resection in rats provides a good AHF model<sup>[57]</sup>, whilst a less than 90% hepatectomy is the upper limit for a liver regeneration research model in mice, as higher values produce mortal hepatic failure<sup>[58]</sup>. A potentially reversible model using pigs has been described which combines partial hepatectomy (70%) with porta-caval derivation and produces death from AHF after an interval which is sufficiently prolonged

**Table 1 Main AHF animal models in different species**

<b>Animal model</b>	<b>Species</b>	<b>Advantages/disadvantages</b>
Surgical		
Total/partial hepatectomy	Pig, dog, rabbit, rat, mouse	Hepatic encephalopathy; reproducible/no reversibility; no long-term survival
Total/partial devascularization	Pig, dog, rabbit, rat	Hepatic encephalopathy; reproducible/no reversibility; no long-term survival
Chemical		
Acetaminophen	Pig, dog, rabbit, rat, mouse	Hepatic encephalopathy; no hazard/non-reproducible; variable interval between damage and death; species and age variability
Amanitin	Pig	Hepatic encephalopathy; specific toxic effects; large animal
Azoxymethane	Mouse	Hepatic encephalopathy; reproducible/small size; hazard
Carbon tetrachloride	Pig, rabbit, rat, mouse	Hepatic encephalopathy/non reproducible; extrahepatic toxicity; small time window before death
Concanavalin A	Rat, mouse	Hepatic encephalopathy/small size
Galactosamine	Pig, dog, rabbit, rat, mouse	Hepatic encephalopathy; biochemical markers/non-reproducible; hazard; variable interval between damage and death; species differences
Lipopolysaccharide	Rat, mouse	Hepatic encephalopathy/non-reproducible; small size; hazard; small time window before death
Thioacetamide	Rabbit, rat, mouse	Hepatic encephalopathy; reproducible; large time window before death/hazard
Viral		
Rabbit hemorrhagic disease	Rabbit	Hepatic encephalopathy; reproducible; no hazard

**Table 2 Main criteria for an AHF animal model (according to Terblanche and Hickman (1991))**

Reversibility	Suitable treatment may reverse and improve survival
Reproducibility	Reproducible end-points are required to standardize the model
Death from liver failure	Should reflect biochemical, histological and clinical changes including death from AHF
Therapeutic window	Time for treatment should be available between insult and death
Adequate animal size	Size large enough to allow blood and tissue analysis to take place serially
Minimal hazard to personnel	Minimum risk for operators and associated staff

to enable studies of hepatic support measures to be carried out. In addition, the animal is of an appropriate size, and the technique does not represent a health hazard<sup>[59]</sup>.

The partial hepatectomy models are equivalent to patients who have undergone large liver resections for liver tumors. It has been demonstrated by DNA analyses of rats subjected to various levels of partial hepatectomy that induced AHF is a consequence of both an increased rate of apoptosis and a reduction in liver regeneration<sup>[60]</sup>. Moreover, models of partial hepatectomy have been used to test the usefulness of different support systems. Thus, intraperitoneal transplant of syngeneic-bioencapsulated bone marrow cells, which can transdifferentiate into hepatocyte-like cells in the peritoneal cavity of 90% hepatectomized rats, increases the survival rate of these animals<sup>[61]</sup>. Examination of the effects of a series of allogenic hepatocyte transplantations in rats with subtotal hepatectomy indicates that intrasplenic hepatocyte transplantation 1 d before liver surgery shows the best results in terms of survival<sup>[62]</sup>. The usefulness of an artificial liver module having a liver lobule-like structure has been recently tested in rats with combined partial hepatectomy and hepatic ischemia, demonstrating that in treated

rats the increase in blood ammonia was completely suppressed and all animals recovered<sup>[63]</sup>.

The clinical equivalent of liver total hepatectomy is the massive liver damage due to liver trauma or a primary graft failure<sup>[51]</sup>. Main disadvantages are the absence in circulation of the toxic substances and inflammatory factors which play a role in the pathogenic mechanisms of AHF. Advantages are related to replicability and its usefulness in the *in vivo* study of artificial support devices in the absence of toxic products eliminated or produced by the damaged liver. Despite the disadvantages indicated, total hepatectomy has been used on rats to study hepatic regeneration<sup>[64]</sup>, and with pigs as a replicable model for testing the effectiveness and function of various temporary support device systems<sup>[65,66]</sup>. A new surgical model for hepatectomy in pigs, requiring prior to *en bloc* hepatectomy a Y-shaped bypass starting with end-to-side anastomosis between the vena cava and the portal vein, followed by anastomosis to the intrathoracic vena cava has been recently described. This model permits total hepatectomy with minimal blood loss under stable circulation without requiring an extracorporeal bypass<sup>[67]</sup>.

**Devascularization:** Complete devascularization of the liver may be achieved by portacaval shunt followed by occlusion of the hepatic artery, and in most cases also occlusion of the common bile duct and accessory hepatic vessels<sup>[68,69]</sup>. Depending on the time of temporary occlusion of the hepatic artery the model is more or less reversible. These techniques have been successfully used to induce a reproducible hepatic failure in pigs, which could be useful in the study of different artificial and/or bioartificial hepatic support devices<sup>[70,71]</sup> or to test the effects of antioxidant molecules such as N-acetylcysteine<sup>[72]</sup>. For example, a reproducible model has been developed using dwarf pigs for the study of reversible devascularization through hepatic artery ligation and porto-caval anastomosis, where intracranial pressure was monitored in addition to other classic parameters

indicative of AHF. This model provides an 8-h therapeutic window, enabling tests on different bioartificial support systems to be carried out<sup>[73]</sup>. In fact, with the use of a similar model in pigs, albumin dialysis using the molecular adsorbents recirculating system (MARS) has been reported to attenuate extracellular brain ammonia and lactate levels<sup>[74]</sup>. Hepatic devascularization in pigs has also allowed the demonstration that endothelium-dependent hyperpolarization of vascular smooth muscle contributes to the development of hyperdynamic circulation in AHF<sup>[75]</sup>.

A model using total clamping of the portal triad in dogs demonstrated that the damage caused by ischemia-reperfusion as a consequence of the surgical procedure was reduced following administration of a bradykinin  $\beta 2$  receptor antagonist<sup>[76]</sup>. Dogs were also used in another AHF model employing porto-caval derivation combined with bile duct ligation, in order to test a new system of bioartificial liver by inoculation of hepatocytes. This model was configured by inoculating porcine hepatocyte spheroids into the cell circuit of a hollow fiber bioreactor<sup>[77]</sup>. Recently a new pig model has been developed in which a 75%-80% liver resection is combined with an ischemia period<sup>[78]</sup>.

Studies carried out on survival time, technical ease, safety and reproducibility of AHF surgical models have reported that devascularization was more useful for studying the development and treatment of AHF caused by ischemia and related side effects, whilst partial hepatectomy was the most suitable technique for studying liver deficiency status and AHF treatment via bioartificial support devices<sup>[79]</sup>.

### **Chemical models**

The use of chemical agents such as acetaminophen, thioacetamide or galactosamine may reproduce a number of important AHF clinical characteristics, such as hypoglycemia, encephalopathy, and increased blood levels of hepatic enzymes, and hepatotoxic chemical agents are still frequently used as a model for AHF. However, repeated administration or a support therapy may be required in some models. In addition, intracranial hypertension, one of the main characteristics of human AHF, is absent in some chemical models whilst in other cases, an increase in toxins involved in hepatic encephalopathy and cerebral edema in human AHF cannot always be demonstrated<sup>[49]</sup>.

**Acetaminophen:** Acetaminophen (paracetamol) is a commonly used drug which can produce hepatic damage. In fact, it is the drug most frequently used to commit suicide in the United Kingdom despite the existence of the antidote acetylcysteine. Acetaminophen overdoses are the number one causes of AHF in USA, United Kingdom, and most of Europe, accounting for nearly 50% of USA cases<sup>[13]</sup>. Acetaminophen toxicity is dose-dependent, but its effects can be exacerbated by fasting, cytochrome P-450 inducer drugs and especially by alcohol. Studies on both hepatocyte cultures and mice have shown that c-jun kinases (JNK) play a major role in

the toxic effect of the drug<sup>[80]</sup>. More recently, it has been shown that apoptosis signal-regulating kinase 1 (ASK1), a member of the mitogen-activated protein kinase kinase family, is activated by acetaminophen overdose in mice, most likely *via* a mechanism involving thioredoxin-ASK1 dissociation, and that it plays a role in acetaminophen-induced liver injury through JNK activation<sup>[81]</sup>. The fact that JNK inhibition is not protective in acute carbon tetrachloride-mediated or anti-Fas antibody-mediated hepatic injury, suggests specificity for the role of JNK in the pathogenesis of acetaminophen-induced liver failure, thereby identifying JNK as an important therapeutic target in the treatment of acetaminophen hepatotoxicity<sup>[82]</sup>.

The results of numerous studies with animal models using acetaminophen to induce AHF have produced heterogeneous results due to the existence of significant variations in the hepatic detoxifying metabolism of the drug related to species and age<sup>[83,84]</sup>. Under normal conditions, acetaminophen hepatic metabolism is produced by glucuronidation and sulfation reactions, with formation of metabolites which are later excreted through the kidney. When an excess of the drug is present, normal detoxifying pathways are saturated and the drug is metabolized through cytochrome P-450 to N-acetyl-p-benzoquinoneimine which, unless conjugated with glutathione, is thought to interrupt mitochondrial calcium flux and to induce cell damage by the formation of hydroxyl radicals, nitrites, and nitrates, leading to apoptosis and cell necrosis<sup>[85]</sup>. Therefore, in order to potentiate acetaminophen toxicity, inducers of the cytochrome P-450 systems such as phenobarbitone and 3-methylcholanthrene, glutathione depletion induced by the glutathione synthetase inhibitor buthionine sulfoximine or a combination of both systems are used<sup>[84,86]</sup>.

Other important aspects which have not been standardized in acetaminophen models, and which produce variable results, include the optimal drug dose, the most suitable method of administration and the necessity or not of induction of the cytochrome P-450 system<sup>[86,87]</sup>. Lack of standardization is the origin of some of the major disadvantages of these models, specifically, their lack of reproducibility and the variable interval between inducing damage and the death of the research animals<sup>[49,52]</sup>. Furthermore, in some rodents significant differences have been found in concentrations of the main coagulation factors compared to those found in human AHF<sup>[88]</sup>.

Acetaminophen-induced animal models of AHF are widely used to improve our insight into the metabolic and physiological derangements of AHF and to facilitate the development of new therapeutic modalities. Thus, implantation of encapsulated lentivirally immortalized human hepatocytes rescue mice from lethal doses of acetaminophen, confirming that lentiviral vectors represent tools of choice for immortalization of non-dividing primary cells and that immortalized human hepatocytes are promising reagents for cell-based therapy of acute liver failure<sup>[89]</sup>. More recently, it has

been found that adult-derived mononuclear bone marrow fraction is capable of significantly increasing the survival rate of rats with acetaminophen-induced AHF<sup>[90]</sup>. Research has also shown that acetaminophen-induced hepatocellular damage is associated with increased circulating catecholamines, which may contribute to the pathophysiology of acetaminophen-induced hepatotoxicity by compromising hepatic perfusion, and that toxicity may be abolished by the use of  $\alpha$ (1) antagonists<sup>[91]</sup>.

**Galactosamine:** D-galactosamine is a molecule which, when metabolized *via* the galactose pathway in the liver, causes serious metabolic alterations and hepatic necrosis through depletion of different uridine intracellular mediators<sup>[49]</sup>, and has therefore been used to develop AHF models. In one of the first models using rabbits<sup>[92]</sup>, death occurred between 21 and 44 h, following a coma lasting on average 2.6 h, with histologic and biochemical findings compatible with AHF. Furthermore, it was possible to show that in this same species, hepatotoxin did not cross the hematoencephalic barrier<sup>[93]</sup>. More recently, galactosamine has been used on anesthetized dogs. This model also displays the characteristic effects of human AHF, such as an increase in blood levels of liver enzymes, bilirubin, ammonium or lactate and the associated coagulopathy, hypoglycemia, coma and increase in intracranial pressure<sup>[94]</sup>. However, the effects were not the same in dogs without anesthesia, probably due to the added effect of the anesthetic. A reproducible model has been developed with pigs which, because of their size, are suitable for the assessment of different support systems designed for treating AHF in humans<sup>[95]</sup>. Significant differences in galactosamine sensitivity across different species exist. Furthermore, the interval between damage caused and death is not uniform, the agent is expensive to use in large-scale models, and lastly, it carries health risks<sup>[50]</sup>.

Galactosamine models have been used to investigate the renal damage which accompanies AHF<sup>[96]</sup> and the liver metabolic pathways involved<sup>[97]</sup>. In addition, the potential protective effects of substances such as the chimeric protein hyper-IL-6<sup>[98]</sup> or 1,6 diphosphate fructose have been investigated in rats<sup>[99]</sup>. Cardiotrophin 1 may improve the outcome of D-galactosamine-induced AHF through its effects on anti-apoptosis and cell repair<sup>[100]</sup>. Blocking of N-methyl-D-aspartate receptors prevents ammonia-induced death<sup>[101]</sup> and also prevents or delays death of rats by galactosamine-induced AHF<sup>[102]</sup>. Moreover, this model has been used to identify the contribution of cytosolic polypyrimidine tract-binding protein to the mechanisms of hyperinsulinemia by stabilization of mRNA encoding insulin and its secretory granule proteins<sup>[103]</sup>. D-galactosamine models have also allowed testing of different extracorporeal hepatic support devices<sup>[104]</sup> and bioartificial systems, including hepatocytes transfected with the human gene interleukin-1 receptor antagonist in rats<sup>[105]</sup>, the use of a nonwoven fabric bioreactor containing porcine hepatocytes<sup>[47]</sup>, or the study of the

potential effects of cerebrospinal fluid drainage and cranial decompression in rats<sup>[106]</sup>.

A combination of D-galactosamine and lipopolysaccharide has also been widely used to induce AHF in rats. This model has allowed the demonstration of the potential therapeutic role of vascular endothelial growth factor<sup>[107]</sup>. Using this approach, evidence for a direct link between tumour necrosis factor (TNF)- $\alpha$  and Fas/FasL in mediating hepatocyte apoptosis has been provided<sup>[108]</sup>, it has been reported that type I inositol 1, 4, 5-triphosphate receptors increase in the kidney<sup>[109]</sup>, and it has been demonstrated that transcription factor early growth response (Egr)-1 plays an important role in acceleration of hepatic inflammation, apoptosis, and subsequent mortality in acute liver injury<sup>[110]</sup>. Research with this model has also found that the expression and activity of both leukotriene C4 synthase and microsomal glutathione-S-transferase are up-regulated, being partly responsible for cysteinyl leukotriene hepatic accumulation<sup>[111]</sup>, and that a combination of 5-hydroxyindole acetic acid, glucose,  $\beta$ -hydroxybutyrate, and phosphate concentrations in the plasma is a potential marker for AHF, as well as for the early prognosis of AHF<sup>[112]</sup>. Studies using SP600125, a small molecule JNK-specific inhibitor have confirmed the role of JNK as a critical apoptotic mediator in galactosamine/lipopolysaccharide-induced AHF<sup>[113]</sup>. Very recently it has been demonstrated that in mice challenged with D-galactosamine and lipopolysaccharide, deficiency of uncoupling protein-2, which plays a role in liver cell death through its involvement in the production of reactive oxygen species and adenosine, provides protection under endotoxic stress conditions, underlining the significant role of the bioenergetic status in critical illness<sup>[114]</sup>.

**Carbon tetrachloride:** Carbon tetrachloride has been widely used to induce chronic liver damage, especially as a model of primary hepatic cirrhosis. Nevertheless, its use to induce AHF has been very limited due to low reproducibility and wide variation between species<sup>[50,115]</sup>. The mechanism of action is produced in the endoplasmic reticulum by formation of reactive intermediates through isoenzymes of cytochrome P-450<sup>[116]</sup>. This mechanism also involves significant alterations to mitochondrial calcium homeostasis and is dose-dependent<sup>[117]</sup>.

A relatively uniform model was developed using pigs which induced coma and death between 12 and 52 h through a combination of pretreatment with phenobarbital and a 2-h interruption of arterial blood flow followed by intragastric administration of the toxin<sup>[118]</sup>. The administration of carbon tetrachloride in rats has been shown to simultaneously induce both severe damage processes and hepatic regeneration<sup>[119]</sup>. Depending on the dose administered, exposure time, the presence of exacerbating agents, or the age of the organism affected, regeneration can occur and lead to the total recovery of the damaged liver<sup>[120,121]</sup>.

Rats have been used for the study of intrasplenic transplant of hepatocytes<sup>[122]</sup>, and to investigate the mechanisms involved in compensatory liver regeneration which avoids progressive toxic damage<sup>[43]</sup>. Carbon

tetrachloride-induced AHF has also allowed the demonstration in rats of the therapeutical efficacy of Gabexate mesilate, a synthetic protease inhibitor<sup>[123]</sup>, the sulfated polysaccharide extracted from brown algae fucoidan<sup>[124]</sup>, or naringenin-loaded nanoparticles<sup>[125]</sup>, but not of granulocyte colony stimulating-factor<sup>[126]</sup>.

Criticisms of these models include the fact that carbon tetrachloride mainly affects the central zone of the hepatic acinus, and the characteristic massive necrosis of human AHF is not present. Furthermore, carbon tetrachloride is not completely metabolized in the liver and some of the non-metabolized toxin affects and damages other organs, especially the lungs and kidneys<sup>[52]</sup>. Finally, there is a wide variation in species and age sensitivity, basically due to different levels of development and effectiveness of the cytochrome P-450 detoxifying system<sup>[49]</sup>.

**Thioacetamide:** Thioacetamide causes hepatocellular necrosis following biotransformation by monooxygenases<sup>[127]</sup>, and has been used to explore the role of reactive oxygen species<sup>[128]</sup>, and the protective effect of antioxidants such as curcumin<sup>[129]</sup>, pro-regenerative substances<sup>[130]</sup>, or the worsening of encephalopathy following long-term treatment with substances such as indometazine<sup>[131]</sup>. Using the thioacetamide model of AHF, it has been recently shown that cannabinoids and capsaicin improve liver function<sup>[132]</sup> and that *Ginkgo biloba* ameliorates hepatic damage most probably due to its free radical-scavenging effects<sup>[133]</sup>. Simvastatin improves encephalopathy and survival in thioacetamide-treated rats, an effect that is offset by N(G)-nitro-L-arginine methyl ester (L-NAME), a non-selective inhibitor of nitric oxide synthase (NOS), which supports the role of nitric oxide in liver damage and encephalopathy<sup>[134]</sup>. Moreover, the fact that L-NAME administration, but not L-canavanine (specific inhibitor of inducible NOS), had detrimental effects on the severity of hepatic damage and motor activities in thioacetamide-treated rats, suggests that constitutive NOS activities play a major protective role<sup>[135]</sup>.

**Azoxymethane:** Azoxymethane administration induces in mice alterations similar to those encountered in human AHF<sup>[136]</sup>. In fact, it has been shown that mice present decreased locomotor activity followed by loss of righting and corneal reflexes, are hyperammonemic, and develop spontaneous hypothermia and brain amino acid profiles typical of AHF in other species including humans. These findings demonstrate that azoxymethane treatment affords a reproducible model which may be suitable for the study of the cerebral complications of AHF<sup>[137]</sup>. Induction of AHF in C57BL/6J mice by using azoxymethane has recently allowed the observation that altered expression of zonula occludens-2 precedes increased blood-brain barrier permeability, suggesting that zonula occludens-2 may play an important role in the pathogenesis of brain edema in AHF<sup>[138]</sup>.

**Concanavalin A:** A single injection of concanavalin A has also been proposed as a model of AHF as it induces

hepatocellular destruction<sup>[139]</sup> through mechanisms which appear to involve participation of immune cells, including macrophages and activated CD4+ T cells<sup>[140]</sup>. The use of this animal model has demonstrated that suppressor of cytokine signaling-1 (SOCS1) plays an important negative role in fulminant hepatitis and that forced expression of SOCS1 is therapeutic in preventing the disease<sup>[141]</sup>. In concanavalin A-treated mice it has been reported that TNF- $\alpha$  levels are not affected by adiponectin, whereas IL-10 production is increased. Therefore, adiponectin might play a role in the control and limitation of inflammation in the liver, and a contribution has been suggested for IL-10 in adiponectin-mediated hepatoprotection<sup>[142]</sup>. siRNA delivery for osteopontin, which has been implicated in various helper T cell type 1 immunity-mediated diseases, has therapeutic potential in concanavalin A-mediated AHF<sup>[143]</sup>.

**Other models:** AHF has also been induced through the use of poisons such as the derivative of *Amanita phalloides* which, although not a frequent cause of poisoning, has well-known effects on humans. In fact, the effect of amatoxins is due to ARN polymerase induction, producing cell toxicity in hepatocytes, intestinal mucosal cells and kidney tubular cells and they have been used in combination with lipopolysaccharide to develop an animal model of AHF using pigs<sup>[144]</sup>. Models with pigs have also been reported which combine amanitine with lipopolysaccharide, with the aim of studying survival following orthotopic liver transplant and tacrolimus administration<sup>[145]</sup>.

Other models employ parenteral administration of *Propionebacterium acnes* and lipopolysaccharide in mice to study the inhibition of the acquired immune response<sup>[146]</sup>, and intraportal administration of  $\alpha$ -amanitine and lipopolysaccharide in pigs to study bioartificial liver support devices<sup>[147]</sup>.

Intrahepatic upregulation of the immunoactivating molecules CD40 and CD40 ligand (CD40L) are early mechanisms for liver cell damage in human and murine AHF. The use of a model based on intrahepatic overexpression of CD40L by adenoviral-mediated gene transfer (AdCD40L) in mice, has led to the demonstration that CD40-CD40L interaction can induce liver damage, and that CD40L-induced AHF depends on competent lymphocytes<sup>[148]</sup>.

Although their use has been limited, various models combining drug administration and surgical procedures have been described, such as a combination of 70% liver resection and endotoxin administration in rats<sup>[149]</sup>, or resection of all of three hepatic arteries combined with intraportal injection of carbon tetrachloride<sup>[150]</sup>.

### Viral models

Despite the fact that viral hepatitis is a main cause of AHF in many countries, the use of infective agents to develop animal models of AHF has in general been unsuccessful and only the use of transgenic mice overexpressing virus B hepatitis proteins (HBV) or BALB/cj mice infected with MHV-3<sup>[151,152]</sup> has shed some light

on virus-induced AHF mechanisms. However, these murine models display significant limitations as regards the absence of intracranial pressure measurements, or the lack of data concerning toxins involved in hepatic encephalopathy and cerebral edema, as well as the small size of the models used which renders testing of new liver support systems impossible<sup>[49,50]</sup>.

More recently, our research group has described a new animal model of AHF using experimental infection of rabbits with 104 hemagglutination units of an isolate of the rabbit hemorrhagic disease virus (RHDV)<sup>[153]</sup>. First reported two decades ago, RHDV is a member of the *Caliciviridae* family which causes an acute and highly fatal disease in wild and domestic rabbits<sup>[154]</sup>. Rabbit hemorrhagic disease (RHD) is a viral hepatitis which displays surprising clinical, anatomopathological and transmission mode similarities to fulminant human viral hepatitis B, C, and E<sup>[155]</sup>. The virus does not replicate in any other vertebrate<sup>[156]</sup> and to date there is no indication that it can be transmitted to humans, even among those populations most exposed to the virus.

It has been shown that the viral antigen can already be found in hepatocytes at 12 h postinfection (p.i.) and that at 36 h and 48 h p.i., it is localised in 60%-80% of hepatocytes<sup>[157]</sup>. RHD is characterized by a high morbidity and a mortality rate that approaches 90%<sup>[153,158]</sup>. Rabbits die within 36 to 54 h p.i. with clinical signs characteristic of progressive AHF and coma. In addition, the interval between infection and death, in the majority of animals, provides a wide therapeutic window which indicates that our model complies with another of the essential prerequisites of a good AHF animal model, that is, the existence of a sufficiently prolonged interval between intervention and death to enable research into various treatment methods or liver support technologies. In addition, the use of a medium-sized animal facilitates serial collection of blood samples, and makes easier monitoring of intracranial pressure and biochemical alterations produced during the course of the infection<sup>[153]</sup>.

This model reproduces representative biochemical and histological parameters and clinical signs of human AHF. Thus, significant increases in blood transaminase and lactate dehydrogenase activities, and in blood bilirubin concentrations, are detected. Moreover, blood concentration of aromatic amino acids increases significantly, with a decrease in the Fischer index and hypoglycemia. Prolonged prothrombin time, a prognostic element in AHF, and exhaustion of factor V and VII are systematic findings. These effects could occur as a consequence of diminished synthesis of clotting factors and the development of disseminated intravascular coagulation<sup>[153]</sup>. In addition to biochemical and histological abnormalities, infected rabbits demonstrate a clinical picture consistent with AHF. Prostration and side recumbency are present at later stages and neurologic symptoms (convulsions, ataxia, and posterior paralysis) rapidly progress to coma and brain death in the terminal phases. In our model, intracranial pressure rises progressively

in the terminal phases, suggesting a loss of intracranial compliance, and short episodic spikes are also observed. The rise in intracranial pressure in RHDV-infected animals is accompanied by an increase in plasma ammonia levels<sup>[153]</sup>.

Histological and immunohistochemical examination reveals necrotic areas associated with hemorrhages and neutrophil infiltration, and large apoptotic areas with a high caspase 3 expression, mainly in the periportal areas of hepatic acini<sup>[159]</sup>. A significant increase in inducible nitric oxide synthase expression and TNF- $\alpha$  activity, similar to those reported in AHF<sup>[160]</sup>, are also observed in infected rabbits<sup>[161]</sup>. TNF- $\alpha$  may lead to cell proliferation or to apoptosis, and its over-expression correlates with both apoptosis and hepatic regeneration in AHF<sup>[162]</sup>. Balance between proliferation and apoptosis may be influenced by an excess of reactive oxygen species that, if not neutralized by glutathione and antioxidant enzymes, may cause mitochondrial damage and cytosol release of cytochrome c, causing caspase activation and cell death<sup>[163]</sup>. This also happens in RHDV-infected rabbits, which show impaired glutathione levels and antioxidant enzyme activities<sup>[161]</sup>, with a marked activation of the apoptotic intrinsic pathway<sup>[159]</sup>.

Therefore, RHDV experimental infection induces an AHF in rabbits which has a number of physiological and biochemical features seen clinically in humans, is highly reproducible, has a long therapeutic window and generates intracranial hypertension and an associated-encephalopathy. Thus, it is the first successful model using infective agents and satisfies the criteria applicable to an animal model of AHF. This model could provide a useful tool for the study of AHF and the evaluation of new liver support technologies in humans.

## CONCLUSION

AHF is a potentially devastating syndrome whose treatment has been limited by the lack of satisfactory animal models. The potential disadvantages of surgical models are that they do not offer reversibility or recovery, they are difficult to replicate, they depend on surgical skill, many of the clinical and biochemical parameters typical of human AHF are not present, and that they do not reproduce an environment complicated by the release of inflammatory mediators and products of cell necrosis. Thus, their usefulness is limited to the evaluation of various liver support systems. Models using hepatotoxins do not suffer from the above limitations, but nevertheless they may present disadvantages, such as the necessity for adjusting dosage and the potential health hazard which in most cases such chemical agents represent. As for the only viral model developed to date which has proved to be viable, induced RHDV infection in rabbits, it is reproducible and presents characteristics similar to human AHF. The only limitation is that the only susceptible species is the rabbit, although this could also be considered an advantage as it does not represent a health hazard to researchers.

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# Molecular characteristics and stages of chronic hepatitis B virus infection

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## Abstract

Hepatitis B virus (HBV) is a common viral pathogen that causes a substantial health burden worldwide. Remarkable progress has been made in our understanding of the natural stages of chronic HBV infection. A dynamic balance between viral replication and host immune response is pivotal to the pathogenesis of liver disease. Knowledge of the HBV genome organization and replication cycle can unravel HBV genotypes and molecular variants, which contribute to the heterogeneity in outcome of chronic HBV infection. Most HBV infections are spontaneously resolved in immunocompetent adults, whereas they become chronic in most neonates and infants at a great risk of developing complications such as cirrhosis and hepatocellular carcinoma (HCC). Those with chronic HBV infection may present in one of the four phases of infection: immune tolerance, immune clearance [hepatitis B e antigen (HBeAg)-positive chronic hepatitis B (CHB)], inactive carrier state, and reactivation (HBeAg-negative CHB). Understanding the dynamic nature of chronic HBV infection is crucial in the management of HBV carriers. Long-term monitoring and optimal timing of antiviral therapy for chronic HBV infection help to prevent progression of HBV-related liver disease to its later stage, particularly in patients with higher risk markers of HCC, such as serum DNA concentration, HBeAg status, serum aminotransferase, HBV genotypes, and pre-core or core mutants.

## INTRODUCTION

Hepatitis B virus (HBV) infection is a major health problem worldwide. Some individuals can develop acute HBV infection and achieve complete immune clearance of virus, yielding a life-long immunity, while others can develop chronic HBV infection depending on the host immune response. Chronic HBV infection is associated with a wide range of clinical manifestations, from an asymptomatic carrier state with a normal liver histology to severe and chronic liver diseases, including cirrhosis and hepatocellular carcinoma (HCC)<sup>[1,2]</sup>.

There is a particular concern in the Asia-Pacific region, where chronic HBV infection is prevalent, with a carrier rate of approximately 10% of chronic HBV carriers. About 25%-40% of them will eventually die of liver disease (cirrhosis with and without HCC) with a death rate of 50% for male carriers and 15% for female carriers, respectively. Chronic HBV infection is a dynamic process with a replicative or a non-replicative (or low replicative) phase based on virus-host interaction which is pivotal to the pathogenesis of liver disease. Understanding the dynamic nature of chronic HBV infection is crucial in the management of HBV carriers. Long-term monitoring and optimal timing of antiviral therapy for chronic HBV infection patients can help to prevent progression of HBV-related liver disease to its later stage<sup>[3,4]</sup>.

## PATHOLOGY OF HBV INFLAMMATORY REACTION

Viral hepatitis, characterized by diffused inflammatory

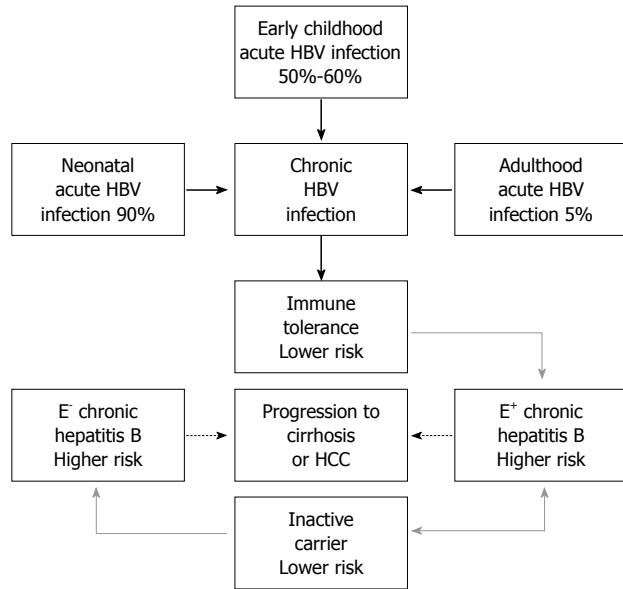
reaction, is associated with cell damage and death. It has been recently reported that HBV replication is associated with cell death, which is different from the widely accepted non-cytopathic characteristics of HBV<sup>[5]</sup>. The mechanism of cell damage is generally defined as the result of cytotoxic T-lymphocyte (CTL)-mediated immune responses to viral infection<sup>[6]</sup>. Another typical process causing cell death is apoptosis. It has been shown that HBV proteins, such as HBx and HBsP, can induce apoptosis<sup>[7]</sup>. A careful light microscopic examination of HBV genotypes A-C transfected cells can reveal rounded up and death cells which are apoptotic signs. To identify the observed cell death, FAC is used because apoptotic cells can show phosphatidylserine on cell membrane. HepG2 cells can be transfected with HBV genotypes A-C. Cells observed under a phase contrast microscope, can be stained with apoptosis markers and analyzed by flow cytometry. HBsP expression can be detected by Western blotting assay. BH3 sequences can be aligned and analyzed with the vector NT1. HBV genotypes A-C transfected cells display cell death which has been further proved as apoptosis. HBsP, a pro-apoptotic protein, is detectable during transfection of virus genomes. Different apoptotic effects are correlated with the expression of different genomes. Alignment and analysis of the HB3 domains of three virus genomes can reveal a slight variance. It has been reported that variant HBsP expression and BH3 sequence of HBV genotype may be involved in differential apoptotic effects on transfected cells<sup>[8]</sup>. However, HBV can also directly cause death of hepatocytes<sup>[6]</sup>.

## HBV TRANSMISSION AND INFECTION

In high endemic regions, such as Asia, Africa, Pacific Islands and the Arctic, early perinatal and horizontal infection in childhood is the main route of HBV transmission with a hepatitis B surface antigen (HBsAg) positive rate of 8%-15%, while in low endemic areas, such as Western countries, HBV is a predominant disease in adolescents and adults due to high risk sexual behaviors or drug injections, with a HBsAg positive rate of less than 2%<sup>[9]</sup>.

The vast majority of early perinatal or horizontal infections in childhood are the main route of HBV transmission in untreated infants whose mothers are hepatitis B e antigen (HBeAg) positive, and over 90% of them will become chronic HBV carriers. In contrast, about 90% of HBV infections may occur as acute infection and only 5%-10% may occur as chronic infection in adults. This dramatic difference in chronic rates is believed to reflect the host immunologic status and the time of infection.

Although infants whose mothers are HBeAg-positive HBV carriers are at a high risk of developing infection and subsequently become viral persistence, the age of infants at the time of HBV infection is inversely correlated with the chronic rate<sup>[10,11]</sup>. A HBc/HBe-specific Th-cell tolerance model can show the reversibility of T-cell tolerance. It has been shown that a single prenatal dose of HBeAg can result in apparent T-cell tolerance



**Figure 1 Phases of chronic hepatitis B virus (HBV) infection.** Thick arrows indicate HBV infection rates in different age groups, dotted arrows indicate changes in histology, grey arrows indicate changes at risk of progressing to cirrhosis or HCC (E+: HBeAg positive; E-: HBeAg negative).

in mice at the age of 8-12 wk, but the tolerance may disappear at the age of 16 wk<sup>[12]</sup>, suggesting that T-cell tolerance can be maintained and HBcAg/HBeAg will continuously present. HBc/HBe-specific thymocytes are absent in thymus and this “repertoire renewal process” requires about 16 wk. Similarly, human fetus may be exposed to tolerogenic HBeAg in uterus not infected at birth. The longer the elapsed time before HBV infection is, the greater the probability of renewing HBc/HBe-specific T-cell repertoire is, because the neonate would no longer be exposed to HBeAg<sup>[13]</sup>.

There is an obvious difference between patients infected with HBV in adolescence or adulthood immediately entering immune clearance phase, and short duration and tendency quiescent after seroconversion from HBeAg to antibody against HBeAg (anti-HBe). Such patients are termed “healthy” carriers. In contrast, patients with early HBV infection have a prolonged immune tolerance phase and a prolonged immune clearance phase, indicating that their diseases tend to progress after HBeAg seroconversion.

## STAGES OF HBV INFECTION

Remarkable progress has been made in our understanding of the four natural stages of chronic hepatitis B (CHB): immune tolerance stage, immune clearance stage, inactive HBsAg carrier stage, and reactivation stage. However, not all CHB infection patients go through all the four stages (Figure 1).

### Immune tolerance stage

Patients with perinatal or early childhood-acquired HBV infection have an initial tolerance stage characterized by the presence of HBeAg, high serum DNA level, normal serum aminotransferase level, and minimal or

no inflammation on liver biopsy<sup>[14]</sup>. Such manifestations can rarely be seen in those who are infected with HBV in later childhood or adulthood and whose infection subsequently develop into chronic HBV infection<sup>[14]</sup>.

Although a high serum DNA level in liver disease patients with minimal or no inflammation is considered as a sequela of immune tolerance to HBeAg, it has been shown that HBeAg may promote HBV chronicity by functioning as an immunoregulatory protein<sup>[15]</sup>. For example, in transgenic mice, transplacental transfer of maternal HBeAg may preferentially behave as a tolerogen and inactive HBe/HBc-specific Th cells through at least central deletion of high-affinity HBe/HBcAg-specific CD4 T cells or clonal ignorance and anergy in periphery blood, resulting in ineffective cytotoxic T cell lysis of infected hepatocytes. Such a mechanism may be responsible for the high chronic HBV infection rate (-90%) observed in babies infected by their HBeAg positive mothers accounting for the inability of infants to clear perinatal HBV infection. After neonatal or prenatal HBV infection (absent in uterus tolerance), secretion of monomeric HBeAg in the relatively Th2-biased neonatal immune system may also have an anti-inflammatory influence on nucleoprotein-specific immune response by eliciting Th2-like cytokines. Secreted HBeAg can also enter thymus. It has been subsequently reported that HBeAg specific Th2-like cells can preferentially survive tolerance production to a greater extent than HBeAg-specific Th1-like cells<sup>[16]</sup>. Therefore, chronicity resulting from vertical transmission of HBV characterized by the predominance of HBeAg-specific Th2-like cells and secretion of anti-inflammatory cytokines, such as IL-4, IL-5, and IL-10, can enhance antibody production, and viral persistence would characterize the HBeAg-specific T-cell response. If the *status quo* of various clonal tolerance phenotypes could be maintained as long as the HBeAg concentration and/or the non-inflammatory hepatic environment remain unchanged, the immune tolerance would last 1-4 decades. During this phase, the rate of spontaneous or treatment-induced HBeAg seroconversion is less than 5%<sup>[17,18]</sup>. Patients in the immune tolerance phase are considered at a low risk of progressing to cirrhosis or HCC based on serial monitoring of virological, clinical and ultrasonographic assessments.

Although antiviral therapy is not recommended for immune tolerance patients, they should be closely monitored for progression to the immune clearance phase. Once this occurs, antiviral therapy should be considered more diligently after 6-12 mo if HBeAg seroconversion does not occur because disease progression can occur in the immune clearance phase.

### **Immune clearance stage**

As the host immune system matures, a nonspecific increase in hepatic inflammation or decrease in HBeAg serum concentration, perhaps due to the emergence of core promoter region or pre-core region mutants, may allow activation of intermediate-or low-avidity HBeAg-specific T cell clones that are not physically deleted and/or reverse the anergic state of others<sup>[17]</sup>. Therefore,

treatment modalities for chronic HBV infection should be directed at activating the relatively low-avidity HBeAg-specific T cells. Such a shift from HBeAg-specific Th2 cell tolerance to Th1 cell activation may recognize HBV-related epitopes on hepatocytes, and immune-mediated hepatocellular injury ensues, the so called clearance phase of CHB infection. Then, IL-2, INF and tumor necrosis factor are secreted following inflammation. The HBcAg-specific T-cell response is characterized by CTL induction, liver injury and inhibition of viral replication<sup>[6]</sup>. In patients with prenatally acquired HBV infection, transition from immune tolerance phase to immune clearance phase occurs during the second or third decade of life. Although HBV replication and viremia continue in the liver, the serum virus level becomes lower in immune clearance phase than in immune tolerance phase when viral replication is completely unopposed. The active phase of CHB is often marked by increased levels of alanine aminotransferase (ALT), necrotic inflammatory activity, and cycling HBV-DNA and HBeAg due to liver injury. Pre-core minus mutants and mutations within the core gene begin to accumulate at the time of ALT flare up because they have a better ability to evade immune clearance<sup>[19]</sup>, suggesting that nucleoprotein antigens are the major immune attacking foci during chronic HBV infection, perhaps because the nucleoprotein-specific T-cell repertoire has been eroded to a lesser extent than the envelope-specific repertoire simply due to the lower concentration of HBe/HBcAgs. CD4<sup>+</sup> HBeAg-specific T cells, identified in HBeAg-single-Tg and TCR-HBeAg-Tg mice, are not deleted or anergized and remain quiescent in the presence of serum HBeAg, but can mediate seroconversion and liver injury once they are activated. These HBeAg-specific T cells escape tolerance induction due to their low avidity and/or low TCR density<sup>[18]</sup>.

This active phase is characterized by the presence of HBeAg, high serum HBV DNA and aminotransferase levels, as well as active inflammations and fibrosis in the liver. A key event in the natural history of HBeAg positive CHB patients is HBeAg seroconversion<sup>[20]</sup>. Several studies have shown that seroconversion with a marked reduction in HBV replication is associated with biochemical and histological remission of inflammatory activity in the majority of patients<sup>[2,10,20]</sup>. Most studies showed that the mean annual rate of spontaneous HBeAg seroconversion ranges 8%-15% in children or adults with an elevated ALT level<sup>[20]</sup>. Although the ALT level is normal in most Asian children, their spontaneous HBeAg seroconversion rate is less than 2% during the first 3 years of age and then increases to 4%-5%. In some cases, spontaneous flare up of hepatitis is not frequently recognized because it is usually asymptomatic. Since subsequent HBeAg seroconversion would not occur in such flare up of hepatitis, it can thus be viewed as an abortive attempt at seroconversion. However, some patients present with a symptomatic flare up of hepatitis that mimics acute hepatitis and even present with fulminant hepatic failure. Regression of fibrosis occurs several months or years after HBeAg seroconversion. These flare ups of hepatitis may precede the disappearance

of HBeAg and development of HBeAg antibody, culminating in the remission of hepatitis activity. It has been recognized that the duration of immune clearance phase and the frequency and severity of flare ups are correlated with the risk of progressing to cirrhosis and HCC<sup>[21-23]</sup>.

### **Inactive HBsAg carrier stage**

This inflammatory phase of HBV infection also leads to HBeAg seroconversion and enters into inactive HBsAg carrier status. Inactive carriers form the largest group of chronic HBV infection patients. After seroconversion, most patients remain negative for HBeAg and positive for anti-HBe antibody with an undetectable or a low HBV DNA level, while the minority have undetectable viral loads. Biopsy findings can range from mild inflammation and minimal fibrosis to inactive cirrhosis if the disease is severe during immune clearance<sup>[20]</sup>.

The progress of inactive HBsAg carrier state is usually benign. A long-term follow-up (up to 18 years) of these carriers can indicate a sustained biochemical remission and a very low risk of developing cirrhosis or HCC in them<sup>[19]</sup>. Patients even with no cirrhosis may develop liver cancer in their inactive HBsAg carrier state. In addition, approximately 20%-30% of inactive HBsAg carriers may undergo spontaneous reactivation of hepatitis B during the follow-up. Multiple episodes of reactivation or sustained reactivation can cause progressive liver damage and even decompensation. HBV reactivation is usually asymptomatic but can occasionally mimic acute viral hepatitis. Some carriers eventually become HBsAg negative and develop anti-HBs. The estimated incidence of delayed HBsAg clearance is 1%-2% per year in Western countries where HBV infection is acquired in adulthood, and 0.05%-0.8% per year in endemic areas where HBV infection is mostly acquired perinatally or in early childhood. Prognosis can be improved by loss of HBsAg as liver disease is inactive or non-progressive, but HBsAg clearance does not completely prevent occurrence of liver decompensation or HCC in patients with cirrhosis<sup>[23-25]</sup>.

### **Reactivation stage**

Chronic HBeAg-negative patients can be divided into chronic inactive HBsAg carriers and CHB patients with biochemical and intermittent virological activity<sup>[5]</sup>. HBeAg-negative chronic hepatitis may recur in one third of inactive HBV carriers without serum reversion of HBeAg<sup>[22,23]</sup>.

It is believed that seroconversion of HBeAg to HBeAb is accompanied with cessation of HBV replication and remission of liver disease. However, HBsAg-negative CHB has been recognized as an important form of chronic hepatitis, and e-antigen negativity is due to mutations in pre-core and core promoter regions. The most frequent pre-core mutation is a G-A change in nucleotide 1896 (G1896A) which creates a stop codon<sup>[23]</sup> and the most common core promoter mutation involves a substitution of nucleotides 1762 and 1764, which can result in loss of HBeAg synthesis. Loss of circulating HBeAg can decrease the induction of HBeAg-specific Th2 cell activity and result in a predominance of inflammatory

Th1-like cells<sup>[16]</sup>. HBeAg-negative CHB (pre-core mutant) occurs as the predominant species during typical HBV infection with wild-type virus which is selected during the immune clearance phase (HBeAg seroconversion). Several studies have shown that HBeAg may be a target antigen on HBV-infected hepatocytes<sup>[5,15,18]</sup>. Failure to produce a target antigen may be a means of evading immune clearance. The clonal heterogeneity of HBeAg-specific T-cell tolerance may explain how a primarily tolerogenic protein can exert its pressure on the immune response to the selection of HBeAg negative mutant. For example, high-avidity HBeAg-specific T-cell clones may be tolerized and low-activity T-cell clones may be activated and involved in selecting HBeAg-negative mutant in the same patient<sup>[18,24-26]</sup>. The occurrence of HBeAg-negative mutants during chronic active HBV infection, especially in the presence of a high viral load, is correlated with an exacerbation of liver injury and a worse prognosis. Serum HBeAg can act as an efficient T-cell tolerogen which reduces the frequency of liver injury and down-regulates anti-HBc production. Anergy of HBc/HBeAg and HBeAg specific T-cells depends on HBeAg concentration and is reversible in the absence of HBeAg, which may explain the correlation between pre-core and core promoter mutations and severe liver injury<sup>[19,22,27,28]</sup>.

Progress to this phase occurs spontaneously or to inactive carriers during immune suppression. Some patients can progress directly from HBeAg positive to HBeAg negative CHB. Identification of pre-core/core promoter mutations and recognition of HBeAg negative CHB indicate that the disease occurs after HBeAg seroconversion<sup>[19]</sup>. Age is significantly higher in HBeAg-negative patients than in HBeAg-positive patients. ALT and HBV DNA levels are significantly lower in e-antigen negative patients than in e-antigen positive patients. However, spontaneous recovery is rarer, long-term prognosis is poorer, and histological lesions are more severe in HBeAg-negative patients than in HBeAg-positive patients. Necrotic inflammatory activity is almost identical in both HBeAg-negative and positive patients. However, fibrotic activity is higher in e-antigen negative patients than in e-antigen positive patients. The estimated annual incidence of cirrhosis is 2%-6% in HBeAg positive CHB patients and 8%-10% in HBeAg negative CHB patients. The higher incidence of cirrhosis in HBeAg-negative patients is related to age and fibrosis stage, suggesting that HBeAg-negative chronic hepatitis can progress to cirrhosis and HCC in the natural history of HBV infection rather than *de novo* infection with HBV variants that do not produce HBeAg<sup>[29-31]</sup>. HBeAg-specific T cell tolerance is reversible in the absence of tolerogen. Since antiviral treatment can reduce HBeAg and viral load possibly in combination with HBc/HBeAg-specific immunization, it can alleviate chronic HBV infection by shifting the cytokine profile from Th2 to Th0/1<sup>[26,32]</sup>.

### **Occult HBV infection**

Occult HBV infection is defined as the existence of HBV DNA in serum, although it is not considered as a phase of CHB<sup>[33,34]</sup>.

In addition to a symptomatic and serologically

**Table 1 Characteristics of chronic hepatitis B at different stages**

Phase	ALT	HBsAg	HBeAg	HBeAb	HBV DNA (IU/mL)	Th cell biased	Liver histology
Immune tolerance	Usually normal	Present	Present	Absent	≥ 20000	Th <sub>2</sub> cell	Normal or mild inflammation
Immune clearance	Elevated	Present	Present	Absent	≥ 20000	Th <sub>2</sub> /Th <sub>1</sub> cell	Active inflammation
Inactive HBsAg carrier	Usually normal; can have flares	Present	Absent	Present	> 20000	Th <sub>1</sub> cell	Mild inflammation or inactive cirrhosis
HBeAg <sup>-</sup> CHB	Period flares	Present	Absent	Present	> 20000 < 20000	Th <sub>1</sub> cell	Active inflammation
Occult hepatitis B	Rarely elevated	Absent	Absent	Present when HBV recovered	< 20000	Th <sub>1</sub> cell	From normal to cirrhosis HCC

evident infection, occult persistent HBV carriage has been identified since nucleic acid amplification assay enhances its sensitivity to hepadnaviral genomes and their replicative intermediates. There is evidence that occult HBV infection is a common and long-term consequence of acute hepatitis B resolution. This form of residual infection is termed as secondary occult infection (SOI). The data from the woodchuck model of HBV infection indicate that exposure to a small amount of hepadnavirus can also cause primary occult infection where virus genome but not serological makers of virus exposure are detectable without liver involvement. However, both forms of virus replicate at a low level in the lymphatic system. Serological testing for SOI can reveal the presence of antibodies to HBV core antigen (anti-HBc), which has been recognized not only as a valuable marker of prior HBV exposure but also as an indicator of progressing occult HBV infection<sup>[35]</sup>. It has been recently reported that up to 20% of individuals with occult HBV carriage are not reactive to anti-HB<sub>x</sub> or any other serological indicators of HBV exposure, and detection of naturally acquired antibodies to HBsAg (anti-HBc) does not exclude the existence of occult HBV infection<sup>[33,36]</sup>.

The severe consequences of occult HBV infection have not been fully recognized. There is evidence that occult HBV can be a source of virus contamination in blood and organ donations, as well as a reservoir from which full blown hepatitis can arise<sup>[37]</sup>. Case reports also indicate that immunosuppression caused by chemotherapy or immunomodulatory agents or immunodeficiency due to HIV infection or hematological malignancies can induce reactive occult infection<sup>[38,39]</sup>. Mild necrotic inflammation has been documented in liver samples obtained from acute hepatitis B patients many years after recovery<sup>[40]</sup>. Liver fibrosis and cirrhosis of unknown origin have been explained by occult HBV infection in many retrospective studies<sup>[35,41,42]</sup>. The oncogenic potency of occult HBV persistence becomes progressively evident and is further elevated in alcoholics and patients with other liver ailments like hepatitis C<sup>[41,42]</sup>. No reports are available on the treatment of occult HBV infection (Table 1).

## CONSEQUENCES OF CHRONIC HBV INFECTION

Individuals with chronic HBV infection are at an increased risk of developing end-stage liver diseases

including cirrhosis, hepatic failure, and HCC. It has recently been estimated that about 53% of HCC cases in the world are related to HBV infection. The lifetime risk of developing HCC is increased even in patients with cleared HBsAg or occult HBV infection. Further risk factors include chronic HCV infection, exposure to aflatoxin B<sub>1</sub>, alcohol abuse, obesity and diabetes<sup>[4,43]</sup>. Thus, it is important to identify HBV-infected patients at a higher risk of progressing to HCC.

The reason why some CHB patients progress to HCC remains unknown. Host factors, such as immune response to HBV, genetic predisposition to HCC, high HBV replication rate, mutations within the HBV genome, are related with HCC. Many observations revealed that the major factor for the development of HBV-associated HCC is the immune system<sup>[41,43,45]</sup>. Development of hepatitis, chronic hepatitis, and HCC could be exclusively observed in mice reconstituted with bone marrow and in non-transgenic animals, but not in controls, suggesting that ineffective immune response is the principle oncogenic factor during chronic HBV infection of human beings. In other words, the same T-cell response has different effects. If T cell response is strong enough, HBV can be eliminated from the liver. If not, a pro-carcinogenic effect can be induced by triggering necrotic inflammatory disease without final eradication of HBV from the liver. It can, thus, be concluded that the immune system-mediated chronic inflammation of the liver, continuous cell death and subsequent cell proliferation may increase the frequency of genetic alteration and the risk of developing cancer. However, the molecular basis of inflammatory liver carcinogenesis caused by HBV remains largely unsolved. Cytokines modulate inflammation and the presence of inflammatory cells with the production of inflammatory cytokines activates cellular oxidant-generating pathways. Reactive oxygen species that are generated in inflammatory conditions induce oxidative DNA damage and increased oxidative stress caused by chronic inflammation can produce genetic mutations and gross chromosomal alterations<sup>[4,44,45]</sup>. Extensively oxidative DNA damage has been detected in hepatocytes of HBV-transgenic mice and humans with chronic hepatitis<sup>[46]</sup>.

HBV genotype C infection is associated with a higher risk of developing HCC than HBV genotype B infection<sup>[29]</sup>. The BCP A1762T/G1764A mutant is associated with an increased risk of developing HCC

compared with the double wild type variant, whereas the pre-core G1896A mutation is associated with a decreased risk of developing HCC compared with the wild-type variant. Several mechanisms of liver carcinogen are related to the BCP A1762T/G1764A mutation which may enhance HBV virulence by increasing host immune response and viral replication, or by altering the coding region of the X antigen. Mutant BCP may augment the host immune response to HBV-infected hepatocytes by diminishing circulating HBeAg and increasing hepatocyte apoptosis and regeneration, thus leading to liver injury<sup>[47,48]</sup>. The BCP mutation appears to enhance the efficacy of viral replication either by modulating the relative levels of pre-core and core RNAs or by creating a transcription factor binding site for hepatocyte nuclear factor 1. Mutations in the BCP region over lapping the coding sequence of the X antigen of HBV may result in changes of amino acids, K130M and V131I, in the X gene. These amino acid changes may interfere with cell growth control and DNA repair, thus leading to HCC<sup>[49,50]</sup>. There is experimental evidence that HBx, a multifunctional protein with oncogenic potentials, can interact with a large number of cellular factors and modulate their normal function, thus leading to deregulation of normal cell activities and HCC<sup>[46,51]</sup>. Despite its importance in HCC development, the clinical significance of genetic variability in the X genetic region still remains poorly understood<sup>[52]</sup>.

Several factors, including age, male gender, repeated episodes of severe acute exacerbation, and HBV reactivation after HBeAg seroconversion, are related with the risk of developing advanced liver disease in patients with CHB. Previous studies showed that HBV genotype C infection is associated with later HBeAg seroconversion and multiple episodes of acute exacerbation without HBeAg seroconversion than genotype B HBV infection<sup>[9,21,25,30,49]</sup>. The delayed HBeAg seroconversion may prolong the inflammation process and subsequently result in more severe liver damage<sup>[50]</sup>. Several nucleotide mutations in the pre-core and core promoter regions may reduce HBeAg production and are associated with advanced liver disease<sup>[47]</sup>. In Asia, genotype C and T1762 and A1764 mutants may play a role in HBV-related liver cirrhosis, and can be used in predicting the clinical outcome of patients with chronic HBV infection.

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ORIGINAL ARTICLES

## Constructive thinking, rational intelligence and irritable bowel syndrome

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### Abstract

**AIM:** To evaluate rational and experiential intelligence in irritable bowel syndrome (IBS) sufferers.

**METHODS:** We recruited 100 subjects with IBS as per Rome II criteria (50 consulters and 50 non-consulters) and 100 healthy controls, matched by age, sex and educational level. Cases and controls completed a clinical questionnaire (including symptom characteristics and medical consultation) and the following tests: rational-intelligence (Wechsler Adult Intelligence Scale, 3rd edition); experiential-intelligence (Constructive Thinking Inventory); personality (NEO personality inventory); psychopathology (MMPI-2), anxiety (state-trait anxiety inventory) and life events (social readjustment rating scale). Analysis of variance was used to compare the test results of IBS-sufferers and controls, and a logistic regression model was then constructed and adjusted for age, sex and educational level to evaluate any possible association with IBS.

**RESULTS:** No differences were found between IBS cases and controls in terms of IQ ( $102.0 \pm 10.8$  vs  $102.8 \pm 12.6$ ), but IBS sufferers scored significantly lower in global constructive thinking ( $43.7 \pm 9.4$  vs  $49.6 \pm 9.7$ ). In the logistic regression model, global constructive thinking score was independently linked

to suffering from IBS [OR 0.92 (0.87-0.97)], without significant OR for total IQ.

**CONCLUSION:** IBS subjects do not show lower rational intelligence than controls, but lower experiential intelligence is nevertheless associated with IBS.

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**Key words:** Constructive thinking; Intelligence tests; Intelligence; Irritable bowel syndrome

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### INTRODUCTION

Irritable bowel syndrome (IBS) is a common disease worldwide, with a prevalence in the general population ranging from 2% to 15%, depending on the definition criteria used<sup>[1,2]</sup>. Its pathophysiology is unknown, yet several biological factors have been implicated<sup>[3]</sup>; and aside from these biological factors, psychosocial factors have long been known to be involved in IBS. While anxiety has consistently been associated with IBS<sup>[4]</sup>, to what precise degree it is a cause or consequence remains unresolved<sup>[5]</sup>. Personality traits, and neuroticism in particular, have been linked to IBS, although it is a matter of debate whether they are associated with consultation behavior<sup>[6]</sup> or with the disease itself<sup>[7]</sup>.

Cognition (beliefs, interpretation and expectations) plays a pivotal role in the interaction of subjects with

the environment, and cognitive abilities should drive this interaction on a daily basis and for long-term success. Several items of information suggest that cognitive processes may be important in IBS. Abnormal illness behavior which has been associated with many chronic diseases, including non-consulters' IBS<sup>[8]</sup>, is to a great extent the result of a cognitive process<sup>[9]</sup>, involving appraisal and interpretation, and is likely associated with worry<sup>[10]</sup>. Hypervigilance as to abdominal perceptions may be viewed as a cognitive process<sup>[11,12]</sup>. IBS patients use less effective coping styles<sup>[6,13]</sup>, which are indeed modulated (appraisal of threat, adapting the response to the situation) by the use of cognitive abilities.

Although intelligence is not easy to define<sup>[14]</sup>, it may be construed as the ability to solve problems<sup>[15]</sup>. Problems arising from life may be categorized as abstract problems, which call for an analytical approach and a slow response, and daily life problems, which call for automatic analysis and a quick response. Solving abstract problems is a task performed by rational intelligence and can be measured by IQ<sup>[14]</sup>. IQ has been found to be associated with health<sup>[16]</sup> and longevity<sup>[17]</sup>, likely through an influence on health knowledge and health behavior<sup>[18]</sup>. Attree *et al*<sup>[19]</sup> recently found a lower IQ in IBS subjects *vs* controls. Rational intelligence might enhance one's ability to identify environmental factors precipitating bowel symptoms and change one's lifestyle accordingly.

Solving daily problems is not a rational task but rather relies on cognitive abilities to interpret events efficiently. Epstein proposed the concept of constructive thinking (experiential intelligence), defined as automatic thoughts in daily life to survive at a minimum cost of stress<sup>[20]</sup>. According to his cognitive-experiential theory, constructive thinking operates passively and automatically at a preconscious level, thinking in terms of associations and broad categories, is closely connected with emotions, interpreting experience, and guiding conscious thoughts and behaviour. It can be measured by the Constructive Thinking Inventory (CTI), which is independent of IQ and moderately related to success in life and physical well-being<sup>[21]</sup>. Under the hypothesis of stress-driven changes in brain-gut interaction (IBS as emotional motor system output)<sup>[22]</sup>, it might be hypothesized that the lower a person's constructive thinking, the higher the stress generated by daily life and, by extension, the higher the possibility of suffering from IBS symptoms.

Accordingly, this study sought primarily to assess whether IBS sufferers might be different to healthy non-IBS sufferers in terms of intelligence (rational and experiential), and, secondarily, whether there might be a link between intelligence and IBS-related medical consultation.

## MATERIALS AND METHODS

### Study design

Case-control study including IBS sufferers and matched healthy subjects.

### Study population

Cases were defined as subjects, both consulters and non-consulters, who met the Rome II IBS criteria. An IBS consulter was defined as any currently symptomatic subject who had consulted a physician due to bowel symptoms and had been diagnosed with IBS after an appropriate work-up. An IBS non-consulter was defined as any subject from the general population with symptoms of IBS who had not consulted a physician in this connection.

Controls were defined as subjects from the general population without recurrent abdominal pain or bowel symptoms, who suffered no relevant chronic diseases and had undergone no relevant abdominal surgery.

### Recruitment, selection and inclusion

IBS consulters were recruited from primary care and secondary-level gastroenterologist offices at the 7th Health Area of Madrid (Spain), which provides medical attention to approximately 515 000 inhabitants. Patients diagnosed with IBS in accordance with the above definition, were invited to participate. Patients were not enrolled at a tertiary-care facility so as to better represent the population of patients with IBS.

IBS non-consulters and healthy controls were recruited from the general population residing in the same geographical area as patients. Members of the public were directly approached at corporate offices, leisure centers or department stores and invited to participate in the study. Relatives of recruited IBS patients and patients or patients' relatives attending medical facilities were excluded. After initial agreement, all subjects were briefly interviewed about any medical conditions (to exclude relevant chronic diseases), recurrent abdominal pain, bowel-related symptoms (to classify them as potential IBS subjects as per Rome II criteria or as subjects free of bowel-related symptoms), and prior medical consultation on account of such symptoms (to classify them as non-consulters): subjects with bowel-related symptoms who failed to meet the Rome II criteria as well as subjects who met the Rome II criteria but reported consulting a physician in this regard were not selected.

All subjects selected-IBS consulters, non-consulters, and controls-completed a clinical questionnaire, which included questions on sociodemographic data, symptoms and medical resource utilization, including number of physician visits due to bowel symptoms in the prior year. This questionnaire was successfully used in a population-based IBS study<sup>[2]</sup>, and enables Rome II criteria as well as consultation behavior in the preceding year to be verified. Inclusion criteria for IBS subjects (patients and non-patients) were age 18-65 years and Rome II criteria checked by the clinical questionnaire. Healthy controls were matched to IBS patients by age ( $\pm$  5 years), sex, and educational level (junior school, high school, and university). Exclusion criteria were history of psychiatric disease requiring treatment; significant visual or hearing deficit; and inability to complete the set of instruments used in the study.

## Instruments

In addition to the clinical questionnaire, the study design included instruments that measured IBS severity, rational intelligence, non-intellectual intelligence, personality traits, psychopathology, and life events. These instruments were respectively.

### **Functional bowel disease severity index (FBDSI)**

The FBDSI was developed by Drossman *et al*<sup>[23]</sup> and has been shown to correlate with symptoms interference with daily functioning and health related quality of life<sup>[24,25]</sup>. It comprises three variables, namely: current pain [evaluated by a visual analog scale (VAS)]; diagnosis of functional chronic abdominal pain (chronic pain without bowel dysfunction); and the number of medical visits in the preceding 6 mo. Severity was classified as mild (< 37), moderate (37-110) or severe (> 110). Since severity in IBS subjects as rated by the complete index included medical consultation, both the complete FBDSI and current pain assessed by VAS were analyzed.

### **Wechsler Adult Intelligence Scale, 3rd edition (WAIS-III)**

The WAIS-III was designed as a comprehensive test of cognitive ability for adults. It contains 11 subtests and three additional subtests. Variables obtained are three IQ scores (total IQ, verbal IQ, and performance IQ). It has been adapted and validated for use in the Spanish population, and results were scored using normative values<sup>[26]</sup>. The test was taken in quiet surroundings and all subjects underwent a brief psychological interview beforehand; special care was taken to minimize potential anxiety regarding test performance. Moreover, it was clearly stated at the time of inclusion that test results would in no case be linked to any care change in IBS subjects or to any specific psychological intervention. Subjective scoring of the WAIS-III was done blinded to subjects' study group.

## CTI

The CTI is a self-administered test developed by Epstein under the conceptual framework of Cognitive-Experiential Theory<sup>[20]</sup>. Constructive thinking is a set of automatic habitual thoughts used in daily life, and is regarded as a measure of experiential intelligence<sup>[21]</sup>. CTI provides a global measure of constructive thinking, six subscales (Emotional coping, Behavioral coping, Personal superstitious thinking, Categorical thinking, Esoteric thinking, and Naïve optimism), and two validity subscales (Defensiveness scale and Validity scale). This test has been adapted and validated for use in the Spanish population, normative values for which are available<sup>[27]</sup>. T-scores were obtained using a computerized scoring software program. Under the rules of the Spanish Manual, tests were deemed invalid when the T-score was above 70 on the defensiveness scale or below 30 on the validity scale.

### **NEO personality inventory (NEO-PI)**

The NEO-PI is a widely used personality inventory designed to measure personality based on the "big five"

theory. The Spanish version is a validated instrument and normative data are available. T-scores for the five traits (neuroticism, extroversion, openness, agreeableness, and conscientiousness) were obtained using a computerized scoring software program.

### **State-trait anxiety inventory (STAI)**

A widely used questionnaire with a validated Spanish version, the STAI is a 40-item self-report measure designed to assess anxiety. Subjects indicate how they generally or right now feel, by rating the frequency of their feelings of anxiety on a 4-point scale ranging from 1 (almost never) to 4 (almost always).

### **Minnesota multiphasic personality inventory 2 (MMPI-2)**

The MMPI-2 is a widely used inventory for the assessment of psychopathological personality. Normative values for the Spanish population are available. Clinical scales were included (hypochondriasis, depression, hysteria, psychopathic deviate, paranoia, psychasthenia, schizophrenia, hypomania, and social introversion).

### **Social readjustment rating scale (SRRS)**

The SRRS is a measure of stressful life events developed by Holmes and Rahe in 1967<sup>[28]</sup>, consisting of a 61-item list of significant life events. A validated Spanish version exists, which includes a scale of each item's emotional impact scored from 0 to 100<sup>[29]</sup>. On the 61-item checklist, the participants marked events that they had experienced in the previous year. The number of stressful life events and their total emotional impact in the preceding year were obtained for each subject.

## Statistical analysis

Primary analysis was intended to disclose differences in intelligence (rational and non-intellectual) between IBS subjects (both patients and non-patients) and healthy controls. For this purpose, analysis of variance was used to perform univariate comparisons of WAIS-III and CTI measures, and sociodemographic data, personality traits and life events were also compared between the respective groups. To evaluate to what extent global constructive thinking and IQ variables might be associated with suffering from IBS, a logistic regression model was constructed (model 1), adjusting for age, sex and educational level; a full model was constructed to adjust for variables showing significant differences between IBS and control subjects, excluding those with correlation coefficients of 0.7 or higher with Global CTI score or IQ.

A secondary analysis was performed to evaluate the possible implication of measured factors in medical consultation sought because of IBS symptoms. To this end, IBS consulters and non-consulters were compared, using the same statistical model. To evaluate illness behavior, a multiple regression model was constructed (stepwise forward method), with the number of physician visits in the last year as a dependent variable and global constructive thinking, total IQ, neuroticism, number of life stressful events, clinical scales of MMPI-2

and state and trait anxiety as independent variables. Data are expressed as mean  $\pm$  SD, unless otherwise stated. Statistical analysis was performed using the SPSS version 13 computer software package.

### **Composition of case group and calculation of sample size**

We chose a ratio of 1:1 for IBS consulters and non-consulters in the case group, which may well represent the entire IBS population in this country (49% of IBS subjects as defined by the Rome II criteria had visited a physician in the previous year)<sup>[30]</sup>.

As there were no experimental data at the time of protocol design (January 2003) for making assumptions in similar settings, a five-point difference in the overall CTI scale was taken as relevant. This five-point estimate was based on the Spanish correction manual, which suggests that T-scores of 45 to 55 be regarded as normal, and 35 to 44 as moderately low. Since the mean T score for the general population could be expected to be 50, the minimum value for inferring a relevant lower score would be 44 (a five-point margin being sufficient to detect relevant differences). Hence, assuming a standard deviation of 10 (since this is a T-score), and with  $\alpha$  and  $\beta$  risks set at 0.05 and 0.1 respectively, 85 subjects would be needed in each group.

In so far as IQ was concerned, a difference of 10 points was estimated as relevant. Assuming a standard deviation of 15, then, with the same  $\alpha$  and  $\beta$  risk, the sample size would be 56 subjects in each group. Accordingly, a sample of 85 subjects in each group would be enough to enable a mean difference of eight points or higher to be detected. Allowing for the possibility of a 15% data loss, the final sample size was set at 100 subjects per group.

For the secondary analysis, assuming the same relevant differences in mean scores and the same standard deviations, a sample size of 50 per group (consulters and non-consulters) would provide a power of 80% with an  $\alpha$  risk of 0.1.

### **Ethics**

The study was formally approved by the Institutional Ethics Committee, and prior informed consent was obtained in writing from all participants.

## **RESULTS**

A total of 73 IBS consulters and 81 IBS non-consulters were recruited. Twenty-three IBS consulters were not included because of psychiatric diagnosis requiring treatment (four patients), not accomplishing Rome II criteria (two patients), incomplete information (one patient) or lack of adequate matching control (16 patients). Thirty-one IBS non-consulters were not included due to not accomplishing Rome II criteria (10 subjects), incomplete information (one subject) or lack of adequate matching control (20 subjects).

The study covered 100 subjects with IBS (50 consulters and 50 non-consulters) and 100 matching healthy controls. Of these, 70 were female in each case. The mean age of

**Table 1 Comparison of intelligence, personality, and life events between controls and IBS subjects**

	<b>Healthy subjects (n = 94)</b>	<b>IBS subjects (n = 94)</b>
Rational intelligence (WAIS-III)		
Total IQ	102.8 $\pm$ 12.6	102.0 $\pm$ 10.8
Verbal IQ	103.4 $\pm$ 11.6	102.6 $\pm$ 10.7
Performance IQ	102.4 $\pm$ 14.2	101.6 $\pm$ 11.6
Experiential intelligence (CTI)		
Global CTI score	49.6 $\pm$ 9.7	43.7 $\pm$ 9.4 <sup>b</sup>
Personality traits (NEO-PI)		
Neuroticism	55.0 $\pm$ 10.6	60.9 $\pm$ 8.7 <sup>b</sup>
Extroversion	44.6 $\pm$ 11.0	45.4 $\pm$ 10.9
Openness	49.6 $\pm$ 12.6	50.6 $\pm$ 12.2
Agreeableness	45.3 $\pm$ 7.9	44.1 $\pm$ 9.0
Consciousness	37.8 $\pm$ 9.0	35.9 $\pm$ 8.2
Psychopathology (MMPI-2)		
1-Hypochondriasis	49.6 $\pm$ 9.2	62.2 $\pm$ 12.1 <sup>b</sup>
2-Depression	48.7 $\pm$ 9.6	52.9 $\pm$ 10.9 <sup>a</sup>
3-Hysteria	52.2 $\pm$ 9.6	60.5 $\pm$ 11.5 <sup>b</sup>
4-Psychopathic deviate	49.8 $\pm$ 9.2	54.8 $\pm$ 11.0 <sup>b</sup>
5-Masculinity-femininity	52.2 $\pm$ 10.6	50.3 $\pm$ 10.0
6-Paranoia	48.2 $\pm$ 8.2	52.3 $\pm$ 9.2 <sup>a</sup>
7-Psychastenia	46.8 $\pm$ 8.7	51.7 $\pm$ 8.3 <sup>b</sup>
8-Schizophrenia	47.0 $\pm$ 8.1	51.5 $\pm$ 10.0 <sup>b</sup>
9-Hypomania	49.1 $\pm$ 9.7	52.3 $\pm$ 10.3 <sup>a</sup>
0-Social introversion	47.4 $\pm$ 8.9	48.6 $\pm$ 8.3
Anxiety (STAI)		
State anxiety	33.4 $\pm$ 8.8	40.5 $\pm$ 11.4 <sup>b</sup>
Trait anxiety	37.1 $\pm$ 9.2	43.7 $\pm$ 10.8 <sup>b</sup>
Stressful life events (SRRS)		
Number in prior 12 mo	8.5 $\pm$ 5.1	9.9 $\pm$ 4.8 <sup>a</sup>
Total emotional impact	445.1 $\pm$ 280.4	521.9 $\pm$ 252.5 <sup>a</sup>

IBS: Irritable bowel syndrome. <sup>a</sup>P < 0.05 (ANOVA), <sup>b</sup>P < 0.001 (ANOVA) vs controls.

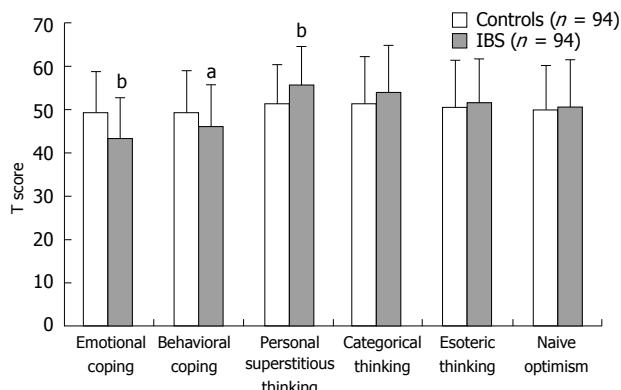
IBS subjects was 37.2  $\pm$  12.6 years, and there were no differences vis-à-vis healthy controls (37.2  $\pm$  13.1). Both groups reported 18 subjects with junior school, 23 with high school, and 59 with university education. Controls and IBS subjects were similar in terms of: marital status (single 50 and 53, and married 43 and 40, respectively); work status (student 10 and 9, gainfully employed 85 and 84, retired or unemployed 7 and 7, respectively); and family monthly income (€ 2684  $\pm$  1469 and € 2354  $\pm$  2272, respectively).

### **IBS features**

Among IBS sufferers, 31 were classified as per the Rome II criteria as diarrhea-predominant, 26 as constipation-predominant, and 43 as alternating. With respect to severity of IBS, 55 subjects were classified as mild, 37 as moderate, and eight as severe, with a mean FDSI score of 47.0  $\pm$  49.4, and a mean current VAS pain of 29.2  $\pm$  24.7. Seventy-seven subjects reported IBS symptoms of more than 2 years duration.

### **Intelligence, personality, psychopathology, anxiety and life events in IBS subjects**

Comparison of intelligence, personality, and life events between controls and IBS subjects were shown in Table 1. Six subjects (four controls, one IBS consulter, and one IBS non-consultor) produced invalid CTI scores, so that they and their matched counterparts were excluded from the analysis. IBS subjects registered similar IQ test results



**Figure 1** Subscales of constructive thinking in IBS and controls. <sup>a</sup>P < 0.05, <sup>b</sup>P < 0.01 vs controls.

**Table 2** Regression models to evaluate the association of Global CTI and IQ with suffering IBS

	Model 1	Full model
Global CTI score	0.93 (0.90-0.97) <sup>d</sup>	0.92 (0.87-0.97) <sup>b</sup>
Total IQ	0.99 (0.96-1.03)	0.99 (0.97-1.04)
Stressful life events (SRRS)		1.05 (0.97-1.15)
State anxiety		1.05 (0.99-1.10)
1-Hypochondriasis		1.19 (1.11-1.27) <sup>d</sup>
2-Depression		0.95 (0.90-1.00)
3-Hysteria		0.96 (0.90-1.02)
4-Psychopathic deviate		1.02 (0.96-1.08)
6-Paranoia		1.04 (0.99-1.10)
7-Psychasthenia		1.01 (0.93-1.09)
8-Schizophrenia		0.93 (0.85-1.01)
9-Hypomania		1.02 (0.97-1.07)

Adjusted by age, sex and educational level. <sup>b</sup>P < 0.01 (ANOVA); <sup>d</sup>P < 0.001 (ANOVA). Hosmer and Lemeshow goodness-of-fit test for the full model: P = 0.16. CTI: Constructive Thinking Inventory.

to the controls, without differences in total, verbal and performance IQ. However, IBS subjects scored lower in global constructive thinking and higher in neuroticism, and reported more stressful life events in the prior year than did controls. The detailed CTI subscale scores obtained by healthy controls and IBS subjects are shown in Figure 1.

Global CTI score was correlated to neuroticism ( $r = 0.71$ ) and trait anxiety ( $r = 0.71$ ), so these last variables were not included in the model. Table 2 shows the results of the logistic regression model. When neuroticism and trait anxiety were included, scale 1 of MMPI-2 (hypochondriasis) [OR 1.19 (1.11-1.27)] and global CTI score [OR 0.93 (0.87-0.99)] remained independently related, at the same magnitude, to suffering from IBS, without significant OR for any other variable.

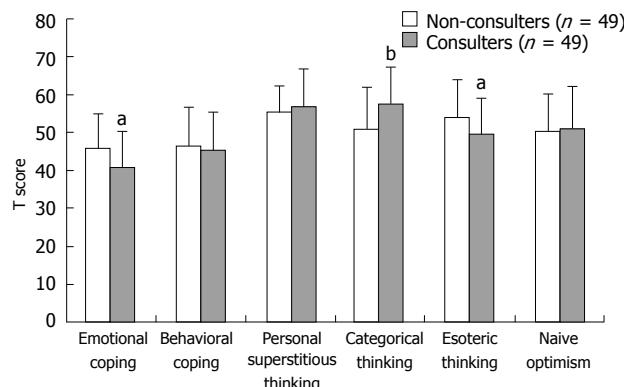
### IBS consulters vs non-consulters

Two subjects (one IBS consultor and one IBS non-consultor) produced invalid CTI scores and were thus excluded from the analysis. There were no significant differences between IBS consultors and non-consultors in terms of age, sex, educational level, marital status, work status and family monthly income. Furthermore, IBS features (distribution of subtypes, evolution of

**Table 3** Comparison of intelligence, personality, and life events between IBS consultors and non-consultors

	IBS non-consultors (n = 49)	IBS consultors (n = 49)
<b>Rational intelligence (WAIS-III)</b>		
Total IQ	103.8 ± 11.2	99.3 ± 10.5 <sup>a</sup>
Verbal IQ	104.4 ± 11.0	100.1 ± 10.4 <sup>a</sup>
Performance IQ	103.0 ± 11.7	99.1 ± 11.6
<b>Experiential intelligence (CTI)</b>		
Global CT score	46.6 ± 9.2	41.1 ± 9.0 <sup>d</sup>
<b>Personality traits (NEO-PI)</b>		
Neuroticism	59.7 ± 7.5	62.1 ± 9.6
Extroversion	46.6 ± 11.5	43.3 ± 10.2
Openness	52.5 ± 11.0	48.6 ± 13.0
Agreeableness	45.5 ± 8.4	42.7 ± 9.2
Conscientiousness	35.9 ± 7.4	35.7 ± 8.9
<b>Psychopathology (MMPI-2)</b>		
1-Hypochondriasis	60.1 ± 12.0	64.6 ± 11.8
2-Depression	50.4 ± 9.8	55.8 ± 11.2 <sup>a</sup>
3-Hysteria	58.6 ± 12.3	62.7 ± 10.5
4-Psychopathic deviate	52.5 ± 9.7	57.3 ± 11.9 <sup>a</sup>
5-Masculinity-femininity	49.2 ± 11.2	50.9 ± 8.4
6-Paranoia	51.4 ± 9.5	53.1 ± 8.8
7-Psychasthenia	49.8 ± 8.4	54.1 ± 8.0 <sup>a</sup>
8-Schizophrenia	50.5 ± 9.3	52.9 ± 10.4
9-Hypomania	51.7 ± 9.1	52.6 ± 11.3
0-Social Introversion	47.9 ± 8.5	49.4 ± 8.0
<b>Anxiety (STAI)</b>		
State anxiety	37.2 ± 10.0	43.6 ± 11.6 <sup>a</sup>
Trait anxiety	41.4 ± 9.5	45.8 ± 11.4 <sup>a</sup>
<b>Stressful life events (SRRS)</b>		
Number in prior 12 mo	10.3 ± 5.3	9.5 ± 4.4
Total emotional impact	539.2 ± 274.4	500.8 ± 233.3

<sup>a</sup>P < 0.05 (ANOVA), <sup>b</sup>P < 0.001 (ANOVA) vs non-consultors.



**Figure 2** Subscales of constructive thinking in IBS consultors and non-consultors. <sup>a</sup>P < 0.05, <sup>b</sup>P < 0.01 vs non-consultors.

bowel symptoms) were similar in both groups, except for severity, with differences between consultors and non-consultors in FDSI scores ( $70.8 \pm 57.8$  vs  $23.5 \pm 21.7$ ;  $P < 0.001$ ) and current pain on VAS ( $34.4 \pm 25.5$  vs  $23.5 \pm 21.7$ ;  $P < 0.05$ ). IBS consultors displayed lower total and verbal IQ and lower global constructive thinking than did IBS non-consultors, without differences in personality traits and stressful life events in the preceding year (Table 3). CTI subscale scores obtained by IBS consultors and non-consultors are shown in detail in Figure 2.

In the logistic regression model, adjusted for age, sex and educational level, no variable was independently

**Table 4** Results of stepwise multiple linear regressions to predict number of physician visits among IBS consulters

Dimension	Variables entered in the model	Standardized coefficient ( $\beta$ )	Model
Step 1	1-Current pain (VAS)	-0.38 <sup>b</sup>	$r^2 = 0.13^d$
Step 2	1-Current pain (VAS) 2-Age	-0.39 <sup>b</sup> -0.33 <sup>a</sup>	$r^2 = 0.22^d$

<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ ; <sup>d</sup> $P < 0.001$ .

associated with IBS-related health care seeking. Among those who had sought health care, the number of physician visits correlated with age ( $r = 0.31$ ;  $P < 0.05$ ), current pain ( $r = 0.38$ ;  $P < 0.01$ ) and hypochondriasis ( $r = 0.29$ ;  $P < 0.05$ ). The number of physician visits among IBS consulters was predicted by current pain, evaluated by VAS, and age in the stepwise multiple linear regression model (Table 4).

## DISCUSSION

The main finding of our study is that experiential rather than rational intelligence is associated with IBS *per se*, supporting the role of cognitive factors, specifically through generation of stress from daily life events.

Our results reject the hypothesis of IBS subjects having a lower rational intelligence. Attree *et al*<sup>[19]</sup> recently reported that IBS subjects had a full and verbal IQ slightly lower than controls and a deficit in verbal vs performance IQ. Differences in our results may be due to differences in the study sample. We studied a convenience sample of subjects with IBS covering the whole spectrum of the disease, without interest in psychological management or prior psychiatric diagnosis, while Attree *et al*<sup>[19]</sup> included a group of IBS subjects who had previously expressed an interest in participating in research at a school of psychology. Indeed, our finding that consulters displayed a slightly lower full and verbal IQ than non-consulters points to this explanation. Although a subtle cognitive impairment in some IBS patients cannot be ruled out, this would not seem to be true of the majority of IBS subjects.

Our results support the hypothesis of a lower level of constructive thinking among IBS subjects. The main CTI measure is defined as the ability to adapt the way of thinking to different situations (flexibility in thinking), while one of the main scales (emotional coping) is primarily described as the ability to appraise situations as a challenge rather than a threat. The relationship between physical symptoms and constructive thinking seems to be mediated by stress, mostly self-produced, through the generation of negative emotions<sup>[31]</sup>. Moreover, in a common stressful situation such as pregnancy, constructive thinking was shown to impact both on cognitive appraisal of stress (reducing the need for coping and adjustment) and on active coping responses, once the situation has been perceived as stressful<sup>[32]</sup>. Low constructive thinking suggests that subjects with IBS have a higher probability of experiencing daily life events

that are not intrinsically stressful, as being stressful, due to cognitive appraisal.

Stress is thought to play a pre-eminent role in generating and sustaining IBS symptoms. Over 50% of IBS subjects-consulters<sup>[33]</sup> and non-consulters alike<sup>[34]</sup> report that stressful events precipitate or worsen their symptoms. While some previous studies have observed that IBS subjects experience a slightly greater number of stressful life events than do controls or patients with other digestive disease<sup>[35,36]</sup>, other studies have observed no differences in this regard<sup>[6,37]</sup>. In a prospective study, Levy *et al*<sup>[38]</sup> failed to find differences between IBS subjects and controls in the number of positive or negative daily events, but self-reported daily stress was higher among IBS subjects. Hence, daily events are perceived as being more stressful by IBS subjects, and indeed, a recent Japanese study showed that IBS subjects scored higher on a perceived stress scale<sup>[39]</sup>.

Emotional distress plays a role in IBS and may also impact on daily stress. The association between low constructive thinking and IBS does not seem to be explained by emotional distress, as an association in multivariate analysis persisted even when adjusting for emotional distress measures, like MMPI clinical scales. Although we excluded subjects with a psychiatric diagnosis requiring treatment, our sample of IBS subjects still suffer higher emotional distress than control subjects, to a level similar to that reported by Drossman *et al*<sup>[6]</sup>.

Constructive thinking correlated to neuroticism; a correlation has been previously reported with the CTI emotional coping subscale, which has been deemed to represent the cognitive component of neuroticism<sup>[21]</sup>. Neuroticism is a broad concept that includes cognitive, affective, and behavioral traits<sup>[40]</sup>. Most<sup>[5,41-44]</sup>, although not all<sup>[36]</sup> studies have reported an association between neuroticism and IBS. However, constructive thinking seems to account for the relationship between neuroticism and IBS, suggesting the relevance of cognitive factors in IBS.

Several studies have shown that IBS is associated with less effective strategies to cope with stress<sup>[45,46]</sup>, specifically with symptoms. Thus, the difference between constructive thinking and coping response merits some comment. Constructive thinking would be mostly implicated in the automatic and holistic cognitive appraisal of any event, prior to the occurrence of emotion, and operates at a preconscious level in a way in which a person is unaware<sup>[47]</sup>. Coping is usually defined as cognitive and behavioural efforts to manage a troubled person-environment relationship; it is the reaction to a conscious appraisal of the situation<sup>[48]</sup>. Thus, interpretational activity may occur outside awareness and may be the non-conscious trigger of an emotional response<sup>[49]</sup>. Such a process is likely involved in the activation of the anterior cingulate cortex during subliminal and supraliminal stimulation<sup>[50]</sup>. Moreover, hypnotherapy is effective in IBS and, although the underlying mechanism is unknown<sup>[51]</sup>, it might be hypothesized that it operates partly through a change in preconscious appraisal of internal events<sup>[52,53]</sup>.

Another interesting finding is the association between constructive thinking and IBS-related medical consultation. In the 1980s and 1990s, psychological factors were regarded as predictors of health care sought by IBS subjects<sup>[6,54]</sup>, but recent population-based studies suggest that such factors are not quite so important<sup>[7,39,55]</sup>. Our results are closer to these latter studies, inasmuch as severity of symptoms is seen as the leading factor accounting for medical consultation, without any role for psychological factors. Nevertheless, univariate analysis showed a lower level of constructive thinking in those who had sought health care compared to those who did not; consulters displayed lower emotional coping and higher categorical thinking scores than non-consulters, a finding that points to appraisal of events (i.e. symptoms) as a threat and a more rigid mindset inclined to simplistic solutions as the main differences in thinking between those seeking care and non-consulters. However, constructive thinking does not seem to have a significant role in illness behavior, in view of the lack of correlation with the number of physician visits.

The main limitation of our study lies in the fact that subjects were not extracted from the general population strictly at random. We opted for a semi-random approach owing to the difficulties posed by recruiting our target population in a purely random fashion. There were four requirements for inclusion, namely: presence of bowel symptoms as per Rome II or, alternatively, absence of such symptoms; absence of any psychiatric diagnosis requiring treatment; consent to undergo in-depth evaluation, including a 2-h WAIS-III interview and a number of self-administered questionnaires; and, the need to be matched by age, sex and educational level. The direct-invitation approach allowed for a brief 5-min conversation to assess these criteria, and subjects were recruited in different environments (work and leisure centers) unrelated to medical facilities. Indeed, results from control subjects support this approach, since test profiles proved quite similar to data expected from the general population (mean T-scores of around  $50 \pm 10$ ; IQ around  $100 \pm 15$ ). In the case of IBS non-consulters, the data were comparable to the results of Mearin *et al*<sup>[2]</sup> based on a random Spanish-population sample. Thus, the results in no way suggest that there was any selection bias.

## COMMENTS

### Background

Irritable bowel syndrome (IBS) has been related to psychological distress and consequences of the stress driven emotional outputs. Cognitive abilities drive the interaction of the subject with the environment, which is the basis for stress, and may participate in abnormal illness behaviour, hypervigilance, and less effective coping abilities. One study suggested a lower intelligence in IBS patients.

### Research frontiers

This study is the first to comprehensively evaluate intelligence (rational and experiential intelligence) in an adequate sample of cases and controls.

### Innovations and breakthroughs

The potential role of intelligence in the pathophysiology of IBS is tested. This arm (cognition ability) of the subject-environment interaction has not been formally tested.

### Applications

This study provides support for the role of experiential intelligence in IBS.

Moreover, experiential intelligence may be improved with psychological therapies.

### Peer review

The authors exposed patients and controls to eight different psychological tests in an effort to identify correlations/associations for IBS and psychological phenotypes. The article is well written and the contents are credible.

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ORIGINAL ARTICLES

# Enhancement patterns of pancreatic adenocarcinoma on conventional dynamic multi-detector row CT: Correlation with angiogenesis and fibrosis

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**RESULTS:** The absolute enhanced value in the arterial phase correlated with the level of VEGF and MVD ( $P = 0.047$ ,  $P = 0.001$ ). The relative enhanced value in arterial phase and tumor-aorta enhancement ratio (arterial) correlated with MVD ( $P = 0.003$ ,  $P = 0.022$ ). Tumor-aorta enhancement ratio (arterial) correlated negatively with the extent of fibrosis ( $P = 0.004$ ). The tumors with greater MVD and higher expression of VEGF tended to show high enhancement in the arterial dominant phase. On the other hand, the tumors with a larger amount of fibrosis showed a negative correlation with the grade of enhancement during the arterial phase.

**CONCLUSION:** Enhancement patterns on dynamic CT correlated with angiogenesis and may be modified by the extent of fibrosis.

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**Key words:** Computed tomography; Contrast media; Pancreatic cancer; Angiogenesis

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## Abstract

**AIM:** To evaluate retrospectively the correlation between enhancement patterns on dynamic computed tomography (CT) and angiogenesis and fibrosis in pancreatic adenocarcinoma.

**METHODS:** Twenty-three patients with pancreatic adenocarcinoma underwent dynamic CT and tumor resection. In addition to the absolute and relative enhanced value that was calculated by subtracting the attenuation value on pre-contrast from those on contrast-enhanced CT in each phase, we defined one parameter, "tumor-aorta enhancement ratio", which was calculated by dividing enhancement of pancreatic cancer by enhancement of abdominal aorta in each phase. These enhancement patterns were correlated with the level of vascular endothelial growth factor (VEGF), microvessel density (MVD), and extent of fibrosis.

## INTRODUCTION

Pancreatic cancer is one of the leading causes of cancer-related death, with an overall 5-year survival rate of < 5%<sup>[1]</sup>. Surgical resection is still the only potentially curative treatment for pancreatic cancer. However, the resection rate is < 40%<sup>[2,3]</sup> because of the difficulty in achieving early detection. In addition, the results of other treatment methods including radiation therapy and chemotherapy are also poor.

Angiogenesis is the development of new blood vessels and is required for tumor growth. In the 1970s, Folkman reported that the development of neoplasms is angiogenesis-dependent<sup>[4,5]</sup>, with this process induced by angiogenic factors such as vascular endothelial growth factor (VEGF). As a result, microvessel density (MVD) increases in neoplasms. Recently, it has been clarified that the grade of tumor angiogenesis is a useful prognostic marker in human cancers<sup>[6-9]</sup>, including pancreatic cancer<sup>[10-14]</sup>. Generally, tumors with strong expression of angiogenesis show a poor prognosis. Therefore, anti-angiogenic treatment may be effective in improving the prognosis of patients with neoplasms including pancreatic cancer.

Evaluation of the grade of angiogenesis is important as a prognostic marker and is necessary for deciding the indications and evaluating the effect of anti-angiogenic treatment. For this, biopsy is necessary. However, because repeated biopsy is often difficult and invasive, and the specimen obtained does not always reflect the entire tumor, to establish the grade of tumor angiogenesis by non-invasive imaging may be important clinically. There have been several reports evaluating the correlation between angiogenesis and imaging findings in several types of cancers<sup>[15-18]</sup>, but only a few such reports on pancreatic adenocarcinoma<sup>[19,20]</sup>. Recently, perfusion computed tomography (CT) has been used to measure the hemodynamic characteristics of various tumors, and many authors have reported the results of perfusion CT in this context<sup>[21-23]</sup>. The correlation of perfusion CT findings and MVD in lung cancer<sup>[24,25]</sup> and the evaluation of the effect of anti-angiogenic therapy by perfusion CT<sup>[26,27]</sup> have been described. However, this method requires an additional procedure for conventional CT examination and a special CT machine or software. In addition, its usefulness for pancreatic cancer is now under investigation. For the present, anti-angiogenesis agents are still not approved for the treatment of pancreatic cancer. However, as a preliminary investigation for future clinical application, to predict the grade of angiogenesis by conventional dynamic multidetector CT (MDCT), most commonly performed for the diagnosis of pancreatic cancer, would be useful clinically.

The purpose of the present study was to evaluate the validity of conventional dynamic MDCT findings to predict angiogenesis in pancreatic cancer. We analyzed retrospectively the correlation between the enhancement on CT and the histopathological findings, including the grade of tumor angiogenesis, with special reference to MVD and expression of VEGF, and the extent of fibrosis in surgically resected pancreatic adenocarcinoma.

## MATERIALS AND METHODS

### Patients

Thirty-six patients with pancreatic cancer underwent surgical resection between January 2003 and October 2004. Among them, 10 patients did not receive dynamic CT examination and were excluded from the study.

Additionally, two with adenosquamous carcinoma and one with mucinous carcinoma were excluded. Finally, 23 patients (15 men and eight women; age range, 34-79 years; mean age, 62.6 years) with tubular adenocarcinoma of the pancreas were evaluated. All patients underwent dynamic CT, surgical resection, and histopathological examination. The range of tumor sizes was 20-48 mm and the mean was 40.5 mm.

Our institutional review board approved this retrospective study and informed consent for the use of medical records was obtained from the patients.

### CT imaging

CT images were obtained using a multi-detector row CT scanner (LightSpeed Ultra 16; GE Medical Systems, Milwaukee, WI, USA). The scanning parameters were 2.5 mm section thickness, pitch of 1.5, 120 kV, and auto mA. After pre-contrast CT scans, arterial dominant phase images of dynamic CT were obtained starting 30 s after the beginning of the intravenous bolus injection (3 mL/s) of 100 mL of iodized contrast medium at 300 mg/mL. The pancreatic phase and the late phase (near equilibrium phase) were also obtained, starting at 50 and 90 s after injection, respectively.

### Imaging analysis

Two radiologists (Y.H., T.G., one with > 7 years and the other with > 23 years of experience in pancreatic imaging) evaluated retrospectively all images and determined a decision with consensus circular regions of interest (ROI) were decided in the most enhanced area of the tumor in the pancreatic phase, excluding cystic or necrotic areas and adjacent pancreatic parenchyma, and in the abdominal aorta of the same slice, and ROIs in the other phases were drawn on the same site. Attenuation values were measured in Hounsfield units (HU) (absolute enhanced value). The relative enhanced value was calculated by subtracting the attenuation value on pre-contrast CT from those on contrast-enhanced CT in each phase. Furthermore, we defined one parameter as follows. The “tumor-aorta enhancement ratio” was calculated by dividing the attenuation value (HU) of pancreatic cancer by that of the abdominal aorta in each phase of contrast-enhanced CT, as a parameter of the grade of tumor enhancement. The ratio in arterial dominant phase was defined as the “tumor-aorta enhancement ratio (arterial)”, and in the pancreatic phase “tumor-aorta enhancement ratio (pancreatic)” and in late phase “tumor-aorta enhancement ratio (late)”, respectively.

### Immunohistochemical staining

Surgical specimens of 5 mm thickness were cut in the axial plane for pancreatic head cancer and the sagittal plane for pancreatic body and tail cancer. Formalin-fixed, paraffin-embedded tissues were sectioned at 4 µm thickness. The sections were stained with hematoxylin and eosin. Immunohistochemical and elastica van Gieson (EVG) staining was performed in the section that corresponded to the particular CT slices employed for the evaluation of enhancement pattern of the tumor. Immunohistochemical

staining was performed using the dextran polymer system (EnVision+ System; DAKO, Glostrup, Denmark). Color development was performed using 3,3'-diaminobenzidine tetrahydrochloride (DAKO), followed by hematoxylin counterstaining. For the detection of VEGF, which is an angiogenic factor, we used rabbit polyclonal anti-VEGF antibodies (A-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:100. The sections were heated in citrate buffer (pH 6.0; 10 mmol/L) using microwaves at 95°C for 20 min, and incubated at 4°C overnight in humid chambers with primary antibodies. For the detection of CD34 expressed on small-vessel endothelial cells, we used mouse monoclonal anti-CD34 antibodies (clone GBEnd/10; IMMUNOTECH, Marseilles, France) at a dilution of 1:200. The sections were incubated at room temperature for 1 h with primary antibodies. Islets of Langerhans and endothelium of arterial branches were used as internal positive controls for VEGF and CD34, respectively.

### Histopathological analysis

One author (Y.H.) evaluated the anonymous histological specimens without any information about the radiological images under assistance of one pathologist (Y.N., with > 30 years of experience). The level of VEGF staining was scored in comparison with that in the islets of Langerhans as a positive control: score 1, extremely weak; score 2, weak; score 3, mildly weak; score 4, almost equal. Each CD34-stained slide was scanned at a low magnification ( $\times 40$ ) to determine five "hot spot" areas of the largest number of microvessels. MVD was determined according to the mean number of microvessels counted in the five hot spots at high magnification ( $\times 200$ ). The extent of fibrosis was scored according to the ratio of fibrosis in the tumor with EVG staining in which elastic fibers were stained dark brown and collagen fibers were stained pink, with a score of 1, 0%-25%; 2, 25%-50%; and 3, 50%-100%.

### Statistical analysis

Statistical software (Dr. SPSS II for windows; SPSS, Chicago, IL, USA) was used for statistical analysis. The extent and dynamics of enhancement on dynamic CT were correlated with the level of VEGF, MVD and extent of fibrosis, to analyze whether the dynamic CT parameters defined above reflect the histopathological findings, including tumor angiogenesis. In addition, we also analyzed the correlation among the expression of VEGF, MVD and fibrosis. For these analyses, Spearman's rank correlation test was used.  $P < 0.05$  was considered to indicate a significant difference.

## RESULTS

### Correlation between absolute and relative enhanced values and histopathological findings

Table 1 shows the averages of the absolute attenuation value (HU) of the tumor and abdominal aorta in each phase of dynamic CT, and Table 2 shows the averages of the relative enhanced value (HU).

**Table 1 Absolute attenuation value (HU)**

	Pre-contrast	Arterial phase	Pancreatic phase	Late phase
Pancreatic cancer				
mean $\pm$ SD	41 $\pm$ 4	61 $\pm$ 13	80 $\pm$ 13	86 $\pm$ 11
Median	41	56	81	85
Range	32-48	41-93	60-104	64-106
Abdominal aorta				
mean $\pm$ SD	45 $\pm$ 4	284 $\pm$ 50	185 $\pm$ 40	139 $\pm$ 21
Median	45	295	171	136
Range	38-51	205-409	127-307	107-208

**Table 2 Relatively enhanced value (HU)**

	Arterial phase	Pancreatic phase	Late phase
Pancreatic cancer			
mean $\pm$ SD	20 $\pm$ 14	39 $\pm$ 15	45 $\pm$ 13
Median	19	38	43
Range	6-54	15-70	23-74
Abdominal aorta			
mean $\pm$ SD	239 $\pm$ 51	140 $\pm$ 40	94 $\pm$ 22
Median	248	125	91
Range	157-365	86-269	69-171

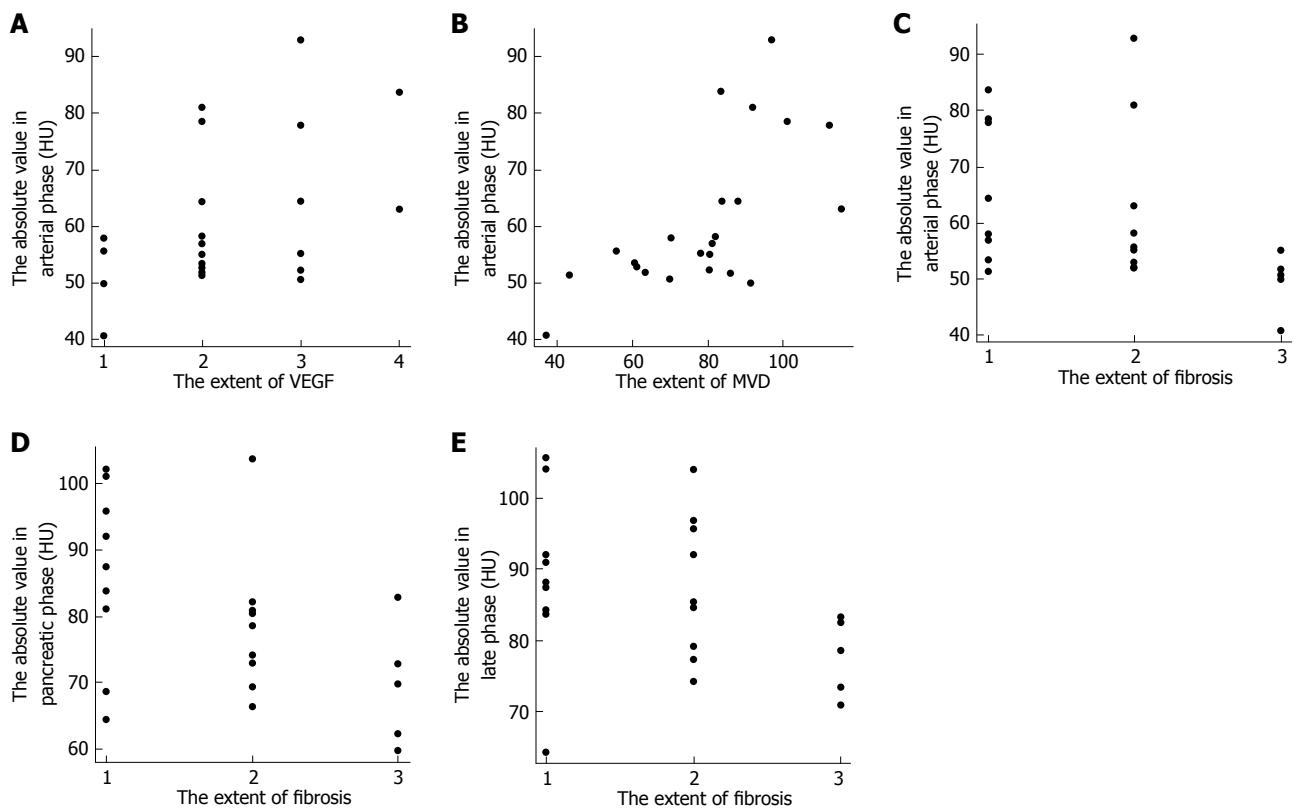
**Table 3 Tumor-aorta enhancement ratio**

	mean $\pm$ SD	Median	Range
Tumor-aorta enhancement ratio (arterial)	0.835 $\pm$ 0.053	0.066	0.029-0.216
Tumor-aorta enhancement ratio (pancreatic)	0.291 $\pm$ 0.12	0.244	0.139-0.569
Tumor-aorta enhancement ratio (late)	0.487 $\pm$ 0.129	0.483	0.295-0.816

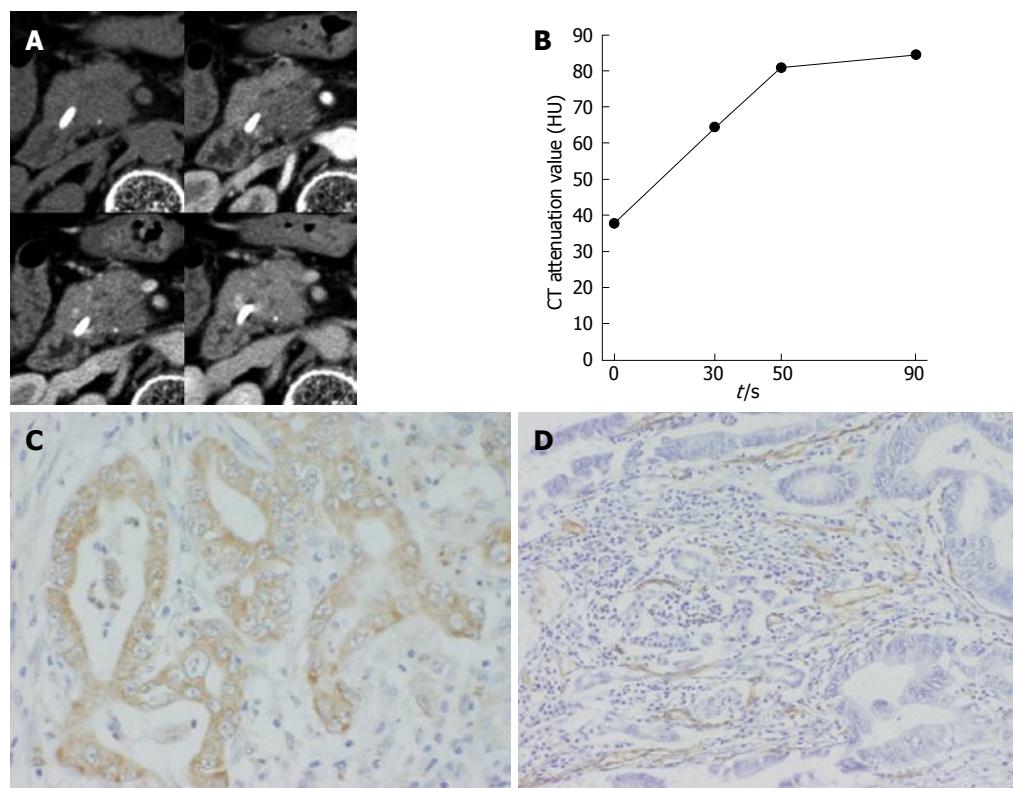
The absolute value of pre-contrast CT correlated significantly with none of the histopathological findings, or the level of VEGF, MVD or fibrosis. The absolute value in the arterial phase correlated significantly with the level of VEGF and MVD ( $P = 0.047$ ,  $P = 0.001$ ) (Figure 1A and B, Figures 2 and 3). The absolute value in the arterial, pancreatic and late phases correlated significantly and negatively with the extent of fibrosis ( $P = 0.006$ ,  $P = 0.018$ ,  $P = 0.035$ ) (Figure 1C-E and Figure 4). None of the relatively enhanced values in any phase correlated significantly with the level of VEGF. The relatively enhanced value in the arterial phase correlated significantly with MVD ( $P = 0.003$ ) (Figure 5A). All of the relatively enhanced values in the arterial, pancreatic and late phase correlated significantly and negatively with the extent of fibrosis ( $P = 0.003$ ,  $P = 0.020$ ,  $P = 0.039$ ) (Figure 5B-D).

### Correlation between tumor-aorta enhancement ratio and histopathological findings

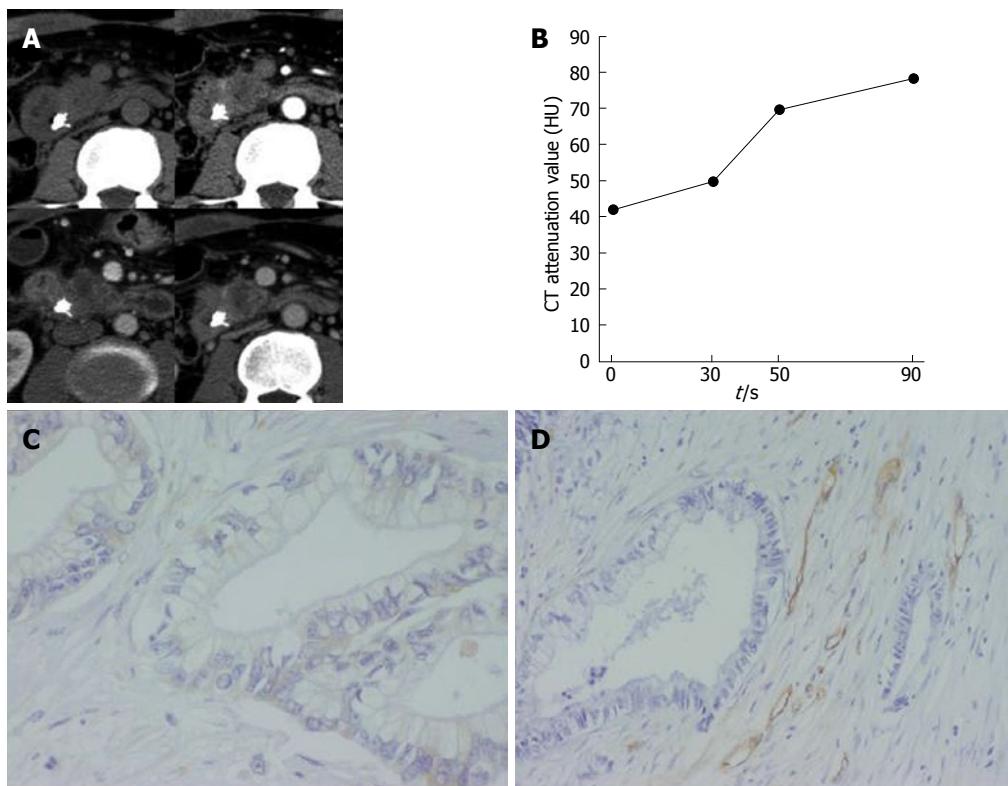
The averages of the tumor-aorta ratio are shown in Table 3. None of tumor-aorta enhancement ratios in any phase correlated significantly with the level of VEGF. Tumor-aorta enhancement ratio (arterial) was correlated significantly with MVD ( $P = 0.022$ ) (Figure 6A), and significantly and negatively with the extent of fibrosis ( $P = 0.004$ ) (Figure 6B).



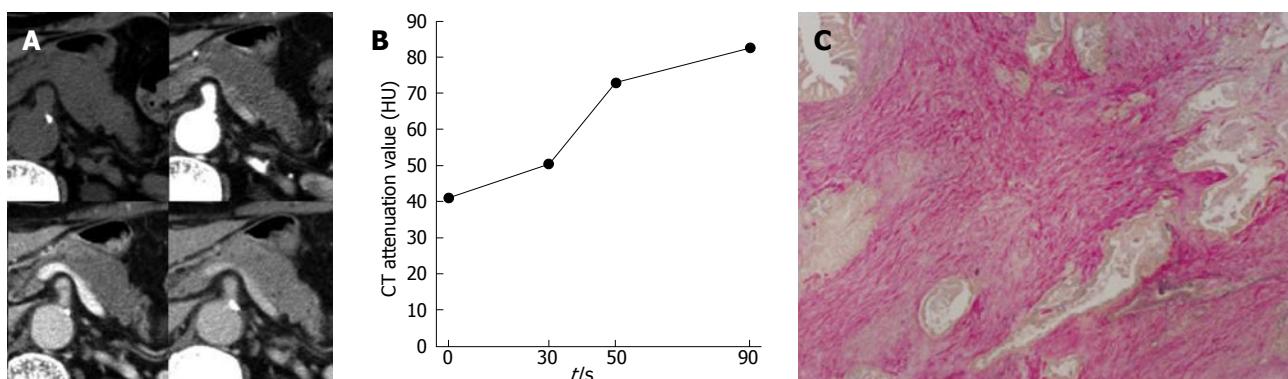
**Figure 1** Scatter plots showing correlation between absolute values and histopathological findings. A: The absolute value in the arterial phase correlated significantly with the level of VEGF ( $r = 0.418, P = 0.047$ ); B: The absolute value in the arterial phase correlated significantly with MVD ( $r = 0.649, P = 0.001$ ); C: The absolute value in the arterial phase correlated significantly and negatively with the extent of fibrosis ( $r = -0.556, P = 0.006$ ); D: The absolute value in the pancreatic phase correlated significantly and negatively with the extent of fibrosis ( $r = -0.488, P = 0.018$ ); E: The absolute value in the late phase correlated significantly and negatively with the extent of fibrosis ( $r = -0.442, P = 0.035$ ).



**Figure 2** Moderately differentiated tubular adenocarcinoma in a 73-year-old woman. A: Transverse dynamic CT images; B: Time-attenuation curve. Dynamic CT scans showing marked enhancement in the arterial phase; C: Photomicrograph showing immunoreactivity to VEGF, which is depicted as brown cytoplasm. The score was 4 (high expression) (Anti-VEGF stain; original magnification,  $\times 400$ ); D: Photomicrograph showing abundant microvessels and depicting vessel walls that appeared brown (Anti-CD34 stain; original magnification,  $\times 200$ ).



**Figure 3 Well-differentiated tubular adenocarcinoma in a 44-year-old man.** A: Transverse dynamic CT images; B: Time-attenuation curve. Dynamic CT scans showing low enhancement in the arterial phase; C: Photomicrograph showing immunoreactivity to VEGF, which is depicted as brown cytoplasm. The score was 1 (extremely weak) (Anti-VEGF stain; original magnification,  $\times 400$ ); D: Photomicrograph showing few microvessels and depicting vessel walls, which appear brown (Anti-CD34 stain; original magnification,  $\times 200$ ).



**Figure 4 Moderately differentiated tubular adenocarcinoma in a 79-year-old man.** A: Transverse dynamic CT images; B: Time-attenuation curve. Dynamic CT scans showing gradual enhancement; C: Photomicrograph showing abundant fibrosis and collagen fibers, which appear pink. The score was 3 (EVG stain; original magnification,  $\times 40$ ).

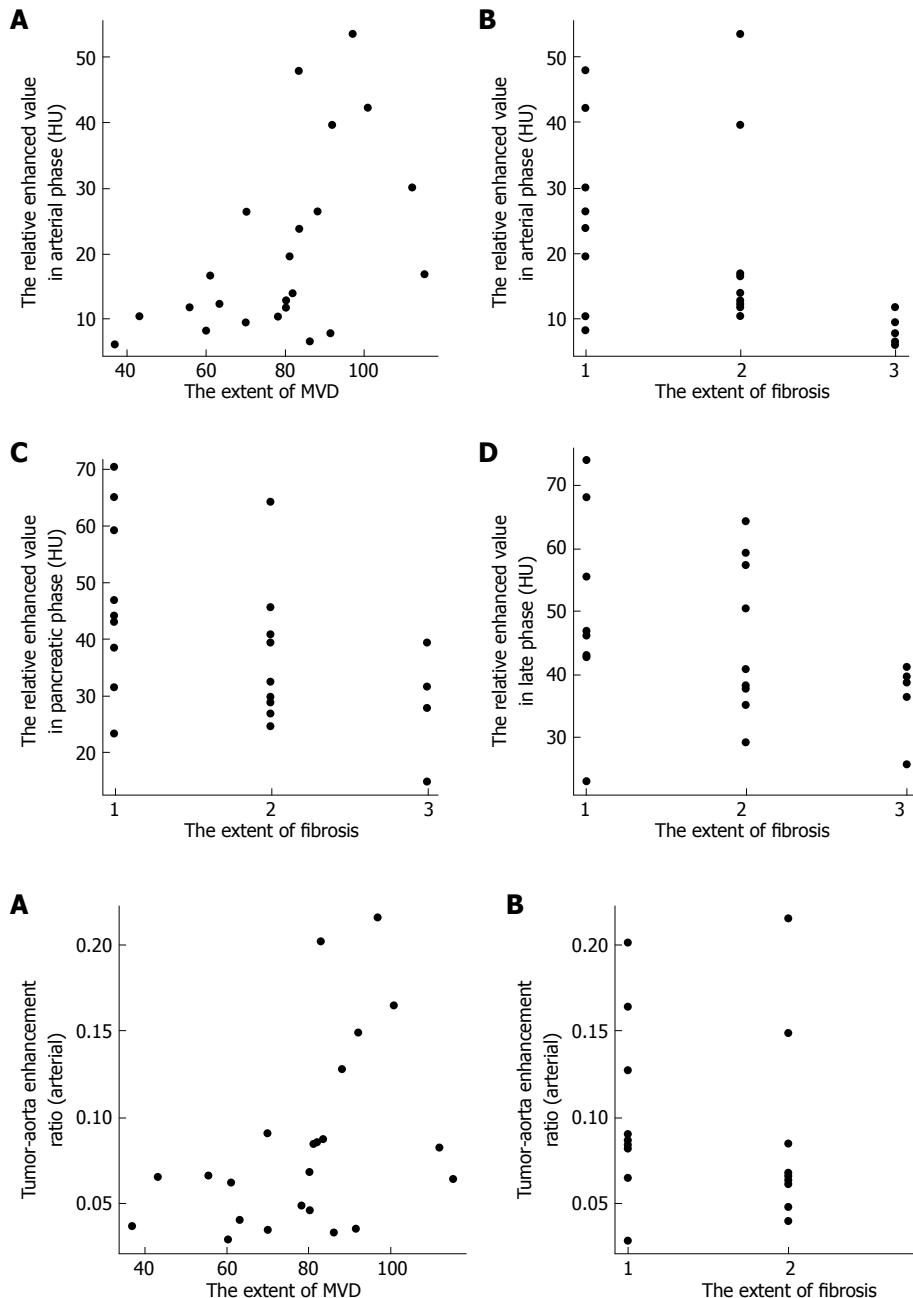
### Correlation among histopathological findings

The level of VEGF was correlated significantly with MVD ( $P = 0.037$ ). The extent of fibrosis was not correlated significantly with the level of VEGF and MVD.

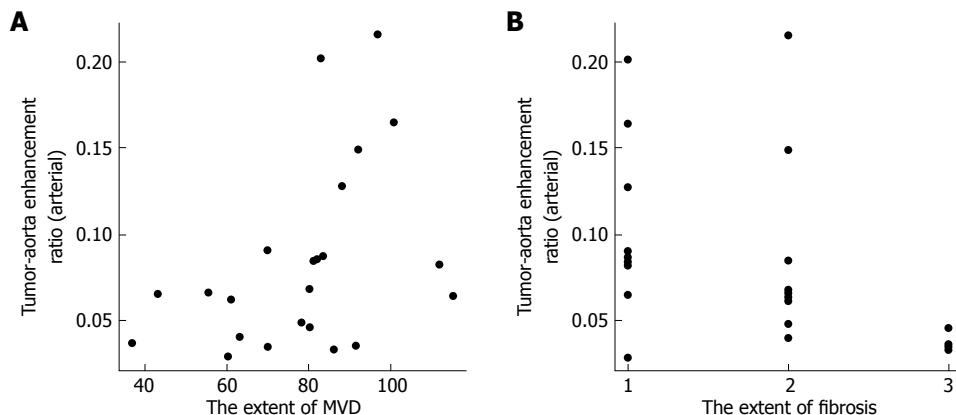
### DISCUSSION

The correlation between conventional dynamic MDCT findings and angiogenesis in lung<sup>[15]</sup> and renal cell cancer has been reported previously. These studies have revealed that the attenuation value of the peak enhancement of the tumor and the enhancement ratio (peak enhancement value divided by time) are correlated

positively with the extent of angiogenesis. However, it is not realistic to apply these results to pancreatic cancer, which usually has abundant fibrosis and tends to show gradual enhancement with the peak enhancement in the equilibrium phase<sup>[28,29]</sup>. To overcome this important problem in the common type of pancreatic cancer, we analyzed the correlation with enhancement of each phase and angiogenesis and fibrosis. In general, contrast agents have two-compartment pharmacokinetics with intravascular and extravascular-extracellular (interstitium) components. The enhancement of the tumor depends on the concentration of the injected agent, blood flow, blood volume, permeability, and extravascular-



**Figure 5** Scatter plots showing correlation between the relative enhanced values and histopathological findings. A: The relatively enhanced value in the arterial phase correlated significantly with the extent of MVD ( $r = 0.593, P = 0.003$ ); B: The relatively enhanced values in the arterial phase correlated significantly and negatively with the extent of fibrosis ( $r = -0.590, P = 0.003$ ); C: The relatively enhanced values in the pancreatic phase correlated significantly and negatively with the extent of fibrosis ( $r = -0.483, P = 0.020$ ); D: The relatively enhanced values in the late phase correlated significantly and negatively with the extent of fibrosis ( $r = -0.433, P = 0.039$ ).



**Figure 6** Scatter plots showing correlation between tumor-aorta enhancement ratio and histopathological findings. A: Tumor-aorta enhancement ratio (arterial) was correlated positively with MVD ( $r = 0.477, P = 0.022$ ); B: Tumor-aorta enhancement ratio (arterial) was correlated negatively with the extent of fibrosis ( $r = -0.575, P = 0.004$ ).

extracellular components. The contrast agents in the arterial dominant phase are predominantly the intravascular component. In the pancreatic phase (near portal dominant phase), they pass into the extravascular-extracellular components. The enhancement in this phase is considered to be a mixture of intravascular and extravascular-extracellular components. Tissues with adequate blood supply generally show the highest enhancement in this phase. The contrast agents in late phase (equilibrium phase) are both intravascular and extravascular-extracellular components, and the enhancement depends mainly on the extravascular-extracellular components. In addition to absolute and relative enhanced values, we employed the tumor-aorta enhancement ratio, which was calculated by dividing

the attenuation value (HU) of pancreatic cancer by that of the abdominal aorta in each phase of contrast-enhanced CT, as a parameter of the grade of tumor enhancement. This parameter was decided in order to exclude the influence of the intravascular concentration of an injected contrast agent that is dependent on cardiac output and circular blood volume. We think that this parameter reflects tumor enhancement more exactly than the absolute and relative attenuation values. Tumor-aorta enhancement ratio (arterial) is considered to reflect mainly the amount of arterial blood flow and volume of intratumoral blood spaces. Tumor-aorta enhancement ratio (pancreatic) may depend on vascular permeability in addition to intratumoral blood flow and/or blood volume. Tumor-aorta enhancement ratio (late) may

reflect mostly the extravascular-extracellular component.

In the present study, several findings of dynamic CT showed significant correlations with the histopathological findings. MVD in the tumor correlated significantly with the absolute value in the arterial and pancreatic phases, relative enhanced value in the arterial phase and the tumor-aorta enhancement ratio (arterial). This may have resulted from the increased vascular space and/or increased blood flow in tumors with increased MVD. The absolute value in the arterial phase also correlated significantly with the level of VEGF, probably for the same reason as in the case of MVD. On the other hand, the absolute value and relatively enhanced value in the arterial and pancreatic phases and tumor-aorta enhancement ratio (arterial) correlated significantly and negatively with the extent of fibrosis. This may have resulted from the smaller intratumoral blood spaces and blood flow in the tumors, with abundant fibrosis resulting in an absolutely lower volume of contrast inflow into this kind of tumor. Both the absolute value and relatively enhanced value in the late phases correlated significantly and negatively with the extent of fibrosis. It is known that the tumors with abundant internal fibrosis show prominent delayed enhancement in the late-phase of dynamic CT because of an increased extravascular-extracellular component. Therefore, the results obtained in our tumor were not consistent with previous speculations. This may also have been caused by the smaller amount of blood inflow into the tumors with more abundant fibrosis. We need to study this issue further with a more delayed phase on dynamic CT.

Histologically, the extent of VEGF expression was correlated significantly with MVD. However, the extent of fibrosis was not correlated significantly with the level of VEGF or MVD. These results support the similarity of the findings of dynamic CT between tumors with increased MVD and expression of VEGF. However, these findings may be modified by the extent of intratumoral fibrosis, which has no direct correlation with VEGF expression and MVD.

There are several limitations in the present study. First, the protocol of dynamic CT was not entirely appropriate. The late phase was earlier than the widely accepted equilibrium phase of dynamic CT of the pancreas. We used a fixed amount of contrast material. The grade of enhancement might be influenced by several factors such as patient weight, cardiac output, and CT tube wear<sup>[30,31]</sup>. Since we analyzed the validity of conventional dynamic CT retrospectively and preliminarily, we did not adopt these factors in the present study. Second, the attenuation value (HU) reflects not only the vascular characteristics of immature vessels formed by tumor angiogenesis, but also those of pre-existing mature vessels. Third, there are several other factors that affect the enhancement pattern of pancreatic cancers, such as the shape of intratumoral blood spaces and vasoactive elements. Fourth, the attenuation value (HU) in each phase on conventional dynamic MDCT may reflect various levels of blood flow, blood volume, vascular permeability and extravascular-

extracellular components. In particular, new capillaries formed by tumor angiogenesis are immature and have greater permeability than normal capillaries<sup>[32]</sup>. Further investigation, including by perfusion CT, is needed in this regard. In spite of these limitations, we think that our results provide some useful indication for the estimation of angiogenesis and intratumoral fibrosis in pancreatic cancer. After clinical application of anti-angiogenesis agents for pancreatic cancer, evaluation of the results obtained this study should be performed.

In conclusion, there was a significant correlation between the enhancement in conventional dynamic CT and angiogenesis and fibrosis in pancreatic adenocarcinoma. The tumors with greater MVD and expression of VEGF tended to show high enhancement in the arterial dominant phase. On the other hand, the tumors with a larger amount of fibrosis showed a negative correlation with the grade of enhancement during the arterial phase. Dynamic CT features that are caused by angiogenesis may be modified by the extent of intratumoral fibrosis.

## COMMENTS

### Background

Prognosis of pancreatic cancer is poor. Recently, it has been clarified that the grade of tumor angiogenesis is a useful prognostic marker in human cancer, including pancreatic cancer. Therefore, evaluation of the grade of angiogenesis by imaging may be important as a prognostic marker and is necessary for deciding the indications and evaluating the effect of anti-angiogenic treatment.

### Research frontiers

To establish the grade of tumor angiogenesis by non-invasive imaging may be important clinically. However, there are only a few such reports on pancreatic adenocarcinoma. In the present study, the authors analyzed the correlation between enhancement on dynamic multidetector computed tomography (MDCT) and histopathological findings, including the grade of tumor angiogenesis, and the extent of fibrosis in surgically resected pancreatic adenocarcinoma.

### Innovations and breakthroughs

This study predicted the grade of angiogenesis by conventional dynamic MDCT that is performed most often for the diagnosis of pancreatic cancer. The tumors with strong angiogenesis tended to show high enhancement in the arterial dominant phase. On the other hand, tumors with a larger amount of fibrosis showed a negative correlation with the grade of enhancement during the arterial phase.

### Applications

Anti-angiogenesis agents are still not approved for the treatment of pancreatic cancer at present. However, as a preliminary investigation for future application, prediction of the grade of angiogenesis by conventional dynamic MDCT would be useful clinically.

### Terminology

Angiogenesis is the process of new blood vessel formation. This consists of endothelial sprouts of preexisting vessels and is stimulated by angiogenic factors such as vascular endothelial growth factors. Recently, angiogenesis has been recognized as an important factor of tumor growth and metastasis.

### Peer review

The author retrospectively evaluated the correlation between enhancement patterns on dynamic computed tomography and angiogenesis and fibrosis in pancreatic adenocarcinoma.

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BRIEF ARTICLES

## Microscopic colitis: A large retrospective analysis from a health maintenance organization experience

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376 patients with LC and 171 patients with CC. The female/male ratio was 3:1 in CC and 2.7:1 in LC patients. Celiac disease ( $P < 0.001$ ), irritable bowel syndrome (IBS) ( $P < 0.001$ ), and thyroid diseases ( $P < 0.001$ ) were found to have a higher occurrence in MC compared to the control group. No statistical differences in the occurrence of colorectal cancer, diabetes and IBD were found between the MC group and the control group.

**CONCLUSION:** This is the largest group of patients with MC known to the authors that has been studied to date. Conditions such as celiac disease, IBS, and thyroid diseases were found to be related to MC. Furthermore, neither an increased risk of colorectal cancer nor IBD was associated with MC in this study.

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**Key words:** Collagenous colitis; Inflammatory bowel disease; Lymphocytic colitis; Microscopic colitis

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### Abstract

**AIM:** To examine the demographic data on a large multi-ethnic population of patients with microscopic colitis (MC) in Southern California and to determine the association of MC with inflammatory bowel disease (IBD) and colorectal cancer.

**METHODS:** All patients diagnosed with MC by colonic biopsy from 1996-2005 were identified utilizing a pathology database. All biopsies were reviewed by experienced pathologists utilizing standard histologic criteria. Patients' medical records were reviewed and data regarding patient age, co-morbidities, sex, ethnicity, and medications were analyzed. An age- and sex-matched standard control group was also generated. Chi-square test was used to evaluate the associations of co-morbidities between lymphocytic colitis (LC), collagenous colitis (CC) and the control group.

**RESULTS:** A total of 547 cases of MC were identified,

### INTRODUCTION

Microscopic colitis (MC) is a condition characterized by chronic, watery diarrhea with normal endoscopic appearance of colonic mucosa, yet abnormal histology. Further classification of this condition based on histology reveals two distinct entities: collagenous colitis (CC) and lymphocytic colitis (LC). Although both entities demonstrate colonic intraepithelial lymphocytosis, increased inflammatory cells within the lamina propria and preserved crypt architecture, the presence of a

thickened sub-epithelial collagen band is characteristic of CC.

The incidence of CC is approximately 0.6–2.3/100 000 per year with a prevalence of 10–15.7/100 000, while LC has an incidence of 3.1/100 000 per year and a prevalence of 14.4/100 000<sup>[1,2]</sup>. However, more recently published data from Olesen *et al*<sup>[3]</sup> suggests that the incidence of microscopic colitides are on the rise. Observational studies have also suggested associations between MC and various autoimmune diseases including thyroid, rheumatoid, celiac, and inflammatory bowel disease (IBD)<sup>[4–7]</sup>. Various medications have also been linked with MC in published case reports. Currently, little is known regarding cancer risk associated with MC.

With the number of reported cases clearly increasing, surprisingly, its etiology is still mostly unknown. Furthermore, the relationship between collagenous and LC and their association with other diseases has yet to be clearly defined. Should a connection between MC and colorectal cancer or IBD exist, this would have huge ramifications on the management of MC. Most of the currently available data on MC are from European studies with relatively small and homogeneous populations. There are even fewer comparative studies on the subtypes of MC.

The purpose of this study was to observe and report the demographic data on a large multi-ethnic population in Southern California, USA, with MC. We also report on the pattern of concurrent diseases and cancers in this group of patients.

## MATERIALS AND METHODS

All patients diagnosed with MC (both collagenous and LC) from 1996 to 2005 by colonic biopsy were identified using the pathology database at Kaiser Permanente (a large health maintenance organization) in Southern California and the search words “MC, LC and CC”. This region includes eight major Kaiser Permanente medical centers that serve over three million members throughout Southern California.

The histological criteria for inclusion in this study were defined as follows: (1) An increase in intraepithelial lymphocytes (more than 10 lymphocytes/100 epithelial cells); (2) Surface epithelial damage; (3) Absent or minimal crypt architectural damage; (4) An increase in sub-epithelial collagen band > 10 micrometers was required for the diagnosis of CC (Figure 1).

Experienced gastrointestinal pathologists (JM, SW, NK, RZ, MK, JP, KG) at Kaiser Permanente reviewed all biopsies.

Patients with ulcerations, erythema or any other visible abnormalities seen on the colonic mucosa during the endoscopic exam were excluded from the study. We included patients with a history of colorectal cancer.

We reviewed medical records using the Kaiser Permanente Database System and recorded data regarding patient age at diagnosis, sex, ethnicity, and concurrent medical conditions. All medications taken by patients, within 1 year prior to the diagnosis of collagenous or LC,

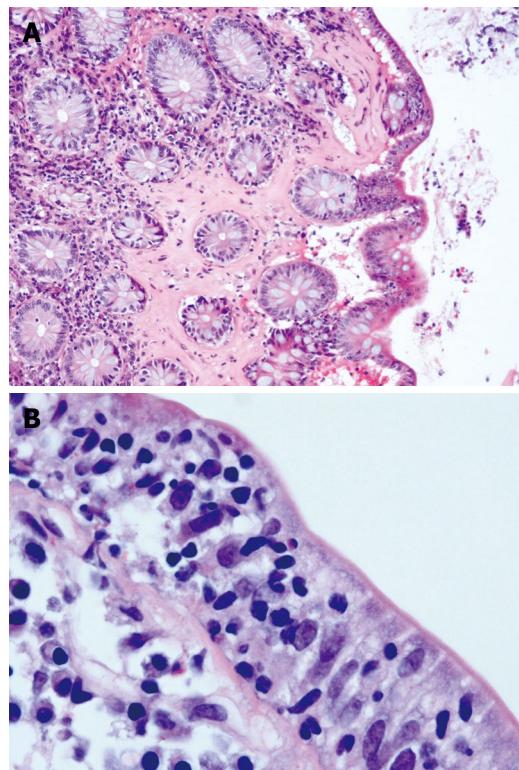


Figure 1 Collagenous colitis (A) and lymphocytic colitis (B).

were also recorded for analysis.

Demographic data such as age at diagnosis, sex and ethnicity is presented as means and standard deviation. An age- and sex-matched standard control group was also randomly generated from the same Kaiser Permanente database. We used a chi-square test to evaluate the associations of co-morbidities between LC, CC and the control group.

Differences in the clinical characteristics between LC and CC were also evaluated using the chi-square test. Differences were assumed as significant at a *P* value of less than 0.05.

The study protocol was approved by the Internal Review Board of Kaiser Permanente in Southern California.

## RESULTS

From January 1996 to July 2005 a total of 547 patients were identified with MC from our database. There were 27.1% male subjects, while 72.9% were female. The average age at diagnosis was 61.7 years for both men and women. We were able to identify the ethnicity in 465 cases. The percentage of each ethnic group was as follows: 72.4% Caucasian, 6.9% Hispanic, 2.6% Asian, 2.9% African American, and 0.2% Middle Easterners.

## CC

**Demographics:** A total of 171 patients were identified as having CC. The average age at diagnosis in these patients was 63.8 years with a standard deviation of 13.5 years. The range for age at diagnosis was between

**Table 1 Comparison of our demographic data against published demographic data in collagenous colitis (mean  $\pm$  SD)**

	Total number	Age at diagnosis (yr)	Gender ratio M/F
Goff <i>et al</i> <sup>[8]</sup> 1997	31	66	5/26
Fernandez-Banares <i>et al</i> <sup>[1]</sup> 1999	23	57.8 $\pm$ 2.9	4/19
Agnarsdottir <i>et al</i> <sup>[9]</sup> 2002	71	66.1 $\pm$ 14.3	8/63
Olesen <i>et al</i> <sup>[3]</sup> 2004	51	Not reported	6/45
Koskela <i>et al</i> <sup>[18]</sup> 2004	30	56.5 $\pm$ 12.7	10/20
Kao <i>et al</i> 2006	171	63.8 $\pm$ 13.5	42/129

29 to 93 years. The sex distribution was 24.6% male and 75.4% female. The ratio between male and female ratio was about 1:3. Of the 171 patients identified, we were able to identify the ethnicity of 152 patients. The percentage of each ethnic group was as follows: 80.7% Caucasian, 5.3% Hispanic, 2.3% African American and 0.6% Asian. No patients identified as Middle Easterners were observed<sup>[8,9]</sup> (Table 1).

**Associated diseases:** Of the CC population identified, the most common endocrinopathies were as follows: hyperlipidemia (42.7%), diabetes (14%) and thyroid disease, including hyperthyroidism, hypothyroidism and Grave's disease (21%). The three most common rheumatologic and other inflammatory disorders were rheumatoid arthritis (7%), fibromyalgia (3.5%) and Raynaud/CREST syndrome (2.9%). Other diseases diagnosed in these patients included giant cell/temporal arteritis ( $n = 3$ , 1.75%), polymyalgia rheumatica ( $n = 2$ , 1.16%), systemic lupus erythematosus ( $n = 1$ , 0.58%) and scleroderma ( $n = 1$ , 0.58%). Only five patients in the CC group were diagnosed with celiac disease (2.92%). One of these patients had the diagnosis of celiac disease prior to the diagnosis of CC. Thirty patients were diagnosed with irritable bowel syndrome (IBS) prior to the diagnosis of CC, while only one patient was diagnosed with irritable bowel after the diagnosis of CC was made. We observed one patient with an established diagnosis of IBD prior to the diagnosis of CC. During a median follow up of 3 years, none of our CC patients have subsequently been diagnosed with IBD.

Compared with the control group, patients with CC had higher rates of thyroid disease ( $P < 0.001$ ). We also observed a higher rate of celiac disease ( $P < 0.01$ ), IBS, ( $P < 0.001$ ) rheumatoid arthritis ( $P < 0.001$ ) and Raynaud/CREST syndrome ( $P < 0.01$ ) in our patients with CC (Table 2).

**Cancer:** The three most common cancers found in CC patients were breast ( $n = 4$ , 2.34%), lung ( $n = 3$ , 1.75%) and colorectal cancer ( $n = 5$ , 2.82%). Four patients were diagnosed with cancer prior to the diagnosis of CC. Only one patient was diagnosed with colorectal cancer following the diagnosis of CC. This patient was a 79-year-old female diagnosed with colorectal cancer by screening colonoscopy 5 years after the diagnosis of CC was made.

**Table 2 Comparison of concurrent diseases between collagenous colitis and the control group**

Disease	Number of patients affected	Percentage (%)	Compared to control group
Hyperlipidemia	73	43.5	NS
Diabetes type I and II	24	15.8	NS
Thyroid diseases <sup>1</sup>	36	21	$P < 0.001$
Rheumatoid arthritis	12	7	$P < 0.001$
Fibromyalgia	6	3.51	NS
Raynaud/CREST syndrome	5	2.92	$P < 0.01$
Giant cell/temporal arteritis	3	1.75	NS
Polymyalgia rheumatica	2	1.16	NS
SLE	1	0.58	NS
Scleroderma	1	0.58	NS
Celiac disease	5	2.92	$P < 0.010$
IBS	30	17.5	$P < 0.001$
IBD	1	0.58	NS

<sup>1</sup>Thyroid diseases including hypothyroidism, hyperthyroidism and Grave's disease. SLE: Systemic lupus erythematosus.

**Table 3 Comparison of our demographic data against published demographic data in lymphocytic colitis (mean  $\pm$  SD)**

	Total number	Age at diagnosis (yr)	Gender ratio M/F
Fernandez-Banares <i>et al</i> <sup>[1]</sup> 1999	37	65.3 $\pm$ 2.4	10/27
Agnarsdottir <i>et al</i> <sup>[9]</sup> 2002	54	68.7 $\pm$ 12.7	5/45
Olesen <i>et al</i> <sup>[3]</sup> 2004	199	59	59/140
Koskela <i>et al</i> <sup>[18]</sup> 2004	54	55.4 $\pm$ 13.2	8/46
Kao <i>et al</i> 2006	376	60.7 $\pm$ 16.1	106/270

No cancer demonstrated a statistical difference compared with the control group, including colorectal cancer.

## LC

**Demographics:** A total of 376 patients were identified as having LC. The average age at diagnosis in this group was 60.7 years, with a standard deviation of 16.1 years. The age range at diagnosis was between 19 to 98 years. Sex distribution was 28.1% male and 71.8% female. The ratio between men and women was about 1:2.5. Of the 376 patients identified, ethnicity was determined in 313 cases. The percentage of each ethnic group was as follows: 68.8% Caucasian, 7.71% Hispanic, 3.45% Asian, 3.19% African American and 0.26% Middle Eastern (Table 3).

**Associated diseases:** Of the total LC population, the most common endocrinopathies were as follows: hyperlipidemia = 44.1%, diabetes = 14.6% and thyroid disease (including hyperthyroidism, hypothyroidism and Grave's disease) = 18.8%. Rheumatoid arthritis was seen in 3.99% of the LC patients. Fibromyalgia was observed in 4.52%, while Raynaud/CREST syndrome was observed in 1.33% of the LC population. Others diseases such as giant cell/temporal arteritis ( $n = 2$ , 0.53%), polymyalgia rheumatica ( $n = 6$ , 1.6%), systemic lupus erythematosus ( $n = 3$ , 0.8%) and scleroderma ( $n = 1$ , 0.27%) were also

**Table 4 Comparison of concurrent diseases between lymphocytic colitis and the control group**

Disease	Number of patients affected	Percentage (%)	Compared to control group
Hyperlipidemia	166	44.10	NS
Diabetes type I and II	55	14.60	NS
Thyroid diseases <sup>1</sup>	71	18.80	P < 0.01
Rheumatoid arthritis	15	3.99	P < 0.01
Fibromyalgia	17	4.52	P < 0.01
Raynaud/CREST syndrome	5	1.33	P < 0.01
Giant cell/temporal arteritis	2	0.53	P < 0.025
Polymyalgia rheumatica	6	1.60	NS
SLE	3	0.80	P < 0.025
Scleroderma	1	0.27	NS
Celiac disease	13	3.46	P < 0.001
IBS	43	11.40	P < 0.001
IBD	1	0.27	NS

<sup>1</sup>Thyroid diseases including hypothyroidism, hyperthyroidism and Grave's disease.

observed. Thirteen patients were diagnosed with celiac disease (3.46%) and six of these patients had a diagnosis of celiac disease prior to the diagnosis of LC. Forty three patients were thought to have IBS prior to the diagnosis of LC, while seven were diagnosed with IBS after the diagnosis of LC was made. Overall, 11.4% of patients with LC were diagnosed with IBS. We observed one patient with an established diagnosis of IBD prior to the diagnosis of LC. During a median follow up of 3 years, none of our LC patients have subsequently been diagnosed with IBD.

Patients with LC had a higher rate of thyroid diseases ( $P < 0.01$ ) compared with the control group. Diseases thought to be autoimmune-related, such as rheumatoid arthritis ( $P < 0.01$ ), Raynaud/CREST syndrome ( $P < 0.01$ ), SLE ( $P < 0.025$ ), fibromyalgia ( $P < 0.01$ ), and temporal arteritis ( $P < 0.025$ ) also had statistically higher rates of occurrence in patients with LC. IBS ( $P < 0.001$ ) and celiac disease ( $P < 0.001$ ) also occurred more often in patients with LC (Table 4).

**Cancer:** The three most common cancers found in our LC patients were breast ( $n = 20$ , 5.31%), prostate ( $n = 7$ , 1.86%) and lung ( $n = 4$ , 1.06%). Five patients were diagnosed with colorectal cancer prior to the diagnosis of LC. None were diagnosed with colorectal cancer after the diagnosis of LC had been made.

No cancer was noted to have a statistical difference compared with the control group, including colorectal cancer.

### CC vs LC

Despite a significant population size difference, we found little statistical difference between collagenous and LC, in terms of their associated diseases. Interestingly, we found a higher rate of irritable bowel disease diagnosed concurrently with CC than with LC ( $P < 0.05$ ).

## DISCUSSION

Increasingly, MC has become more and more recognized worldwide, yet little is known about its pathophysiology and the long term outcome of the disease. Currently, multiple hypotheses exist for the pathophysiology of MC and include immune dysregulation/autoimmunity. This hypothesis is further supported by findings that human leukocyte antigen haplotype is increased in A1 and DRW53 in LC<sup>[10]</sup>. While others have found increases in DQ2 and DQ1, three in LC and CC<sup>[11]</sup>. Autoantibodies such as ANA have also been found to be higher in patients with MC<sup>[12]</sup>. Indeed, multiple retrospective clinical studies have noted a higher incidence of autoimmune diseases in patients diagnosed with MC. This suspected relationship between immune dysregulation/autoimmunity and MC has led to the question of whether MC is a pre-IBD entity? If so, does the patient with MC require a higher level of surveillance and more aggressive treatment to prevent IBD from developing? These concerns were further illustrated by multiple case reports that seemed to convincingly suggest that MC may be the initial phase of a full blown IBD. However, the above theory has never been formally addressed in a large population study. In our investigation we were able to validate the previously known association between LC, CC and various autoimmune diseases, celiac disease and thyroid disorders in a large population study. Yet, interestingly, we found no significant correlation between IBD and CC/LC. Therefore, LC and CC may not be an indicator for intense surveillance to detect progression to IBD. To date, none of our LC or CC patients have been diagnosed with IBD.

Another theoretic risk for patients with MC is colorectal cancer. This concern is based on the IBD model where chronic inflammation can lead to increased dysplasia and malignancy. Thus far, to the authors' knowledge, only one study has evaluated the risk of colorectal cancer in MC. Chan *et al*<sup>[13]</sup> studied 117 patients diagnosed with CC during a mean follow-up period of 7 years to examine the risk of colorectal cancer. A similar risk of colorectal cancer was found in MC patients and the general population in that study. Only one patient was diagnosed with colorectal cancer after being diagnosed with MC. This was confirmed in our study as no increase in colorectal cancer occurrence was found between our patients with MC and the control group.

Interestingly, we found that IBS was associated with MC in approximately 13% of our MC patients. Other studies have also shown a correlation between IBS and MC, with IBS diagnosed in up to 23% of patients with LC<sup>[14,15]</sup>. The relationship is unclear, however it could be due to a similar clinical presentation between MC and IBS, as suggested by Barta *et al*<sup>[16]</sup>. There is also increasing evidence to support an inflammatory process in the pathogenesis of IBS. Furthermore, studies have shown that increasing amounts of intraepithelial lymphocytes can be seen in patients diagnosed with post infectious

IBS<sup>[17]</sup>. Usually, the number of lymphocytes does not reach that needed for the diagnosis of MC in most post infectious IBS patients, however, there have been cases where patients with a clinical history of post infectious IBS have been diagnosed with MC. To accurately distinguish these entities is important, as their management and treatment may be very different. For example, currently, colonoscopy is rarely performed for the diagnosis of IBS. However, as a result of our findings, colonoscopy with random biopsy may be warranted as a part of the workup for suspected diarrhea predominant IBS in the future.

Endocrinopathies such as thyroid dysfunction and diabetes have been known to have correlations with MC as noted in previously published studies. Several studies showed that diabetes was found in up to 8.3% of CC patients and in up to 13.5% of LC patients<sup>[18,19]</sup>. Despite reports of these associations, our study did not show a statistical significance when compared with our control group. One possible explanation for this is that LC, CC and diabetes tend to occur more frequently in elderly populations and thus could easily be thought of as related diseases. In a recent published study by Williams *et al*<sup>[20]</sup> after risk stratification of their data by age, the risk association between MC and the general population was no longer significant. Another possible explanation is that most of the previous studies were smaller in size which may have led to sampling error. However, the same argument can not be used to explain the high occurrence of thyroid dysfunction previously reported<sup>[21]</sup>. Indeed, we found a statistically higher occurrence of thyroid dysfunction in LC and CC patients when compared to the control group. The association between thyroid dysfunction, LC and CC is still unknown, although some suggest autoimmunity as a possible cause.

There are several limitations to our study. For example, because routine screening for other disorders that may be related to MC (such as celiac disease) were not performed in a prospective format, the prevalence of these associated disorders may be underestimated. Another limitation is the lack of long term follow up. Because of this lack of long term follow up, it is possible that colorectal cancers and IBD may not have had sufficient time to manifest. Due to this, we continue to actively follow these patients and long term data should be forthcoming in the future. Another limitation involves the retrospective nature of this study for which selection bias is commonly seen. However, we hope that the large patient population would minimize such bias.

Our analysis produced many interesting observations and provoked thoughtful questions regarding the etiology, disease mechanism, and associations with other diseases and medications in the largest study to date on MC. Several of these findings have been previously noted, however, some other relationships have yet to be reported. We believe that the large number of patients in our study makes this study less vulnerable to sampling error and provides a good representation of MC.

## COMMENTS

### Background

The prevalence of microscopic colitis (MC) is on the rise worldwide, yet little is known about the natural history and etiology of this disease. Currently, it is believed that the disease follows a benign course; however, cases have been reported in association with inflammatory bowel disease (IBD). Its association with colorectal cancer is also poorly understood.

### Research frontiers

Case reports have suggested that MC can evolve into IBD, but no studies have been carried out to thoroughly evaluate this relationship.

### Innovations and breakthroughs

This is the largest study published to-date that addresses the important question of whether patients with MC have a higher prevalence of IBD compared to the general population. It also provided additional clinical information regarding this rarely-studied disease.

### Applications

This study, which evaluated the largest population to-date, demonstrated that there is no increase in the incidence of IBD or colorectal cancer in patients with MC. It also showed that MC can mimic symptoms of irritable bowel syndrome which should be considered during evaluation.

### Terminology

MC is a condition characterized by chronic, watery diarrhea with normal endoscopic appearance of the colonic mucosa. It is further divided into lymphocytic colitis and collagenous colitis based on its histologic appearance.

### Peer review

This is an interesting paper addressing a topic which often remains scarcely investigated. The article is well balanced and the discussion is clear and exhaustive.

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BRIEF ARTICLES

## Portal hypertension secondary to myelofibrosis with myeloid metaplasia: A study of 13 cases

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### Abstract

**AIM:** To describe the clinical presentation and complications of portal hypertension (PH) secondary to myelofibrosis with myeloid metaplasia (MMM).

**METHODS:** Medical records for 123 patients with MMM were reviewed.

**RESULTS:** Thirteen patients with PH secondary to MMM were identified. Median ages at time of MMM and PH diagnosis were 61 and 66 years, respectively. The interval from MMM diagnosis to presentation with one of the PH features ranged from 1 to 11 years. Variceal bleeding and ascites were the most common presentations. Of the eight patients who presented with variceal bleeding, six patients underwent endoscopic variceal ligation (EVL) with no variceal recurrence or hematological worsening during a 12-mo follow up period.

**CONCLUSION:** Patients with MMM might develop PH. Exact mechanisms leading to PH in MMM are still controversial. As in other etiologies, variceal bleeding and ascites are the most common presentations. Anemia may correlate with, and/or predict, the severity of the PH presentation in these patients. EVL can successfully control variceal bleeding in MMM. Further clinical studies are required.

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### INTRODUCTION

The term “myelofibrosis with myeloid metaplasia (MMM)” is usually referred to for patients with chronic idiopathic myelofibrosis (CIM), also known as angiogenic myeloid metaplasia (AMM), and for those with advanced phases of polycythemia vera [post polycythemic myeloid metaplasia (PPMM)] and essential thrombocythemia [post thrombocythemic myeloid metaplasia (PTMM)]. All three represent chronic stem cell-derived clonal myeloproliferative diseases that, in the case of myeloid metaplasia, are accompanied by an intense reactive bone marrow fibrosis that leads to ineffective erythropoiesis and extramedullary hematopoiesis in multiple organs, predominantly the spleen. However, extramedullary hematopoiesis may also occur at sites other than the spleen, including lymph nodes causing lymphadenopathy and also in the liver resulting in hepatomegaly and possibly, with other contributing factors, portal hypertension (PH)<sup>[1,2]</sup>.

Patients with MMM present with variable clinical and histomorphologic features. The typical clinical features include hypermetabolic symptoms (fever, fatigue and weight loss), marked splenomegaly and anemia<sup>[3]</sup>. PH with subsequent ascites and gastrointestinal hemorrhage from ruptured varices has been described in patients with MMM<sup>[4-8]</sup>. Only a few published studies have discussed this issue extensively. Furthermore, these reports described and focused on PH in CIM which represents a subgroup of MMM. In this retrospective study, we describe 13 patients with PH secondary to MMM.

## MATERIALS AND METHODS

After obtaining approval from the Institutional Review Board of our institution, the study patients were identified through the use of a comprehensive institutional database of medical diagnoses and procedures. Between January 1, 1995 and December 31, 2007, an estimated number of 123 patients with MMM were evaluated at the Mayo Clinic, Rochester, MN. The diagnosis of MMM was confirmed on the basis of traditional criteria that included bone marrow fibrosis associated with splenomegaly and leukoerythroblastosis (immature granulocytes and nucleated red cells). Patients with bone marrow fibrosis resulting from other disorders were excluded. This included patients with myelodysplastic syndrome, acute myelofibrosis, or chronic myelogenous leukemia.

From this sample, a subgroup of 13 patients with clinical evidence of PH was identified. Although the gradient between wedged and free hepatic venous pressure was not assessed in many patients, the diagnosis of PH was based on clinical criteria [e.g. esophageal varices by endoscopy, ascites with Serum-Ascites Albumin Gradient (SAAG) > 1.1] in conjunction with imaging studies. Patients with liver disease and patients having risk factors for liver disease such as chronic alcohol consumption were excluded. Patients with PH due to other diseases were also excluded. The medical records for these 13 patients with PH secondary to MMM were comprehensively reviewed. Pertinent clinical and laboratory variables were recorded for all patients.

## RESULTS

Thirteen patients with PH secondary to MMM were identified. Table 1 shows their pertinent clinical characteristics, presentation and laboratory values within 1 wk of PH diagnosis. Nine male (69%) and four female patients (31%) were identified: six patients (46%) had AMM, six patients (46%) had PPMM, and one patient (8%) had PTMM. The age of the patients ranged from 49 to 88 years (median, 67 years). Median ages and range at time of MMM and PH diagnoses were 61 (45-79) years and 66 (47-84) years respectively. The interval from MMM diagnosis to presentation with one of the PH features ranged from 1 to 11 years (median, 5 years).

Variceal bleeding and ascites were the most common presentations. Six patients (46%) initially presented with GI bleeding (five patients presented with acute upper GI bleeding and one presented with melena). Among those six patients; three presented with GI bleeding only, two presented with both GI bleeding and ascites, and one presented with GI bleeding and jaundice. All the six patients were diagnosed as having mild to moderate esophageal varices (Grade 1 to 2). Five patients (38%) presented initially with ascites. Among these five patients; three had only ascites at presentation, two presented with GI bleeding with the ascites, and one patient presented with abdominal

**Table 1 Clinical characteristics, presentation and laboratory values within 1 wk of PH diagnosis for the 13 patients**

Variable	
Age (yr), median (range)	
All patients	67 (49-88)
At time of diagnosis of MMM	61 (45-79)
At time of diagnosis of PH	66 (47-84)
Interval till presentation with PH	5 (1-11)
Sex n (%)	
Male	9 (69)
Female	4 (31)
Type of MMM n (%)	
AMM	6 (46)
PPMM	6 (46)
PTMM	1 (8)
Initial presentation n (%)	
GI bleeding only	3 (23)
Ascites only	3 (23)
GI bleeding and ascites concurrently	2 (15)
Jaundice (with GI bleeding)	1 (8)
Encephalopathy	0 (0)
Abdominal pain (with ascites)	1 (8)
Splenomegaly	13 (100)
Hepatomegaly	9 (69)
Portal vein thrombosis	3 (23)
CBC, median (range)	
Hemoglobin (g/dL)	10 (6.6-13.9)
WBC ( $\times 10^9/L$ )	8.8 (2.1-49)
Platelet count ( $\times 10^9/L$ )	225 (47-694)
Liver function, median (range)	
AST (U/L)	69 (26-172)
Alkaline phosphatase (U/L)	338 (74-850)
Total bilirubin (mg/dL)	0.9 (0.3-2.2)
PT (s)	12.4 (10.8-14.1)
Albumin (g/dL)	4 (2.8-4.4)
SAAG	2 (1.9-2.4)

PT: Prothrombin time.

pain in addition to ascites. SAAG was calculated in patients with ascites; all had SAAG above 1.1 which was considered as indicative of PH. Amongst all 13 patients, 12 of them (92%) eventually developed at least one episode of ascites within 6 mo from initial presentation. Jaundice was never the sole presenting feature. One patient presented with jaundice in addition to GI bleeding. Another patient developed jaundice afterwards, during the course of the disease. None presented with encephalopathy.

All patients (100%) had splenomegaly; nine patients (69%) had hepatomegaly and consequently nine patients (69%) had both. Eleven patients (84%) had elevated alkaline phosphatase levels at time of PH diagnosis, nine patients (69%) had elevated aspartate aminotransferase levels, and eight patients (62%) had both enzyme levels elevated. Median and range values for all liver tests are displayed in Table 1. Hyperbilirubinemia was present in four patients (31%) at time of PH diagnosis but jaundice was uncommon. Four patients (31%) had low albumin, with a median albumin level of 4 g/dL (2.8-4.4 g/dL). Ten patients (77%) were anemic at the time of PH diagnosis. Median hemoglobin level was 10 mg/dL (6.6-13.9 mg/dL). Three patients (23%) were diagnosed with portal vein thrombosis at time of

PH diagnosis, indicated by either abdominal doppler ultrasound (US) or CT or MRI. Among these three patients with portal vein thrombosis, one patient had high white blood cell count and low hemoglobin, while the other two patients had white blood counts and hemoglobin levels within normal limits but had decreased portal blood flow velocity indicated by abdominal doppler US. Among the 10 patients who did not have thrombosis, eight patients have undergone liver biopsy: all eight showed non cirrhotic liver parenchyma with varying degrees of extramedullary hematopoiesis, and infiltration of liver sinusoids with hematopoietic cells (myeloid metaplasia). Two of these eight patients also had mild fibrotic changes but none of them had truly cirrhotic features. All patients, including those without thrombotic etiology, had splenomegaly. Of the eight patients presenting with variceal bleeding, six patients underwent endoscopic variceal ligation (EVL) requiring 1-4 sessions. These six patients were followed up by endoscopies at 1, 3 and 12 mo to inspect for the re-appearance of varices. Varices were completely obliterated with no recurrence at the 12 mo time point. No hematologic worsening was recorded during the 12 mo follow-up period. The other two patients underwent endoscopic variceal sclerotherapy (EVS) but were not followed up. Interestingly, three patients (23%) had pulmonary hypertension concurrent with PH, with no cardiopulmonary causes.

## DISCUSSION

PH has been reported in 7%-18% of AMM, which represents a subgroup of MMM<sup>[8,9]</sup>. In this study, simple mathematical calculation revealed an 11% prevalence of symptomatic PH in MMM. However, as in other most etiologies, PH secondary to MMM is usually asymptomatic and diagnosis of PH in these patients is often not made until they become symptomatic, which sometimes does not become apparent for up to 11 years. In the present study we found that the median interval for our patients to present with one of the PH features is 5 years, ranging from 1-11 years. Taking into consideration that the median survival for patients with MMM is 4 years<sup>[10-15]</sup>, this suggests that many MMM patients die due to the underlying disease before they become symptomatic from PH. Therefore, we believe that a prevalence of 11% represents the prevalence of symptomatic PH and that the true prevalence of PH in MMM, including the asymptomatic PH, is higher.

When patients become symptomatic, they usually present either with acute upper GI bleeding from ruptured varices or in the form of melena, or they present with ascites. Other presentations such as jaundice and encephalopathy are unlikely.

Exact mechanisms leading to PH in MMM are still controversial. In the absence of portal and/or hepatic vein thromboses two theories have been proposed. The first theory states that PH develops in MMM patients due to sinusoidal narrowing and intrahepatic obstruction

caused by extramedullary hematopoiesis and infiltration of the liver by myeloid cells leading to increased intrahepatic resistance<sup>[6,16,17]</sup>, while the other theory states that PH develops in such patients due to increased portal blood flow through the enlarged spleen<sup>[8,18,19]</sup>.

Portal vein thrombosis is a major cause of PH in hematological disorders<sup>[6,16,18]</sup>. It is still unclear whether portal vein thrombosis in MMM is caused by a hypercoagulable state and hyperviscosity related to underlying disease or as a consequence of stasis due to elevated sinusoidal pressure. In our study, PH in three of our patients (23%) was due to thrombosis of the portal and/or hepatic veins as indicated by imaging studies. The three patients had prothrombin time within normal limits. However, one of these patients had a very high white blood cell count (49 000 cells/mL), possibly contributing to thrombosis by increasing the blood viscosity. When thrombosis is absent, PH has been related to an increased intrahepatic resistance and sinusoidal narrowing due to the myeloid metaplasia<sup>[6,16,17]</sup> and/or due to a marked increase in portal flow as a consequence of marked splenomegaly<sup>[8,18,19]</sup>. In our report, the 10 patients who did not have portal vein thrombosis had splenomegaly; six of them had dilated splenic veins with increased portal blood flow indicated by abdominal doppler ultrasound. The increase in portal flow itself may explain PH. In fact, few cases of PH in myelofibrosis patients secondary to increased splenic and/or portal flow with minimal hematopoiesis have been reported<sup>[20,21]</sup>. On the other hand, Sikuler *et al*<sup>[22]</sup> experimentally demonstrated that in the absence of structural alteration of the liver, PH does not develop as a consequence of an increased portal flow. They proposed that the main contributory factor is the increased intrahepatic resistance caused by obstruction due to extramedullary hematopoiesis of the liver. In our group of patients, liver biopsy in those six patients with increased portal flow and another two patients without increased portal flow showed infiltration of liver sinusoids with hematopoietic cells and myeloid metaplasia. Therefore six of our patients, without thrombotic etiology, have both enhanced portal flow and increased intrahepatic resistance as contributing factors. Hence, it seems that in absence of thrombosis, both enhanced portal flow from the enlarged spleen and intra-hepatic sinusoidal obstruction have synergistic effects, so that even a slight increase in resistance in the face of enhanced portal flow might produce clinically significant PH.

Patients with PH characteristically exhibit a hyperdynamic circulation with increased cardiac output and decreased peripheral resistance<sup>[23]</sup>. Overactivity of some vasodilator factors has been proposed and there is a growing body of evidence suggesting that endogenous NO accounts for much of this activity<sup>[24-26]</sup>. Anemia, which is a very common feature in patients with MMM, has been shown to further worsen the hyperdynamic circulation associated with PH<sup>[27-29]</sup>. In this group of patients, 10 patients (77%) were anemic at the time of PH

diagnosis. At presentation, a total of four patients had a picture of severe PH. Three of these four patients had profound anemia (hemoglobin 6.6, 6.9 and 7.1 mg/dL) suggesting that anemia, possibly by worsening the hyperdynamic circulation, might have a role in exacerbating PH and that anemia may correlate with and/or predict the severity of the PH presentation in these patients. Experimental studies have demonstrated that increasing blood hemoglobin levels partially correct PH hyperdynamic circulation in rats<sup>[30]</sup>. However, clinical data focusing on the role of anemia on the hyperdynamic state are scant and more studies are needed.

The optimal management of PH secondary to MMM and management of subsequent variceal hemorrhage and ascites have not been well established. Based on the theory of increased portal flow due to splenomegaly as a mechanism for the PH in MMM patients, splenectomy would be a reasonable choice. In fact, splenectomy, which is commonly performed for patients to relieve abdominal discomfort and early satiety caused by the mass effect of the enlarged spleen, has effectively reversed PH in selected patients<sup>[4,31]</sup>. However, increased blood flow from an enlarged spleen is not the sole mechanism for development of PH in MMM patients. Also, the enlarged spleen may possibly be the main or even the only site for red cell production in patients with advanced MMM. Moreover, there are some unique post-operative complications such as massive hepatomegaly due to extramedullary hematopoiesis in 16%-24% of splenectomized MMM patients leading to liver failure in some cases, post-splenectomy extreme thrombocytosis in up to 50% of splenectomized MMM patients, and the major concern of leukemic transformation. All these factors, together with the knowledge that splenectomy has not been shown to improve overall survival in MMM patients, must be strongly considered before proceeding for splenectomy to manage PH<sup>[3,32-34]</sup>.

PH caused by intra-hepatic or portal obstruction requires interventional or surgical portosystemic shunting. Relief of intra-hepatic PH in patients with MMM can be accomplished by implantation of a transjugular intrahepatic portosystemic shunt (TIPS)<sup>[35-37]</sup>. TIPS is an effective and well established procedure that involves creation of a side-to-side portacaval shunt in the liver and it has very good efficacy for intractable ascites. However, such a procedure needs ideal candidates who display normal liver synthetic function with little interventional risk. Only a few reports have been published regarding the use of TIPS for PH secondary to MMM, but these have proved to be effective<sup>[3,35-39]</sup>. Few data exist regarding the outcomes of this procedure in MMM patients and more studies are needed to examine whether it prolongs survival or just alleviates variceal hemorrhage and recurrent ascites.

Managing the acute bleeding episodes consists of general resuscitative measures such as volume and blood replacement, and specific measures to stop bleeding. EVL<sup>[40-42]</sup> as well as EVS<sup>[7]</sup> have been utilized successfully

for the management of GI bleeding in MMM patients. EVL has been reported to have very good efficacy, with fewer therapeutic sessions and complications when compared to EVS in variceal bleeding due to other etiologies<sup>[43]</sup>. In our report, six out of eight patients who had variceal bleeding underwent EVL with no variceal recurrence or hematological worsening during a brief 12 mo follow up period. Nevertheless, there is a paucity of data regarding the use of EVL in patients with MMM-associated PH and further studies are required. We report our successful experience with EVL in this small group of patients.

In conclusion, patients with MMM might develop PH. Exact mechanisms leading to PH in MMM are still controversial. In the absence of portal vein thrombosis, both increased intrahepatic resistance due to sinusoidal narrowing caused by extramedullary hematopoiesis and a rise in portal pressure, via an increase in portal blood flow secondary to increased splenic blood flow from an enlarged spleen, might play a role in the pathogenesis of PH. Clinical presentation is similar to PH due to other etiologies with variceal bleeding and ascites being the most common presentations. Anemia may correlate with, and/or predict the severity of, the PH presentation in these patients. EVL can successfully control variceal bleeding in MMM. Further clinical studies are required.

## COMMENTS

### Background

Patients with myelofibrosis with myeloid metaplasia (MMM) present with variable clinical features. The typical clinical features include constitutional symptoms, splenomegaly and progressive anemia. Portal hypertension (PH) with subsequent variceal bleeding and ascites has been described.

### Research frontiers

Few published studies have discussed PH in patients with MMM. However, these small reports described and focused on PH in chronic idiopathic myelofibrosis (CIM) which represents a subgroup of MMM.

### Innovations and breakthroughs

In this retrospective study, the authors describe clinical presentation and complications of 13 patients with PH secondary to MMM. The article also discusses the possible mechanisms leading to PH in these patients and the possible treatment options.

### Applications

Patients with MMM might develop PH. As in other etiologies, variceal bleeding and ascites are the most common presentations. Exact mechanisms leading to PH in MMM are still controversial. Interestingly, anemia may correlate with and/or predict the severity of the PH presentation in those patients. Endoscopic variceal ligation can successfully control variceal bleeding in these patients. However, further clinical studies and trials are required.

### Terminology

PH is an increase in the pressure within the portal vein and its tributaries. It is defined as a portal pressure of 12 mmHg or more compared with a normal figure of 5-8 mmHg. Myelofibrosis with myeloid metaplasia is a term referred to for patients with CIM, also known as angiogenic myeloid metaplasia, and advanced phases of polycythemia vera and essential thrombocythemia.

### Peer review

This is a retrospective study of patients with myelofibrosis and PH. The authors describe the clinical presentation and complications of this unusual condition. The paper is well written and includes a discussion of the pathogenesis of PH in myelofibrosis.

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BRIEF ARTICLES

## Serum bile acid profiling reflects enterohepatic detoxification state and intestinal barrier function in inflammatory bowel disease

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acid (BA) levels in inflammatory bowel disease (IBD) subgroups with defined clinical manifestations.

**METHODS:** Comprehensive serum BA profiling was performed in 358 IBD patients and 310 healthy controls by liquid chromatography coupled to electrospray ionization tandem mass spectrometry.

**RESULTS:** Serum levels of hyodeoxycholic acid, the CYP3A4-mediated detoxification product of the secondary BA lithocholic acid (LCA), was increased significantly in Crohn's disease (CD) and ulcerative colitis (UC), while most other serum BA species were decreased significantly. Total BA, total BA conjugate, and total BA glycoconjugate levels were decreased only in CD, whereas total unconjugated BA levels were decreased only in UC. In UC patients with hepatobiliary manifestations, the conjugated primary BAs glycocholic acid, taurocholic acid, and glycochenodeoxycholic acid were as significantly increased as the secondary BAs LCA, ursodeoxycholic acid, and taurooursodeoxycholic acid compared to UC patients without hepatobiliary manifestations. Finally, we found that in ileocecal resected CD patients, the unconjugated primary BAs, cholic acid and chenodeoxycholic acid, were increased significantly compared to controls and patients without surgical interventions.

**CONCLUSION:** Serum BA profiling in IBD patients that indicates impaired intestinal barrier function and increased detoxification is suitable for advanced diagnostic characterization and differentiation of IBD subgroups with defined clinical manifestations.

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**Key words:** Bile acids; Liquid chromatography; Tandem mass spectrometry; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis

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Gnewuch C, Liebisch G, Langmann T, Dieplinger B, Mueller T, Haltmayer M, Dieplinger H, Zahn A, Stremmel W, Rogler G, Schmitz G. Serum bile acid profiling reflects enterohepatic

### Abstract

**AIM:** To determine free and conjugated serum bile

detoxification state and intestinal barrier function in inflammatory bowel disease. *World J Gastroenterol* 2009; 15(25): 3134-3141 Available from: URL: <http://www.wjgnet.com/1007-9327/15/3134.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.3134>

## INTRODUCTION

The pathophysiology of inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC) is not yet completely understood. In recent years, it has become obvious that genetic, immunological, environmental and microbial factors contribute to the etiology of IBD<sup>[1-5]</sup>. The concept of multilevel dysfunction of the intestinal detoxification system is accepted as an important aspect of the pathophysiology of IBD<sup>[6]</sup>. Intestinal epithelial cells are of major importance as a physiological barrier against components of the intestinal lumen such as bacteria, nutrients and toxins. Protective mechanisms that maintain intestinal barrier function include detoxification and biotransformation of luminal substances, as well as the control of junctional proteins in cell-cell contacts. These processes are influenced by lipids and the availability of adequate cellular lipid processing<sup>[7]</sup>.

Bile acids (BAs) are involved in these processes. First of all, intestinal reabsorption is a critical step in enterohepatic circulation (EHC) of BAs<sup>[8]</sup>. Once synthesized in the liver and secreted via the bile duct into the duodenum, BAs are effectively absorbed in the distal ileum and transported back to the liver via the portal vein, which contributes to the pool of BAs in the blood<sup>[9,10]</sup>. Absorption in the distal ileum may be hampered in CD by terminal ileitis or by ileocecal resection, which tends towards decreased fasting and slightly increased postprandial blood BA levels<sup>[11-15]</sup>. There have also been indications of abnormal blood BA levels in UC<sup>[16,17]</sup>. Furthermore, EHC and enterohepatic detoxification of BAs depend on a carefully adjusted regulatory network of BA-binding transcription factors, including farnesoid X receptor (FXR) and pregnane X receptor (PXR)<sup>[9,18]</sup>. For instance, like ursodeoxycholic acid (UDCA), the endogenous toxic lithocholic acid (LCA) belongs to a group of potent PXR agonists<sup>[19-21]</sup> that comprises steroid hormones, vitamins and β-carotene<sup>[22]</sup>. Several detoxification genes and ATP-binding cassette transporters are down-regulated in intestinal cells of IBD patients<sup>[23]</sup>, and PXR as a major transcriptional regulator of these detoxification genes is decreased in UC patients<sup>[23]</sup>. Finally, despite their potential toxic activities, BAs can also confer gut mucosal protection against bacteria and other cell damaging constituents of the gut lumen by two mechanisms<sup>[10]</sup>. In the proximal small intestine, BAs inhibit bacterial growth directly via their pharmacological properties, whereas in the distal small intestine, BAs mediate their antimicrobial effect indirectly via activation of FXR<sup>[24,25]</sup>. For instance, binding of chenodeoxycholic acid (CDCA) to FXR and

subsequent activation of the receptor is followed by up-regulation of genes that are involved in the prevention of bacterial overgrowth and promotion of epithelial integrity<sup>[26]</sup>.

In the present multicenter study, serum BA profiling was performed retrospectively in 358 IBD patients and in 310 age-matched healthy controls to assess the influence of different IBD phenotypes with various clinical manifestations on BA composition. The results further elucidate the intestinal contribution to BA homeostasis and detoxification, which is much less understood compared to corresponding processes in the liver<sup>[24,27]</sup>.

## MATERIALS AND METHODS

### **Patients and specimens**

Blood samples of IBD patients and healthy volunteers were from the University Hospitals of Regensburg and Heidelberg (Germany), the Konventhospital Barmherzige Brüder Linz (Austria), and the Innsbruck Medical University (Austria). Informed consent was obtained from all patients, and the study was approved by the respective ethics committees. Samples of the Regensburg patients were from the serum bank of the German IBD network of excellence ("Kompetenznetz CED"). Blood samples were collected irrespective of the individual diet. For BA analysis, sera and clinical data were obtained from 197 CD patients (62% females; aged 16-84 years, mean age, 40 years; 46 with active disease, 43 with chronic active disease, 108 in remission) and 161 UC patients (63% females; aged 17-90 years, mean age, 40 years; 42 with active disease, 40 with chronic active disease, 79 in remission). The diagnosis was based on clinical, radiological and pathological criteria according to the guidelines of the German Gastroenterological Association (DGVS). CD patients were subgrouped according to the Vienna Classification with respect to disease behavior and localization. A CD activity index (CDAI) > 150 was regarded as "active CD", duration of CDAI > 150 for > 3 mo as "chronic active CD", and CDAI < 150 as "CD in remission". UC patients were classified according to the Truelove-Witts index: A Truelove-Witts index > 6 was regarded as "active UC", a Truelove-Witts index > 6 for > 3 mo as "chronic active UC", and a Truelove-Witts index < 6 as "UC in remission". Sera from 310 healthy blood donors (60% females; aged 19-66 years, mean age, 40 years) served as controls. All samples were stored frozen at -20°C until analysis.

### **Materials for BA analysis**

Cholic acid (CA), CDCA, deoxycholic acid (DCA), LCA, UDCA, hyodeoxycholic acid (HDCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), glycdeoxycholic acid (GDCA), glycolithocholic acid (GLCA), glycoursoxycholic acid (GUDCA), glycohydeoxycholic acid (GHDCA), taurocholic acid (TCA), taurochenodeoxycholic acid (TCDC), taurodeoxycholic acid (TDCA), tarolithocholic acid (TLCA), tauro-

sodeoxycholic acid (TUDCA), taurohyodeoxycholic acid (THDCA) standard substances, as well as deuterated BA internal standard (IS) substances ( $D_4$ -CA, -CDCA, -DCA, -LCA, -UDCA, -GCA, -GCDCA) were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany), Steraloids Inc. (Newport, RI, USA), Campro Scientific GmbH (Berlin, Germany), Larodan Fine Chemicals AB (Malmö, Sweden), and were at least of 95% purity. Ammonium acetate (98%), hydrochloric acid (p.a.), as well as HPLC grade methanol and acetonitrile were purchased from VWR Int. GmbH (Darmstadt, Germany). Ultra pure water (18.2 MΩcm) was obtained from a Milli-Q Plus system (Millipore GmbH, Schwalbach, Germany).

### **BA extraction**

For serum BA extraction, the method of Tagliacozzi *et al*<sup>[28]</sup> was applied with some modifications. Twenty-five microliters of a mixed IS BA solution (6-140 μmol/L in methanol) was pipetted into a 1.5-mL reaction tube and vacuum-evaporated. Two hundred and fifty microliters serum and 30 μL 1 mol/L hydrochloric acid were added (pH < 1), and the mixture was shaken for 1 min. After addition of 1 mL acetonitrile, the mixture was shaken for 2 min and centrifuged at 14000 g for 15 min. The acetonitrile supernatant was transferred to a new reaction tube and vacuum-evaporated. The residue was dissolved in 250 μL methanol/water 1:1 (v/v) that contained 10 mmol/L ammonium acetate by shaking and sonication, and centrifuged at 14000 g for 5 min. Ten microliters of the clear methanolic supernatant was used for analysis. Calibration samples were prepared by spiking pooled control serum with 0, 25, 50, 75 and 100 μL of a combined BA standard solution that contained appropriate amounts of each BA (0.5-70.5 μmol/L).

### **Liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) determination of BAs**

BAs were analyzed by LC-ESI-MS/MS using the following instrumentation. An HTS PAL autosampler (CTC Analytics AG, Zwingen, Switzerland), an Agilent 1100 series binary HPLC pump (Agilent Technologies Sales & Services GmbH & Co KG, Waldbronn, Germany) combined with a Micromass Quattro Ultima tandem MS (Waters GmbH, Eschborn, Germany) operated in negative-ion mode. BA separation was performed on a Symmetry C18 reversed-phase HPLC column (50 mm × 2.1 mm, 3.5 μm particle size; Waters GmbH) by gradient elution at a flow rate of 0.3 mL/min. The 25-min elution cycle consisted of a stepwise linear change from 90% solvent A (methanol/water 1:1, v/v, 10 mmol/L ammonium acetate) + 10% solvent B (methanol, 10 mmol/L ammonium acetate) to 100% B; in detail: 0-6 min A/B = 90/10; 6-12 min A/B = 72/28; 12-16 min A/B = 60/40; 16-22 min A/B = 0/100; 22-25 min A/B = 90/10. The mass spectrometer was operated in multiple-reaction monitoring (MRM) with a cone voltage of 80 V and a collision gas pressure

of 250 kPa argon. Unconjugated BAs were detected unfragmented using a collision energy of 10 eV. Glycine- and taurine-conjugated BAs were analyzed by their specific product ions at m/z 74 and 80 using collision energies of 35 and 85 eV, respectively. In detail, the retention times and MRM transitions were as follows: TCA (6.0 min, 514→80), TUDCA (4.0 min, 498→80), THDCA (4.8 min, 498→80), TCDCA (9.3 min, 498→80), TDCA (10.2 min, 498→80), TLCA (13.7 min, 482→80), GCA (6.2 min, 464→74), GUDCA (4.1 min, 448→74), GHDCA (4.9 min, 448→74), GCDCA (9.5 min, 448→74), GDCA (10.4 min, 448→74), GLCA (13.9 min, 432→74), CA (7.1 min, 407→407), UDCA (5.1 min, 391→391), HDCA (6.0 min, 391→391), CDCA (11.0 min, 391→391), DCA (11.8 min, 391→391), LCA (14.8 min, 375→375). Quantification was performed by peak ratios of BA peak areas and corresponding IS peak areas. BAs without identical deuterated ISs were related to the IS with the nearest retention time, as well as the similar MRM transition.

### **Statistical analysis**

The significance of differences in BA concentrations was determined between cohorts with  $n \geq 10$  by Mann-Whitney U test for non-normally distributed data using SPSS for Windows version 14.0 (SPSS Inc., Chicago, IL, USA). A two-sided P value < 0.05 was considered statistically significant.

## **RESULTS**

### **Differentiation of CD, UC and controls by characteristic BA profiles**

Based on previous findings on BA metabolism in IBD and our earlier results on dysregulation of xenobiotic nuclear receptors including PXR in IBD<sup>[23]</sup>, serum BA levels and composition were determined in the two major IBD phenotypes, CD and UC, and in healthy controls. The most significant differences in serum BA concentrations were found in comparison to controls. BA concentrations were decreased predominantly in both IBD subgroups, CD and UC (Tables 1-3). Considering individual BA species, as in CD, most BAs were decreased significantly in UC patients compared with controls, but several BA conjugates, for instance TCDCA, GCDCA and GDCA, were decreased more significantly in CD than in UC patients (Table 1).

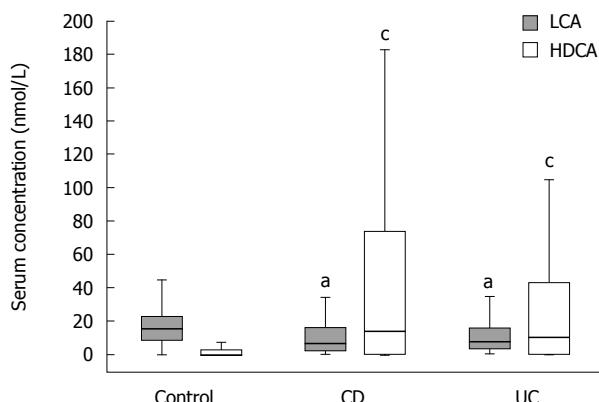
While serum levels of LCA, known to be the strongest PXR agonist, were significantly lower, serum levels of HDCA, the CYP3A4-mediated detoxification product of LCA, were always significantly higher in CD and UC patients compared to controls (Figure 1).

If total serum BA levels were considered (Table 4), we found that total unconjugated and total BA tauroconjugate levels, respectively, were decreased significantly in UC patients, but other than in CD patients, there was no decrease in total BA, total BA conjugate, and total BA glycoconjugate levels compared to controls. Moreover, total BA conjugate levels, as well as total BA glycoconjugate and

**Table 1 Serum bile acids and conjugates in CD, UC and control cohorts (nmol/L)**

BA class	BA/BA-conjugate	Control	CD	UC
Primary	CA	62.5	72.0	58.0
	TCA	23.6	0.0 <sup>a</sup>	0.0 <sup>a,b</sup>
	GCA	383.5	234.0 <sup>a</sup>	377.0 <sup>b</sup>
	CDCA	196.5	190.0	145.0 <sup>a,b</sup>
	TCDCA	230.5	46.0 <sup>a</sup>	93.0 <sup>a,b</sup>
	GCDCA	1446.0	848.0 <sup>a</sup>	1243.0 <sup>a,b</sup>
Secondary	DCA	239.8	53.0 <sup>a</sup>	64.0 <sup>a</sup>
	TDCA	48.2	0.0 <sup>a</sup>	0.0 <sup>a</sup>
	GDCA	238.6	26.0 <sup>a</sup>	80.0 <sup>a,b</sup>
	LCA	15.0	6.2 <sup>a</sup>	8.0 <sup>a</sup>
	TLCA	0.0	0.0 <sup>a</sup>	0.0 <sup>a</sup>
	GLCA <sup>1</sup>	17.4	0.0 <sup>a</sup>	0.4 <sup>a</sup>
	UDCA	28.5	22.0 <sup>a</sup>	17.0 <sup>a</sup>
	TUDCA	0.0	0.0 <sup>a</sup>	0.0 <sup>b</sup>
Tertiary	GUDCA <sup>1</sup>	137.9	75.6 <sup>a</sup>	60.6 <sup>a</sup>
	HDCA	0.0	16.0 <sup>a</sup>	10.0 <sup>a</sup>
	THDCA	0.0	0.0 <sup>a</sup>	0.0 <sup>a</sup>
	GHDCA <sup>1</sup>	0.0	0.0 <sup>a</sup>	0.0 <sup>a</sup>

Control: n = 310; CD: n = 197, n = 73<sup>1</sup>; UC: n = 161, n = 44<sup>1</sup>. Data expressed as medians. <sup>a</sup>P < 0.05 vs control, <sup>b</sup>P < 0.05 vs CD (Mann-Whitney U test).



**Figure 1 Decrease of serum LCA but increase of serum HDCA in CD and UC vs healthy control cohorts.** LCA and HDCA were analyzed using LC-ESI-MS/MS. Box plots represent interquartile ranges containing medians (boxes) and minimum/maximum bars. <sup>a</sup>P < 0.05 vs LCA in controls; <sup>c</sup>P < 0.05 vs HDCA in controls (Mann-Whitney U test).

tauroconjugate levels alone, were increased significantly in UC vs CD patients. However, if levels relative to total BA conjugate levels were considered, total BA tauroconjugate levels were increased significantly but total BA glycoconjugate levels were decreased significantly in UC vs CD patients. In addition, UC patients were characterized by a significantly decreased ratio of total deoxy-BA, LCA, and LCA conjugate levels to total BA levels compared to CD patients and controls (Table 4).

#### Hepatobiliary manifestations influence BA composition in UC

IBD is often accompanied by extraintestinal manifestations (EMs), such as hepatobiliary diseases, and eye, joint and skin affections<sup>[29,30]</sup>. We therefore investigated whether IBD patients with EMs showed different serum

**Table 2 Effect of extraintestinal manifestations on serum bile acids and conjugates in UC cohorts (nmol/L)**

BA class	BA/BA-conjugate	Control	No EM	Arthralgia/ arthritis	Hepatobiliary diseases
Primary	CA	62.5	67.5	88.5	59.0
	TCA	23.6	0.0	0.0	67.9 <sup>b,c</sup>
	GCA	383.5	360.0	408.5	722.5 <sup>a,b</sup>
	CDCA	196.5	37.5	175.5	252.0
	TCDCA	230.5	140.0 <sup>a</sup>	92.5 <sup>a</sup>	163.0 <sup>c</sup>
	GCDCA	1446.0	1327.0	1281.5	2403.0 <sup>b,c</sup>
Secondary	DCA	239.8	78.0 <sup>a</sup>	28.0 <sup>a</sup>	62.5 <sup>a</sup>
	TDCA	48.2	0.0 <sup>a</sup>	0.0 <sup>a</sup>	0.0 <sup>a</sup>
	GDCA	38.6	82.2 <sup>a</sup>	14.0 <sup>a</sup>	131.7 <sup>c</sup>
	LCA	15.0	8.0 <sup>a</sup>	6.1 <sup>a</sup>	18.0 <sup>b,c</sup>
	TLCA	0.0	0.0 <sup>a</sup>	0.0 <sup>a</sup>	0.0
	GLCA <sup>1</sup>	17.4	2.0 <sup>a</sup>	0.0	6.5
	UDCA	28.5	18.0 <sup>a</sup>	7.0 <sup>a,b</sup>	131.5 <sup>b,c</sup>
	TUDCA	0.0	0.0	0.0 <sup>a,b</sup>	30.9 <sup>a,b,c</sup>
Tertiary	GUDCA <sup>1</sup>	137.9	96.5	54.1	75.1
	HDCA	0.0	9.5 <sup>a</sup>	8.0 <sup>a</sup>	28.0 <sup>a</sup>
	THDCA	0.0	0.0 <sup>a</sup>	0.0	0.0
	GHDCA <sup>1</sup>	0.0	0.0	0.0	0.0

Control: n = 310; No EM: n = 50, n = 21<sup>1</sup>; Arthralgia/arthritis: n = 30, n = 7<sup>1</sup>; Hepatobiliary diseases: n = 16, n = 6<sup>1</sup>. Data expressed as medians. <sup>a</sup>P < 0.05 vs control, <sup>b</sup>P < 0.05 vs no EM, <sup>c</sup>P < 0.05 vs arthralgia/arthritis (Mann-Whitney U test).

BA profiles. While we found no influence of EMs on serum BA levels in CD patients (data not shown), UC patients with hepatobiliary diseases, e.g. primary sclerosing cholangitis (PSC), hepatitis, or cholelithiasis, had significantly increased BA concentrations compared to UC patients without EMs, especially levels of the primary BAs TCA, GCA and GCDCA, as well as the secondary BAs LCA, UDCA and TUDCA (Table 2).

#### Previous bowel resection influences BA composition in CD

Intestinal reabsorption of BAs is a physiological function of the terminal ileum, therefore, surgical interventions in this region may influence serum BA levels caused by impaired EHC. While there were no UC patients with surgical interventions included in the present study, CD patients showed significant variations in serum BA concentrations correlated to previous bowel resection (Table 3). Overall, compared to controls and CD patients without surgical interventions, ileocecal resection alone was associated with the most intensive decrease of primary and secondary BA conjugates, such as TCDCA, TDCA, GCA and GDCA, as well as a marked increase in the unconjugated primary BAs, CA and CDCA. In addition, CD patients with ileocecal resection and other surgical interventions, e.g. ileostomy, sigmoidostomy, transversostomy, fistula excision, and hemicolectomy, had significantly decreased TCDCA compared to controls and CD patients without surgical interventions. Furthermore, in CD patients with colectomy, TCDCA, as well as the secondary BAs GDCA and DCA, were decreased significantly compared to those in controls and patients without surgical interventions.

**Table 3** Effect of surgical interventions on serum bile acids and conjugates in CD cohorts (nmol/L)

BA class	BA/BA-conjugate	Control	No surgery	Ileocecal resection	Colectomy	Other surgery	Ileocecal resection + other surgery
Primary	CA	62.5	52.0	160.0 <sup>a,b</sup>	71.0	57.5 <sup>c</sup>	89.0 <sup>b</sup>
	TCA	23.6	0.0 <sup>a</sup>	0.0 <sup>a,b</sup>	9.5 <sup>c</sup>	8.0 <sup>c</sup>	0.0
	GCA	383.5	240.5 <sup>a</sup>	156.0 <sup>a,b</sup>	375.0 <sup>c</sup>	212.0	221.5 <sup>a</sup>
	CDCA	196.5	144.0 <sup>a</sup>	505.0 <sup>a,b</sup>	174.5	153.0	121.0
	TCDDA	230.5	81.0 <sup>a</sup>	19.0 <sup>a,b</sup>	37.5 <sup>a,b</sup>	55.5 <sup>a</sup>	17.0 <sup>a,b</sup>
	GCDCA	1446.0	848.5 <sup>a</sup>	767.0 <sup>a</sup>	1059.0	663.5 <sup>a</sup>	772.0
Secondary	DCA	239.8	90.5 <sup>a</sup>	11.0 <sup>a</sup>	6.5 <sup>a,b</sup>	17.5 <sup>a</sup>	53.0 <sup>d</sup>
	TDCA	48.2	5.9 <sup>a</sup>	0.0 <sup>a,b</sup>	0.0 <sup>a</sup>	0.0 <sup>a,b</sup>	0.0 <sup>a</sup>
	GDCA	238.6	64.0 <sup>a</sup>	0.0 <sup>a,b</sup>	0.0 <sup>a,b</sup>	0.0 <sup>a,b</sup>	43.5 <sup>a</sup>
	LCA	15.0	7.0 <sup>a</sup>	5.9 <sup>a</sup>	3.0 <sup>a</sup>	4.7 <sup>a</sup>	2.9 <sup>a</sup>
	TLCA	0.0	0.0	0.0 <sup>a,b</sup>	0.0 <sup>a,b</sup>	0.0	0.0
	GLCA <sup>1</sup>	17.4	3.6 <sup>a</sup>	0.0 <sup>a</sup>	0.0	2.3	0.0
Tertiary	UDCA	28.5	14.5 <sup>a</sup>	26.0 <sup>b</sup>	12.7	53.5	22.5
	TUDCA	0.0	0.0	0.0 <sup>a</sup>	0.0 <sup>a</sup>	0.0 <sup>a</sup>	0.0 <sup>a</sup>
	GUDCA <sup>1</sup>	137.9	95.2 <sup>a</sup>	59.7	92.1	323.5	75.6
	HDDCA	0.0	27.0 <sup>a</sup>	10.0 <sup>a</sup>	9.5 <sup>a</sup>	9.0 <sup>a</sup>	2.0 <sup>a</sup>
	THDCA	0.0	0.0 <sup>a</sup>	0.0	0.0	0.0	0.0
	GHDCA <sup>1</sup>	0.0	0.0	0.0	0.0	2.0	0.0

Control:  $n = 310$ ; No surgery:  $n = 64$ ,  $n = 31^1$ ; Ileocecal resection:  $n = 41$ ,  $n = 16^1$ ; Colectomy:  $n = 22$ ,  $n = 8^1$ ; Other surgery:  $n = 12$ ,  $n = 4^1$ ; Ileocecal resection + other surgery:  $n = 12$ ,  $n = 9^1$ . Data expressed as medians. <sup>a</sup> $P < 0.05$  vs control, <sup>b</sup> $P < 0.05$  vs no surgery, <sup>c</sup> $P < 0.05$  vs ileocecal resection, <sup>d</sup> $P < 0.05$  vs colectomy (Mann-Whitney *U* test).

**Table 4** Total serum bile acids and conjugates in CD, UC and control cohorts

	Control	CD	UC
Total BAs (nmol/L)	3752.0	2563.3 <sup>a</sup>	3010.2
Total unconjugated BAs <sup>1</sup> (nmol/L)	644.1	631.1	471.0 <sup>a,b</sup>
Total conjugated BAs (nmol/L)	2763.6	1526.9 <sup>a</sup>	2529.6 <sup>b</sup>
Total BA glycoconjugates (nmol/L)	2423.6	1407.6 <sup>a</sup>	2298.9 <sup>b</sup>
Total BA glycoconjugates/total conjugated BAs (%)	87.4	95.7 <sup>a</sup>	92.7 <sup>a,b</sup>
Total BA taurourconjugates <sup>1</sup> (nmol/L)	344.2	68.0 <sup>a</sup>	145.0 <sup>a,b</sup>
Total BA taurourconjugates/total conjugated BAs (%)	12.6	4.3 <sup>a</sup>	7.3 <sup>a,b</sup>
(Deoxy-BAs + TLCA + GLCA + LCA)/total BAs (%)	83.7	84.1	77.4 <sup>a,b</sup>

Control:  $n = 310$ ; CD:  $n = 73$ ,  $n = 197^1$ ; UC:  $n = 44$ ,  $n = 161^1$ . Data expressed as medians. <sup>a</sup> $P < 0.05$  vs control, <sup>b</sup> $P < 0.05$  vs CD (Mann-Whitney *U* test).

#### No effects of disease activity and medical treatment on BA composition in CD and UC

We also investigated whether serum BA composition was influenced by disease activity and different therapeutic medications in IBD patients, since mucosal inflammation, as well as pharmacologically induced changes in the inflammatory process in IBD may influence BA reabsorption, which results in changed serum BA profiles. Overall, serum BA composition in CD and UC were independent of disease activity and medical treatment (data not shown).

#### DISCUSSION

During the past three decades, BA analysis in IBD has been achieved in serum, bile or feces from small patient cohorts by radioimmunoassay or gas-liquid chromatography detecting total BAs and selected

individual BAs, respectively<sup>[17,31-34]</sup>. In the present study, we applied a sensitive high-throughput LC-ESI-MS/MS method with minimal sample preparation steps for simultaneous determination of 18 different BA species as serum BA profiles in a large cohort of IBD patients and controls. We analyzed the main unconjugated human primary, secondary and tertiary BAs, i.e. CA, CDCA, DCA, LCA, UDCA, HDCA, and their respective glycine and taurine conjugates. Comparing a whole profile of BA subspecies in various IBD phenotypes may reflect intestinal malfunction and disease states more sensitively than just considering total or selected individual BA levels.

Thus, we showed that decreased serum BA levels were not restricted to CD alone, as previously reported<sup>[13-15,35]</sup>, but were also found in UC if a defined set of specific BAs were considered. This is contradictory to the few reports on abnormal blood BA levels in UC patients. Ejderhamm and Strandvik<sup>[17]</sup> have reported increased primary serum BAs, CA and CDCA, in juvenile active UC patients compared to healthy controls, while there were no significant differences for CA, but decreased CDCA levels in our UC cohort compared to controls. Kostic *et al*<sup>[16]</sup> have reported decreased total plasma BA levels in CD and UC patients compared to controls, but we did not find significant differences in total BA, total BA conjugate, and total BA glycoconjugate levels between UC patients and the control group. Only total BA taurourconjugate and unconjugated BA levels were reduced significantly in UC patients *vs* controls. However, because of their relatively low contribution to total BA and BA conjugate levels compared to the most abundant BA glycoconjugates, this effect was not dominant and may explain the missing reports on decreased total BA levels in UC patients. In summary, our data confirm studies that have shown a decrease in serum

BA levels in CD patients, which reflects the strongest impact on intestinal BA reabsorption during EHC. This can be explained by the fact that BA reabsorption takes place predominantly in the distal small intestine<sup>[10]</sup>, which is usually more affected in CD in contrast to colon-restricted UC. Therefore, most serum BA levels in UC patients are not decreased as much as in CD patients.

Furthermore, it is noteworthy that the levels of the unconjugated primary BAs, CA and CDCA, in CD and UC patients were not significantly different from the controls, except for decreased CDCA in UC patients. The explanation that there is an increased compensatory synthesis of primary BAs in IBD, as suggested by Rutgeerts *et al*<sup>[13]</sup> in Crohn's ileitis, assumes an accelerated bacterial deconjugation of the respective glyco- and tauroconjugates in the intestine. Indeed, the reduced serum levels of TCA, TCDCA, GCA, and GCDCA shown in Table 1 support this assumption, which is in agreement with previous findings of unusually high intestinal BA deconjugation in CD and UC<sup>[36,37]</sup>. Apart from deconjugation, the quantitatively most important bacterial biotransformation of BAs is 7α-deoxidation of CA and CDCA by *Eubacteria* in the colon, which yields DCA and LCA, respectively<sup>[38,39]</sup>. In UC patients, we found significantly reduced ratios of total deoxy-BA (including DCA, LCA, and its conjugates) to total BA levels compared to those in CD patients and controls, which may reflect an abnormal colonic bacterial flora with reduced deoxidation capacity. In addition, bacterial overgrowth in the small intestine and colon of IBD patients may enhance the described BA biotransformation processes and contribute to the imbalance of BA species distribution in the EHC. Decreased intestinal BA levels, especially of conjugated BAs, may promote bacterial overgrowth because of a loss of their antimicrobial properties<sup>[10,26]</sup>. Since IBD patients included in this study were not stratified according to the use of antibiotics, this effect has not been evaluated and needs further systematic investigation in well-defined patient cohorts.

The invariably increased HDCA and decreased LCA in IBD compared to control sera, irrespective of the clinical findings (EMs, surgical interventions, disease activity or medication), indicates accelerated enterohepatic LCA detoxification *via* CYP3A4<sup>[18]</sup>. Whether serum HDCA elevation is additionally caused by increased intestinal reabsorption or impaired hepatic excretion cannot be resolved by the present data and has to be further investigated.

Moreover, the influence of hepatobiliary EMs on serum BA levels in IBD is demonstrated clearly in UC patients, who showed a significant increase in primary and secondary BAs compared to EM-free patients. This confirms previous observations when elevated serum levels of total primary BA conjugates have been seen in IBD patients with liver diseases<sup>[31,40]</sup>. Although serum LCA levels in UC patients with hepatobiliary EMs were found to be normal compared to controls, in accordance with

the findings of Dew *et al*<sup>[40]</sup>, they were significantly higher compared to those in UC patients without EMs and with arthralgia/arthritis. Elevated BA levels are particularly found in patients with PSC, which is often associated with IBD<sup>[29,41]</sup>. However, it cannot be ruled out that the significantly increased serum levels of TUDCA, UDCA and LCA found in our UC cohort with hepatobiliary EMs were caused by therapeutic administration of UDCA, which is being used increasingly for the treatment of cholestatic liver diseases<sup>[42-44]</sup> and PSC-associated UC<sup>[45,46]</sup>. UDCA medication not only causes increased primary BA biosynthesis, but UDCA is also metabolized to additional TUDCA and LCA<sup>[47]</sup>, which yields increased serum levels in these patients. Nonetheless, we assume that disturbed EHC of BAs in IBD is highly susceptible to additional hepatobiliary EMs.

With surgical interventions predominantly appearing in CD patients, we found that ileocecal resection exerts the strongest impact on serum BA levels in CD patients, since BA reabsorption is located predominantly in the terminal ileum<sup>[10]</sup>. Compared to patients without surgical interventions, the finding that patients with ileocecal resection showed significantly decreased conjugated BAs but increased unconjugated primary BAs, i.e. CA and CDCA, may be explained by an increased compensatory synthesis of primary BAs in IBD, as previously suggested<sup>[13]</sup>, associated with an enhanced bacterial deconjugation of the respective glyco- and tauroconjugates in the remaining intestinal sections<sup>[36,37]</sup>. In addition, concerning the elevated CA levels in patients with ileocecal resection, we confirm the previous findings of Tougaard *et al*<sup>[48]</sup>. As expected, unlike ileocecal resection, the influence of colectomy on serum BA levels in CD was less significant, since only small amounts of BAs are reabsorbed in this intestinal region.

To summarize, using mass spectrometric BA species profiling instead of total BA determination, we showed the characteristic impact of different IBD phenotypes with intestinal and hepatobiliary manifestations on BA homeostasis and detoxification. Further prospective studies on prominent BAs in well-defined IBD cohorts are necessary to confirm their diagnostic and prognostic value.

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## COMMENTS

### Background

Inflammatory bowel disease (IBD) is a multifactorial disorder with as yet incompletely elucidated causes. Since bile acids (BAs) derived from the liver are directly involved in intestinal processes primarily by facilitating lipid digestion, IBD has an impact on BA metabolism. This correlation may be reflected in unusual BA blood levels that differentiate between the two clinical IBD phenotypes, Crohn's disease (CD) and ulcerative colitis (UC), as well as between CD and UC subgroups with diverse clinical manifestations.

## Research frontiers

Besides their digestive functions, BAs have recently been found to play an important regulatory role in numerous metabolic processes, e.g. energy and lipid balance and elimination of harmful substances. They are mediated by binding appropriate nuclear receptors in the cell that depend on the molecular type of BA, which can be differentiated by means of high performance mass spectrometry. Thus, quantifying diverse BAs simultaneously, a characteristic profile of main and rare BAs is available that reflects medical conditions far better than measuring total BA levels or individual abundant BAs.

## Innovations and breakthroughs

Applying BA profiling in IBD patients, the authors showed that most but not all BA species are decreased in CD and UC patient sera, but with different intensity. BA decrease is highly pronounced in CD patients with surgical interventions in the gut except for unconjugated primary BAs. On the other hand, UC patients with additional liver and gallbladder diseases show clearly increased levels of primary and secondary BAs. Finally, the authors found a marked decrease in the toxic BA lithocholic acid, together with a marked increase in its physiological detoxification product, hyodeoxycholic acid, irrespective of the IBD phenotype or clinical manifestation, which shows accelerated detoxification activity in IBD patients.

## Applications

Serum BA profiling may serve as an additional diagnostic tool for IBD characterization and differentiation. In combination with expression profiles of pregnane X receptor (PXR)-regulated genes, it may allow us to estimate the BA detoxification potential of IBD patients.

## Terminology

Primary BAs are directly synthesized in the liver and secondary BAs are derived from primary BAs by biochemical modification by intestinal bacteria. BAs can be conjugated, mainly with the amino acids glycine and taurine, or unconjugated. The enterohepatic circulation leads to a maximum physiological recycling of BAs and comprises liver synthesis, intestinal excretion via the bile duct, intestinal reabsorption, and return transport to the liver via the portal vein. Liquid chromatography tandem mass spectrometry is a sensitive analytical method for simultaneous determination of structural related biomolecules like BAs. The nuclear BA receptors farnesoid X receptor and PXR mediate most of the physiological effects of BAs, e.g. expression of detoxification genes by PXR.

## Peer review

This work expand the knowledge about the role of BA metabolism in IBD. It is a well-conducted study.

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BRIEF ARTICLES

## Human papilloma virus and esophageal carcinoma in a Latin-American region

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### Abstract

**AIM:** To investigate the presence of high-risk human papilloma virus (HPV) in esophageal squamous cell carcinomas (ESCCs) in a non-selected Mexican population.

**METHODS:** Cases with a pathological diagnosis of squamous cell carcinoma of the esophagus were obtained from Department of Pathology files, at the National Cancer Institute in Mexico City during the period between 2000 and 2008. Slides from each case were reviewed and cases with sufficient neoplastic tissue were selected for molecular analysis. DNA was extracted from paraffin-embedded tissue samples for polymerase chain reaction analysis to detect HPV DNA sequences. Demographic and clinical data of each patient were retrieved from corresponding clinical records.

**RESULTS:** HPV was detected in 15 (25%) of ESCCs. HPV-16 was the most frequently observed genotype, followed by HPV-18; HPV-59 was also detected in

one case. Unfortunately, HPV genotype could not be established in three cases due to lack of material for direct sequencing, although universal primers detected the presence of HPV generic sequences. No low-risk HPV genotypes were found nor was HPV-16/18 co-infection. HPV presence in ESCC was not significantly associated with gender, age, alcohol consumption, smoking, anatomic location, or histologic grade. All patients belonged to low and very low socioeconomic strata, and were diagnosed at advanced disease stage. Male patients were most commonly affected and the male:female ratio in HPV-positive ESCC increased two-fold in comparison with HPV-negative cases (6.5:1 vs 3.1:1).

**CONCLUSION:** High prevalence of high-risk HPV in ESCC in Mexico does not support the hypothesis that HPV-associated ESCC is more common in areas with higher ESCC incidence rates.

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**Key words:** Esophagus; Human papilloma virus; Squamous cell carcinoma; High-risk human papilloma virus

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### INTRODUCTION

With regard to incidence and prevalence, esophageal cancer exhibits striking geographical variations due to unknown factors between countries, as well as between different regions of the same country. According to the World Health Organization, incidence-rate spectra are located between Western Africa -at the low-risk end, and China at the high-risk end, including the so-called "Asian esophageal cancer belt"<sup>[1]</sup>.

Among Latin American countries, Mexico and Peru have the lowest mortality rates for esophageal carcinoma, in both males and females, whereas Brazil, Argentina and Chile, have the highest mortality rates<sup>[2]</sup>. Mexico has a low esophageal cancer risk, with mortality rates in male and female inhabitants *ca* 2 and *ca* 1 per 100 000, respectively<sup>[3]</sup>.

Esophageal carcinoma has been related to tobacco, alcohol, fungal toxins, nutritional deficiencies, and hot food and beverages<sup>[4]</sup>. In addition, human papilloma virus (HPV), a major cause of carcinoma of the cervix uteri throughout the world, is suspected of being related to the development of this carcinoma. Meta-analyses by Syrjänen<sup>[5]</sup> (2002) showed that HPV DNA was detected in 15.2% of esophageal squamous cell carcinomas (ESCCs). To date, total or partial sequencing identified > 200 genotypes of HPV, which are categorized into the following four cervical oncogenicity-based risk groups: high, probably high, low and undetermined risks<sup>[6]</sup>. High-risk HPV is considered to cause neoplastic transformation of normal epithelial cells, through expression of early transforming viral proteins E6 and 7, resulting in cell cycle-machinery deregulation and expression of several transforming oncogenes<sup>[7]</sup>.

High-risk HPV-associated ESCC is hypothesized to be consistently more common in countries with higher ESCC risk<sup>[8]</sup>. In a recent study by Castillo *et al*<sup>[9]</sup>, HPV DNA was detected in 34% and 19% of esophageal carcinomas in Colombia and Chile, respectively; HPV-16 was the most frequent genotype in both countries. It is noteworthy that the HPV detection rate in ESCCs was found to be two-fold higher in Colombia than in Chile, whereas the esophageal cancer mortality rate showed an inverse relationship.

The aim of the present study was to investigate the presence of high-risk HPV DNA in ESCCs, among a non-selected Mexican population.

## MATERIALS AND METHODS

### Subjects

Cases with a pathological diagnosis of squamous cell carcinoma of the esophagus were obtained from Department of Pathology files, at the National Cancer Institute in Mexico City during the period between 2000 and 2008. Slides from each case were reviewed and cases with sufficient neoplastic tissue were selected for molecular analysis. Because the majority of the cases of esophageal cancer seen at our Institution correspond to advanced clinical stages, elective surgery is not performed. Subsequently, DNA was extracted from paraffin-embedded tumor samples, obtained during a panendoscopic procedure; adjacent normal esophageal mucosa was not sampled. Demographic and clinical data of each patient were retrieved from corresponding clinical records.

### DNA extraction and HPV detection

Twenty micrometer sections of formalin-fixed and paraffin-embedded tumors were de-waxed by incubation

with N-octane and washings with 100% ethanol. This process was repeated twice, and the pellet was dried. Deparaffinized samples were digested with 1 mL of lysis buffer (Tris-HCl 10 mmol/L pH 8.0, EDTA 0.1 mol/L pH 8.0, SDS 0.5%, Proteinase K 200 µg/mL, RNase A 20 µg/mL) at 55°C for 3 h. DNA was extracted with phenol/chloroform precipitations as described by Sambrook *et al*<sup>[10]</sup>. To test DNA suitability for polymerase chain reaction (PCR) amplification the DNA obtained was amplified for: β-globin gene (PCO4/GH2O) under conditions described by Resnick *et al*<sup>[11]</sup>. Samples were later submitted to HPV amplification with three sets of the following universal primers recognizing distinct size fragments of L1 gene: L1C1/L1C2, MY09/MY11, and GP5/GP6<sup>[12-14]</sup>. HPV type-specific amplification was also performed with primers designed to amplify the E6 gene of HPV type 16 and 18 as described by Lizano *et al*<sup>[15]</sup>.

HPV PCR products were electrophoresed in a 1.2% agarose gel and visualized by ethidium bromide staining. HPV typing was carried out by direct sequencing of PCR products by means of the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The resulting sequences were analyzed in BLAST data bank for comparison with known HPV sequences. HPV 16 and 18 specific amplification was conducted for every DNA sample. DNA extracted from CaSki and Hela HPV containing cell lines were used as positive controls.

### Statistical analysis

Fisher's exact test was applied for categorical variables. A probability value  $P < 0.05$  was considered statistically significant.

## RESULTS

In 60 cases of ESCC, DNA quality was adequate for PCR analysis to detect HPV DNA sequences, as demonstrated by β-globin gene amplification. There were 47 male and 13 female patients, with a mean age of 62.7 years (range, 27-85 years) and 61.2 years (range, 47-80 years), respectively. Thirty-six subjects consumed alcohol (ranging from occasional to 60 years drinkers) but frequency and alcoholic-beverage type could not be precisely assessed. In 14 subjects, previous history of alcohol consumption was unknown and ten patients denied alcohol consumption. Smoking habit was recorded in 28 patients (from occasional to up to 60 years of smoking; frequency was not known), whereas in 16 subjects smoking habit was unknown; 16 patients did not smoke. Clinical history of esophageal achalasia was not recorded in any case, and there were previous symptoms of gastro-esophageal reflux disease (GERD) in two patients. Finally, oesophageal surgery was performed in one patient 26 years previously due to direct trauma. All patients belonged to low and very-low socioeconomic strata, the majority of these were agricultural workers.

All patients were diagnosed at advanced disease stage. Among these, 42 patients (70%) were treated with chemo- and/or radiotherapy and three subjects underwent esophagectomy immediately after neoadjuvant

**Table 1** HPV detection frequency according to subjects' demographic factors and lifestyles, and the clinicopathological features of esophageal carcinoma

	Total (%)	HPV- (%)	HPV+ (%)	P value
Total	60 (100)	45 (75)	15 (25)	
Gender				0.485
Female	13 (100)	11 (85)	2 (15)	
Male	47 (100)	34 (72)	13 (28)	
Alcohol consumption				0.377 (0.420)
No	10 (100)	9 (90)	1 (10)	
Yes	36 (100)	27 (75)	9 (25)	
Unknown	14 (100)	9 (64)	5 (36)	
Smoking				0.274 (0.450)
No	16 (100)	14 (88)	2 (12)	
Yes	28 (100)	21 (75)	7 (25)	
Unknown	16 (100)	10 (63)	6 (37)	
Esophageal location				0.330
Upper third	14 (100)	9 (64)	5 (36)	
Upper/middle third	4 (100)	2 (50)	2 (50)	
Middle third	28 (100)	23 (82)	5 (28)	
Middle/lower third	10 (100)	7 (70)	3 (30)	
Lower third	4 (100)	4 (100)	0 (0)	
Histologic grade				0.222
Basaloid/poorly differentiated	1 (100)	1 (100)	0 (0)	
Poorly differentiated	20 (100)	17 (85)	3 (15)	
Moderately-differentiated	38 (100)	27 (71)	11 (29)	
Well-differentiated	1 (100)	0 (0)	1 (100)	

P values in parentheses are those without "unknown" category.

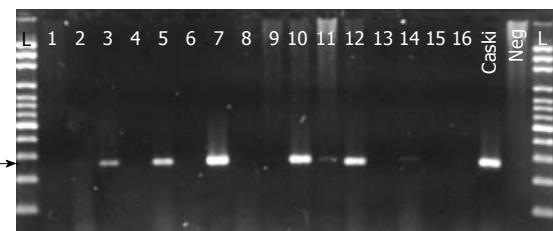
**Table 2** HPV genotypes detected in ESCCs

HPV genotype	n (%)
HPV-16	6 (40)
HPV-18	5 (33)
HPV-59	1 (7)
Unknown	3 (20)
Total	15 (100)

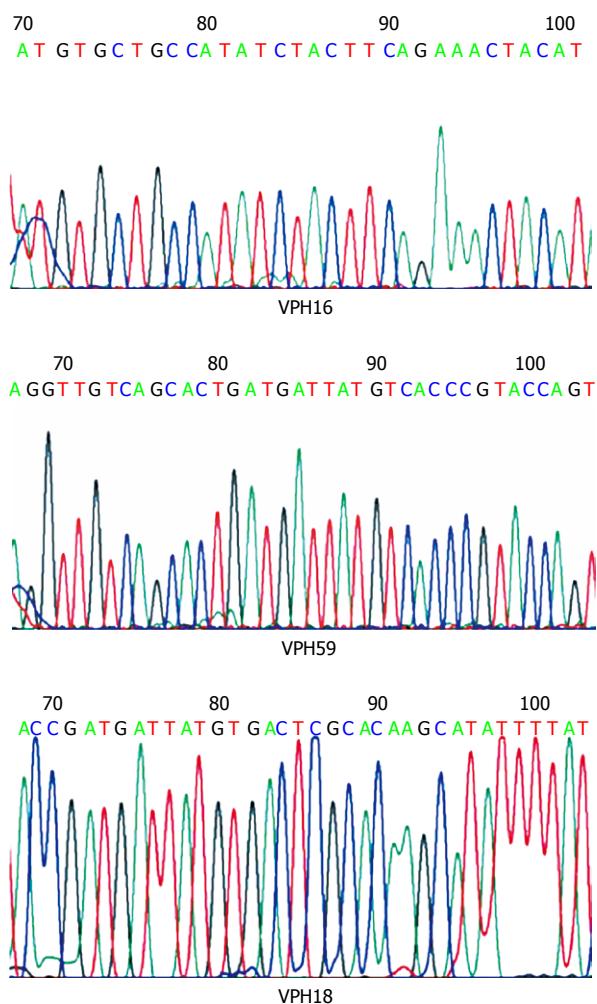
ESCCs: Esophageal squamous cell carcinomas.

therapy. Finally, 18 patients (30%) refused any treatment. During follow-up, seven patients (11.7%) died of oesophageal carcinoma, whereas one of these died of causes not related to the neoplastic disease. The majority of patients were alive but with disease at 1 year or less of follow-up, but did not return for subsequent medical care. Regardless of the presence of HPV, all patients with ESCCs belonged to low and very-low socioeconomic strata, the majority of these were agricultural workers (data not shown). HPV presence in ESCCs was also unrelated to anatomic location, histologic grade or patient condition (alive or dead) at hospital discharge.

HPV universal primers and HPV-16 and -18 specific primers detected the presence of HPV DNA in 15 (25%) of 60 ESCCs. Cases of HPV DNA consisted of 13 males and two females with a mean age of 63.4 years (range, 37-76 years) and 62.5 years (range, 47-78 years), respectively. There were no statistical differences or associations ( $P > 0.05$ ) between HPV status and gender, age, previous history of alcohol consumption and smoking, anatomic location, histologic grade or patient



**Figure 1** Identification of HPV with general primers L1C1/C2. PCR products were run in 2% agarose gel. Lanes 1-16 correspond to representative positive and negative cases. DNA extracted from Caski cell lines were used as a positive control. Neg: PCR mixture without DNA; L: 100 bp ladder.



**Figure 2** Representative electropherograms showing fragments of HPV-16, -18 and -59 genomic sequences, obtained from the analysis of PCR products L1C1/C2 from HPV-positive samples.

condition (alive or dead) at hospital discharge (Table 1).

Direct PCR-product sequencing detected 12 cases of high-risk HPV. As summarized in Table 2, HPV-16 was the most frequently detected genotype, followed by HPV-18; interestingly HPV-59 was also detected in one case (Figures 1 and 2). In three of 15 HPV-positive cases (20%), HPV genotype could not be specified although universal primers detected the presence of HPV generic sequences. Low-risk HPV DNA sequences were not detected and we found no HPV-16/18 co-infections among the cases under study.

## DISCUSSION

PCR analysis in this study demonstrated the presence of high-risk HPV in 25% of ESCCs from a small set of Mexican patients; HPV-16 was the most frequent viral genotype followed by HPV-18. Interestingly, HPV-59 was also detected in one case; to our knowledge, this HPV genotype has not been previously reported in other ESCC studies. No low-risk HPV genotype was detected. In addition, HPV-16/18 co-infection was not found.

High-risk HPV-genotypes distribution in Mexican ESCCs observed in the present study is in agreement with that reported in the studies of cervical carcinomas in Mexico, in which HPV-16 is the most frequent genotype, followed by HPV-18, then by other high-risk HPV genotypes<sup>[16]</sup>. A similar high-risk HPV distribution was observed in a South American study of ESCC in Colombia and Chile<sup>[9]</sup>, as well as a small Mexican series of lung carcinoma<sup>[17]</sup>. HPV-16 is not only the most prevalent high-risk genotype in cervical carcinomas<sup>[18]</sup> and in young females with normal Papanicolaou smears<sup>[19]</sup>, but also in HPV-associated ESCC, worldwide<sup>[5]</sup>.

HPV prevalence in Mexican women, as analyzed in cervical smears, has been reported to range 16.7%-23%, depending on the age group<sup>[20]</sup>, and detected in 92% of cervical cancers<sup>[21]</sup>. A study of HPV prevalence in men showed a 62% HPV global positivity in samples from external genitalia of Mexican men, with 13% oncogenic types<sup>[22]</sup>.

Since the Syrjänen<sup>[5]</sup> studies in the early 1980s, several studies have been conducted in different countries and in different geographical regions of the same country, to identify HPV DNA in ESCC. Utilizing molecular methods, the majority of these studies have shown the presence of high-risk HPV in a variable proportion of cases<sup>[23-27]</sup>; others, however, have failed to demonstrate HPV traces in EC, even from highly prevalent regions<sup>[28,29]</sup>. Differences in such figures could be attributed, first, to the sensitivity and specificity of molecular methods employed to detect HPV DNA<sup>[5]</sup>; it is widely accepted that PCR is the most sensitive method for detecting HPV DNA, and can detect as few as 20 copies or less<sup>[30]</sup>.

According to the concept of “Condemned Mucosa Syndrome” proposed by Pillai and Nair<sup>[31]</sup>, HPV promotes neoplastic transformation in a previously damaged mucosa with the aid of other carcinogenic agents. With regard to alcohol consumption and/or smoking, there were no substantial differences between HPV-positive and HPV-negative ESCCs, in this study. The present study also examined the possible association between the presence of HPV DNA and esophageal disorders involving membrane damage, such as achalasia and GERD. However, patients in the present study had no clinical history of esophageal achalasia. In two cases of ESCC in which there were previous symptoms of GERD and in another case with direct trauma-related esophageal surgery 26 years previously, HPV DNA was not detected.

The majority of our Mexican patients suffering

from ESCC, were native to rural and semi-urban areas of Mexico, thus indicating low yearly income and a low level of education. Indeed, low socioeconomic status is characterized by, among others, nutritional deficiencies, poor hygiene habits and lack of health and public services, including drinking water supply, conditions that taken together could also predispose to esophageal carcinogenesis. Low socioeconomic status has also been associated with an increased risk of cervical intraepithelial neoplasia and cervical invasive carcinoma, among HPV-positive Mexican women<sup>[32]</sup>.

Interestingly, the male:female ratio in HPV-positive ESCC increased two-fold in comparison with HPV-negative cases (6.5:1 vs 3.1:1). This ratio is higher than that reported from Colombia and Chile (0.75:1 vs 1.1:1)<sup>[9]</sup>. However, the gender-ratio between HPV-positive and -negative cases was not statistically significant. This finding does not allow us to draw any conclusion, due in part, to the small number of cases under study. No such comparison has been explored in other ESCC series, but its meaning warrants further research.

The etiological role of HPV in mucosal malignancy development has been supported on the basis of high-risk HPV localization and expression of viral oncoproteins in neoplastic cell nuclei, the characteristic squamous and basaloid morphology of malignant tumors, the occurrence of HPV-associated malignant neoplasms in anatomic sites where HPV is known to cause benign papillomas and where HPV direct exposure is suspected, and the elevation of serum antibodies against E6/E7 in patients harboring HPV-associated invasive cancers, among others<sup>[33]</sup>. The etiological significance of HPV detected in ESCCs is yet to be established. It is noteworthy that the present study was unable to find the association of basaloid morphology with HPV presence in ESCC. Although HPV-positive ESCC frequency was high, the mere presence of viral DNA does not necessarily imply its etiological involvement. It is necessary to conduct further studies to examine viral DNA integration into host carcinoma cells, localization in malignant cells, and monoclonality of HPV-positive cells.

The HPV transmission route detected in ESCCs is also of interest. HPV infection of the esophageal mucosa is highly suspected to occur in a direct fashion. In a recent case-control study<sup>[34]</sup>, HPV oral infection was strongly associated with a sub-group of oropharyngeal squamous cell carcinomas, in which high-risk sexual behaviors (i.e. oral, vaginal) were recorded, regardless of alcohol and tobacco use. Notwithstanding the association between oral HPV infection and sexual behavior, a Finnish HPV Family Study<sup>[35]</sup> has shown that persistent high-risk HPV infection in a mother is a major risk factor for oral and genital infections by this virus in her offspring; this susceptibility appears to be modulated by the immune system. Thus, it could be argued that previous high-risk HPV oral infection, may predispose asymptomatic carriers for further ESCC development.

In conclusion, one fourth of ESCCs diagnosed in a Mexican population were found to harbor high-risk

HPV DNA. Elevated high-risk HPV prevalence of ESCC in Mexico, where ESCC incidence is relatively low, does not support the hypothesis that HPV-associated ESCC is more common in areas with higher ESCC incidence rates. Further studies are warranted to evaluate the etiological significance of HPV detected in Mexican ESCCs.

## COMMENTS

### Background

Esophageal carcinoma is a dismal disease which exhibits striking geographical variations in incidence and prevalence between countries and between different regions of the same country, due to unknown factors. Western Africa has the lowest incidence rates and China the highest. Among Latin America countries, Mexico and Peru have low mortality rates for esophageal carcinoma, whereas Brazil, Argentina and Chile have the highest. In Mexico, mortality rates in male and female inhabitants are ca 2 and ca 1 per 100 000, respectively.

### Research frontiers

Esophageal carcinoma is multifactorial in origin; tobacco, alcohol, fungal toxins, nutritional deficiencies, hot food and beverages, as well as infectious agents, are related to esophageal carcinogenesis. Among infectious agents, high risk human papilloma virus (HPV), a major cause of carcinoma of the cervix uteri throughout the world, is strongly implicated in the etiology of esophageal carcinoma. High-risk HPV DNA sequences have been detected in approximately 15.2% of esophageal squamous cell carcinomas (ESCCs) worldwide, HPV-16 and -18 being the most frequent genotypes.

### Innovations and breakthroughs

In this study, the authors found the presence of high-risk HPV DNA in 25% of ESCCs. HPV-16 and -18 were the most frequent genotypes, but they also demonstrated the presence of HPV-59 in one of the cases, a genotype not previously reported in ESCCs anywhere. On the other hand, the results do not support the hypothesis that HPV-associated ESCC is more frequent in areas with higher ESCC incidence rates, because ESCC is not a common cancer in Mexico. Interestingly, the male:female ratio in HPV-positive ESCC increased two-fold in comparison with HPV-negative cases (6.5:1 vs 3.1:1).

### Applications

By knowing the prevalence of high-risk HPV-associated ESCC, this study among others, may contribute to the design of future strategies for the prevention of HPV-related malignancies, through the development of effective vaccines.

### Terminology

HPVs are DNA viruses that infect basal skin and mucosal cells. HPVs are categorized according to their cervical oncogenicity-based risk, with high, probably high, low and undetermined risks.

### Peer review

This study examined HPV infection in ESCCs in Mexican patients. This is an interesting study and the manuscript is written well.

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BRIEF ARTICLES

## Is percutaneous endoscopic gastrostomy tube placement safe in patients with ventriculoperitoneal shunts?

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**CONCLUSION:** Complications following PEG placement in patients with VP shunts were infrequent in this study.

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**Key words:** Percutaneous endoscopic gastrostomy; Ventriculoperitoneal shunt; Complication; Ventriculoperitoneal shunt infection; Prophylactic antibiotic

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Kim JS, Park YW, Kim HK, Cho YS, Kim SS, Youn NR, Chae HS. Is percutaneous endoscopic gastrostomy tube placement safe in patients with ventriculoperitoneal shunts? *World J Gastroenterol* 2009; 15(25): 3148-3152 Available from: URL: <http://www.wjgnet.com/1007-9327/15/3148.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.3148>

### Abstract

**AIM:** To investigate whether percutaneous endoscopic gastrostomy (PEG) tube placement is safe in patients with ventriculoperitoneal (VP) shunts.

**METHODS:** This was a retrospective study of all patients undergoing PEG insertion at our institution between June 1999 and June 2006. Post-PEG complications were compared between two groups according to the presence or absence of VP shunts. VP shunt infection rates, the interval between PEG placement and VP shunt catheter insertion, and long-term follow-up were also investigated.

**RESULTS:** Fifty-five patients qualified for the study. Seven patients (12.7%) had pre-existing VP shunts. All patients received prophylactic antibiotics. The complication rate did not differ between VP shunt patients undergoing PEG (PEG/VP group) and non-VP shunt patients undergoing PEG (control group) [1 (14.3%) vs 6 (12.5%),  $P = 1.000$ ]. All patients in the PEG/VP group had undergone VP shunt insertion prior to PEG placement. The mean interval between VP shunt insertion and PEG placement was 308.7 d (range, 65-831 d). The mean follow-up duration in the PEG/VP group was 6.4 mo (range, 1-15 mo). There were no VP shunt infections, although one patient in the PEG/VP group developed a minor peristomal infection during follow-up.

### INTRODUCTION

Percutaneous endoscopic gastrostomy (PEG) tube placement has been widely used for long-term nutritional support in patients with severe neurological impairment ever since it was first described by Gauderer *et al*<sup>[1]</sup> in 1980. However, patients requiring PEG tube placement may have concomitant hydrocephalus requiring insertion of a ventriculoperitoneal (VP) shunt, and VP shunts themselves are frequently associated with complications, such as shunt infection, obstruction, and migration with or without erosion into nearby structures. Shunt infection is a relatively common complication, occurring in 3% to 29% of patients<sup>[2-6]</sup>; its mortality rate is 30% to 40%<sup>[6]</sup>. There are a number of factors that can expose intraperitoneally placed catheters to bacterial pathogens in PEG patients with pre-existing VP shunts. Therefore, the presence of a VP shunt in a patient requiring PEG placement raises concerns about potential life-threatening complications such as VP shunt infection and VP shunt malfunction. A few studies have evaluated the safety of PEG placement in patients with VP shunts, but the results have been inconclusive. Furthermore, the study design, methods of PEG placement, control groups, and the use of antibiotics in these studies have been highly diverse.

Thus, to date, controversy still exists as to whether PEG

placement is safe in patients with VP shunts. This study was therefore designed to report our single center experience with PEG placement in patients with VP shunts, looking specifically at PEG-related complications and VP shunt infections. Relevant publications were also reviewed.

## MATERIALS AND METHODS

### Study design and patients

We performed a retrospective study on all patients who underwent PEG tube placement for enteral feeding at Uijeongbu St. Mary's Hospital between June 1999 and June 2006. A preliminary chart review identified the subset of patients with endoscopic records indicating PEG tube placement. A total of 55 patients were identified. Those patients with VP shunts were identified and assigned to the combined PEG and VP shunt (PEG/VP) group. The patients undergoing PEG tube placement (but without VP shunts) were assigned to the control group. A more detailed chart review was performed, evaluating patient ages at the time of the procedure, underlying disorders, comorbid diseases, number of PEG placements, and PEG-related complications. Adjustment for comorbidity was carried out for patients in this study using Charlson's comorbidity index<sup>[7]</sup>. Post-PEG placement complications were compared between the two groups. Furthermore, the incidence of VP shunt infections, interval between PEG placement and VP shunt catheter insertion, position of the abdominal shunt catheter, follow-up duration, and outcome of long-term follow-up were investigated in the PEG/VP group.

The requirement for informed consent was waived, because the study design was retrospective.

### PEG tube placement

All PEGs were placed by gastroenterologists. A commercially available gastrostomy tube (US Endoscopy, Mentor, Ohio, USA) was introduced by standard pull-through technique. Enteral feeding was discontinued 12 h before PEG tube placement. All patients received prophylactic or perioperative antibiotics and received intravenous sedation and topical pharyngeal anesthesia. In each patient, the stomach was endoscopically inflated with air, and following satisfactory transillumination of the stomach in the left hypochondrium or epigastrium, the needle was passed through this site directly into the stomach. A guide wire was advanced through the needle, and the commercially available gastrostomy tube was placed over the wire from the aerodigestive tract, through the stomach, to the abdominal wall. In each patient with a pre-existing VP shunt, the shunt tract was carefully demarcated so it could be avoided during PEG tube placement.

### Statistical analysis

With respect to demographic data and complications in the two groups, continuous variables were compared using Student's *t*-test, and discrete variables were compared using the Chi-square test or Fisher's exact probability test.

**Table 1** Baseline patient characteristics *n* (%)

	PEG/VP ( <i>n</i> = 7)	Control ( <i>n</i> = 48)	<i>P</i> value
Age (yr)	55.3 ± 12.3	61.0 ± 16.6	0.387
Sex (M/F)	5/2	31/17	1.000
Primary diagnosis			0.897
Cerebrovascular disease	7 (100)	36 (75)	
Amyotrophic lateral sclerosis		4 (8.3)	
Hypoxic brain damage		2 (4.2)	
Parkinson's disease		2 (4.2)	
Malignancy		2 (4.2)	
Aspiration pneumonia		1 (2.1)	
Pharyngeal paralysis		1 (2.1)	
Diabetes mellitus	2 (28.6)	10 (20.8)	0.639
Tracheostomy	6 (85.7)	25 (52.1)	0.122
Mean number of PEG placements	1.3 ± 0.5	1.6 ± 1.1	0.459
Charlson's index score	3.0 ± 1.6	3.5 ± 1.9	0.504

PEG: Percutaneous endoscopic gastrostomy; VP: Ventriculoperitoneal; PEG/VP: Patients with PEG tubes and VP shunts; Control: Patients with PEG tubes alone.

A probability value of  $< 0.05$  was considered statistically significant. All data were analyzed using SPSS 11.0 (SPSS Inc, Chicago, Illinois, USA).

## RESULTS

Over a 7-year period, 55 patients underwent PEG tube placement at our hospital. Selected clinical characteristics of the patients are provided in Table 1. Seven patients (12.7%) had pre-existing VP shunts at the time of PEG placement (PEG/VP group), and 48 patients had no VP shunts (control group). There was no difference in the mean age between the PEG/VP and control groups ( $55.3 \pm 12.3$  vs  $61.0 \pm 16.6$  years,  $P = 0.387$ ) and no difference in the sex ratio between the two groups (male/female: 5/2 vs 31/17,  $P = 1.000$ ). The primary diagnosis in all patients in the PEG/VP group was cerebrovascular disease, and all patients underwent VP shunt placement for hydrocephalus secondary to cerebral hemorrhage. In the control group, reasons for PEG tube placement included cerebrovascular disease in 36 patients (75%), amyotrophic lateral sclerosis in 4 (8.3%), hypoxic brain damage in 2 (4.2%), Parkinson's disease in 2 (4.2%), malignancy in 2 (4.2%), aspiration pneumonia in 1 (2.1%), and pharyngeal paralysis in 1 (2.1%). There were two patients (28.6%) with diabetes mellitus in the PEG/VP group and 10 (20.8%) in the control group ( $P = 0.639$ ). Six patients (85.7%) in the PEG/VP group had tracheostomies at the time of PEG tube placement, as did 25 patients (52.1%) in the control group ( $P = 0.122$ ). A total of 88 PEG tube placements were performed in 55 patients. The mean number of PEG placements per patient was  $1.3 \pm 0.5$  in the PEG/VP group and  $1.6 \pm 1.1$  in the control group ( $P = 0.459$ ). There was no difference in Charlson's comorbidity index score between the two groups ( $3.0 \pm 1.6$  vs  $3.5 \pm 1.9$ ,  $P = 0.504$ ). All patients received prophylactic or periprocedural antibiotics.

There was one complication (14.3%) after PEG tube placement in the PEG/VP group, and there were six complications (12.5%) in the control group ( $P = 1.000$ ).

**Table 2 Diagnosis of complications n (%)**

	<b>PEG/VP (n = 7)</b>	<b>Control (n = 48)</b>	<b>P value</b>
Complications	1 (14.3)	6 (12.5)	1.000
Wound infection	1	3	
Stomal leakage		1	
Bleeding		1	
Gastroesophageal reflux disease		1	
VP shunt infection	No	-	

Complications in the control group included three peristomal infections, one stomal leak, one case of bleeding, and one case of gastroesophageal reflux. There was no post-PEG VP shunt infection, malfunction, neurologic deterioration, or meningitis in patients with pre-existing VP shunts (Table 2). Because no VP shunt infections were identified based on clinical features (signs and symptoms), a detailed definition of shunt infection (CSF culture or leukocyte count) was not needed.

Long-term outcomes in patients with pre-existing VP shunts are shown in Table 3. In the PEG/VP group, the interval between VP shunt insertion and PEG tube placement ranged from 65 to 831 d (mean,  $308.7 \pm 260.5$  d). The abdominal end of the VP shunt catheter was positioned in the right abdomen in five patients and in the left abdomen in two patients. Of the seven patients with pre-existing VP shunts, two had diabetes mellitus and six had tracheostomies. The mean follow-up duration was  $6.4 \pm 4.5$  mo (range, 1-15 mo). One patient in the PEG/VP group had only a minor peristomal infection during follow-up. Four patients did well, and two required PEG tube replacement due to self-removal. One patient resumed eating and was able to have the PEG tube removed 96 d after placement. No patient died during follow-up.

## DISCUSSION

In this study, patients with pre-existing VP shunts accounted for 7 (12.7%) of the 55 patients having PEG tubes inserted over a 7-year period. The incidence of PEG-related complications was 14.3% (1/7) among patients with VP shunts. The incidence of PEG-related complications was 12.5% (6/48) among patients without VP shunts. There was no difference between the groups with regard to complication rate and when disregarding the primary underlying disorder, presence of diabetes mellitus, and tracheostomy state. No VP shunt infections were identified in the patients with both PEG tubes and VP shunts during the mean follow-up duration of 6.4 mo. The mean interval between VP shunt insertion and PEG tube placement was  $308.7 \pm 260.5$  d.

Ever since Gauderer *et al*<sup>[1]</sup> introduced the endoscopic placement of feeding gastrostomy tubes in 1980, clinicians have been able to perform the PEG procedure with a shorter operative time and without the need for laparotomy. This procedure has been shown to have fewer complications and lower cost compared to the traditional open gastrostomy originally described by Stamm

in 1894<sup>[8-10]</sup>. However, PEG-related complications, including wound infection, bleeding, gastric leakage, tube dysfunction, and aspiration pneumonia, occur in approximately 10% of all cases<sup>[10]</sup>. Stomal site infections occur in 2.9% to 8.8% of patients<sup>[11-13]</sup>, and peritonitis occurs in 0.5% to 6.6% of patients<sup>[12,14-16]</sup>. Major complications requiring surgical intervention, including intraperitoneal abscess and fistula formation, occur in 2% to 3% of all patients<sup>[10]</sup>. In this study, the incidence of PEG-related complications was 14.3% in the PEG/VP group and 12.5% in the PEG alone group. All complications were manageable with conservative therapy. Despite the small numbers of patients, especially in the PEG/VP group, these incidences were similar to those seen in previous reports, and no other major complications occurred.

VP shunt placement is the major neurological procedure required in the treatment of hydrocephalus. However, VP shunts are frequently associated with serious complications, including shunt obstruction, meningitis, and intraperitoneal infection. According to the available literature, the rate of shunt infection ranges from 3% to 29% after VP shunting procedures<sup>[2-6]</sup>. Many of these complications occur at the abdominal sites of VP shunts. Patients with indwelling peritoneal shunts could be at risk for infection, even without PEG tubes.

Therefore, we hypothesized that the incidence of VP shunt infection would be higher in those patients with VP shunt catheters and PEG tubes. However, the question is, do PEG tubes increase VP shunt complication rates? To date, there have been seven reports addressing the safety of PEG tubes in patients with VP shunts (Table 4)<sup>[17-23]</sup>. There is only one prospective study in the literature<sup>[17]</sup>. The number of patients in these studies with both PEG tubes and VP shunts ranged from 6 to 55, and the VP shunt infection rate ranged from 0% to 50%. Most patients have had their VP shunts placed first, followed by PEG insertion. Two separate studies looked at VP shunt infection rates in patients undergoing VP shunt placement before PEG tube placement and in patients undergoing PEG tube placement before VP shunt placement. Infection rates were higher in patients undergoing PEG tube placement first, although not to a statistically significant degree<sup>[22,23]</sup>. In the study of Taylor *et al*<sup>[19]</sup>, PEG tubes and VP shunts were simultaneously placed in 16 patients; VP shunt infections occurred in eight patients (50%). Therefore, the investigators recommended that simultaneous PEG tube/VP shunt insertion be avoided. The VP shunt infection rate was higher in tracheostomy patients in the study of Taylor *et al*<sup>[19]</sup>, but it was not higher in our study. With regard to the time interval between PEG tube and VP shunt insertion, Graham *et al*<sup>[17]</sup> insisted that a 1-wk interval is safe. However, this interval has been more than 1 mo in most previous reports, and Nabika *et al*<sup>[22]</sup> recommended a 1-mo interval because three of four patients developing VP shunt infections in their study had PEG tubes and VP shunts placed within 1 mo of each other. In our study, the mean interval between the two was very long (308.7 d). We think this may have contributed to the absence of

**Table 3** Long-term outcomes in patients with PEG tubes and VP shunts

No. of case	Sex/age (yr)	PEG-VP shunt interval (d)	Position of abdominal shunt catheter	DM	Complication	Follow-up (mo)	Outcome
1	F/67	409	Right	-	Wound infection	7	Doing well
2	M/57	65	Right	+	-	15	Doing well
3	M/57	256	Left	+	-	8	PEG change due to self-removal
4	F/62	831	Right	-	-	6	PEG change due to self-removal
5	F/67	274	Right	-	-	5	Doing well
6	F/36	259	Right	-	-	1	Doing well
7	F/42	67	Left	-	-	3	PEG removal

**Table 4** Summary of published data on infections related to gastrostomy placement in patients with ventriculoperitoneal shunts

Investigator	Study design	Method of gastrostomy	Order of PEG & VP shunt	n	VP shunt infection rate	Interval between PEG & VP shunt	Control group	VP shunt infection rate in control group	Antibiotic used
Graham et al <sup>[17]</sup>	Prospective	Percutaneous endoscopic	VP→PEG	15	0%	2.2 wk	None	-	Cefazolin
Sane et al <sup>[18]</sup>	Retrospective	Fluoroscopic	VP→PEG	23	9% (2/23)	At least 4 wk	None	-	None
Taylor et al <sup>[19]</sup>	Retrospective	Percutaneous endoscopic	Simultaneous	16	50% (8/16)	-	VP shunt and tracheostomy without PEG	0% (0/21)	Yes (unspecified)
Baird et al <sup>[20]</sup>	Retrospective	Percutaneous endoscopic	VP→PEG	6	0%	33 d	None	-	Cefazolin
Schulman et al <sup>[21]</sup>	Retrospective	Percutaneous endoscopic	VP→PEG	39	5% (2/39)	43.1 d	None	-	72% received (unspecified)
Nabika et al <sup>[22]</sup>	Retrospective	Percutaneous endoscopic	Both	23	17.4% (4/23)	29.3 d	Only VP shunt	4.9% (6/123)	Cefazolin
			PEG→VP	12	25% (3/12)	27.2 d			
			VP→PEG	11	9.1% (1/11)	39.2 d		(P = 0.0519)	
Roeder et al <sup>[23]</sup>	Retrospective	Percutaneous endoscopic and surgical	Both	55	12.7% (7/55)	-	Only PEG	-	90.9% received (unspecified)
			PEG→VP	30	16.6% (5/30)				
			VP→PEG	25	8% (2/25)				
This study	Retrospective	Percutaneous endoscopic	VP→PEG	7	0% (0/7)	308.7 d	Only PEG	-	Yes (unspecified)

VP shunt infections in our study. Concerning the control group, there have been two studies with VP shunt patients serving as the control group<sup>[19,22]</sup>. The VP shunt infection rates were 50% (8/16) in the PEG/VP group and 0% (0/21) in the control group in one study due to simultaneous insertion<sup>[19]</sup>, but the VP shunt infection rates were 17.4% (4/23) in the PEG/VP group and 4.9% (6/123) in the control group ( $P = 0.0519$ ) in the other study<sup>[22]</sup>. Therefore, except for simultaneous insertion, the VP shunt infection rates of patients with PEG and VP shunts are not significantly different from those seen in control patients with VP shunts. Only one report has addressed the question of mortality<sup>[23]</sup>. In this report, the all-cause mortality at 1 year after PEG tube placement in patients with VP shunts was 21%, and PEG tube placement in patients with VP shunts was not associated with excessive mortality compared to PEG tube placement alone. Prophylactic antibiotics were given in all studies, except for one. That study used percutaneous fluoroscopic antegrade technique in 23 children, 2 (9%) of whom developed VP shunt infections<sup>[18]</sup>.

The limitations of our study are similar to those of previously published studies. Firstly, our study was retrospective. Secondly, the number of study patients was small; specifically, there were only seven patients with PEG tubes and VP shunts. Thirdly, the control group in our study was composed of patients with PEG

tubes alone, not patients with VP shunts. However, despite these limitations, our study and literature review suggest that PEG tube placement is safe in patients with VP shunts, especially those in whom the VP shunt is inserted first, those in whom the interval between PEG tube and VP shunt insertion is greater than 1 mo, and those in whom prophylactic antibiotics are used.

## COMMENTS

### Background

Percutaneous endoscopic gastrostomy (PEG) tube placement has been widely used for long-term nutritional support in patients with severe neurological impairment. These patients requiring PEG tube placement may have concomitant hydrocephalus requiring insertion of a ventriculoperitoneal (VP) shunt. However, the presence of a VP shunt in a patient requiring PEG placement raises concerns about potential life-threatening complications such as VP shunt infection and VP shunt malfunction. Therefore, we aimed to investigate if PEG tube placement is safe in patients with VP shunts.

### Research frontiers

To date, controversy still exists as to whether PEG placement is safe in patients with VP shunts. There have been seven reports addressing the safety of PEG tubes in patients with VP shunts. There is only one prospective study in the literature.

### Innovations and breakthroughs

This study suggests that PEG tube placement is safe in patients with VP shunts, especially those in whom the VP shunt is inserted first, those in whom the interval between PEG tube and VP shunt insertion is greater than 1 mo, and those in whom prophylactic antibiotics are used.

## Applications

To confirm whether PEG placement is safe in patients with VP shunts, a large scale prospective study including a control group which has patients with VP shunts is needed.

## Peer review

This paper presents a series of patients with preexisting ventriculoperitoneal shunt, who needed a percutaneous endoscopic gastrostomy. The authors conclude that percutaneous endoscopic gastrostomy after previous ventriculoperitoneal shunt is safe. The paper may help to support the indication even in this group, if a gastrostomy is needed.

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# What is the most cost-effective strategy to screen for second primary colorectal cancers in male cancer survivors in Korea?

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lifetime costs, life expectancy, and incremental cost-effectiveness ratio (ICER).

**RESULTS:** In the base-case analysis, the non-dominated strategies in cancer survivors were COL5, and COL3. The ICER for COL3 in cancer survivors was \$5593/life-year saved (LYS), and did not exceed \$10000/LYS in one-way sensitivity analyses. If the risk of CRC in cancer survivors is at least two times higher than that in the general population, COL5 had an ICER of less than \$10500/LYS among both good and poor prognosis of index cancer. If the age of cancer survivors starting CRC screening was decreased to 40 years, the ICER of COL5 was less than \$7400/LYS regardless of screening compliance.

**CONCLUSION:** Our study suggests that more strict and frequent recommendations for colonoscopy such as COL5 and COL3 could be considered as economically reasonable second primary CRC screening strategies for Korean male cancer survivors.

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**Key words:** Cost-effectiveness; Second primary colorectal cancer; Screening; Cancer survivor

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## INTRODUCTION

A recent improvement in cancer survival owing to early diagnosis and advances in treatment has raised the issue of second primary cancers (SPCs) in cancer survivors after their primary treatment<sup>[1,2]</sup>. Due to carcinogenic effects of cancer-related treatment, genetic susceptibility

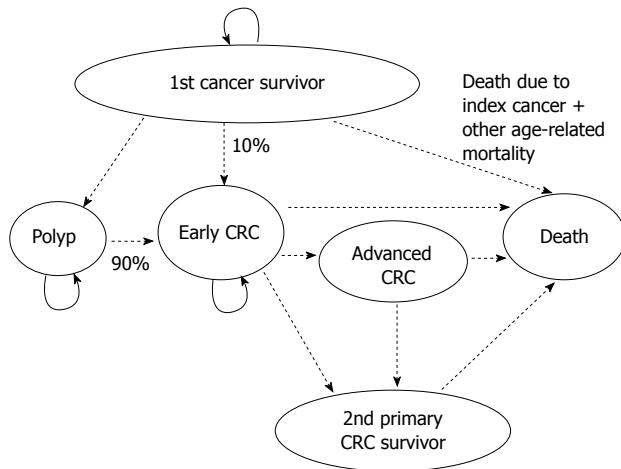
ity or unhealthy behavior such as smoking, alcohol and obesity, cancer survivors are at increased risk for SPCs, not only at the original site but at other sites as well<sup>[3,4]</sup>. Recent studies have shown that the age-standardized incidence rate was 2.3 times higher for an SPC than for a first cancer in the Korean general male population<sup>[5]</sup>. Specifically, the age-standardized incidence rate was about four times higher for second than for first primary colorectal cancers (CRCs)<sup>[5]</sup>. It is well-known that screening for CRC reduces mortality through detection of malignancy at an earlier, more treatable stage as well as by identification and removal of the precursor lesion, the adenomatous polyp<sup>[6]</sup>. These findings suggest that more thorough surveillance and screening for second primary CRC is needed for the cancer survivors.

Many previous studies have focused on the cost-effectiveness (CE) of CRC screening in the general population<sup>[6-9]</sup>, and several panels have recommended CRC screening for the general population<sup>[10-12]</sup>. As the risk of CRC and life expectancy are quite different between cancer survivors and the general population, screening guidelines for the general population could not be applied to the cancer survivors. However, until now, there have been few recommendations for CRC screening for cancer survivors. To suggest a feasible economic strategy of second primary CRC screening for cancer survivors in Korea, we constructed a decision-analytic model, and compared the CE results of cancer screening in cancer survivors and in the average-risk general population.

## MATERIALS AND METHODS

The natural history of a simulated male cancer survivors' cohort was modeled with and without second primary CRC screening until age of 75 years (Figure 1). For simplicity and comparison with results from the general population, we assumed that all male cancer survivors enter at age 50 years, which most guidelines for the general population recommended for starting CRC screening<sup>[10-12]</sup>. We developed a Markov model using TreeAge-Pro 2007 software (TreeAge Software Inc., Williamstown, Massachusetts). The Markov model estimated the clinical and economic consequences of eight different screening strategies as follows: (1) no screening, (2) fecal occult blood test (FOBT) annually (FOBT1), (3) FOBT every 2 years (FOBT2), (4) sigmoidoscopy every 5 years (SIG5), (5) double contrast barium enema every 5 years (DCBE5), (6) colonoscopy every 10 years (COL10), (7) colonoscopy every 5 years (COL5), and (8) colonoscopy every 3 years (COL3).

Individuals were placed into health states defined by the presence or absence of a colorectal polyp or second primary CRC (early or advanced) 1 year after the index cancer diagnosis. Cases of positive screening test results were worked up with a colonoscopy, and individuals diagnosed with polyps underwent polypectomy. The probability of perforation was assigned to colonoscopy, sigmoidoscopy, DCBE and polypectomy<sup>[8,13,14]</sup>. Mortality caused by the risk of perforation was assumed to be



**Figure 1** Markov model of second colorectal cancer (CRC) screening in Korean male cancer survivors.

0.02%<sup>[9,14]</sup>. Colonoscopy was repeated every 3 years for surveillance after polypectomy<sup>[15]</sup>. We assumed that 80% of male cancer survivors underwent the initial screening test, independent of whether they were compliant with past tests. The compliance of follow-up or surveillance colonoscopy was assumed to be 100%. We also assumed that 90% of CRCs develop from polyps<sup>[15,16]</sup>, and the latent period between early stage and advanced stage was assumed to be 2 years<sup>[9]</sup>. The relative risk of CRC in Korean male cancer survivors was assumed to be four times higher than that in the general population<sup>[5]</sup>. Age-specific transition probabilities and prevalence were calculated between normal, polyp, and CRC to yield an incidence rate of polyp and CRC derived from previous literature and the Korea Central Cancer Registry<sup>[7,17-19]</sup>. The stage-specific CRC mortality were applied uniformly to all malignancies regardless of the means of detection (by symptoms or screening) or the state of detection (diagnosed *vs* undiagnosed cancer). Age-specific mortality from index cancer and other causes was estimated based on the above sources combined with statistics published by the National Center for Health Statistics<sup>[20]</sup> and Korea Central Cancer Registry<sup>[21]</sup>. As there have been few studies on mortality from second primary CRC, we calculated the additional yearly probability of dying from a second primary CRC based on cancer stage from previous studies of CRC as the first index cancer<sup>[22-23]</sup>.

We obtained the data on the costs of CRC treatment by stage and time period from the National Health Insurance Corporation (social insurer of the national health insurance (NHI) with a universal coverage of population)<sup>[24]</sup>. Costs of screening tests were obtained from the fee schedule of the National Health Insurance Corporation (the NHI of Korea has a fee schedule applied to all insured services)<sup>[25]</sup>. Costs were expressed in US dollars and the exchange rate was 955 Korean Won for one US dollar in 2006<sup>[26]</sup>. As the indirect costs of cancer screening are not established in Korea, we included only direct medical costs.

Our main outcome measures were discounted lifetime costs, life expectancy, incremental cost-effectiveness

**Table 1** Summary of assumptions

Parameter	Base case value (range)	Ref.
Sensitivity & specificity of colorectal screening tests	Sensitivity of FOBT for colorectal polyps/cancer	0.1/0.5
	Sensitivity of colonoscopy for colorectal polyps/cancer	0.9/0.95
	Sensitivity of double contrast barium enema for colorectal polyps/cancer	0.5/0.8
	Sensitivity of sigmoidoscopy for colorectal polyps/cancer	0.46/0.52
	Specificity of FOBT	0.9
	Specificity of colonoscopy	1
	Specificity of double contrast barium enema	0.9
	Specificity of sigmoidoscopy	0.95
Natural history of colorectal polyp/cancer sequence	Prevalence of polyps at age 50 yr	0.20 (0.1-0.4)
	Annual polyp incidence rate in cancer survivors	Age specific
	Percent of cancers originating as polyps	90%
	Relative risk of colorectal cancer in cancer survivor compared with the general population	4 (1-5)
	Age specific incidence rate of colorectal cancer without polypoid precursors in cancer survivors	Age specific
	Age specific incidence rate of colorectal cancer with polypoid precursors in cancer survivors	Age specific
	Dwelling time of colorectal cancer in early stages	2 yr
	Percent of colorectal cancers detected in early stages with no screening	5% (2%-10%)
	Five-year all cause survival for early 2nd primary colorectal cancer	90% (80%-95%)
	Five-year all cause survival for advanced 2nd primary colorectal cancer	60% (40%-70%)
Natural history of cancer survivors	Five-year survival for index cancer	40% (20%-80%)
	Age specific mortality except the index cancer	Age specific
	Age of cancer survivors for starting colorectal cancer screening, year	50 (40-60)
	Compliance of 2nd colorectal cancer screening	80% (60%-100%)
Complications and unintended consequences	Rate of perforation of colon in colonoscopy	0.20% (0.1%-0.3%)
	Rate of perforation of colon in polypectomy	0.40% (0.2%-0.5%)
	Rate of perforation from sigmoidoscopy	0.01% (0.005%-0.05%)
	Rate of perforation from double contrast barium enema	0.005% (0.001%-0.01%)
	Death rate due to perforated colon	0.2% (0.1%-5%)
Cost (dollar <sup>1</sup> ) & discount rate	Sigmoidoscopy	31.3
	Colonoscopy	61.7
	Double contrast barium enema	68.5
	FOBT	2.7
	Polypectomy, biopsy and pathologic exam	189
	Treatment of early cancer for first year	7330 (5860-8800)
	Treatment of advanced cancer for first year	14660 (10050-15080)
	Treatment of cancer after first year	2094 (1670-2510)
Discount rate	Cost to repair the endoscopic perforation	3141 (2510-3770)
	Discount rate	0.03 (0-0.05)

<sup>1</sup>Exchange rate: 955 Korean Won for one US dollar in 2006; <sup>2</sup>Estimated by calibration to national data on colorectal polyp and cancer incidence.

ratio (ICER), which were compared for different CRC screening strategies. Because there are uncertainties with respect to quality of life associated with CRC screening, colorectal polyp, and second CRC, we conducted the base case analysis using increase in life expectancy as the primary outcome. Incremental CE analysis was performed by ranking the 16 strategies in order of increasing effectiveness. After eliminating strategies that were more or equally costly and less effective than a competing strategy [i.e. ruled out by simple dominance], we calculated the ICER for each strategy (additional cost divided by life-year saved (LYS)] compared with the next least expensive strategy. Strategies exhibiting extended dominance were eliminated from the rank-ordered list, and ICERs of the remaining strategies were recalculated<sup>[27]</sup>. Future costs and life-years were discounted at an annual rate of 3%. We compared the results from male cancer survivors with that from general male population.

Sensitivity analyses were performed to assess the

stability of the results of a plausible range of several parameters, such as prevalence of colorectal polyps at age 50 years, 5-year survival rates of second primary CRC, complications of screening test or polypectomy etc (Table 1). We performed detailed analyses by changing key variables of the index cancer such as 5-year survival rate of first cancer, relative risk of CRC in cancer survivors compared with that in general population. In addition, we evaluated the effects of changing age of subjects for starting second primary CRC screening and compliance rate on the CE of our results.

## RESULTS

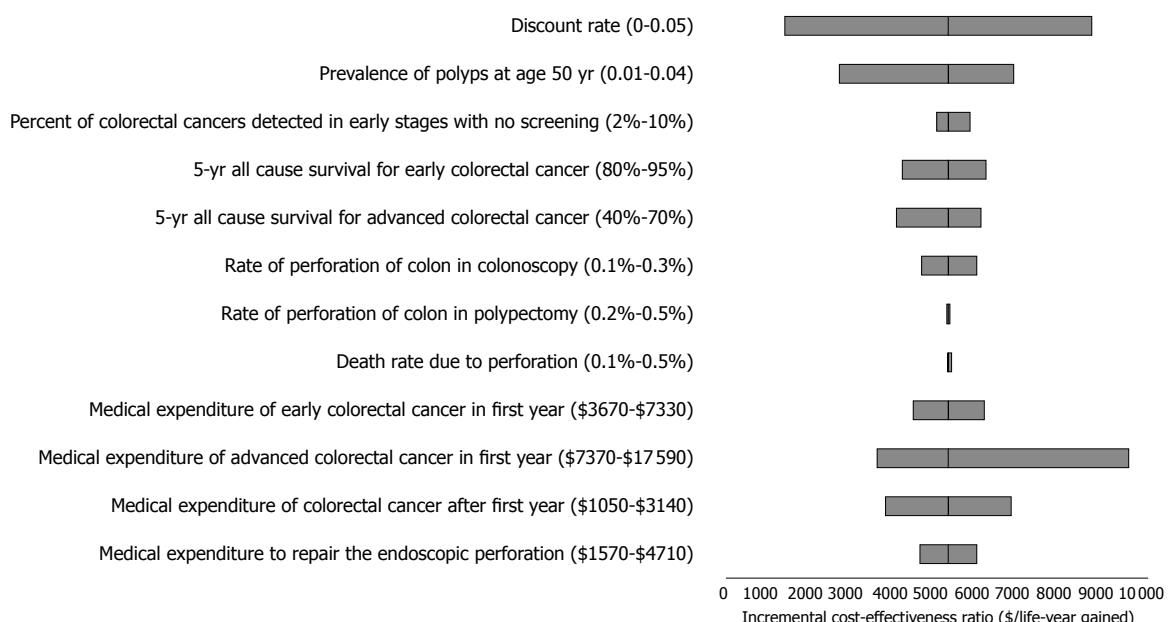
### Base case

In the base-case analysis at 80% screening compliance, all screening strategies extended life expectancy both in male cancer survivors and the general population (Table 2). The strategies which were not ruled out by simple

**Table 2 Cost-effectiveness of colorectal screening strategies among male cancer survivors and the general population in Korea (80% compliance)**

Male general population				Male cancer survivors			
Strategy (abbreviation)	Lifetime cost per person (dollar) <sup>1</sup>	Life expectancy (yr)	Incremental C/E <sup>2</sup> , dollar <sup>1</sup> per life-year gained	Strategy (abbreviation)	Lifetime cost per person (dollar) <sup>1</sup>	Life expectancy (yr)	Incremental C/E <sup>2</sup> , dollar <sup>1</sup> per life-year gained
COL every 10 yr	437.3	17.260	...	COL every 5 yr	463.5	7.572	
No screening	448.0	17.243	...	COL every 10 yr	480.1	7.568	...
COL every 5 yr	478.3	17.263	14456.8	COL every 3 yr	480.2	7.575	5592.9
DCBE every 5 yr	542.3	17.256	...	DCBE every 5 yr	563.4	7.562	...
SIG every 5 yr	542.4	17.255	...	SIG every 5 yr	571.8	7.560	...
COL every 3 yr	554.4	17.265	38876.8	No screening	632.2	7.544	...
FOBT every 2 yr	810.0	17.252	...	FOBT every 2 yr	735.2	7.557	...
FOBT every 1 yr	1130.9	17.257	...	FOBT every 1 yr	842.3	7.564	...

COL: Colonoscopy; SIG: Sigmoidoscopy; DCBE: Double contrast barium enema; FOBT: Fecal occult blood test. Ellipse indicates no data (incremental CR ratios not calculated for these strategies because they were dominated). <sup>1</sup>Exchange rate, 955 Korean Won for one US dollar in 2006; <sup>2</sup>Incremental CE ratio (dollar/year) = Incremental cost per person/incremental years of life gained.

**Figure 2 Sensitivity analyses on cost-effectiveness from the perspective of colonoscopy every 3 years vs colonoscopy every 5 years in male cancer survivors.**

dominance nor extended dominance (non-dominated strategies) in the general population were COL10, COL5, and COL3, while those in cancer survivors were COL5 and COL3. The ICER for COL3 in cancer survivors was \$5593 per LYS. In cancer survivors, the lifetime total cost per person associated with "FOBT annually" was larger than that associated with no screening, while COL5 and COL3 were less costly than no screening.

### Sensitivity analyses

Figure 2 shows the results of one-way sensitivity analyses on CE from the perspective of COL3 vs COL5 in male cancer survivors. In most cases, COL5 and COL3 were non-dominated strategies, and the ICER of COL3 ranged between \$1480 and \$9192.

Table 3 shows the results of two-way sensitivity analyses by changing risk of second CRC and 5-year survival rate of index cancer in Korean male cancer

survivors. If the risk of CRC in cancer survivors was at least three times higher than that in the general population, screening with COL5 in cancer survivors had an ICER of less than \$4000 per LYS in the entire range of 5-year survival of index cancer between 20% and 80%. If the risk of CRC in cancer survivors was two times higher than that in the general population, COL5 in cancer survivors had an ICER of less than \$10 500 per LYS in both types of index cancer with poor and good prognosis. If the risk of CRC in cancer survivors was the same as that in the general population, non-dominated strategies were no screening, COL10, COL5, and COL3, and the ICER of COL5 was more than \$25 000 per LYS, while the ICER of COL10 ranged between \$2315 in index cancer with good prognosis and \$19 650 in index cancer with poor prognosis.

Table 4 shows the results of sensitivity analysis by changing compliance of CRC screening and age of cancer

**Table 3** Two-way sensitivity analysis by changing variables of index cancer such as 5-yr survival rate of first cancer and relative risk of colorectal cancer in Korean male cancer survivors

Relative risk of colorectal cancer in cancer survivor compared with that in general population	5-yr survival of index cancer = 20%				5-yr survival of index cancer = 40%				5-yr survival of index cancer = 80%			
	Non-dominated strategy	Lifetime cost per person (dollar) <sup>1</sup>	Life expectancy (yr)	Incremental C/E <sup>2</sup> (dollar) <sup>1</sup> per life-year gained	Non-dominated strategy	Lifetime cost per person (dollar) <sup>1</sup>	Life expectancy (yr)	Incremental C/E <sup>2</sup> (dollar) <sup>1</sup> per life-year gained	Non-dominated strategy	Lifetime cost per person (dollar) <sup>1</sup>	Life expectancy (yr)	Incremental C/E <sup>2</sup> (dollar) <sup>1</sup> per life-year gained
5	COL5	329.0	4.512	4921.7	COL5	532.5	7.567	2634.4	COL5	957.9	13.829	
	COL3	339.2	4.514		COL3	542.2	7.571		COL3	962.6	13.836	658.2
	COL5	306.7	4.516		COL5	463.5	7.572		COL10	826.0	13.837	
	COL3	329.0	4.517		COL3	480.2	7.575		COL5	831.1	13.843	835.2
4	COL10	266.6	4.517	2685.2	COL10	401.4	7.576	3365.6	COL3	879.7	13.847	11508.2
	COL5	270.3	4.518		COL5	409.6	7.579		COL10	682.9	13.847	
	COL3	295.5	4.519		COL3	447.3	7.580		COL5	701.9	13.851	4104.9
3	No screening	185.4	4.512	27126	COL10	324.4	7.582	22760.2	COL3	761.9	13.854	18922.1
	COL10	224.5	4.52		COL5	340.1	7.583		COL10	533.5	13.857	
	COL5	232.3	4.521		COL3	383.7	7.584		COL5	566.7	13.860	10725.2
	COL3	260.4	4.522		COL3	316.8	7.589		COL3	638.6	13.862	34075.6
2	No screening	96.1	4.519	20568.2	No screening	175.9	7.580	9196.3	No screening	342.6	13.853	
	COL10	180.5	4.523		COL10	244.0	7.587		COL10	377.2	13.867	2424.5
	COL5	192.4	4.524		COL5	267.2	7.588		COL5	425.1	13.869	31064.6
	COL3	223.5	4.524		COL3	316.8	7.589		COL3	509.3	13.870	81681.8

COL10: Colonoscopy every 10 years; COL5: Colonoscopy every 5 years; COL3: Colonoscopy every 3 years; Dominated strategy is a strategy that is more or equally costly and less effective than a competing strategy.<sup>1</sup> Exchange rate, 955 Korean won for one US dollar in 2006; <sup>2</sup> Incremental CE ratio (dollar/year) = Incremental cost per person/Incremental years of life gained.

**Table 4** Two-way sensitivity analysis on cost-effectiveness of 2nd primary colorectal cancer screening by compliance of screening and age of Korean male cancer survivors for starting screening

Age for starting screening	Compliance of 2nd colorectal cancer screening = 60%				Compliance of 2nd colorectal cancer screening = 80%				Compliance of 2nd colorectal cancer screening = 100%			
	Non-dominated strategy	Lifetime cost per person (dollar) <sup>1</sup>	Life expectancy (yr)	Incremental C/E <sup>2</sup> (dollar) <sup>1</sup> per life-year gained	Non-dominated strategy	Lifetime cost per person (dollar) <sup>1</sup>	Life expectancy (yr)	Incremental C/E <sup>2</sup> (dollar) <sup>1</sup> per life-year gained	Non-dominated strategy	Lifetime cost per person (dollar) <sup>1</sup>	Life expectancy (yr)	Incremental C/E <sup>2</sup> (dollar) <sup>1</sup> per life-year gained
40	COL5	370.0	8.633	7584.1	COL10	341.0	8.326	872.5	COL10	352.2	8.328	
	COL3	388.9	8.636		COL5	343.7	8.329		COL5	369.2	8.330	7375.9
50	COL3	479.2	7.665	26483.2	COL3	383.7	8.330		COL3	424.1	8.331	50842.3
	COL5	463.5	7.572		COL5	480.2	7.575		COL10	475.0	7.571	
60	COL3	528.3	7.668	5592.9	COL3	522.1	6.057	330.8	COL5	519.5	6.057	14571.1
	COL5	508.1	7.577		COL5	529.5	6.059		COL3	508.1	7.577	
	COL3	529.5	6.059		COL3	529.5	6.059		COL3	529.5	6.059	4186.7

COL10: Colonoscopy every 10 years; COL5: Colonoscopy every 5 years; COL3: Colonoscopy every 3 years. Dominated strategy is a strategy that is more or equally costly and less effective than a competing strategy.<sup>1</sup> Exchange rate: 955 Korean Won for one US dollar in 2006. <sup>2</sup> Incremental CE ratio (dollar/year) = Incremental cost per person/Incremental years of life gained.

survivors for starting CRC screening. If the age of cancer survivors for starting CRC screening was 50 years or older, COL3 had an ICER of less than \$14 600 per LYS, regardless of the screening compliance. If age for starting CRC screening in cancer survivors was 40 years, the ICER of COL5 was less than \$7400 per LYS saved in all cases of CRC screening compliance.

## DISCUSSION

We constructed a computer simulation to suggest economic strategies of second primary CRC screening

for cancer survivors in Korea, and compared the CE results of CRC cancer screening in cancer survivors and in the average-risk general population. As all non-dominated strategy were those using colonoscopy in both cancer survivors and the general population, more strict and frequent recommendation of colonoscopy such as COL5 and COL3 could be considered economic strategies for male cancer survivors.

Until now, there has been no explicit threshold of CE below which policy makers will consider accepting the strategy. In the US, a figure of \$50 000 per Quality Adjusted Life-Year (QALY) has frequently been quoted

for many years as being cost-effective<sup>[43]</sup>. The World Health Report 2002 suggested that interventions costing less than three times Gross Domestic Product (GDP) per capita for each Disability Adjusted Life-Year (DALY) averted represented good value for money<sup>[44]</sup>, which is usually well in excess of \$50 000 per QALY in many high-income countries<sup>[43]</sup>. In our study, the ICER for COL3 was less than \$6000 per LYS in a base line analysis, and did not exceed \$10 000 per LYS in one-way sensitivity analyses. Our findings also suggest that if the risk of CRC in cancer survivors is at least two times higher than that in the general population, COL5 could be a cost-effective strategy for second primary CRC screening for cancer survivors of both good and poor prognosis of index cancer, with an ICER of less than \$10 500 per LYS. As our primary outcomes are not QALY or DALY, direct comparisons might be difficult. The GDP per capita of Korea was more than \$20 000 in 2006. When we approximately applied these CE thresholds, in most cases, COL5 and COL3 could be considered a cost-effective method for second primary CRC screening for Korean male cancer survivors, regardless of the index cancer. Even if the index cancer were CRC, our finding could be applied and be consistent with the CRC surveillance guidelines of the American Society of Clinical Oncology (ASCO)<sup>[45]</sup>. In ASCO guidelines, routine annual colonoscopies are not recommended for all CRC patients, and colonoscopy every 3–5 years could be sufficient to detect new CRCs and polyps<sup>[45]</sup>.

It is also important to consider the changes in CE of these strategies according to age of starting second primary CRC screening in cancer survivors. Little is known about the CE if cancer survivors start second primary CRC screening above or below age 50, at which most guidelines recommend starting CRC screening in the general population. Our findings suggest that for male cancer survivors in older age, COL3 had more favorable CE results, regardless of screening compliance. For younger cancer survivors aged 40 years old, COL5 could be considered a CE strategy with ICER of less than \$7600 per LYS.

Interestingly, in our study of Korea, COL10, COL5 and COL3 had lower total medical costs than no screening of male cancer survivors. In other countries, screening for CRC usually leads to more costs than no screening. Cost estimates for the medical care of CRC treatment in the US range from \$25 000 to \$70 000 and the cost of COL is about \$1000<sup>[9,16]</sup>. However, in Korea, the cost estimate of CRC treatment in the first year ranges from \$7000 to \$14 000 while the cost of COL was about \$60<sup>[24,25]</sup>. The ratio of treatment cost to COL cost ranges from 25:1 to 70:1 in the US and 120:1 to 230:1 in Korea. Due to the difference in cost structures, screening with colonoscopy might be more cost-effective in Korea than in other countries. However, CRC screenings are not covered by the Korean NHI scheme in either the general population or cancer survivors. Instead, the Korean government started the national cancer screening program (NCSP) in 1999, which was

extended to include CRC screening in 2004<sup>[11]</sup>. The government covers 50% of the screening cost for the insured and 100% for low-income people. However, the primary method for CRC screening in the Korean NCSP is FOBT annually. Our study shows that the strategy of "FOBT annually" is always the dominant strategy in male cancer survivors, and costs more than the strategy of no screening.

The major barrier to promotion of colonoscopy as a primary CRC screening tool is the lack of manpower to deliver colonoscopy to the public in Korea. In these human-resource limited setting, it is important to identify the more vulnerable population who has a greater potential to receive benefits. Our study suggests that cancer survivors who are at increased risk of second primary CRC have a favorable result of CE of CRC screening compared with the general population. Even in younger cancer survivors aged 40 years old, COL5 might be economically feasible, while COL10 is usually recommended for the Korean general population aged 50 years old. Therefore, at least for cancer survivors, CRC screening should be covered by the Korean NHI scheme and screening methods using colonoscopy are needed to be recommended as a primary screening strategy for CRC in this population.

### **Limitations**

Our analysis has several limitations. In the design of the model, we tried to reduce the complex natural history of CRC to a few essential states and to avoid assumptions on treatments for which little or no published data existed. For instance, we assumed that 90% of second primary CRC arose from polyps. We used several sets of data from the general population, such as prevalence of colorectal polyp by age, polyp recurrence rate, and treatment costs of second primary CRC, if there were no published data available in cancer survivors. There were possible differences between these two populations. However, when we performed sensitivity analyses, the CE results were usually insensitive to the plausible range of these uncertain parameters. Second, we calculated only the direct costs and did not take into account the impact of CRC and screening on indirect costs. Third, in our study, recently developed CRC screening strategies such as CT colonoscopy were not included. However, the cost of CT colonoscopy is about four times higher than that of colonoscopy in Korea, while the sensitivity and specificity of CT colonoscopy is not superior to that of colonoscopy<sup>[46]</sup>, and this new method does not seem to be an economically-efficient strategy.

In conclusion, with an increased population of long-term cancer survivors, effective systems for their health promotion are needed. Implementation of the economic SPC screening program might be one of the important interventions to improve their health. Our study showed that COL3 or COL5 might be suggested as a primary strategy for second primary CRC screening in cancer survivors who have a higher risk of CRC than the general population. This study supports the evidence

and rationale for second primary CRC screening in male cancer survivors.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Recent improvement in cancer survival due to early diagnosis and advances in treatment has raised the issue of second primary cancers (SPCs) in cancer survivors after their primary treatment. The age-standardized incidence rate is about four times higher for second primary colorectal cancer (CRC) than for first primary CRC in Korea. However, until now, there have been few recommendations and economic evaluations of CRC screening for cancer survivors.

### Research frontiers

To suggest a feasible economic strategy of second CRC screening for cancer survivors in Korea, the authors constructed a decision-analytic model, and compared the cost-effectiveness results of cancer screening between in male cancer survivors.

### Innovations and breakthroughs

Non-dominated strategies were those using colonoscopy in both cancer survivors and the general population, and more strict and frequent recommendations for colonoscopy (COL) such as COL5 (screening every 5 years) and COL3 (screening every 3 years) could be considered as economic strategies for male cancer survivors.

### Applications

The major barrier to promoting colonoscopy as a primary CRC screening tool is the lack of manpower to deliver colonoscopy to the public in Korea. In these human-resource limited settings, it is important to identify the most vulnerable population who has more potential to receive the benefits. In younger cancer survivors aged 40 years old, COL5 might be economically feasible, while COL10 is usually recommended for the Korean general population aged 50 years old. Therefore, at least for cancer survivors, CRC screening should be covered by the Korean national health insurance scheme and screening methods using colonoscopy should be recommended as a primary screening strategy for CRC in this population.

### Terminology

SPC: A SPC is a new primary cancer developing in a person with a history of cancer.

### Peer review

The authors investigated the cost-effective strategy of CRC screening for cancer survivors in Korea. The article is well written and the contents are reliable.

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# Characterization of clarithromycin resistance in Malaysian isolates of *Helicobacter pylori*

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common occurrence among Malaysian isolates of *H pylori* strains and the mutations A2142G and A2143G detected were associated with low-level resistance.

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**Key words:** Clarithromycin resistance; *Helicobacter pylori*; 23S rRNA mutation; Restriction fragment length polymorphism

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## Abstract

**AIM:** To characterize the types of mutations present in the 23S rRNA genes of Malaysian isolates of clarithromycin-resistant *Helicobacter pylori* (*H pylori*).

**METHODS:** Clarithromycin susceptibility of *H pylori* isolates was determined by E test. Analyses for point mutations in the domain V of 23S rRNA genes in clarithromycin-resistant and -sensitive strains were performed by sequence analysis of amplified polymerase chain reaction products. Restriction fragment length polymorphism was performed using *Bsa* I and *Mbo* II enzymes to detect restriction sites that correspond to the mutations in the clarithromycin-resistant strains.

**RESULTS:** Of 187 isolates from 120 patients, four were resistant to clarithromycin, while 183 were sensitive. The MIC of the resistant strains ranged from 1.5 to 24 µg/mL. Two isolates had an A2142G mutation and another two had A2143G mutations. A T2182C mutation was detected in two out of four clarithromycin-resistant isolates and in 13 of 14 clarithromycin-sensitive isolates. Restriction enzyme analyses with *Bsa* I and *Mbo* II were able to detect the mutations.

**CONCLUSION:** Clarithromycin resistance is an un-

## INTRODUCTION

*Helicobacter pylori* (*H pylori*) is a microaerophilic, Gram-negative bacterium, and is implicated often as the causative agent of chronic gastritis, duodenal and gastric ulcers, and gastric carcinoma<sup>[1,2]</sup>. *H pylori*-associated disorders usually regress or heal completely after treatment with antibiotics<sup>[3]</sup>. Clarithromycin is a macrolide that has been used frequently in combination with other antimicrobial agents for the treatment of *H pylori* infection<sup>[4-6]</sup>. However, resistance to clarithromycin has become one of the major reasons for treatment failure<sup>[7]</sup>. The prevalence of *H pylori* resistance to clarithromycin varies among different countries, such as 12% in Japan, 1.7%-23.4% in Europe and 10.6%-25% in North America<sup>[8,9]</sup>.

Clarithromycin acts by binding to the peptidyl transferase region of 23S rRNA and inhibits bacterial

protein synthesis. Resistance to clarithromycin results from structural changes in the 23S rRNA molecule caused by mutation of the 23S rRNA gene<sup>[10,11]</sup>. Adenine to guanine transitions at positions 2142 and 2143 are the main 23S rRNA mutations in clarithromycin-resistant isolates<sup>[12-14]</sup>. All these mutations have been shown to confer resistance to this macrolide by mutagenesis study<sup>[15]</sup>. Other mutations that have been observed in clarithromycin-resistant *H pylori* isolates are A2515G and T2717C, A2116G, G2141A, A2144T, T2182C, G2224A, C2245T, and T2289C<sup>[16]</sup>. The A2142C/G and A2143G mutation also generate specific restriction sites, namely *Mbo* II and *Bsa* I, and these may be used for the rapid screening of clarithromycin resistance.

In the present study, we determined the prevalence of clarithromycin resistance in our local *H pylori* strains and characterized the types of mutations that occurred in the resistant strains. In addition, we determined whether the Restriction fragment length polymorphism (RFLP) technique was suitable for rapid detection of the mutations among our local isolates.

## MATERIALS AND METHODS

### **Patients**

The patients enrolled in this study were the patients who underwent endoscopy for gastrointestinal symptoms at the Gastroenterology Unit, National University of Malaysia Hospital between 2005 and 2007. Written informed consent was obtained from all patients before biopsies were taken from the antrum and corpus of each patient.

### ***H pylori* culture and antimicrobial susceptibility determination**

Biopsy samples were cultured on Columbia agar supplemented with 10% ox blood. Plates were incubated at 37°C under microaerophilic condition for 5-7 d. Bacterial isolates were identified according to colony morphology, Gram-staining, urease, catalase and oxidase. The cultures were stored at -80°C in Brucella broth supplemented with 15% glycerol and fetal calf serum (Invitrogen, USA).

The MIC of clarithromycin was determined by the E-test method. E tests (AB Biodisk, Sweden) were performed on Columbia agar supplemented with 10% ox blood. The plates were incubated under microaerophilic condition for 3-5 d. Isolates were classified as clarithromycin-resistant if MIC was > 1 µg/mL. *H pylori* strain ATCC43504 was included as a control clarithromycin-sensitive strain.

### **DNA extraction and polymerase chain reaction (PCR) amplification of 23S rRNA gene**

DNA extraction was carried out using the Nucleospin® Tissue Kit (Macherey-Nagel, BD Biosciences, USA) and stored at -20°C until use. The primers used for PCR amplification were Hp5 forward, 5'-GTCGTGCCAA GAAAAGCGTCT-3' (positions 1672-1693; GenBank

accession number U27270), and Hp2 reverse, 5'-TGTG TGCTACCCAGCGATGCTC-3' (positions 2811-2790; GenBank accession number U27270). PCR was carried out on 10 mmol/L dNTP (Promega, USA), 10 pmol of each primer, 1.25 U *Taq* DNA polymerase (Promega), and genomic DNA of *H pylori*. The PCR was performed in a GeneAmp PCR System 2400 thermal cycler (Perkin Elmer, USA) for 30 cycles with the following cycling conditions: 94°C for 1 min, 58°C for 30 s and 72°C for 30 s. The PCR products were then analyzed by electrophoresis using 1% agarose and stained with ethidium bromide.

### **DNA sequencing and sequence analysis**

PCR products were purified using a QIAquick PCR purification kit (Qiagen). Sequencing was performed using BigDye® Terminator v3.1 sequencing kit and analyzed on ABI PRISM® 377 Genetic Analyzer. The sequences were analyzed using free ClustalW software (<http://www.ebi.ac.uk/clustalw>).

### **RFLP analysis**

To detect the A-G point mutations at positions 2142 and 2143, the amplified PCR products were digested with the *Mbo* II and *Bsa* I (MBI Fermentas, Lithuania) restriction enzymes, respectively, and analyzed on 1.6% agarose gels. *Mbo* II-digested PCR products that contained the A2142G mutation were expected to yield two fragments (476 and 662 bp) when digested with *Mbo* II, whereas the PCR products that contained the mutation A2143G were expected to yield three fragments (60, 418 and 666 bp) when digested with *Bsa* I.

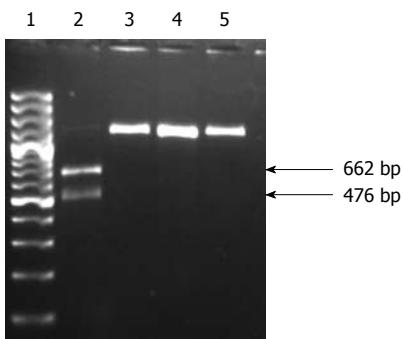
## RESULTS

### **Antimicrobial susceptibility**

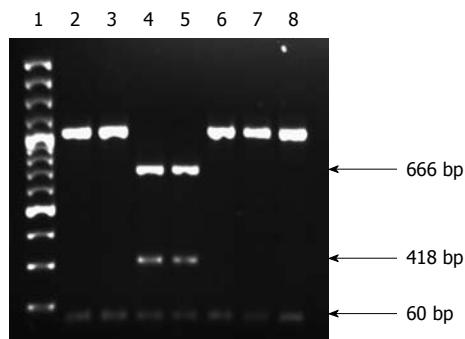
A total of 187 *H pylori* were isolated from gastric biopsies of 120 patients. Four of these isolates (2.1%) from four patients were resistant to clarithromycin, while 183 isolates from 116 patients were sensitive to clarithromycin. The MIC values of all the clarithromycin-resistant isolates ranged from 1.5 to 24 µg/mL (Table 1). The patients with clarithromycin-resistant isolates had never been treated for *H pylori* infection.

### **PCR amplification of 23S rRNA and sequence analysis**

All the clarithromycin-resistant strains and 14 randomly selected clarithromycin-sensitive strains from 14 different patients were subjected to DNA extraction and PCR amplification of domain V of the 23S rRNA gene. The mutations in domain V of 23S rRNA were determined by comparing the sequences of clarithromycin-resistant and -sensitive isolates with the sequences of the reference strain ATCC43504. All of the clarithromycin-resistant isolates were shown to have point mutations of either A2142G or A2143G, while none of the 14 clarithromycin-sensitive isolates had these types of mutations. T2182C mutation was detected in both clarithromycin-resistant and -sensitive isolates.



**Figure 1** RFLP analysis of 23S rRNA to detect the A2142G mutation using *Mbo* II restriction enzyme. Lane 1: 100 bp DNA ladder; Lane 2: Digestion product of resistant isolate with A2142G mutation, showing two fragments of 662 and 476 bp; Lanes 3 and 4: Digestion products of two clarithromycin-resistant isolates with A2143G mutation; Lane 5: Digestion product of a clarithromycin-sensitive isolate with T2182C mutation.



**Figure 2** RFLP analysis of 23S rRNA to detect the A2143G using *Bsa* I restriction enzyme. Lane 1: 100 bp DNA ladder; Lanes 2 and 3: Digestion products of two clarithromycin-resistant isolates with A2142G mutation; Lanes 4 and 5: Restriction analysis of resistant isolates with A2143G mutation, showing three fragments of sizes 666, 418 and 60 bp; Lanes 6-8: Digestion products of clarithromycin-sensitive isolates.

**Table 1** MIC value of clarithromycin and mutations in the domain V 23S rRNA of *H pylori* isolates

Clarithromycin MIC ( $\mu\text{g/mL}$ ) and phenotype	Mutation site	Number of isolate(s)
24 (R)	A2142G	1
24 (R)	A2143G, T2182C	1
3 (R)	A2143G	1
1.5 (R)	A2142G, T2182C	1
< 0.016 (S)	T2182C	13
< 0.016 (S)	No mutation	1

R: Resistant; S: Sensitive.

#### RFLP analysis of clarithromycin-resistant and -sensitive isolates

Sequence analysis of restriction sites of the amplified V domain of the 23S rRNA gene of the resistant strains showed restriction sites that could be digested with *Bsa* I and *Mbo* II. Digestion of the amplified PCR product with *Mbo* II produced two fragments of 662 and 476 bp sizes in strains with the A2142G mutation. No fragment was produced with the A2143G or T2182C mutation (Figure 1). Resistant isolates with the A2143G mutation produced three fragments of 662, 476 and 60 bp when digested with *Bsa* I. No restriction fragment was produced in strains with A2142G and T2182C mutations (Figure 2). None of the clarithromycin-sensitive isolates and the negative control (ATCC43504) produced restriction fragments when digested with *Mbo* II and *Bsa* I, which indicated the absence of A2142G and A2143G mutations.

#### DISCUSSION

The prevalence of clarithromycin resistance was found to be low among the isolates in the present study. To the best of our knowledge, this was the first study that reported and characterized clarithromycin-resistant strains in Malaysia.

Clarithromycin is used worldwide for *H pylori* eradication therapy. *H pylori* strains that are resistant to clarithromycin have been reported increasingly

in several studies<sup>[9,17]</sup>. As resistance will often lead to failure of eradication therapy, knowledge of the current susceptibility patterns of local *H pylori* isolates could help in determining the choice of appropriate treatment for the patient. In the present study, the prevalence of clarithromycin resistance was still low; however, physicians should bear in mind the possibility of clarithromycin resistance if their patient does not respond to this antibiotic.

Versalovic *et al*<sup>[18]</sup> have shown that A2142G and 2143G mutations of 23S rRNA genes in *H pylori* are associated with resistance to clarithromycin. A mutagenesis study performed by Taylor *et al*<sup>[15]</sup> has confirmed that the A2142G and A2143G mutations are associated with clarithromycin resistance of *H pylori*. A review by Mégraud<sup>[8]</sup> of several studies worldwide has shown that 81.5% of the mutations in clarithromycin-resistant isolates were the A2142G or A2143C mutations. In our study, either A2142G or A2143G mutations were found in the clarithromycin-resistant isolates, or they were absent in the clarithromycin-sensitive isolates. The mutations found in our local strains were in accordance with the findings in other countries. The mutations were also associated with low-level resistance.

Another type of mutation detected in our isolates was T2182C. This mutation was detected in most of the strains studied, which suggests a common occurrence among our local strains. This mutation is not new and has been described in previous reports<sup>[19,20]</sup>. This mutation is not associated with clarithromycin resistance<sup>[21]</sup>. However, it has not been discussed whether this type of mutation occurs commonly in *H pylori*. Mutations other than A2142G and A2143G have been reported in other studies<sup>[16,22]</sup>, but among our strains, sequence analysis showed that the mutations were limited to these types only. Although the low number of resistant isolates in our study limit the scope of detection, the results suggest that clarithromycin resistance of *H pylori* in Malaysia can be predicted by detection of mutations at positions 2142 and 2143 of the 23S rRNA gene. The resistance to clarithromycin among the four patients is considered primary resistance

because they were never treated previously for *H pylori* eradication.

Knowledge about the molecular mechanism of resistance is important as it can be used to facilitate the development of other molecular methods to detect resistance. It has been shown that PCR-RFLP can be used to detect mutations in clarithromycin-resistant isolates<sup>[23,24]</sup>. Using this technique in our study, it was demonstrated that only strains with the A2142G and A2143G mutations produced restriction fragments when digested with *Mbo* II or *Bsa* I. This alleviated the need to use the more expensive and time-consuming processes of sequencing and analysis to look for mutation sites.

In conclusion, we showed that there was a low prevalence of clarithromycin resistance in our local strains of *H pylori*. The A2142G or A2143G mutations detected were in accordance with the findings in other countries. These mutations were not found among the analyzed clarithromycin-sensitive strains. T2182C mutation was a common occurrence in our local isolates. Other types of mutations associated with clarithromycin resistance were not observed in our resistant strains. The A2142G or A2143G mutations were detected also by RFLP, thus making it a rapid method for the detection of clarithromycin-resistant strains in our local isolates.

## COMMENTS

### Background

The incidence of clarithromycin-resistant *Helicobacter* is increasing worldwide. This limits the therapeutic options available for eradicating the bacterium. Clarithromycin resistance has been attributed to mutations in the 23S rRNA gene.

### Research frontiers

Clarithromycin acts by binding to the peptidyl transferase region of 23S rRNA and inhibits bacterial protein synthesis. There are many types of mutations observed in the 23S rRNA genes of clarithromycin-resistant *Helicobacter pylori* (*H pylori*). There is little information on the prevalence and characteristics of clarithromycin resistance in *H pylori* strains isolated from Malaysian patients. In the present study, the authors determined the prevalence of resistance and characterized the types of mutations present in their resistant strains.

### Innovations and breakthroughs

The study gave an insight into the low prevalence of clarithromycin resistance among the *H pylori* strains studied. The A2142G and A2143G mutation detected were in accordance with the findings in our other countries. However, other types of mutations associated with clarithromycin resistance were not observed among our resistant strains.

### Applications

The A2142G and 2143G mutations in the 23S rRNA genes in *H pylori* can be detected easily by restriction fragment length polymorphism (RFLP) analysis of the polymerase chain reaction (PCR) product of the genes, using *Mbo* II or *Bsa* I. This eliminates the need to detect these mutations by sequencing.

### Peer review

This is a straightforward study that determined the prevalence of clarithromycin resistance in *H pylori* strains isolated from the authors' institute in Malaysia. The types of mutations that occurred in the resistant strains were further characterized by PCR and sequencing. Finally, the authors evaluated whether the RFLP technique was suitable for rapid detection of the mutations among these isolates. Although the sample size of clarithromycin-resistant isolates was relatively small, the techniques and methodology used were appropriate and standard. The information is potentially useful for the clinical management of *H pylori* infection in Malaysia, and also for our overall appreciation of the global distribution and prevalence of antibiotic resistance of this important pathogen.

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BRIEF ARTICLES

## Cardiopulmonary bypass induced microcirculatory injury of the small bowel in rats

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### Abstract

**AIM:** To investigate microvascular injury quantitatively in the small bowel with respect to cardiopulmonary bypass (CPB) and related mechanisms.

**METHODS:** In 10 male SD rats, normothermic CPB was established and continued with a flow rate of 100-150 mL/kg per minute for 60 min, while another 10 sham-operated animals served as controls. An approximate 10-cm loop of the terminal ileum was exteriorized for observation by means of intravital fluorescence microscopy. The small bowel microcirculatory network including arterioles, capillaries, and collecting venules was observed prior to CPB, CPB 30 min, CPB 60 min, post-CPB 60 min and post-CPB 120 min. The intestinal capillary perfusion, microvascular permeability and leukocyte adherence were also measured.

**RESULTS:** The systemic hemodynamics remained stable throughout the experiment in both groups. In CPB animals, significant arteriolar vasoconstriction, blood velocity reduction and functional capillary density diminution were found. As concomitances, exaggerated albumin extravasation and increased leukocyte accumulation were also noted. These changes were more pronounced and there were no signs of restitution at the end of the observation period.

**CONCLUSION:** CPB induces significant microcirculatory

injury of the small bowel in rats. The major underlying mechanisms are blood flow redistribution and generalized inflammatory response associated with CPB.

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**Key words:** Cardiopulmonary bypass; Functional capillary density; Intestinal microcirculation; Intravital microscopy

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Dong GH, Wang CT, Li Y, Xu B, Qian JJ, Wu HW, Jing H. Cardiopulmonary bypass induced microcirculatory injury of the small bowel in rats. *World J Gastroenterol* 2009; 15(25): 3166-3172 Available from: URL: <http://www.wjgnet.com/1007-9327/15/3166.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.3166>

### INTRODUCTION

Despite excellent improvements, there is increasing evidence to show that cardiopulmonary bypass (CPB) is related to a generalized inflammatory response and splanchnic edema formation in both clinical and experimental investigations<sup>[1-5]</sup>. Impairment of gastrointestinal perfusion during CPB may lead to loss of mucosal vascular integrity and increased permeability. The translocation of microorganisms and endotoxin into the systemic circulation can result in a continuing toxic insult and systemic tissue injury. This is thought to be one of the essential mechanisms in the development of sepsis, shock, post-perfusion complications, and multiple organ failure after CPB<sup>[5-7]</sup>. Identification of rational clinical therapeutic approaches requires a precise elucidation of the underlying pathophysiological processes of the intestinal microvasculature induced by CPB.

However, the exact regional hemodynamic and functional changes of the small intestine in terms of the response to CPB remain unclear. Indirect methods may not be sufficient to delineate the pathomechanisms of intestinal microcirculatory conditions within the

different tissue compartments such as muscle, submucosa, and mucosa<sup>[8-10]</sup>. An appropriate experimental model is needed to investigate the variety of microvascular alterations including capillary perfusion injury, microvascular endothelial integrity loss, and leukocyte activation under both physiological and pathological conditions. In this study, using a rat model of CPB, we directly observed and quantitatively analyzed the microvascular alterations in small bowel by means of fluorescent intravital microscopy, aiming to demonstrate the influence of CPB in the different functional units of the microcirculatory network.

## MATERIALS AND METHODS

### Animal preparation

Adult male Sprague-Dawley rats (450-550 g) served as experimental animals. All animals received humane care in compliance with the principles of laboratory animal care formulated by the National Society of Medical Research and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Publication No. 85-23, revised 1996). Rats were anaesthetized with intraperitoneal administration of ketamine (50 mg/kg) and chlorpromazine (2 mg/kg). After tracheostomy and intubation, the animals were mechanically ventilated with about 10 mL/kg tidal volume, 60 breaths/min respiratory rate, and 100% inspiratory concentration of O<sub>2</sub>. Anesthesia was maintained throughout the experiment with additional doses of intravenous ketamine.

### CPB techniques

A rat CPB model previously developed by our group, with consistent excellent survival, was used in the current study<sup>[11]</sup>. Briefly, the right femoral artery was cannulated with a 24-gauge heparinized catheter for arterial pressure monitoring and blood gas analysis. The homolateral femoral vein was cannulated with a 20-gauge catheter for blood and fluid infusion. Following heparinization (500 U/kg), a 16-gauge catheter, modified to a multi-side-orifices cannula in the forepart, was inserted into the right jugular vein and advanced to the right atrium for blood drainage by gravity and siphon. A 22-gauge catheter was cannulated to the right carotid artery as the arterial infusion line for the CPB circuit. The membrane oxygenator was specially designed with a surface area for gas exchange of 0.05 m<sup>2</sup> and the total assembly dynamic priming volume of 4 mL. A rolling pump was used to drive the blood through silicone arterial inflow tubing and then returned to the right carotid artery. Priming was composed of 8 mL heparinized fresh homologous blood obtained from a donor and 8 mL synthetic colloid (HAES-steril®). The CPB flow-rate was gradually adjusted, stabilized and performed at 100-150 mL/kg per minute throughout the experiment. To ensure the mean arterial pressure (MAP) above 60 mmHg and the hematocrit about 30%-40%, additional whole blood and Ringer's solution was given during the

experiment to supply the blood and fluid loss caused by surgery, sampling, leakage and evaporation. Body central temperature was monitored with a rectal probe and maintained at 36.5-38.0°C by a heating lamp placed above.

### Intravital microscopy

A modified intravital microscope (Shanghai 2XC4TV, China) with a 100 W mercury arc lamp was used to observe the intestinal microcirculation. With I<sub>2/3</sub> and N<sub>2</sub> filter blocks interposed into the light path, the green (530-560 nm) and the blue (450-490 nm) light was selected, respectively for epi-illumination. For *in vivo* microscopy, fluorescein isothiocyanate conjugated to bovine serum albumin (FITC-BSA), which fluoresces under blue light, allowed visualization of the intestinal microvessels. Acridine orange, which fluoresces under green light, stains leukocytes and allows their adhesion in the microvasculature to be monitored. The microscopic images were acquired by a charge-coupled device digital camera (Olympus C-5050, Japan), recorded by a digital video system (Cannon MVX-150i, Japan), and displayed on a high-resolution monitor (Patriot 798HD, China) for off-line analysis. The final magnification of the images can be approximately achieved by 250 × and 650 × with 10 × and 25 × long-distance objectives.

### Experimental protocol

The animals were randomly divided into two groups. In the CPB group, 10 rats were subjected to the CPB circuit and received extracorporeal perfusion for 60 min. After the bypass was terminated, the remaining priming solution was infused gradually to maintain the main arterial pressure around 60 mmHg. Ten sham operated animals which were cannulated, heparinized and subjected to the CPB circuit but without CPB served as controls, and the CPB circuit was weaned 1 h later. The dosage of heparin for anticoagulation was the same in both groups.

In all animals, *via* a small transverse abdominal incision, an approximate 10 cm loop of ileum was exteriorized and placed on a movable stage attached to the microscope. A 10 mm incision along the antimesenteric border was made with an electric microcautery device for visualization of the mucosal surface. The exposed segment was gently extended, held in place by stay sutures, and covered with a glass microscopical cover slide. The tissues were constantly superfused with warm Ringer's lactate at 37°C to avoid drying and local hypothermia.

The intestinal microcirculation was visualized after intravenous administration of FITC-BSA (0.2 mL/100 g body weight, 5%) and acridine orange (0.1 mL/100 g body weight, 0.2%). To avoid damage to the microcirculation due to long periods of light exposure, the recording time varied 30-60 s. The microvasculature of smooth muscle and the submucosa was assessed from the serosal side in the oral closed part of the segment by adjusting the focus level of the microscope. The villous microcirculation was visualized from the mucosal surface

**Table 1** Macrohemodynamic parameters in CPB and sham animals (mean  $\pm$  SD)

Variables	Groups	Prior to CPB	CPB 30 min	CPB 60 min	Post CPB 60 min	Post CPB 120 min
MAP (mmHg)	CPB	74 $\pm$ 8	77 $\pm$ 7	75 $\pm$ 6	75 $\pm$ 5	72 $\pm$ 5
	Sham	73 $\pm$ 5	75 $\pm$ 6	77 $\pm$ 6	75 $\pm$ 7	77 $\pm$ 5
pH	CPB	7.40 $\pm$ 0.02	7.36 $\pm$ 0.02 <sup>a,c</sup>	7.31 $\pm$ 0.04 <sup>b,c</sup>	7.27 $\pm$ 0.04 <sup>b,c</sup>	7.22 $\pm$ 0.03 <sup>b,c</sup>
	Sham	7.40 $\pm$ 0.02	7.39 $\pm$ 0.01	7.40 $\pm$ 0.02	7.39 $\pm$ 0.02	7.39 $\pm$ 0.02
PaO <sub>2</sub> (mmHg)	CPB	414 $\pm$ 16	279 $\pm$ 13 <sup>b,c</sup>	280 $\pm$ 14 <sup>b,c</sup>	370 $\pm$ 21 <sup>b,c</sup>	372 $\pm$ 21 <sup>b,c</sup>
	Sham	404 $\pm$ 16	401 $\pm$ 16	394 $\pm$ 15	406 $\pm$ 14	402 $\pm$ 19
PaCO <sub>2</sub> (mmHg)	CPB	38.8 $\pm$ 4.2	39.3 $\pm$ 2.8	38.9 $\pm$ 2.1	38.8 $\pm$ 3.8	40.2 $\pm$ 4.9
	Sham	39.1 $\pm$ 2.2	40.9 $\pm$ 1.6	38.3 $\pm$ 2.8	38.6 $\pm$ 2.5	40.3 $\pm$ 3.9
Hematocrit (%)	CPB	40.8 $\pm$ 2.2	33.0 $\pm$ 0.9 <sup>b,c</sup>	33.4 $\pm$ 1.0 <sup>b,c</sup>	32.3 $\pm$ 0.9 <sup>b,c</sup>	32.8 $\pm$ 0.8 <sup>b,c</sup>
	Sham	39.7 $\pm$ 1.4	40.8 $\pm$ 2.1	40.2 $\pm$ 1.7	39.3 $\pm$ 1.9	38.5 $\pm$ 1.6

<sup>a</sup>P < 0.05, <sup>b</sup>P < 0.001 vs sham group; <sup>c</sup>P < 0.001 vs baseline.

through the longitudinal incision in the aboral part of the segment.

After stabilization for about 15 min, four non-overlapping regions of interest were defined and measured to obtain representative values for every parameter. The quantitative analysis of intestinal microcirculation was performed by means of the standard technique usually used in intravital microscopic studies in small animals<sup>[12,13]</sup>. A computer-controlled image analysis system (ImageJ 1.30V, National Institutes of Health, USA) was applied in those processes. Microcirculatory parameters were recorded prior to CPB, CPB 30 min, CPB 60 min, post-CPB 60 min, and post-CPB 120 min. In addition, the systemic arterial pressure was monitored continually and the arterial blood gas analyses were performed repeatedly throughout the experiments.

### Statistical analysis

SPSS for Windows 11.0.1 (SPSS Inc. 2001, USA) was used for statistics. All data were expressed as means  $\pm$  SD. Following analysis of variance, the Student's *t* test was used as a test for statistical significance of normal distributed values between the groups. To analyze differences within each group for time effects, paired Student's *t* tests including a correction of the error according to Bonferroni probabilities for repeated measurements were used. *P* < 0.05 was considered statistically significant.

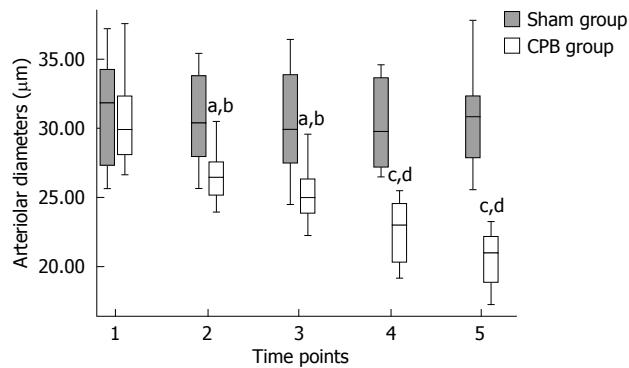
## RESULTS

### Macrohemodynamic parameters

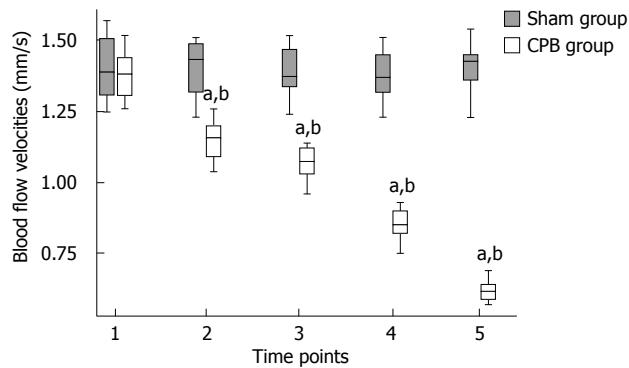
The MAP remained constant and the results of blood gas analysis were within the acceptable range in both groups using this experimental design and CPB technique (Table 1). However, during the entire bypass period, the heart kept beating and lung perfusion persisted. Due to the existence of the shunt, this could result in lower blood oxygenation in the CPB group vs the sham. As is the nature of CPB, hemodilution and a significant decrease in pH were observed in our model similar to that seen in clinical practice.

### Capillary perfusion

In the CPB group, a significant pathological segmental



**Figure 1** Diameter of arterioles in mucosa. Values are expressed as mean  $\pm$  SD, <sup>a</sup>P < 0.01, <sup>b</sup>P < 0.001 vs baseline; <sup>c</sup>P < 0.01, <sup>d</sup>P < 0.001 vs sham group. Time points: 1: Baseline; 2: 30 min of CPB; 3: 60 min of CPB; 4: 60 min after weaning off CPB; 5: 120 min after weaning off CPB.



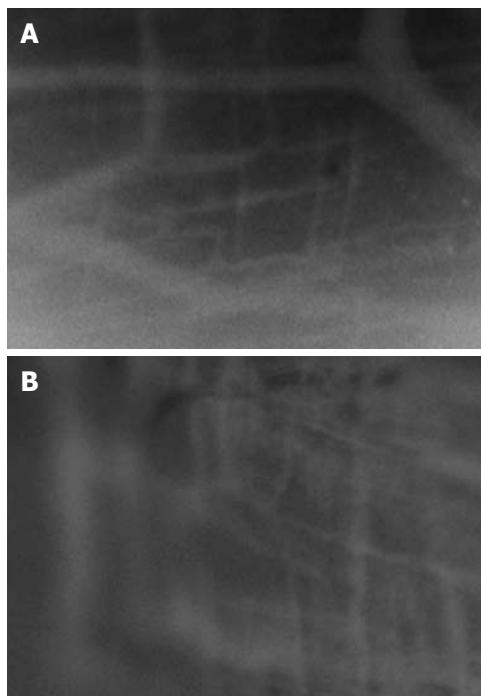
**Figure 2** Blood cell velocities in collecting venules in submucosa. Values are expressed as means  $\pm$  SD, <sup>a</sup>P < 0.001 vs baseline; <sup>b</sup>P < 0.001 vs sham group (Time points: see Figure 1).

vasoconstriction not ascribed to undulating vasomotion was detected in small vessel segments. Mean arteriolar diameters of single arterioles in the mucosal layer were found to be diminished from 30.82  $\pm$  3.57 prior to CPB to 20.59  $\pm$  1.97  $\mu$ m (*P* < 0.001) at the end of the experiment (Figure 1). Blood flow was apparently reduced in the collecting venules in the submucosa, with the red blood cell flow velocities reduced from 1.38  $\pm$  0.08 mm/s prior to initiation of CPB to 0.62  $\pm$  0.04 mm/s (*P* < 0.001) at 2 h after bypass (Figure 2). As a consequence, a concomitant hemoconcentration, typical sludge phenomena and cell aggregates within

**Table 2** Changes in functional capillary density in CPB and sham animals (mean  $\pm$  SD)

Variables	Groups	Prior to CPB	CPB 30 min	CPB 60 min	Post-CPB 60 min	Post-CPB 120 min
FCD in mucosa ( $\text{cm}^{-1}$ )	CPB	511 $\pm$ 17	406 $\pm$ 12 <sup>a,b</sup>	328 $\pm$ 11 <sup>a,b</sup>	219 $\pm$ 13 <sup>a,b</sup>	150 $\pm$ 10 <sup>a,b</sup>
	Sham	504 $\pm$ 17	510 $\pm$ 20	499 $\pm$ 18	513 $\pm$ 14	507 $\pm$ 16
FCD in muscle ( $\text{cm}^{-1}$ )	CPB	221 $\pm$ 13	152 $\pm$ 10 <sup>a,b</sup>	125 $\pm$ 9 <sup>a,b</sup>	102 $\pm$ 8 <sup>a,b</sup>	64 $\pm$ 7 <sup>a,b</sup>
	Sham	218 $\pm$ 14	221 $\pm$ 12	212 $\pm$ 17	217 $\pm$ 13	211 $\pm$ 14

<sup>a</sup> $P < 0.001$  vs sham group; <sup>b</sup> $P < 0.001$  vs baseline.

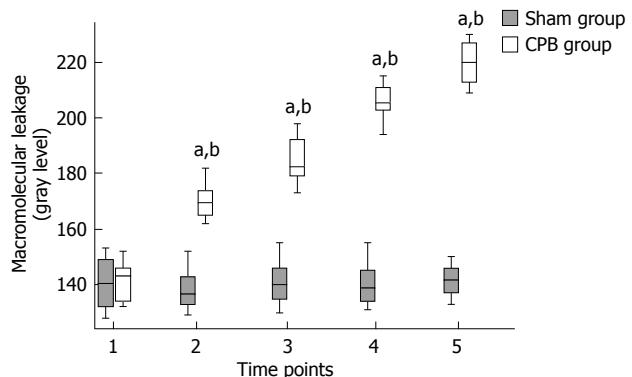


**Figure 3** Interstitial fluorescence after administration of FITC-BSA in muscular layer. A: Control animals. FITC-BSA was contained within the vessels and the capillaries are easily identified; B: CPB animals. FITC-BSA extravasated from the capillaries into the interstitium, and a brighter flare surrounded the microvasculature (Magnification,  $\times 650$ ).

these vessels could be observed. In the control animals, the arterioles showed no changes in diameter and the blood cell velocities remained constant throughout the observation period. Functional capillary density (FCD) was expressed as the length of FITC-BAS perfused capillaries per observational area. Both the intestinal mucosa and the smooth muscle layer were analyzed. In the sham group, the perfusion was homogeneous in both compartment capillary networks (Table 2). However, in the CPB group, the FCD values decreased significantly during and after bypass and reached only 30% of initial values at the end of the observation period.

#### Microvascular permeability

A significant increase in macromolecular leakage induced by CPB was observed in this study. Under circumstances resulting in increased microvascular permeability to macromolecules, FITC-BSA was seen to leak from the vasculature, appearing as a flare in the perivascular interstitium. The interstitial fluorescent intensity was proportional to the degree of FITC-BSA leakage from the vessels. In control animals, the vascular



**Figure 4** Macromolecular leakage of intestinal microcirculation in muscular layer. FITC-BSA leakage was represented as the gray level of Intestinal fluorescent intensity. Values are expressed as means  $\pm$  SD, <sup>a</sup> $P < 0.001$  vs baseline; <sup>b</sup> $P < 0.001$  vs sham group (Time points: see Figure 1).

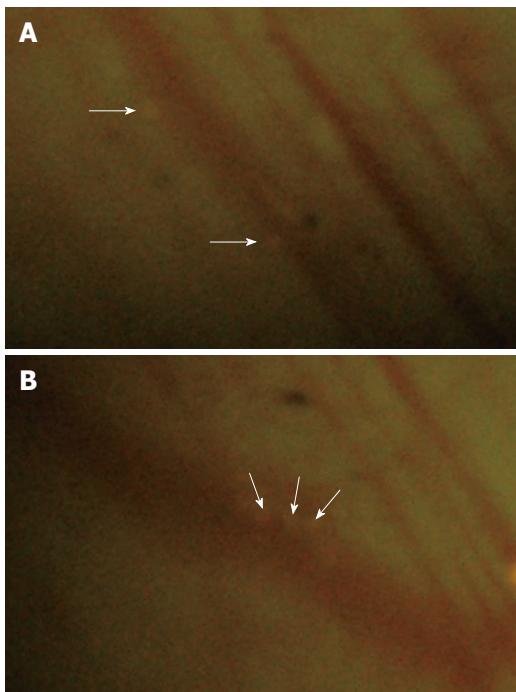
integrity was maintained with no observed increases in interstitial fluorescence during the experimental period (Figures 3 and 4). In the CPB group, the interstitial fluorescence increased at all time points from a gray level of  $141.8 \pm 7.3$  at the beginning of CPB to  $219.9 \pm 7.1$  at 2 h after weaning off bypass ( $P < 0.001$ ).

#### Leukocyte adhesion

Leukocyte adhesion was analyzed within postcapillary venules of the intestinal submucosa. A leukocyte was considered to be adherent in each vessel segment if it did not move or detach from the endothelial lining for at least 30 s. Stationary leukocytes that had migrated to the interstitium but remained in close proximity to the capillaries were also regarded as adherent. Data was expressed as the number of adherent leukocytes per  $100 \mu\text{m}$  length of venular endothelial surface. In the sham group, only occasional adherent leukocytes ( $2.7 \pm 0.9$  cells/ $100 \mu\text{m}$ ) were found firmly adhered to the microvascular endothelium throughout the experimental period (Figures 5 and 6). In the CPB group, a rapid, sustained, and significant increase in leukocyte adherence (about 10-fold at the end of experiments) was induced within the submucosal capillaries ( $P < 0.001$ ) during and after CPB.

## DISCUSSION

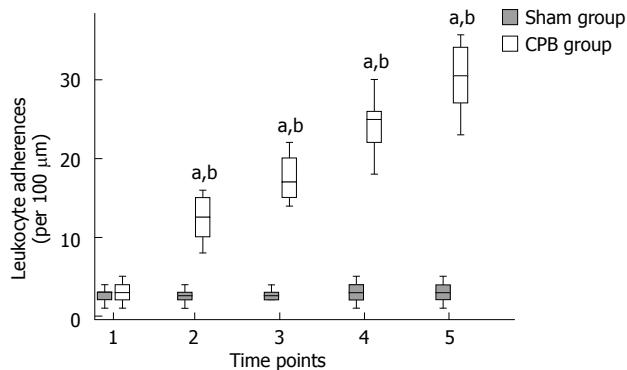
Nowadays, due to various improvements, CPB has made cardiovascular surgery more and more practical and safe even in high-risk patients. However, there are still many complications which are thought to result from



**Figure 5 Leukocyte adhesion in intestinal submucosa.** A: Control animals. Few adherent leukocytes (arrows) were observed within the microcirculation; B: CPB animals. Numerous adherent leukocytes (arrows) were observed within the capillaries.

a systemic inflammatory response and non-occlusive visceral ischemia<sup>[5,7,14,15]</sup>. Recent research has suggested that the intestinal mucosa is extremely sensitive to ischemia-reperfusion (I/R) injury and the gut is both the source and the target of inflammatory mediators<sup>[16,17]</sup>. These changes might contribute to a severe loss of intestinal function with significant complications or even multiorgan failure and systemic shock as a pathological consequence. In clinical practice, the incidence of gastrointestinal complications is low (0.58%-2.0%), however, they are associated with an unacceptable high mortality (15%-63%), especially in patients with pre-existing impairment of bowel perfusion<sup>[15,18,19]</sup>. With the aim of identifying effective preventive and therapeutic approaches, studies on the underlying pathomechanisms of CPB in small bowel capillary perfusion seem to be imperative.

Methods such as laser Doppler flow measurements and tonometry have been described in this area, but they are both indirect measures which do not allow quantitative analysis in different segments of the microvascular bed<sup>[9,20,21]</sup>. In contrast, intravital microscopy allows direct observation and quantitative analysis of the tissues and organs at the level of microcirculation and acquires more information on the pathologic role of CPB. The mesentery has been used frequently in studies of the intestinal microvasculature, but it has very little in common with intestinal microvascular networks<sup>[22,23]</sup>. In our experiments, the use of intravital microscopy employing different fluorescent dyes, allowed the simultaneous quantitative investigation of the microcirculation within all layers of the small intestine, i.e. subserosa, smooth muscle, submucosa and



**Figure 6 Quantitative analysis of leukocyte adhesion in intestinal submucosa.** Adherent leukocytes per 100  $\mu\text{m}$  length of venule. Values are expressed as means  $\pm$  SD. <sup>a</sup>P < 0.001 vs baseline; <sup>b</sup>P < 0.001 vs sham group (Time points: see Figure 1).

mucosa. For the first time, we directly investigated the intestinal microcirculatory changes within different layers relating to CPB by intravital microscopy.

The majority of studies on CPB relating to pathophysiological processes have been performed in large animal models<sup>[24]</sup>. These models have substantial limitations, mainly in that their costs are extremely high, instruments are very complex and they are too labor intensive. Rats are one of the most standard models for intravital microscopy and have been extensively used for the experimental investigation of multiple organ injury in various areas<sup>[12,13,22]</sup>. In our experiments, a rat CPB model with full flow perfusion was established. This model also reduced the cost of animals and equipment.

In the CPB group, the blood pressures were kept constant at a physiological level throughout the experiments without vasoactive drugs and the systemic temperatures were stable at around 37°C. Despite the consistent condition of systemic hemodynamics, microvascular perfusion impairments of the small bowel were clearly demonstrated. Arteriolar vasoconstriction, the reduction in blood cell velocities, together with the consequence of intravascular hemoconcentration and sludge formation, reflected hypoperfusion at the microvascular level. The reduction in the FCD values at the end of the observation period to approximately 30% of the pre-bypass values revealed that the capillary bed had been severely injured. The extravasation of FITC-BSA showed a distinct increase in the permeability of the vascular wall which might result in a loss of the natural barrier function against toxins and microorganisms. This might aggravate the injury to capillary perfusion further. Enhanced leukocyte adherence to the venular wall within submucosal vessels was also demonstrated in this study, which indicated that activated leukocytes may be the primary mediators and may have a causative role in the following pathophysiological sequelae.

The potential underlying pathogenesis of the intestinal injury induced by CPB includes several pathophysiological mechanisms<sup>[1,14,15,25]</sup>, such as a transient reduction in systemic blood pressure and low cardiac outflow (LCO), hypotemperature in the bypass process,

redistribution of local organ blood flow, reactions of inflammatory mediators or vasoconstrictive agents, and obstruction of arterioles with fragments of cells or tissues. With our model, due to the fact that the flow rate of the bypass circuit was kept constant and the arterial blood pressure remained unchanged, the influences of macrohemodynamics could be avoided. This allowed the conclusion that the observed changes in microvascular perfusion in the small bowel were not only a result of systemic hypoperfusion or LCO syndrome. By keeping the systemic and tissue temperatures in the normal range, we could exclude the effects of temperature on the microvascular perfusion in our study. Redistribution of blood flow and induction of inflammatory response seemed to be the major mechanisms responsible for the observed results<sup>[2,26]</sup>. Non-physiological patterns of perfusion originating from the rolling pump results in maldistribution of blood flow away from visceral organs as a reaction to CPB which serves as the primary contributor to microvascular impairments. It is important to note that the degree of damage to the microcirculation reached a maximum even after CPB removal for 2 h, suggesting that the ensuing inflammatory process as a consequence of CPB could be the dominant mechanism. The contact of blood and blood cells with the bypass circuit surfaces, the trauma induced by mechanical stress from the pump, together with the I/R injury of the gastrointestinal tract resulting from the transient decrease in local blood flow can induce the release of inflammatory mediators (e.g. cytokines, thromboxane, and histamine, etc), secretion of proteases (e.g. collagenase and elastase), activation of complements, extravasation of leukocytes, and the generation of oxygen free radicals<sup>[6,12,23]</sup>. These substances may lead to vasoconstriction, hemoconcentration and sludge formation, endothelial cell swelling and the consequent occlusion of vessels, and an increase in vascular permeability.

In conclusion, using intravital microscopy, progressive microvascular injury in the small bowel was demonstrated in a rat CPB model. The ensuing injury response was associated with microvascular perfusion failure, vascular permeability increase, and promotion of circulating leukocyte adherence. These changes in microvessels were even more prominent after the bypass period and no signs of restitution could be observed. The observed phenomena may have resulted from the redistribution of blood flow in different organs and the generation of inflammatory metabolites with potential negative effects on microvascular perfusion during and after CPB. The information obtained from our experiments will facilitate further investigations of different therapeutic regimens aimed at ameliorating microvascular damage due to CPB.

## COMMENTS

### Background

Impairment of intestinal microvascular perfusion induced by cardiopulmonary bypass (CPB) is thought to be related to mucosal edema formation and

microvascular barrier injury, which may result in postoperative gastrointestinal complications and multiple organ failure. A better understanding of the microvascular alterations during and after CPB will facilitate the identification of therapeutic strategies.

### Research frontiers

In general, experimental investigations on the intestinal injury induced by CPB are scarce. The identification of rational clinical therapeutic approaches requires a precise elucidation of the underlying pathophysiological processes of intestinal microvasculature induced by CPB.

### Innovations and breakthroughs

With a novel rat model of CPB, the authors quantitatively investigated the microvascular alterations post CPB in small bowel by means of fluorescent intravital microscopy, which allows the simultaneous quantitative investigation of the microcirculation of the small intestine.

### Applications

By understanding pathomechanisms of CPB in small bowel capillary perfusion, this study may help to identify effective preventive and therapeutic approaches.

### Terminology

Functional capillary density (FCD) is one of the parameters obtained by intravital microscopy using epi-illumination of the tissue surface or transillumination of thin tissue layers. FCD, defined as the length of red cell-perfused capillaries per observation area ( $\text{cm}^{-1}$ ), has been used as an indicator of the quality of tissue perfusion in various animal models. Quantitative analysis of FCD in randomly selected regions of the tissue is performed by means of a computer-assisted video analysis system which allows calculation of the length of RBC-perfused capillaries.

### Peer review

In this paper, the authors used intravital microscopy for assessment of intestinal microcirculation in rats during and after CPB. The authors confirmed arteriolar vasoconstriction, blood velocity reduction and FCD diminution, exaggerated albumin extravasation and increased leukocyte accumulation in intestine in rats during and after CPB.

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# Translation and validation of the Nepean Dyspepsia Index for functional dyspepsia in China

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**CONCLUSION:** The Chinese version of the NDI is a reliable and valid scale for measuring health-related quality of life and disease severity in Chinese patients with FD.

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**Key words:** Nepean Dyspepsia Index; Functional dyspepsia; Health-related quality of life; Validation studies; Questionnaires

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## Abstract

**AIM:** To assess the reliability and validity of the translated version of Nepean Dyspepsia Index (NDI) in Chinese patients with documented functional dyspepsia (FD).

**METHODS:** The translation process included forward translation, back translation, pretest and cross-cultural adaptation. Reliability and validity of the translated version were examined by asking 300 subjects to complete the Chinese version of the NDI. The mean age of subjects was 39.24 years and 68.7% of the subjects were women. Internal consistency analysis with Cronbach's  $\alpha$  was performed to test the reliability. Correlation analysis was used to assess the content validity. Factor analysis and structural equation models were used to assess the construct validity.

**RESULTS:** The Cronbach's  $\alpha$  coefficients ranged 0.833-0.960, well above the acceptable level of 0.70. Correlation analysis showed that each item had a strong correlation with the corresponding domain, but a weak correlation with other domains. Confirmatory factor analysis indicated that the comparative fit index was 0.94, higher than the acceptable level of 0.90.

## INTRODUCTION

Functional dyspepsia (FD) is a common clinical condition characterized by chronic or recurrent upper abdominal pain associated with a variety of gastrointestinal symptoms in the absence of organic disease<sup>[1]</sup>. An epidemiological survey in Western countries showed that 19%-41% of the population have symptoms of FD<sup>[2]</sup>. The report on the incidence of FD in citizens of Tianjin, China, revealed that the number of patients with FD accounts for 23.29% of the total population<sup>[3]</sup>. Since FD greatly affects the quality of life (QOL) of patients<sup>[4-6]</sup>, and contributes to a higher medical cost<sup>[7,8]</sup>, it represents an important healthcare problem in modern society. However, treatment of FD is still controversial and no single therapy is uniformly effective, partially due to lack of a reliable evaluation instrument<sup>[9]</sup>. Many difficulties exist in the single treatment approach to FD<sup>[10]</sup>. A combined approach, which includes herbal medicine, acupuncture and massage therapy, may be more effective than any single treatment<sup>[11]</sup>.

In addition to symptom evaluation, assessment of QOL has been recognized as a major factor for overall

health outcome assessment in FD clinical studies. Nepean Dyspepsia Index (NDI) was designed to measure both symptom scores and impairment of the dyspepsia-specific health-related QOL (H-QOL). Since the development of NDI in 1998<sup>[12]</sup>, it has been used as an outcome assessment in many FD clinical researches<sup>[13,14]</sup> and translated into several languages including French, Dutch, Italian, German, Spanish, and American English<sup>[15,16]</sup>. NDI has proven vital in the diagnosis of FD<sup>[12]</sup>, and its utility has been validated<sup>[4,17]</sup>. A number of studies about the effect of FD on QOL in Western patients are available<sup>[7,18,19]</sup>. However, the effect of FD on the QOL in Asian patients has not received great attention.

Since no validated disease-specific questionnaire is currently available for assessing the impact of FD on the QOL, we translated the original version of NDI (25 items) into Chinese. We also assessed the reliability and validity of this translated version in Chinese patients with documented FD in order to provide researchers, clinicians, and patients with a suitable questionnaire to assess FD.

## MATERIALS AND METHODS

### Ethics

This study was approved by the Ethics Committee of Chengdu University of Traditional Chinese Medicine. All subjects provided their written consent before participation in this study.

### Demographics

Four hundred and six Chinese outpatients with a suspected diagnosis of FD were screened between March 20, 2007 and June 1, 2007. One hundred and six patients were excluded and the symptom scores for the remaining 300 patients enrolled in our study were  $2.970 \pm 3.515$  for burping/belching,  $4.690 \pm 3.770$  for fullness after eating or slow digestion,  $1.800 \pm 2.886$  for nausea,  $2.367 \pm 3.421$  for inability to finish a regular meal,  $1.540 \pm 3.038$  for bad breath,  $3.050 \pm 3.575$  for bitter/sour tasting fluid coming up into mouth or throat,  $0.670 \pm 1.855$  for vomiting,  $5.790 \pm 2.939$  for discomfort in upper abdomen,  $0.786 \pm 2.185$  for cramps in upper abdomen,  $1.306 \pm 2.803$  for burning sensation in upper abdomen,  $5.130 \pm 3.567$  for pain or ache in upper abdomen,  $1.206 \pm 2.601$  for pressure in upper abdomen,  $5.300 \pm 3.516$  for bloating in upper abdomen,  $0.903 \pm 2.380$  for burning sensation in chest (heartburn), and  $0.640 \pm 2.045$  for pain or ache in chest. The mean age of subjects was 39.24 years and 68.7% of the subjects were women.

The study was performed at Chengdu, Wuhan and Changsha medical centers, which comprise five hospitals in total. Before inclusion in the trial, all patients underwent physical examination, abdominal ultrasonography and upper gastrointestinal endoscopy to exclude organic gastrointestinal cause for their symptoms. Patients at the age of 18 years and older were considered eligible in the study. If their endoscopy was either normal or showed only chronic superficial gastritis, they were diagnosed with FD according to

Rome-III criteria<sup>[20]</sup> with no cholecystitis or cholelithiasis on their sonogram. Patients with a history of upper gastrointestinal surgery, or who were pregnant or lactating, were excluded. Mental deficiency, psychiatric, cardiovascular, hepatic, and renal diseases were the additional exclusion criteria. The investigation was carried out in a quiet environment. Specialists explained each item to the patients so that the patients could understand and complete the questionnaire correctly.

### Chinese version of NDI

The original 25-item NDI<sup>[17]</sup> consists of three parts: a symptom checklist that measures the frequency, intensity and level of discomfort of 15 upper gastrointestinal symptoms over the prior 14 d, 25 items designed to assess QOL, and an 11-item questionnaire designed to measure the relevance or importance of the above items.

The QOL includes four domains, namely interference (13 items), know/control (seven items), eat/drink (three items), and sleep/disturb (two items). The scores were determined with a five-point Likert scale which ranges "not at all"-“extremely”. The NDI for QOL score ranges 0-99, higher scores indicate poorer QOL. Symptoms were scored separately from questions representing the QOL. The higher the score of each individual symptom is, the severer the symptom is.

After permission, the original version of NDI was translated into Chinese according to the WHO-QOL methodology of cross-culture adaptation for QOL<sup>[21]</sup>. Forward translation from the original English version was performed independently by two gastroenterologists with good English. Both forward versions were then reconciled and incorporated into the Chinese version. Back translation was carried out by an English teacher understanding the Chinese language with no knowledge of NDI. The semifinal version was derived from reconciliation of the original, back and forward translations. A pretest, NDI for five patients with FD was performed. The final version was obtained after cross-cultural adaptation.

### Statistical analysis

The reliability of NDI was evaluated for internal consistency using Cronbach's  $\alpha$ . Correlation analyses were used to assess the content validity. Factor analysis and structural equation models were used to assess the construct validity.

All data were analyzed with SPSS 13.0 version. Data derived from descriptive statistical analysis were presented in the form of percentages including categorical variables and calculation of the mean.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Checklist of symptoms

The total score for each symptom on the checklist was calculated by adding its corresponding frequency, severity and level of discomfort. As stated above, higher scores were elicited for symptoms of discomfort, bloating, and

**Table 1 Internal consistency of items in each domain**

Factor	Domain	Cronbach's $\alpha$
1	Interference	0.944
2	Know/control	0.906
3	Eat/drink	0.833
4	Sleep disturb	0.960

**Table 2 Pearson item-dimension correlation coefficients**

Item	Domains			
	Interference	Know/control	Eat/drink	Sleep disturb
01	0.626	0.531	0.461	0.354
02	0.701	0.817	0.450	0.376
03	0.666	0.812	0.492	0.396
04	0.522	0.493	0.906	0.457
05	0.457	0.476	0.840	0.454
06	0.589	0.484	0.852	0.389
07	0.473	0.476	0.472	0.982
08	0.511	0.536	0.509	0.980
09	0.822	0.640	0.521	0.464
10	0.828	0.638	0.520	0.399
11	0.826	0.677	0.455	0.437
12	0.818	0.635	0.446	0.403
13	0.815	0.568	0.482	0.342
14	0.800	0.575	0.432	0.397
15	0.794	0.564	0.471	0.289
16	0.771	0.715	0.467	0.442
17	0.753	0.832	0.424	0.430
18	0.742	0.843	0.505	0.528
19	0.746	0.774	0.482	0.374
20	0.824	0.827	0.554	0.458
21	0.751	0.624	0.477	0.370
22	0.557	0.771	0.368	0.347
23	0.506	0.710	0.362	0.321
24	0.709	0.814	0.527	0.487
25	0.631	0.577	0.327	0.294

pain or ache in upper abdomen, and fullness after eating or slow digestion.

### Reliability

The scores were evaluated using Cronbach's  $\alpha$  coefficient. An  $\alpha$  score  $> 0.7$  was considered internally consistent as previously described<sup>[22]</sup>. The Cronbach's  $\alpha$  coefficient ranged 0.833-0.960 (Table 1).

### Validity

The content validity of 25 items and four-field scores were regarded as 29 independent variables. Pearson item-dimension correlation coefficient was employed to evaluate the content validity. Most of the coefficients were higher than 0.6 ( $P < 0.01$ , Table 2).

### Construct validity

The values of the four preceding factors were above 1.0, and their cumulative factor loading rate was 69.287%. The rotated component matrix showed that component 1 had more loadings on items 1, 9-16, 20, 21 and 25; component 2 had more loadings on items 2, 3, 17-20, 22-24; component 3 had more loadings on items 4-6; component 4 had more loadings on items 7 and 8 (Table 3). The confirmatory factor analysis indicated that

**Table 3 Rotated component matrix**

Item	Component			
	1	2	3	4
22	0.777	0.101	0.154	
23	0.735		0.145	
19	0.725	0.352	0.201	
17	0.715	0.417		0.223
18	0.685	0.367	0.178	0.312
20	0.684	0.448	0.261	0.184
24	0.640	0.332	0.266	0.257
03	0.634	0.312	0.336	
02	0.612	0.413	0.263	
16	0.598	0.445	0.156	0.225
25	0.536	0.374		
13	0.206	0.853	0.200	
14	0.216	0.835	0.117	0.164
15	0.225	0.828	0.204	
10	0.354	0.695	0.270	0.122
12	0.395	0.689	0.107	0.202
09	0.341	0.659	0.284	0.215
11	0.449	0.630	0.132	0.248
21	0.458	0.501	0.258	0.129
01	0.335	0.378	0.377	0.124
04	0.182	0.235	0.834	0.188
05	0.277		0.762	0.248
06	0.171	0.399	0.713	
07	0.197	0.180	0.225	0.908
08	0.255	0.192	0.256	0.878

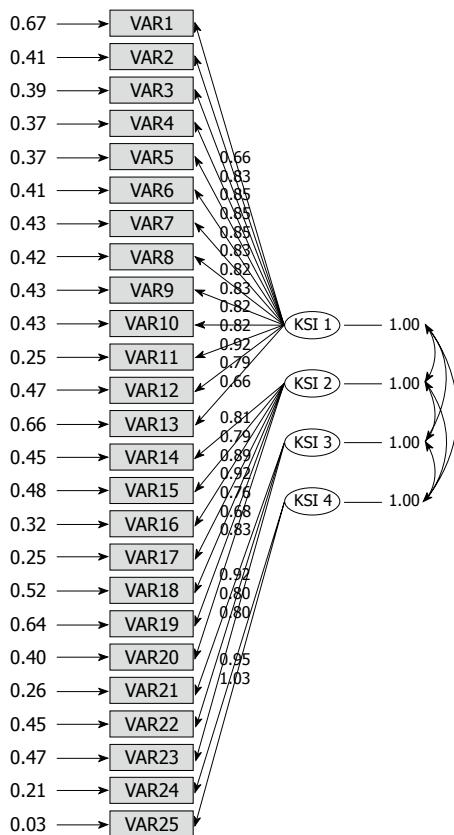
degrees of freedom = 269, minimum fit function chi-square = 1703.32 ( $P < 0.0001$ ), normal theory weighted least chi-square = 1809.13 ( $P < 0.0001$ ), comparative fit index (CFI) = 0.94, non-normal fit index = 0.94. A structural equation model of construct validity is illustrated in Figure 1.

## DISCUSSION

QOL has received increasing attention as more foci are placed on individual satisfaction as an important health outcome in clinical studies. In addition, QOL is particularly significant in diseases lacking of obvious biological or clinical markers, such as FD<sup>[23,24]</sup>. FD greatly affects the QOL of patients. However, treatment of FD is still controversial and no single therapy is uniformly effective, in part, due to the absence of a reliable evaluation instrument.

From our symptom checklist scores, symptoms with the highest scores were discomfort, bloating, and pain or ache in upper abdomen and fullness after eating or slow digestion, all of which are congruent with the main symptoms of FD according to Rome-III criteria<sup>[25]</sup>. However, heartburn, a major symptom in the Rome-III criteria, had a lower score in our study, possibly due to lack of patient comprehension or lack of adequate explanation by the investigator.

In clinical trials, Leeds dyspepsia questionnaire (LDQ) and MOS 36-item short-form health survey (SF-36) have been applied as an evaluation instrument for FD<sup>[26-28]</sup>. LDQ is a valid, reliable and responsive instrument for measuring the presence and severity of dyspepsia, but it lacks of QOL assessment<sup>[29]</sup>. As it is



**Figure 1** Structural equation model of construct validity.

known that, in addition to symptoms, QOL assessment represents a major part of health outcome assessment in clinical studies of FD. However, as different diseases cause different symptoms which necessitate disease-specific H-QOL instruments. SF-36, a generic QOL measurement, contains a large number of questions, the majority of which are often irrelevant to a particular disease. As a result, it may be insensitive to changes in the relevant items due to interference from the irrelevant items. Thus, the evaluation of FD should contain two aspects, namely symptom measure and disease-specific H-QOL assessment. The NDI addresses both aspects.

The original NDI consists of 42 questions, and is shortened to 25 items, excluding those items with a negative response rate of over 60%. The remaining 25 items represent the clinically relevant QOL concepts in subjects with FD<sup>[17]</sup>. Our study was based on the revised version of 25 items.

In order to maintain the sensitivity of the original version, our questionnaire considered differences in language, cultural diversities, and life style. After our version of the NDI was established, its reliability and validity were evaluated using Cronbach's  $\alpha$ . We found that all domains were above 0.80, demonstrating that our scale has an excellent reliability. Correlation analysis demonstrated that each item had a strong correlation with the corresponding domain, but a weak correlation with other domains, supporting that our questionnaire is valid. In addition, our results are in accordance with the original NDI design, representing superior construct

validity. Confirmatory factor analysis also indicated that the CFI was higher than the acceptable level of 0.90<sup>[25,30]</sup>, demonstrating that our scale has a good construct validity.

In the past, FD research in China was limited by the absence of a valid assessment instrument for the disease. Our results suggest that the Chinese version of NDI with excellent psychometrics would promote the clinical study of FD and align its medical course in China with international practice in future.

Our study has some limitations, including lack of extensive demographic characteristics, analysis of multi-dimensional factors (such as education, career, financial and social status), "test-retest" in reliability assessment, and discriminating validity in validity assessment, all of which need to be further studied.

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## COMMENTS

### Background

Functional dyspepsia (FD) is a common non-organic disease in the world. However, treatment of FD is still controversial, partially due to lack of a reliable evaluation instrument. Since 1998, the Nepean Dyspepsia Index (NDI) has been designed to diagnose FD and proven to be valid in measuring both symptom scores and impairment of dyspepsia-specific health-related quality of life (H-QOL) in FD patients.

### Research frontiers

In addition to symptom evaluation, assessment of QOL has been recognized to be more and more important in overall health outcome assessment in FD clinical studies. In this study, the reliability and validity of the Chinese NDI translated from its English version which reflects the dyspepsia-specific H-QOL in Chinese FD patients, were assessed.

### Innovations and breakthroughs

Studies on validation of NDI for FD in Western countries are widely available. However, NDI has not been translated and validated in China, since much attention is not paid to the effect of FD on the QOL of patients. This is the first study to translate the original version of NDI (25 items) into Chinese and to assess its reliability and validity in Chinese FD patients.

### Applications

By validating the translated version of NDI, this study may provide researchers, clinicians, and patients with a suitable questionnaire for the assessment of FD.

### Terminology

Reliability is the extent to which the measurements of a test remain consistent over repeated tests of the same subject under identical conditions. Validity, containing construct, content and convergent validity, refers to the degree to which evidence and theory support the interpretations of test scores entailed by proposed tests. Assessment of the validity of a scale involves evaluation of the scale in relation to the desired conclusion on the basis of prevailing standards.

**Peer review**

The authors have made a detail description of how to use the Chinese version of NDI to diagnose FD patients in China. The study is clinically important, because a Chinese version of NDI is needed to diagnose and compare FD patients in China and other countries.

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BRIEF ARTICLES

# Specific activation of 2'-5'oligoadenylate synthetase gene promoter by hepatitis C virus-core protein: A potential for developing hepatitis C virus targeting gene therapy

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Wang Y, Mao SS, He QQ, Zi Y, Wen JF, Feng DY. Specific activation of 2'-5'oligoadenylate synthetase gene promoter by hepatitis C virus-core protein: A potential for developing hepatitis C virus targeting gene therapy. *World J Gastroenterol* 2009; 15(25): 3178-3182 Available from: URL: <http://www.wjgnet.com/1007-9327/15/3178.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.3178>

## INTRODUCTION

Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma worldwide<sup>[1]</sup>. Currently, there is no vaccine to prevent the infection and no specific antiviral drug directed against the disease. Gene therapy has emerged as a novel approach to combat HCV infection in the last few years<sup>[2,3]</sup>. However, one of the most important obstacles to overcome is “targeting”: the appropriate genes must be delivered to and expressed in HCV infected hepatocytes without harming normal tissues. This problem could be addressed by using a promoter that has a higher level of activity in HCV infected liver cells *vs* normal cells.

HCV is a member of the Flaviviridae family, containing approximately 9.5 kb of positive strand RNA. The viral genome encodes a large precursor polyprotein which is cleaved into functional proteins such as core, envelope (E1, E2) and non-structural proteins (NS2-NS5)<sup>[4,5]</sup>. The viral core protein consists of 191 amino acids and has an apparent molecular mass of 21 kDa. In addition to being the viral capsid protein, it also functions as a transcriptional regulator of various viral and cellular promoters. Some groups have reported that HCV-core protein activates the human

## Abstract

**AIM:** To examine whether 2'-5'oligoadenylate synthetase (*OAS*) gene promoter can be specifically activated by hepatitis C virus (HCV)-core protein.

**METHODS:** Human embryo hepatic cell line L02 was transfected with pcDNA3.1-core plasmid and selected by G418. Expression of HCV-core was detected by reverse transcription polymerase chain reaction and Western blotting. The *OAS* promoter sequence was amplified from the genomic DNA and inserted into pGL3-basic vector. The resultant pGL3-OAS-Luci plasmid was transiently transfected into L02/core cells and luciferase activity was assayed.

**RESULTS:** L02/core cell line stably expressing HCV-core protein was established. The pGL3-OAS-Luci construct exhibited significant transcriptional activity in the L02/core cells but not in the L02 cells.

**CONCLUSION:** HCV-core protein activates the *OAS* gene promoter specifically and effectively. Utilization of *OAS* gene promoter would be an ideal strategy for developing HCV-specific gene therapy.

c-myc, Erk, IL-2, and SV40 early promoters, and trans-suppresses some cellular promoters such as p53 and p21<sup>[6-12]</sup>. Though the mechanisms of these regulations are still unclear, it is possible that these properties of HCV-core protein influence host cell growth, survival, and carcinogenesis. Therefore, utilization of a promoter that is predominantly active in HCV-core positive hepatocytes would be an ideal strategy for developing HCV-specific gene therapy.

During the investigation of interferon (IFN)-inducible JAK-STAT signaling affected by HCV, Naganuma *et al*<sup>[13]</sup> found that HCV-core protein specifically activated the 2'-5'oligoadenylate synthetase (*OAS*) gene promoter in human hepatocyte cells, while the E1, E2, and NS5A proteins did not activate the *OAS* gene promoter. These data indicate that *OAS* promoter might be used to restrict gene expression in HCV-core positive liver cells. However, the role of HCV-core protein in modulating *OAS* gene expression is much controversial, some other groups have reported that HCV-core protein does not affect the activation of IFN-responsive genes (including *OAS* gene), although it modulates the JAK/STAT signaling pathway<sup>[14-16]</sup>. So whether this viral core protein interferes with the *OAS* promoter and whether the promoter could be used to target gene therapy remain to be demonstrated. For these reasons, in the present study, we cloned the *OAS* promoter and examined its activity in the HCV-core expressing liver cells *in vitro*.

## MATERIALS AND METHODS

### Cell culture

Human embryo hepatocyte derived L02 cell line was obtained from Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences (Shanghai, China). The cells were routinely grown in DMEM medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg /mL streptomycin.

### Stable transfection and establishment of stable cell line

The pcDNA3.1-core, which contains the complete coding region of HCV-core protein (1b genotype) under the control of cytomegalovirus (CMV) immediate early promoter, was kindly provided by Professor Jun Cheng (Institute for Epidemic Disease Research, Beijing Ditan Hospital, China). The L02 cells were seeded in 6-well culture plates at 70% confluence 24 h before transfection. Cells were transfected with 2 µg of pcDNA3.1-core plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For establishment of stable cell line, the transfected cells were subjected to selection medium containing 400 mg/L G418 until several G418-resistant clones (L02/core) were obtained, and then the cells were maintained in G418 at a concentration of 200 mg/L. Non-transfected L02 cells were used as parallel control.

### Reverse transcription polymerase chain reaction (RT-PCR) analysis for core-specific gene expression

Total RNA was isolated from L02/core cells with TRIzol reagent (Invitrogen) and reversely transcribed using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas). PCR was subsequently performed with HCV core-specific primers: 5'-ATGAGCACCAATCCTAAA C-3' (forward), and 5'-GGCTGAAGCGGGCAC-3' (reverse). The cycle parameters were as follows: 95°C for 1 min, 52°C for 50 s, and 72°C for 1 min; 30 cycles. The PCR products were electrophoresed in a 1.5% agarose gel containing ethidium bromide and visualized by UV illumination. The expected size of the PCR product for HCV-core mRNA was 572 base-pairs.

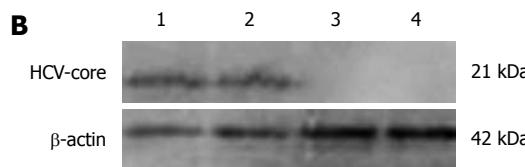
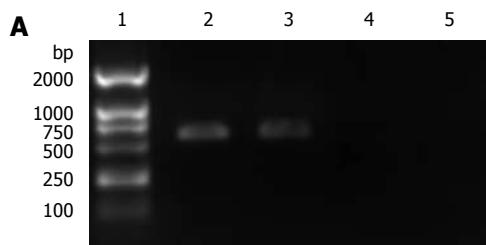
### Western blotting analysis for core protein

The L02/core cells were collected, washed with PBS and lysed in ice-cold lysis buffer (20 mmol/L Tris HCl, pH 7.5, 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Nonidet P-40, 1 mg/L Aprotinin, 100 mg/L PMSF). The protein concentration of the whole cell extract was measured using Bio-Rad DC protein assay kit. Equal amounts of cell lysates (60 µg protein) were separated by 12% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked for more than 1 h in PBS containing 2% nonfat milk and 0.25% Tween-20 and then immunoblotted using anti-HCV core monoclonal antibody (1:500, Santa Cruz). Immunoreactive bands were detected using horseradish-peroxidase-conjugated secondary antibodies (1:1000) in conjunction with an enhanced chemiluminescent system.

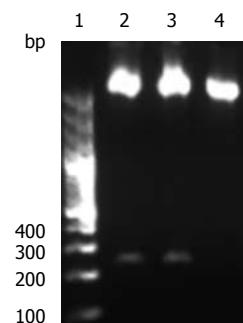
### Promoter cloning and luciferase assay

Template genomic DNA was extracted from human whole blood using DNAzol BD reagent (Invitrogen). The *OAS1* promoter sequence (-157 to +82) was amplified by PCR from the genomic DNA. The primers incorporating *Sac*I and *Hind*III restriction sites were: 5'-CCGAGCTCGGGATCAGGGAGTGT-3' (forward) and 5'-CCCAAGCTTGATCGGGAACACG-3' (reverse). The PCR fragment was digested and cloned into the *Sac*I / *Hind*III sites of pGL3-basic vector (Promega) to generate pGL3-OAS-Luci. Negative and positive control constructs were pGL3-basic lacking any promoter sequence, and pGL3-promoter containing the SV40 promoter sequence.

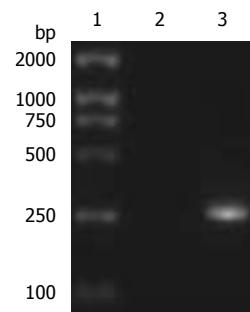
These luciferase reporter plasmids were transiently transfected into L02/core and L02 cells using Lipofectamine 2000 (Invitrogen). The cells were harvested 48 h after the transfection. Luciferase assays were performed according to the manufacturer's protocols (Promega). Briefly, cells were lysed with reporter lysis buffer, and the luciferase activity was determined using a luminometer. A β-galactosidase expression plasmid (pSV-β-galactosidase; Promega) was co-transfected to allow normalization for transfection efficiency. All experiments were performed at least three times in each plasmid and represent the relative luciferase activity as average.



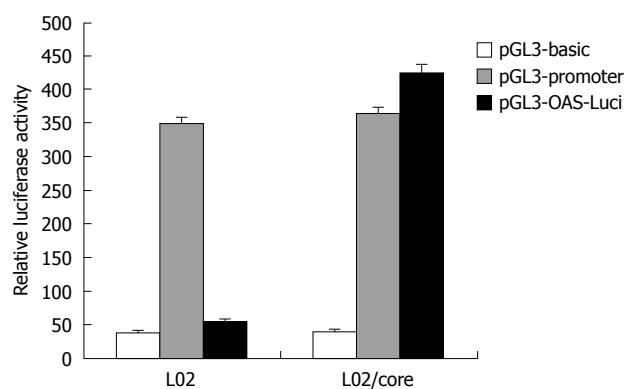
**Figure 1 Expression of HCV-core in the stable cell line L02/core.** A: Detection of HCV-core mRNA by RT-PCR. 572 bp visible fragments consistent with the predicted size of HCV-core mRNA were detected in L02/core cell clones. 1: DL2000 marker; 2, 3: L02 cells transfected with pcDNA3.1-core (L02/core); 4: L02 cells transfected with pcDNA3.1 (empty vector); 5: Untransfected L02 cells; B: Detection of HCV-core protein by Western blotting. 1, 2: L02/core cells; 3: L02 cells transfected with empty vector; 4: Untransfected L02 cells.



**Figure 3 The OAS-luciferase construct (pGL3-OAS-Luci) was confirmed by double enzyme digestion with *Sac* I and *Hind* III.** 1: GeneRuler™ 100 bp DNA Ladder; 2-3: Double digestion of pGL3-OAS-Luci; 4: Double digestion of pGL3-Basic.



**Figure 2 PCR product of OAS promoter.** A 240 bp visible fragment consistent with the predicted size was observed by electrophoresis. 1: DL2000 marker; 2: Negative control; 3: PCR product.



**Figure 4 Transcriptional activity of the OAS promoter in L02 and L02/core cells.** The OAS promoter showed significant transcriptional activity in the presence of HCV-core protein (L02/core), but not in the normal liver cells (L02). The data shown are the mean ± SD from three independent experiments.

#### Transcriptional activity of OAS promoter in HCV-core positive or negative cells

The *OAS* promoter sequence was amplified by PCR from human genomic DNA. As shown in Figure 2, a 240 bp visible fragment consistent with the predicted size was obtained by electrophoresis. Sequencing analysis confirmed that the cloned gene was identical with the original sequence in GenBank (accession number NW\_925395). The *OAS* promoter was put upstream of the firefly luciferase gene in the pGL3-basic vector which lacks eukaryotic promoter and enhancer sequences. The resultant pGL3-OAS-Luci plasmid was confirmed by double enzyme digestion with *Sac* I and *Hind* III (Figure 3).

To examine the transcriptional activity of the *OAS* gene promoter, the pGL3-OAS-Luci was transiently transfected into L02/core and L02 cells, and the firefly luciferase activity obtained was compared with those from the pGL3-basic and pGL3-promoter plasmid. As shown in Figure 4, the pGL3-OAS-Luci construct exhibited significantly higher transcriptional activity in the presence of HCV-core protein (L02/core). The relative luciferase activity was almost 1.2 fold of pGL3-Promoter, and 10-15 fold of the baseline activity of the pGL3-basic vector. In contrast, this *OAS* promoter construct resulted in no or little luciferase activity in the normal liver cell line L02. These findings suggest that HCV-core protein strongly activates the *OAS* gene promoter.

## Statistical analysis

All data are shown as the mean ± SD. Statistical analysis was performed using the *t* test. *P* < 0.05 was considered statistically significant.

## RESULTS

### HCV-core expression in stably transfected L02 cells

In order to investigate the effect of HCV-core on the *OAS* promoter, L02 cells were transfected with pcDNA3.1-core plasmid and were selected on the basis of their resistance to G418 for 2 wk. Expression of HCV-core was detected by RT-PCR and Western blotting. As shown in Figure 1A, 572 bp visible fragments, consistent with the predicted size of HCV-core mRNA, were detected in L02/core cell clones. No PCR-amplification product was found in the pcDNA3.1 transfected L02 cells, neither in the nontransfected L02 cells. The expression of HCV-core protein was confirmed by immunoblotting using anti-HCV core monoclonal antibody. As shown in Figure 1B, HCV-core protein of the expected molecular mass of 21 kDa was observed in L02/core cells, whereas no expression was detected in pcDNA3.1 transfected or nontransfected L02 cells. These data demonstrate that hepatocyte line stably expressing HCV-core protein has been established.

## DISCUSSION

To increase the specificity and safety of gene therapy, the expression of the therapeutic gene need to be tightly controlled within the target tissue. This is particularly important for toxic gene strategies because inappropriate transgene expression may lead to severe toxicity. Targeted expression of therapeutic genes can be accomplished at several levels. The first approach for targeting specificity is at the level of vector delivery<sup>[17]</sup>. The strategies include exploiting natural viral tropisms, and incorporating tissue-specific ligands or monoclonal antibodies onto the surface of viral vectors or liposomes. However, there will undoubtedly be some genes delivered to local and distant normal tissues. Therefore, further safeguards must be put in place to ensure that gene delivery to these tissues does not result in significant expression and toxicity. One attractive approach to this problem is to use promoter elements to control gene expression tightly at the transcriptional level.

Many tissue and tumor specific promoters have been developed in target gene therapy. For example, the  $\alpha$ -fetoprotein promoter has been used to drive gene expression in hepatic carcinoma cells, the tyrosinase promoter in melanoma cells, the prostate specific antigen promoter in prostate cancer cells, and the carcinoembryonic antigen promoter in adenocarcinomas<sup>[18]</sup>. The results of these studies have demonstrated the feasibility of using specific promoters for targeting gene therapy in various cancer cell types.

Although several promoters have been identified more active in HCV-core positive hepatocytes, most of these promoters are much weaker than commonly used viral promoters such as the CMV early promoter, the Rous sarcoma virus long terminal repeat, and the SV40 early promoter. Consequently, their applications in gene therapy are hampered by the low expression. In the present study, we cloned the human *OAS* promoter and examined its transcriptional ability in the HCV-core positive hepatocytes and normal liver cells. We found that the luciferase expression driven by *OAS* promoter was markedly increased in the presence of core protein, but not in the normal liver cells. These data strongly suggest that HCV-core protein can activate *OAS* promoter. Since HCV-core protein plays an important role in persistent infection and hepatocellular carcinogenesis, and amino acid sequence of core protein is relatively conserved, utilization of the *OAS* promoter to drive therapeutic gene expression would be an ideal strategy for developing HCV-specific gene therapy.

## COMMENTS

### Background

The current standard therapy for chronic hepatitis C is inadequate for the majority of patients. Gene therapy has emerged as a novel approach to combat hepatitis C virus (HCV) infection in the last few years. However, a specific promoter is required to restrict transgene expression only in HCV infected cells. Since HCV-core protein plays an important role in persistent infection

and hepatocellular carcinogenesis, and amino acid sequence of core protein is relatively conserved, utilization of a promoter that is predominantly active in HCV-core positive hepatocytes would be an ideal strategy for HCV targeting gene therapy.

### Research frontiers

To increase the specificity and safety of gene therapy, the expression of the therapeutic gene need to be tightly controlled within the target tissue. This is particularly important for toxic gene strategies because inappropriate transgene expression may lead to severe toxicity. Targeted expression of therapeutic genes can be accomplished at several levels, including vector targeting and tissue-specific gene expression. However, no HCV-specific gene delivery system has yet been developed. One attractive approach to this problem is to use promoter elements to control gene expression tightly at the transcriptional level.

### Innovations and breakthroughs

In the present study, the authors cloned the 2'-5'oligoadenylate synthetase (*OAS*) promoter and examined its activity in the human embryo hepatocytes expressing HCV-core *in vitro*. They demonstrated that HCV-core protein can activate *OAS* gene promoter specifically and effectively.

### Applications

Utilization of *OAS* gene promoter to drive therapeutic gene expression would be an ideal strategy for developing HCV-specific gene therapy.

### Terminology

L02/core is the human embryo hepatocyte that stably expresses HCV-core protein. *OAS* is a metabolic enzyme originally identified as a regulator of the ribonuclease L (RNase L) pathway during viral infection.

### Peer review

This is a well written and interesting paper.

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# Improved quality of life in patients with gastric cancer after esophagogastrectomy reconstruction

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## Abstract

**AIM:** To compare postoperative quality of life (QOL) in patients with gastric cancer treated by esophagogastrectomy reconstruction after proximal gastrectomy.

**METHODS:** QOL assessments that included functional outcomes (a 24-item survey about treatment-specific symptoms) and health perception (Spitzer QOL Index) were performed in 149 patients with gastric cancer in the upper third of the stomach, who had received proximal gastrectomy with additional esophagogastrectomy.

**RESULTS:** Fifty-four patients underwent reconstruction by esophagogastric anterior wall end-to-side anastomosis combined with pyloroplasty (EA group); 45 patients had reconstruction by esophagogastric posterior wall end-to-side anastomosis (EP group); and 50 patients had reconstruction by esophagogastric end-to-end anastomosis (EE group). The EA group showed the best postoperative QOL, such as recovery of body weight, less discomfort after meals, and less heart burn or belching at 6 and 24 mo postoperatively. However, the survival rates, surgical results and Spitzer QOL index were similar among the three groups.

**CONCLUSION:** Postoperative QOL was better in the EA than EP or EE group. To improve QOL after

proximal gastrectomy for upper third gastric cancer, the EA procedure using a stapler is safe and feasible for esophagogastrectomy.

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**Key words:** Gastric cancer; Proximal gastrectomy; Esophagogastrectomy; Quality of life

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## INTRODUCTION

Although the incidence of gastric carcinoma has been decreasing continuously during the past decade, gastric cancer remains the second most common cause of cancer-related deaths worldwide. Given equivalent results with regards to survival, the impact of anastomotic methods on quality of life (QOL) becomes even more important. It has been reported that QOL is the main outcome for judging the efficacy of treatment modalities when no overall survival differences are demonstrated<sup>[1]</sup>. There is still no consensus on how to choose a reconstruction method for proximal gastrectomy in patients with upper third gastric cancer<sup>[2]</sup>. This study was designed to compare in detail different types of esophagogastrectomy.

Proximal gastrectomy impacts severely on physical and mental health, and has highly negative consequences for QOL at 6 and 24 mo. Although postoperative QOL has been shown to be important in the surgical literature<sup>[3]</sup>, there have only been a few studies on QOL in patients after proximal gastrectomy. For patients undergoing oncological surgery, QOL is generally accepted as an important outcome parameter, in addition to long-term survival, mortality, and complication rates. In order to provide patients with improved postoperative

QOL, many surgeons seek the optimal surgical methods. Compared with total gastrectomy, the advantages of proximal gastrectomy were shorter operation times, less blood loss and convenience of procedure<sup>[4]</sup>. No previous studies have assessed different types of esophagogastrectomy in terms of QOL.

The aim of this study was to assess outcome in terms of QOL in patients after proximal gastrectomy, by comparing three types of esophagogastrectomy, in order to find the optimal reconstruction method that offers the optimal postoperative QOL at 6 and 24 mo.

## MATERIALS AND METHODS

### **Patients**

Between January 2002 and December 2005, 195 patients with proximal gastric cancer were treated at the Department of Surgical Oncology, First Affiliated Hospital of China Medical University, Shenyang, China. Only 149 patients who were in the present prospective randomized study met the following study criteria: (1) underwent curative resection with lymph node dissection; (2) had no history of other organ malignancies; and (3) had more than 15 lymph nodes retrieved and confirmed by a specialist pathologist. The exclusion criteria included: (1) age > 80 years; (2) renal, pulmonary, or heart failure; (3) undergoing palliative surgery; (4) tumor recurrence during the survey; (5) preoperative or postoperative adjuvant chemotherapy or radiotherapy; and (6) albumin level < 3.5 g/dL and a lymphocyte count lower than 1000 lymphocytes/mm<sup>3</sup> in peripheral blood.

### **Surgical procedure**

The standard surgical procedure and extent of lymph node dissection were defined according to the recommendations of the Japanese Research Society for the Study of Gastric Cancer<sup>[5]</sup>. All patients were treated with stapler suture for digestive tract reconstruction after malignancy removal during the primary surgical procedure. Esophagogastrectomy was performed by using a mechanical stapler (Ethicon Endo-Surgery, USA). In addition, a row of external seromuscular sutures with interrupted absorbable stitches was also performed.

### **Esophagogastrectomy procedure and randomization**

All patients underwent esophagogastrectomy with curative intent and were randomized using a table of random numbers into three groups. To perform esophagogastric anterior wall end-to-side anastomosis combined with pyloroplasty (EA procedure), the anastomosis should be 2-3 cm under the gastric incision line, to guarantee the blood supply of the anastomosis. The stapler inserted into the remnant stomach through the gastric antrum and the center rod of the circular stapler was pierced through the center of the anterior wall, and during this stapling, an ischemic area is not created at all. The esophagus was then anastomosed to the anterior wall in the center of the remnant stomach.

A 3-4 mm wide serosal surface of the anterior wall of the remnant stomach strapped the esophagus circularly. After completion of esophagogastrectomy, the left end of the gastric stump was fixed to the diaphragm. Furthermore, the pyloroplasty was done in the standard manner with interrupted sutures<sup>[6-8]</sup>. To perform esophagogastric posterior wall end-to-side anastomosis (EP procedure), the stapler that was inserted into the remnant stomach through the anterior wall, and the center rod of the circular stapler was pierced through the center of the posterior wall. To perform esophagogastric end-to-end anastomosis (EE procedure), the center rod of the circular stapler was pierced through the left end of the staple line of the stomach.

### **Evaluation of QOL and follow-up study**

Functional outcome was assessed using a 24-item survey designed to assess treatment-specific symptoms, largely gastrointestinal function<sup>[9]</sup>. The questions were scaled according to the Eastern Cooperative Oncology Group<sup>[10]</sup>. The Spitzer QOL index, which reflects the patient's postoperative health perception<sup>[11]</sup>, is a global health assessment with a valid questionnaire that includes five items rated on a three-point scale: activity, daily living, health, support of family and friends, and outlook. The answers were analyzed in a quantitative fashion using a scoring system: the scores ranged from 0 (unsatisfactory result with severe symptoms) to 2 (excellent result with no symptoms); low scores reflected more symptoms. Questionnaires were administered at 6 and 24 mo postoperatively and annually thereafter until tumor recurrence. Average scores were calculated for each question. The Ethics Committee approved the study protocol and informed consent was obtained from each patient. Patients were closely followed after surgery until December 2007. The median follow-up duration was 42.5 mo (range, 9-67 mo). At the time of the last follow-up, 115 patients (77.2%) were alive, no patient was lost to follow-up, and 34 (22.8%) had died from recurrence or other causes.

### **Statistical analysis**

All statistical analyses were performed using Statistical Package for Social Science software for Windows version 13.0 (SPSS, Chicago, IL, USA). Data were expressed as mean  $\pm$  SD. Categorical data were compared using the Kruskal-Wallis test, Mann-Whitney U test, analysis of variance and  $\chi^2$  test.  $P < 0.05$  was considered as statistically significant. For calculation of survival rate, the Kaplan-Meier method was used, and compared using the log-rank test.

## RESULTS

### **Patient characteristics**

Table 1 summarizes clinicopathological factors in relation to the three groups. There were 95 (65%) male and 54 (35%) female patients, with a mean age of 61.5  $\pm$  6.5 years. There were no significant differences in the

**Table 1** Clinicopathological factors of the three groups

Clinicopathological factors	EA group (54)	EP group (45)	EE group (50)	P
Age (yr)	62.5 ± 5.4	56.6 ± 7.6	65.1 ± 8.6	0.668
Gender				0.775
Male	36	29	30	
Female	18	16	20	
Tumor size (cm)	4.6 ± 1.6	5.0 ± 1.7	4.8 ± 1.3	0.963
Histological type				0.993
Well-differentiated	18	15	17	
Moderately differentiated	20	17	19	
Poorly differentiated	10	9	8	
Signet ring cell type	6	4	6	
Lauren's classification				0.978
Intestinal	30	24	27	
Diffuse	18	16	17	
Mixed	6	5	6	
Lymph node status				0.989
Negative	38	34	36	
Positive	16	11	14	
Lymphovascular invasion				0.989
Negative	45	38	42	
Positive	9	7	8	
Stage				0.788
I	17	16	18	
II	21	17	20	
IIIa	11	7	9	
IIIb	5	5	3	

clinicopathological features such as sex, age, tumor size, histological type, Lauren's classification, lymph node status, or lymphovascular invasion among the three groups.

### Overall survival rate and surgical results

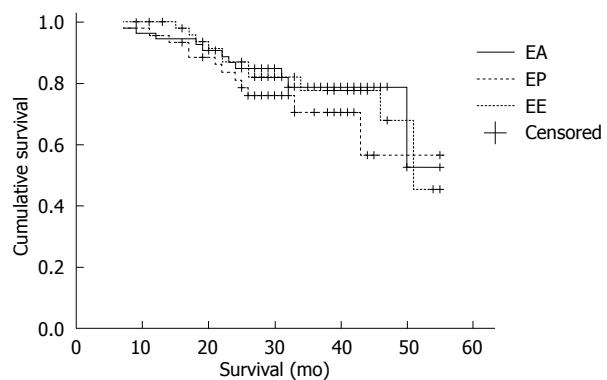
The survival time was defined as the time from diagnosis until last contact, date of death, or the date used as a cutoff for the follow-up database, in which case the survival information was censored. The 2-year survival rate was 79.6%, 73.3% and 78.0% for the EA, EP and the EE group, respectively. The survival rates were similar in all treatment groups ( $P = 0.713$ , Figure 1). The surgical results did not differ among the three groups (Table 2).

### Six months postoperative QOL evaluation

The evaluation scores for eating time were 1.83, 1.58 and 1.42 in EA, EP and the EE groups, respectively. The EA group had a significantly shorter eating time than the EP group (1.83 vs 1.58,  $P = 0.005$ ) and the EE group (1.83 vs 1.42,  $P = 0.000$ ).

The evaluation scores for dietary volume were 1.20, 0.60 and 0.58 in the EA, EP and the EE groups, respectively. The EA group had a significantly better dietary volume than the EP group (1.20 vs 0.60,  $P = 0.000$ ) and the EE group (1.20 vs 0.58,  $P = 0.000$ ).

The evaluation scores for heartburn or belching were 1.37, 0.73 and 0.80 in the EA, EP and the EE groups, respectively. The EA group had significantly less heartburn or belching than the EP group (1.37 vs 0.73,  $P = 0.000$ ) and the EE group (1.37 vs 0.80,  $P = 0.000$ ) (Table 3).



**Figure 1** Kaplan-Meier survival curves for EA, EP and EE patients ( $P = 0.713$ ) (Censored indicate patients who were still alive at follow up).

Most parameters tended to be normalized sooner after surgery in the EA group.

### Twenty-four months postoperative QOL evaluation

The evaluation scores for body weight were 1.48, 1.13 and 1.14 in the EA, EP and the EE groups, respectively. The EA group had significantly better body weight recovery than the EP group (1.48 vs 1.13,  $P = 0.005$ ) and the EE group (1.48 vs 1.14,  $P = 0.030$ ).

The evaluation scores for heartburn or belching were 1.78, 1.73 and 1.38 in the EA, EP and the EE groups, respectively. The EA group (1.78 vs 1.38,  $P = 0.005$ ) and the EP group (1.73 vs 1.38,  $P = 0.023$ ) had significantly less heartburn or belching than the EE group.

The evaluation scores for postprandial discomfort were 1.69, 1.07 and 1.22 in the EA, EP and the EE groups, respectively. The EA group showed less postprandial discomfort than the EP group (1.69 vs 1.07,  $P = 0.000$ ) and the EE group (1.69 vs 1.22,  $P = 0.001$ ) (Table 4).

### Comparison of evaluation scores of postoperative QOL between 6 and 24 mo in the same group

When the QOL scores between 6 and 24 mo were compared in the same group, the frequency of eating, food consistency, food volume, body weight, appetite, and heartburn or belching at 24 mo were all improved significantly in each group compared with at 6 mo. The eating time at 24 mo was significantly shorter than at 6 mo in the EP and EE group (EP,  $P = 0.031$  and EE,  $P = 0.000$ ). The postprandial discomfort at 6 mo was significantly less than at 24 mo in the EE group ( $P = 0.048$ ). Wound pain at 24 mo was significantly less than at 6 mo in the EA group ( $P = 0.031$ ).

It can be seen that, as postoperative time progresses, most symptoms improved, especially in terms eating frequency, food consistency, food volume, body weight, appetite, and heartburn or belching, and the total scores at 24 mo were all improved significantly compared with at 6 mo in the EA ( $P = 0.015$ ), EP ( $P = 0.007$ ) and EE ( $P = 0.011$ ) groups (Table 5).

### Spitzer index evaluation

The data from the Spitzer index (Tables 6 and 7) showed

**Table 2** Surgical results comparison among the three groups

Surgical factors	EA group	EP group	P	EA group	EE group	P	EP group	EE group	P
Additional organ resection			0.609			0.831			0.766
Splenectomy	8	7	0.919	8	7	0.906	7	7	0.832
Splenopancreatectomy	1	2	0.456	1	2	0.515	2	2	0.915
Transverse mesocolicectomy	2	2	0.853	2	3	0.586	2	3	0.736
Wedge liver resection	1	1	0.897	1	2	0.515	1	2	0.623
Margin status			0.670			0.938			0.623
Negative	52	44		52	48		44	48	
Positive	2	1		2	2		1	2	
Lymph node dissection			0.902			0.804			0.721
D1	15	12		15	14		12	14	
≥ D2	39	33		39	36		33	36	
No. of lymph nodes	29.6 ± 7.6	28.5 ± 6.8	0.524	29.6 ± 7.6	30.1 ± 7.2	0.857	28.5 ± 6.8	30.1 ± 7.2	0.657
Operating time (min)	166.3 ± 47.5	156.8 ± 53.1	0.758	166.3 ± 47.5	149.7 ± 61.2	0.564	156.8 ± 53.1	149.7 ± 61.2	0.265
Blood loss (mL)	263.4 ± 112.6	267.3 ± 121.6	0.273	263.4 ± 112.6	276.9 ± 135.5	0.647	267.3 ± 121.6	276.9 ± 135.5	0.798
Postoperative stay (d)	18.0 ± 5.7	20.9 ± 7.8	0.874	18.0 ± 5.7	19.0 ± 5.7	0.953	20.9 ± 7.8	19.0 ± 5.7	0.849
Postoperative complications	12	11	0.795	12	13	0.654	11	13	0.862
Mortality	0	1	0.273	0	1	0.299	1	1	0.940

**Table 3** Comparison of evaluation scores for 6 mo postoperative QOL among the three groups (mean ± SD)

Question	EA group	EP group	P	EA group	EE group	P	EP group	EE group	P
Frequency of eating	0.76 ± 0.799	0.80 ± 0.968	0.957	0.76 ± 0.799	0.78 ± 0.616	0.664	0.80 ± 0.968	0.78 ± 0.616	0.698
Eating time	1.83 ± 0.376	1.58 ± 0.499	0.005 <sup>a</sup>	1.83 ± 0.376	1.42 ± 0.642	0.000 <sup>a</sup>	1.58 ± 0.499	1.42 ± 0.642	0.287
Consistency of food	0.76 ± 0.845	0.71 ± 0.815	0.803	0.76 ± 0.845	0.84 ± 0.792	0.543	0.71 ± 0.815	0.84 ± 0.792	0.391
Volume of food	1.20 ± 0.810	0.60 ± 0.780	0.000 <sup>a</sup>	1.20 ± 0.810	0.58 ± 0.702	0.000 <sup>a</sup>	0.60 ± 0.780	0.58 ± 0.702	0.950
Body weight	0.80 ± 0.711	0.78 ± 0.636	0.969	0.80 ± 0.711	0.66 ± 0.848	0.221	0.78 ± 0.636	0.66 ± 0.848	0.225
Appetite	1.13 ± 0.674	0.87 ± 0.968	0.120	1.13 ± 0.674	1.16 ± 0.817	0.724	0.87 ± 0.968	1.16 ± 0.817	0.118
Difficulty swallowing	1.56 ± 0.664	1.42 ± 0.723	0.333	1.56 ± 0.664	1.54 ± 0.734	0.898	1.42 ± 0.723	1.54 ± 0.734	0.308
Diarrhea	1.83 ± 0.376	1.69 ± 0.733	0.843	1.83 ± 0.376	1.78 ± 0.582	0.852	1.69 ± 0.733	1.78 ± 0.582	0.734
Heartburn or belch	1.37 ± 0.708	0.73 ± 0.863	0.000 <sup>a</sup>	1.37 ± 0.708	0.80 ± 0.756	0.000 <sup>a</sup>	0.73 ± 0.863	0.80 ± 0.756	0.547
Postprandial discomfort	1.46 ± 0.693	1.24 ± 0.957	0.433	1.46 ± 0.693	1.52 ± 0.677	0.646	1.24 ± 0.957	1.52 ± 0.677	0.267
Abdominal pain	1.54 ± 0.770	1.49 ± 0.787	0.721	1.54 ± 0.770	1.60 ± 0.495	0.698	1.49 ± 0.787	1.60 ± 0.495	0.965
Vomiting	1.48 ± 0.771	1.49 ± 0.757	0.993	1.48 ± 0.771	1.68 ± 0.471	0.368	1.49 ± 0.757	1.68 ± 0.471	0.389
General fatigue	1.80 ± 0.528	1.82 ± 0.387	0.792	1.80 ± 0.528	1.92 ± 0.274	0.250	1.82 ± 0.387	1.92 ± 0.274	0.154
Dizziness	1.80 ± 0.562	1.89 ± 0.318	0.687	1.80 ± 0.562	1.74 ± 0.600	0.507	1.89 ± 0.318	1.74 ± 0.600	0.289
Intestinal obstruction	1.65 ± 0.555	1.56 ± 0.693	0.669	1.65 ± 0.555	1.66 ± 0.519	0.997	1.56 ± 0.693	1.66 ± 0.519	0.667
Performance status	1.54 ± 0.719	1.64 ± 0.743	0.240	1.54 ± 0.719	1.58 ± 0.642	0.910	1.64 ± 0.743	1.58 ± 0.642	0.269
Early dumping syndrome	1.54 ± 0.665	1.40 ± 0.720	0.319	1.54 ± 0.665	1.42 ± 0.673	0.307	1.40 ± 0.720	1.42 ± 0.673	0.970
Late dumping syndrome	1.78 ± 0.502	1.73 ± 0.688	0.669	1.78 ± 0.502	1.86 ± 0.452	0.241	1.73 ± 0.688	1.86 ± 0.452	0.532
Physical condition	1.67 ± 0.673	1.80 ± 0.548	0.258	1.67 ± 0.673	1.76 ± 0.517	0.661	1.80 ± 0.548	1.76 ± 0.517	0.450
Wound pain, present	1.57 ± 0.690	1.53 ± 0.815	0.874	1.57 ± 0.690	1.62 ± 0.490	0.809	1.53 ± 0.815	1.62 ± 0.490	0.704
Wound pain, postoperative	1.07 ± 0.866	1.18 ± 0.716	0.600	1.07 ± 0.866	1.22 ± 0.764	0.413	1.18 ± 0.716	1.22 ± 0.764	0.727
Satisfaction with operation	1.57 ± 0.690	1.69 ± 0.557	0.495	1.57 ± 0.690	1.66 ± 0.688	0.357	1.69 ± 0.557	1.66 ± 0.688	0.784
Recommendation to others	1.41 ± 0.714	1.29 ± 0.787	0.484	1.41 ± 0.714	1.46 ± 0.706	0.680	1.29 ± 0.787	1.46 ± 0.706	0.290
Mood or feeling	1.67 ± 0.673	1.67 ± 0.707	0.859	1.67 ± 0.673	1.70 ± 0.463	0.610	1.67 ± 0.707	1.70 ± 0.463	0.507
Total	1.45 ± 0.748	1.36 ± 0.825	0.059	1.45 ± 0.748	1.42 ± 0.743	0.147	1.36 ± 0.825	1.42 ± 0.743	0.389

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

that after all the postoperative and oncological problems were solved, proximal gastrectomy was quite compatible with normal life. From the results of Spitzer index evaluation, we also concluded that patients after proximal gastrectomy may have a nearly normal life with activity and daily living at 6 and 24 mo.

## DISCUSSION

The choice of reconstruction method after proximal gastrectomy remains a controversial issue. Previously, several studies have found no difference in the 5-year

survival among patients with proximal gastrectomy and those with total gastrectomy<sup>[6,7]</sup>. Proximal gastrectomy for early gastric cancer in the upper third of the stomach is an appropriate operation in terms of radical treatment and safety. Proximal gastrectomy was introduced to improve performance status of patients and minimize late postoperative complications such as reflux esophagitis<sup>[8,12-15]</sup>.

It is well known that moderate malnutrition is often associated with gastric cancer surgery<sup>[16]</sup>, and that esophagogastrectomy reconstruction is more favorable to the natural food passage of the duodenum

**Table 4 Comparison of evaluation scores of 24 mo postoperative QOL among the three groups (mean ± SD)**

Question	EA group	EP group	P	EA group	EE group	P	EP group	EE group	P
Frequency of eating	1.67 ± 0.583	1.62 ± 0.614	0.711	1.67 ± 0.583	1.70 ± 0.580	0.693	1.62 ± 0.614	1.70 ± 0.580	0.461
Eating time	1.85 ± 0.359	1.78 ± 0.471	0.472	1.85 ± 0.359	1.88 ± 0.328	0.676	1.78 ± 0.471	1.88 ± 0.328	0.273
Consistency of food	1.65 ± 0.619	1.73 ± 0.618	0.306	1.65 ± 0.619	1.80 ± 0.535	0.105	1.73 ± 0.618	1.80 ± 0.535	0.601
Volume of food	1.69 ± 0.639	1.62 ± 0.684	0.613	1.69 ± 0.639	1.70 ± 0.580	0.915	1.62 ± 0.684	1.70 ± 0.580	0.680
Body weight	1.48 ± 0.637	1.13 ± 0.625	0.005 <sup>a</sup>	1.48 ± 0.637	1.14 ± 0.047	0.030 <sup>a</sup>	1.13 ± 0.625	1.14 ± 0.047	0.837
Appetite	1.57 ± 0.662	1.78 ± 0.560	0.056	1.57 ± 0.662	1.70 ± 0.580	0.290	1.78 ± 0.560	1.70 ± 0.580	0.349
Difficulty swallowing	1.52 ± 0.720	1.49 ± 0.787	0.116	1.52 ± 0.720	1.74 ± 0.600	0.059	1.49 ± 0.787	1.74 ± 0.600	0.136
Diarrhea	1.87 ± 0.339	1.80 ± 0.505	0.654	1.87 ± 0.339	1.82 ± 0.523	0.977	1.80 ± 0.505	1.82 ± 0.523	0.658
Heartburn or belch	1.78 ± 0.502	1.73 ± 0.539	0.651	1.78 ± 0.502	1.38 ± 0.805	0.005 <sup>a</sup>	1.73 ± 0.539	1.38 ± 0.805	0.023 <sup>a</sup>
Postprandial discomfort	1.69 ± 0.609	1.07 ± 0.915	0.000 <sup>a</sup>	1.69 ± 0.609	1.22 ± 0.790	0.001 <sup>a</sup>	1.07 ± 0.915	1.22 ± 0.790	0.445
Abdominal pain	1.65 ± 0.649	1.60 ± 0.720	0.848	1.65 ± 0.649	1.70 ± 0.505	0.968	1.60 ± 0.720	1.70 ± 0.505	0.841
Vomiting	1.69 ± 0.577	1.67 ± 0.674	0.814	1.69 ± 0.577	1.70 ± 0.463	0.786	1.67 ± 0.674	1.70 ± 0.463	0.628
General fatigue	1.76 ± 0.581	1.78 ± 0.420	0.626	1.76 ± 0.581	1.84 ± 0.370	0.800	1.78 ± 0.420	1.84 ± 0.370	0.442
Dizziness	1.83 ± 0.505	1.93 ± 0.252	0.410	1.83 ± 0.505	1.80 ± 0.535	0.671	1.93 ± 0.252	1.80 ± 0.535	0.223
Intestinal obstruction	1.78 ± 0.502	1.64 ± 0.679	0.396	1.78 ± 0.502	1.68 ± 0.513	0.204	1.64 ± 0.679	1.68 ± 0.513	0.771
Performance status	1.63 ± 0.653	1.71 ± 0.661	0.322	1.63 ± 0.653	1.70 ± 0.463	0.951	1.71 ± 0.661	1.70 ± 0.463	0.318
Early dumping syndrome	1.70 ± 0.603	1.62 ± 0.650	0.465	1.70 ± 0.603	1.60 ± 0.728	0.560	1.62 ± 0.650	1.60 ± 0.728	0.901
Late dumping syndrome	1.70 ± 0.537	1.73 ± 0.580	0.567	1.70 ± 0.537	1.72 ± 0.479	0.966	1.73 ± 0.580	1.72 ± 0.479	0.594
Physical condition	1.72 ± 0.627	1.64 ± 0.712	0.616	1.72 ± 0.627	1.76 ± 0.476	0.820	1.64 ± 0.712	1.76 ± 0.476	0.753
Wound pain, present	1.83 ± 0.423	1.73 ± 0.580	0.450	1.83 ± 0.423	1.78 ± 0.465	0.494	1.73 ± 0.580	1.78 ± 0.465	0.910
Wound pain, postoperative	1.07 ± 0.866	1.18 ± 0.716	0.600	1.07 ± 0.866	1.22 ± 0.764	0.413	1.18 ± 0.716	1.22 ± 0.764	0.727
Satisfaction with operation	1.72 ± 0.596	1.76 ± 0.484	0.937	1.72 ± 0.596	1.72 ± 0.056	0.746	1.76 ± 0.484	1.72 ± 0.536	0.807
Recommendation to others	1.59 ± 0.567	1.42 ± 0.690	0.239	1.59 ± 0.567	1.54 ± 0.646	0.795	1.42 ± 0.690	1.54 ± 0.646	0.382
Mood or feeling	1.63 ± 0.708	1.71 ± 0.695	0.371	1.63 ± 0.708	1.66 ± 0.519	0.634	1.71 ± 0.695	1.66 ± 0.519	0.162
Total	1.67 ± 0.612	1.61 ± 0.663	0.116	1.67 ± 0.612	1.65 ± 0.609	0.150	1.61 ± 0.663	1.65 ± 0.609	0.532

<sup>a</sup>P < 0.05.**Table 5 Comparison of evaluation scores of QOL at 6 and 24 mo postoperatively between the same group (mean ± SD)**

Question	EA group		P	EP group		P	EE group		P
	6 mo	24 mo		6 mo	24 mo		6 mo	24 mo	
Frequency of eating	0.76 ± 0.799	1.67 ± 0.583	0.000 <sup>a</sup>	0.80 ± 0.968	1.62 ± 0.614	0.000 <sup>a</sup>	0.78 ± 0.616	1.70 ± 0.580	0.000 <sup>a</sup>
Eating time	1.83 ± 0.376	1.85 ± 0.359	0.793	1.58 ± 0.499	1.78 ± 0.471	0.031 <sup>a</sup>	1.42 ± 0.642	1.88 ± 0.328	0.000 <sup>a</sup>
Consistency of food	0.76 ± 0.845	1.65 ± 0.619	0.000 <sup>a</sup>	0.71 ± 0.815	1.73 ± 0.618	0.000 <sup>a</sup>	0.84 ± 0.792	1.80 ± 0.535	0.000 <sup>a</sup>
Volume of food	1.20 ± 0.810	1.69 ± 0.639	0.001 <sup>a</sup>	0.60 ± 0.780	1.62 ± 0.684	0.000 <sup>a</sup>	0.58 ± 0.702	1.70 ± 0.580	0.000 <sup>a</sup>
Body weight	0.80 ± 0.711	1.48 ± 0.637	0.000 <sup>a</sup>	0.78 ± 0.636	1.13 ± 0.625	0.010 <sup>a</sup>	0.66 ± 0.848	1.14 ± 0.047	0.004 <sup>a</sup>
Appetite	1.13 ± 0.674	1.57 ± 0.662	0.000 <sup>a</sup>	0.87 ± 0.968	1.78 ± 0.560	0.000 <sup>a</sup>	1.16 ± 0.817	1.70 ± 0.580	0.000 <sup>a</sup>
Difficulty swallowing	1.56 ± 0.664	1.52 ± 0.720	0.890	1.42 ± 0.723	1.49 ± 0.787	0.431	1.54 ± 0.734	1.74 ± 0.600	0.112
Diarrhea	1.83 ± 0.376	1.87 ± 0.339	0.590	1.69 ± 0.733	1.80 ± 0.505	0.823	1.78 ± 0.582	1.82 ± 0.523	0.754
Heartburn or belch	1.37 ± 0.708	1.78 ± 0.502	0.001 <sup>a</sup>	0.73 ± 0.863	1.73 ± 0.539	0.000 <sup>a</sup>	0.80 ± 0.756	1.38 ± 0.805	0.000 <sup>a</sup>
Postprandial discomfort	1.46 ± 0.693	1.69 ± 0.609	0.052	1.24 ± 0.957	1.07 ± 0.915	0.321	1.52 ± 0.677	1.22 ± 0.790	0.048 <sup>a</sup>
Abdominal pain	1.54 ± 0.770	1.65 ± 0.649	0.547	1.49 ± 0.787	1.60 ± 0.720	0.481	1.60 ± 0.495	1.70 ± 0.505	0.241
Vomiting	1.48 ± 0.771	1.69 ± 0.577	0.199	1.49 ± 0.757	1.67 ± 0.674	0.188	1.68 ± 0.471	1.70 ± 0.463	0.830
General fatigue	1.80 ± 0.528	1.76 ± 0.581	0.775	1.82 ± 0.387	1.78 ± 0.420	0.600	1.92 ± 0.274	1.84 ± 0.370	0.221
Dizziness	1.80 ± 0.562	1.83 ± 0.505	0.757	1.89 ± 0.318	1.93 ± 0.252	0.461	1.74 ± 0.600	1.80 ± 0.535	0.585
Intestinal obstruction	1.65 ± 0.555	1.78 ± 0.502	0.138	1.56 ± 0.693	1.64 ± 0.679	0.417	1.66 ± 0.519	1.68 ± 0.513	0.834
Performance status	1.54 ± 0.719	1.63 ± 0.653	0.502	1.64 ± 0.743	1.71 ± 0.661	0.740	1.58 ± 0.642	1.70 ± 0.463	0.498
Early dumping syndrome	1.54 ± 0.665	1.70 ± 0.603	0.114	1.40 ± 0.720	1.62 ± 0.650	0.097	1.42 ± 0.673	1.60 ± 0.728	0.072
Late dumping syndrome	1.78 ± 0.502	1.70 ± 0.537	0.377	1.73 ± 0.688	1.73 ± 0.580	0.538	1.86 ± 0.452	1.72 ± 0.479	0.050
Physical condition	1.67 ± 0.673	1.72 ± 0.627	0.637	1.80 ± 0.548	1.64 ± 0.712	0.259	1.76 ± 0.517	1.76 ± 0.476	0.853
Wound pain, present	1.57 ± 0.690	1.83 ± 0.423	0.031 <sup>a</sup>	1.53 ± 0.815	1.73 ± 0.580	0.326	1.62 ± 0.490	1.78 ± 0.465	0.059
Wound pain, postoperative	1.07 ± 0.866	1.07 ± 0.866	1.000	1.18 ± 0.716	1.18 ± 0.716	1.000	1.22 ± 0.764	1.22 ± 0.764	1.000
Satisfaction with operation	1.57 ± 0.690	1.72 ± 0.596	0.197	1.69 ± 0.557	1.76 ± 0.484	0.597	1.66 ± 0.688	1.72 ± 0.536	0.599
Recommendation to others	1.41 ± 0.714	1.59 ± 0.567	0.206	1.29 ± 0.787	1.42 ± 0.690	0.467	1.46 ± 0.706	1.54 ± 0.646	0.606
Mood or feeling	1.67 ± 0.673	1.63 ± 0.708	0.802	1.67 ± 0.707	1.71 ± 0.695	0.636	1.70 ± 0.463	1.66 ± 0.519	0.780
Total	1.45 ± 0.748	1.67 ± 0.612	0.015 <sup>a</sup>	1.36 ± 0.825	1.61 ± 0.663	0.007 <sup>a</sup>	1.42 ± 0.743	1.65 ± 0.609	0.011 <sup>a</sup>

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

than esophagojejunostomy. It has been shown that independent prognostic factors such as ensuring radical cure, maintaining food passage in the duodenum, and less excision of the stomach, are very important for postoperative QOL<sup>[17,18]</sup>. A high incidence of

reflux symptoms after simple esophagogastronomy has prompted the development of several novel reconstructions to prevent reflux<sup>[12,19-21]</sup>. Previous reports have shown that proximal gastrectomy is more likely to produce complications such as heartburn and poor

**Table 6 Comparison of evaluation scores of 6 and 24 mo postoperative Spitzer Index among the three groups (mean ± SD)**

Factors	EA group	EP group	P	EA group	EE group	P	EP group	EE group	P
6 mo									
Activity	1.74 ± 0.556	1.80 ± 0.548	0.403	1.74 ± 0.556	1.76 ± 0.476	0.920	1.80 ± 0.548	1.76 ± 0.476	0.347
Daily living	1.85 ± 0.359	1.78 ± 0.420	0.344	1.85 ± 0.359	1.84 ± 0.422	0.940	1.78 ± 0.420	1.84 ± 0.422	0.326
Health	1.65 ± 0.588	1.62 ± 0.535	0.630	1.65 ± 0.588	1.76 ± 0.517	0.266	1.62 ± 0.535	1.76 ± 0.517	0.115
Support	1.78 ± 0.462	1.71 ± 0.506	0.467	1.78 ± 0.462	1.74 ± 0.487	0.661	1.71 ± 0.506	1.74 ± 0.487	0.767
Outlook	1.76 ± 0.473	1.73 ± 0.539	0.946	1.76 ± 0.473	1.78 ± 0.465	0.790	1.73 ± 0.539	1.78 ± 0.465	0.752
24 mo									
Activity	1.72 ± 0.492	1.71 ± 0.506	0.835	1.72 ± 0.492	1.82 ± 0.388	0.674	1.71 ± 0.506	1.82 ± 0.388	0.802
Daily living	1.87 ± 0.339	1.84 ± 0.367	0.714	1.87 ± 0.339	1.90 ± 0.364	0.724	1.84 ± 0.367	1.90 ± 0.364	0.273
Health	1.76 ± 0.473	1.76 ± 0.484	0.352	1.76 ± 0.473	1.90 ± 0.303	0.557	1.76 ± 0.484	1.90 ± 0.303	0.947
Support	1.80 ± 0.451	1.80 ± 0.457	0.367	1.80 ± 0.451	1.84 ± 0.370	0.839	1.80 ± 0.457	1.84 ± 0.370	0.583
Outlook	1.83 ± 0.376	1.76 ± 0.529	0.892	1.83 ± 0.376	1.70 ± 0.614	0.692	1.76 ± 0.529	1.70 ± 0.614	0.793

**Table 7 Comparison of evaluation scores of Spitzer Index at 6 and 24 mo postoperatively between the same group (mean ± SD)**

Factors	EA group		P	EP group		P	EE group		P
	6 mo	24 mo		6 mo	24 mo		6 mo	24 mo	
Activity	1.74 ± 0.556	1.72 ± 0.492	0.583	1.80 ± 0.548	1.71 ± 0.506	0.164	1.76 ± 0.476	1.82 ± 0.388	0.588
Daily living	1.85 ± 0.359	1.87 ± 0.339	0.782	1.78 ± 0.420	1.84 ± 0.367	0.422	1.84 ± 0.422	1.90 ± 0.364	0.350
Health	1.65 ± 0.588	1.76 ± 0.473	0.342	1.62 ± 0.535	1.76 ± 0.484	0.177	1.76 ± 0.517	1.90 ± 0.303	0.148
Support	1.78 ± 0.462	1.80 ± 0.451	0.813	1.71 ± 0.506	1.80 ± 0.457	0.325	1.74 ± 0.487	1.84 ± 0.370	0.301
Outlook	1.76 ± 0.473	1.83 ± 0.376	0.444	1.73 ± 0.539	1.76 ± 0.529	0.807	1.78 ± 0.465	1.70 ± 0.614	0.701

appetite, and worsen nutritional status compared to other types of gastrectomy<sup>[22]</sup>. To prevent or minimize postgastrectomy complications, proximal gastrectomy with an interposed jejunal pouch has been advocated as an organ-preserving strategy to improve QOL<sup>[13,23-25]</sup>. However, there some studies have shown that patients with an interposed jejunal pouch need a second operation because of food stasis or disordered gastric emptying, and the length of the jejunal pouch is still under discussion<sup>[26-28]</sup>.

To avoid postoperative symptoms, there is no consensus on the need for pyloroplasty after proximal gastrectomy. Our study suggested that pyloroplasty improved gastric emptying and decreased stomach stasis. Other studies have shown that pyloroplasty might significantly relieve gastric distention and speed up gastric emptying<sup>[29,30]</sup>. Our data showed that most patients suffered from heartburn and postprandial abdominal fullness after proximal gastrectomy. However, patients in the EA group showed significantly less heartburn and postprandial abdominal fullness compared to the other groups. Pyloroplasty as a draining procedure helps the patient have fewer complications such as gastric distention, nausea and vomiting, and promotes faster gastric emptying<sup>[31]</sup>. Patients with pyloroplasty may recover 80% of the dietary volume in the short term after surgery. In addition, our study found that pyloroplasty also helped to recover or improve body weight in the long term, postoperatively. Our study suggested that patients treated with an EA procedure had a better QOL than the other groups, however, this was correlated with pyloroplasty and promoted gastric emptying to some extent.

The EA procedure decreased the postoperative reflux symptoms in the long term. This simple and

safe technique does not result in postgastrectomy syndrome. The mortality rate was zero and the absence of early postoperative complications highlighted the safety of this procedure. Thus, proximal gastrectomy reconstruction by EA provided excellent clinical results in patients with proximal gastric cancer.

After proximal gastrectomy, body weight decreased (approximately 5-10 kg) below baseline after the first few postoperative months, and later, the weight stabilized if there were no tumor recurrence, and recovered slowly almost to baseline. Our study showed that even 2 years postoperatively, the EA group showed a significantly better recovered or improved body weight than the other groups. Seven patients in the EA group recovered 100%, and more than two-thirds of patients maintained at least 85% of baseline weight 2 years after surgery. In summary, the EA procedure benefited the patients with upper third gastric cancer in terms of body weight.

Some patients after proximal gastrectomy suffered from reduced meal size, due to microgastrria. To avoid microgastrria, the esophagogastrostomy performed by the EE procedure may help to maintain the longest distance from the pylorus to the anastomotic stoma. The center rod of the circular stapler is pierced through the left end of the staple line of the stomach. The longest distance from the pylorus to the esophagogastrostomy site can be found in this position. The longer distance will reduce microgastrria and also decrease the tension of the stoma.

There were some limitations in our study. Firstly, the patients were not at the same stage in terms of their gastric cancer; 32.2% were early stage patients. Although QOL in the EA group was better than in the other two groups, whether QOL in the EA group is better than that for other reconstruction methods, or if it reaches

the threshold of normal QOL requires further study. Whether QOL in the EA group will remain improved after long-term follow-up (5-year or longer) requires further research.

The clinical implications of this study showed that the EA procedure seems to confer clinical benefit in terms of postoperative QOL, especially in the form of improved meal intake, reduced gastroesophageal reflux, and improved body weight, however, overall survival rate and surgical results are the same using all three procedures. Our data suggest that, to avoid gastroesophageal reflux and improve QOL in patients with upper third gastric cancer after proximal gastrectomy, the EA procedure for reconstruction using a stapler is safe and feasible for esophagogastrectomy. However, better reconstruction methods are still required to decrease postoperative symptoms in the future.

## COMMENTS

### Background

Given equivalent results with regards to survival, the impact of anastomotic methods on quality of life (QOL) becomes even more important. QOL is the main outcome for judging the efficacy of treatment modalities when no overall survival differences are demonstrated.

### Research frontiers

There is still no consensus on how to choose a reconstruction method for proximal gastrectomy in patients with upper third gastric cancer. This study was designed to compare in detail different methods of esophagogastrectomy.

### Innovations and breakthroughs

No previous studies have assessed different methods of esophagogastrectomy in terms of QOL. The aim of this study was to assess outcome in terms of QOL in patients after proximal gastrectomy, by comparing three methods of esophagogastrectomy, in order to find the optimal reconstruction method that offers the optimal postoperative QOL.

### Applications

The clinical implications of this study showed that the anterior wall end-to-side anastomosis combined with pyloroplasty (EA) procedure seems to confer clinical benefit in terms of postoperative QOL, especially in the form of improved meal intake, reduced gastroesophageal reflux, and improved body weight.

### Terminology

The Spitzer QOL Index, which reflects the patient's postoperative status of health perception, is a global health assessment with a valid questionnaire, which includes five items: activity, daily living, health, support of family and friends, and outlook.

### Peer review

The authors evaluated postoperative QOL in patients with upper third gastric cancer, who received proximal gastrectomy with additional esophagogastrectomy. The results suggest that to avoid gastroesophageal reflux and improve QOL for patients with upper third gastric cancer after proximal gastrectomy, the EA procedure of reconstruction using a stapler is safe and feasible for esophagogastrectomy.

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## Enigma of primary aortoduodenal fistula

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### INTRODUCTION

Primary aortoenteric fistula is an extremely rare but potentially fatal cause of gastrointestinal bleeding. We present the case of a 49-year-old man with no past history of abdominal aortic aneurysm, who presented with recurrent gastrointestinal bleeding.

### CASE REPORT

A 49-year-old man was admitted to the emergency room with clinical signs of gastrointestinal bleeding (massive hematemesis, tachycardia of 140 bpm, and blood pressure of 70/40 mmHg). His hemoglobin was 5.5 g/dL. The patient was resuscitated with intravenous fluids and transfusion of 4 U of packed red blood cells. His medical history included multiple sclerosis with minimal neurological deficit, which was treated with Copaxone and Novitropan. He had had no previous surgery or peptic ulcer disease.

An initial evaluation with upper endoscopy and colonoscopy presented as normal. Twenty-four hours later, the patient had recurrent bleeding and required a transfusion of an additional 2 U of packed red blood cells. Repeated upper endoscopy showed blood in the stomach and duodenum, but no source of bleeding could be found. The patient was taken to the angiography suite, where flash aortography and visceral angiography showed no active extravasation of contrast material.

The left gastric artery was embolized empirically with fibered platinum coils, which was tolerated well by the patient. Blood pressure began to normalize immediately after embolization, and the patient's condition stabilized. To evaluate the source of bleeding, he underwent CT examination and capsule endoscopy, which appeared to be normal.

Technetium-99m-red blood cell scintigraphy was used to identify the site of bleeding during the next short episode of hematochezia, but it was inconclusive for bleeding. On a subsequent occasion, the patient displayed an episode of fresh blood in a nasogastric tube. Upper endoscopy (session four) revealed multiple gastric erosions. The patient became unstable with clinical

### Abstract

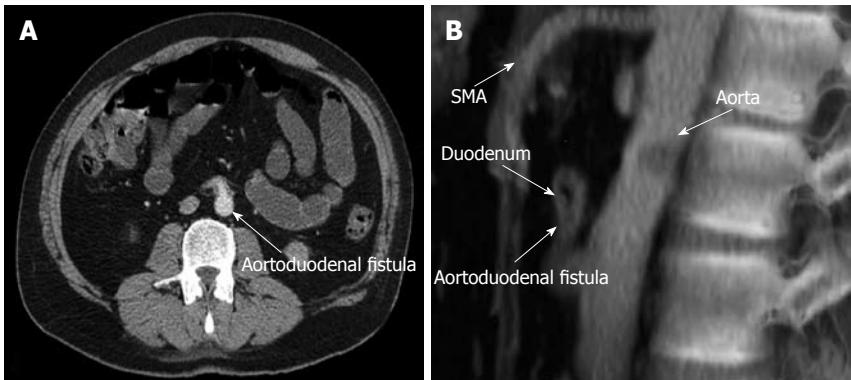
A diagnosis of primary aortoenteric fistula is difficult to make despite a high level of clinical suspicion. It should be considered in any elderly patient who presents with upper gastrointestinal bleeding in the context of a known abdominal aortic aneurysm. We present the case of young man with no history of abdominal aortic aneurysm who presented with massive upper gastrointestinal bleeding. Initial misdiagnosis led to a delay in treatment and the patient succumbing to the illness. This case is unique in that the fistula formed as a result of complex atherosclerotic disease of the abdominal aorta, and not from an aneurysm.

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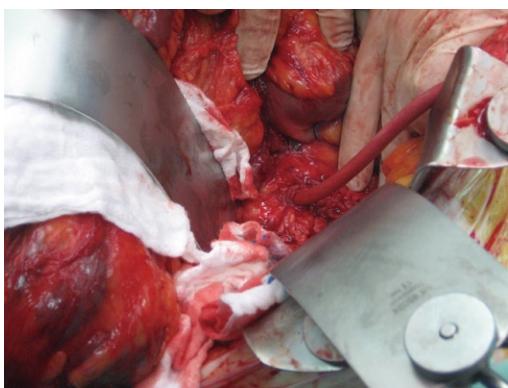
**Key words:** Aortoduodenal fistula; Gastrointestinal hemorrhage; Computed tomography; Aortography

**Peer reviewers:** Dr. Limas Kupcinskas, Professor, Gastroenterology of Kaunas University of Medicine, Mickevicius 9, Kaunas LT 44307, Lithuania; Toru Hiyama, MD, PhD, Health Service Center, Hiroshima University, 1-7-1 Kagamiyama, Higashihiroshima 739-8521, Japan

Bala M, Sosna J, Appelbaum L, Israeli E, Rivkind AI. Enigma of primary aortoduodenal fistula. *World J Gastroenterol* 2009; 15(25): 3191-3193 Available from: URL: <http://www.wjgnet.com>



**Figure 1 CT and lateral aortography.** A: CT showing direct extravasation of contrast material from the aorta into the duodenal lumen (arrow); B: Lateral aortography showing aortoduodenal fistula. Note there is no evidence of abdominal aneurysm.



**Figure 2 Perioperative findings of primary fistula between the third part of the duodenum and aorta.** A Foley catheter was inserted to control the bleeding.

signs of ongoing bleeding. Exploratory laparotomy and gastrotomy were performed with undersuturing of acute gastric ulcers (most were located in the fundus). Intraoperative endoscopy showed no other site of bleeding throughout the stomach, duodenum and 1.5 m of proximal small intestine. Twenty-four hours perioperatively, the patient received 10 red packed cells and other blood supplements and high-dose omeprazole (Omeprazole infusion of 8 mg/h for 72 h, following 40 mg of omeprazole orally per day). The recovery was uneventful and 8 d after surgery, the patient was observed in the regular ward.

Ten days postoperatively, the patient had another episode of bleeding accompanied by syncope with hematochezia and hematemesis. Since he had been receiving fluid resuscitation, blood pressure was undetectable. The patient underwent full cardiopulmonary resuscitation (CPR) and was sent to the operating theater. Repeated angiography and CT revealed an aortoduodenal fistula that had not been detected 3 wk previously (Figure 1).

Exploratory laparotomy was performed and the aortoduodenal fistula was found between the anterior wall of the aorta and the third part of the duodenum. The bowel was full of fresh blood. The fistula was disconnected and a Foley catheter was inserted into the aorta to control the bleeding (Figure 2). This occurred whilst the patient was in prolonged CPR, with a clamp on the descending aorta through left thoracotomy. After 2 h resuscitation in the operating theater, the patient

developed disseminated intravascular coagulation and developed bradycardia and acidosis. His blood pressure and urine output dropped drastically and he died on the operating table from irreversible hemorrhagic shock.

Pathological examination of periaortic tissue revealed non-specific inflammation in the connective tissue, fibrin and blood cells. No postmortem examination was performed.

## DISCUSSION

The diagnosis and treatment of aortoenteric fistulas are difficult. Nevertheless, in a patient with hematemesis and melena who has undergone an aortobifemoral bypass or aortic interposition grafting without esophagogastro-duodenal pathology, a diagnosis of aortoenteric fistula should not be overlooked<sup>[1]</sup>. Primary aortoduodenal fistulas are nearly always associated with abdominal aortic aneurysm, mostly atherosclerotic. In the present clinical case, the available clinical, instrumental and radiological supports made such a diagnosis feasible.

The penetrating aortic ulcer has been recognized recently as an independent pathological entity. It may penetrate through the aortic wall, which leads to fistulas into adjacent organs. Certik *et al*<sup>[2]</sup> have reported the case of a 78-year-old woman who was admitted to hospital with massive gastrointestinal hemorrhage. Endoscopy did not reveal the cause of hemorrhage. The diagnosis was made by CT that showed a primary aortoduodenal fistula without aortic aneurysm, and the patient underwent a successful operation. During urgent surgery, the penetrating atherosclerotic ulcer was found to be the cause of the aortoduodenal communication.

The classic presentation of an aortoduodenal fistula is that of a "herald bleed" (brief, with spontaneous resolution), followed anywhere from hours to weeks later by a massive upper gastrointestinal bleed. A high index of suspicion and rapid diagnosis is imperative as exsanguinating hemorrhage may occur. An aortoduodenal fistula or erosion is rarely seen endoscopically<sup>[3]</sup>. Endoscopy may disclose another cause of bleeding, as was discovered in our patient. Therefore, normal findings, a positive finding of gastritis or ulcers without active bleeding do not rule out aortoduodenal fistula. Careful inspection of the distal duodenum should be performed in the setting of unexplained torrential bleeding. More commonly, fresh blood or clots may be found in the third portion of the duodenum.

CT with contrast is the most suitable diagnostic test and provides a reported detection rate of 30%-61%<sup>[4-6]</sup>. CT may show the abnormal communication between the aorta and the intestine. Angiography is seldom a helpful procedure, unless a pseudoaneurysm is seen<sup>[7]</sup>, and the diagnosis is usually made or confirmed by laparotomy. Surgical repair is the only therapeutic option.

In summary, a diagnosis of primary aortoenteric fistula should be considered in any patient known to have abdominal aortic aneurysm or lower abdominal pain associated with midline mass and upper gastrointestinal bleeding of unexplained etiology. A herald bleed is an indication for prompt intervention. Endoscopy is the first step in diagnosis, and CT may be used for confirmation. An emergency exploratory laparotomy should be performed as soon as the diagnosis is considered.

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LETTERS TO THE EDITOR

## Pentoxifylline: A first line treatment option for severe alcoholic hepatitis and hepatorenal syndrome?

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### Abstract

Although favourable results of pentoxifylline (PTX) used in treatment of severe alcoholic hepatitis patients with a Maddrey discriminant function score  $\geq 32$  have been previously reported, it is not currently recommended as a first line treatment for alcoholic hepatitis owing to lack of evidence for its efficacy as compared to the standard treatment with corticosteroids. In a very recent issue of *World Journal of Gastroenterology*, Dr. De BK and colleagues compared for the first time the two treatment modalities head to head in a randomized controlled study, demonstrating the advantage of PTX over corticosteroids in terms of patients' survival and risk-benefit profile. The advantage of PTX over corticosteroids in survival of patients with severe alcoholic hepatitis was found to be related to the prevention of hepatorenal syndrome in their study. This study raises the question of the use of PTX as a standard treatment for severe alcoholic hepatitis. Considering the fact that PTX presented a spectacular efficiency in prevention of hepatorenal syndrome in their study as well as that previous studies have shown that this effect is possibly related to a primary renoprotective action because it is irrelevant of tumor necrosis factor- $\alpha$  synthesis inhibition or improved liver function, we tempted to speculate that PTX might be an effective option for prevention and/or treatment of hepatorenal syndrome complicating other forms of advanced liver disease. This attractive theory remains to be elucidated by pressing future studies in view of the lack of effective treatment modalities for hepatorenal syndrome.

### TO THE EDITOR

We read with great interest the article recently published by Dr. De and colleagues in *World Journal of Gastroenterology*<sup>[1]</sup>, who evaluated in a randomized double-blind controlled study the advantage of pentoxifylline (PTX) over prednisolone in treatment of severe alcoholic hepatitis [Maddrey discriminant function (DF) score  $\geq 32$ ]. The most important observation was the significantly reduced mortality of patients after treatment with PTX (14.71%) as compared to those after treatment with prednisolone (35.29%,  $P = 0.04$ ). Reduced mortality in patients after treatment with PTX was found to be related to a significant reduction in the development of hepatorenal syndrome. Among patients who died, hepatorenal syndrome developed in 50% of prednisolone-treated patients but in none of PTX-treated patients.

Current guidelines of the American College of Gastroenterology recommend the use of glucocorticosteroids in treatment of patients with severe alcoholic hepatitis as defined by the Maddrey score (DF  $\geq 32$ )<sup>[2,3]</sup>. Primary use of PTX in treatment of severe alcoholic hepatitis patients is not recommended due to the lack of evidence for improvement in patient-oriented outcomes<sup>[2]</sup>. Also, the early switch of corticosteroids to PTX, if no improvement in bilirubin is seen after 7 d of treatment, has been proved to be an inefficient treatment strategy<sup>[4]</sup>. However, a number of French experts in the field consider PTX a reasonable alternative to corticosteroids for severe acute alcoholic hepatitis based on the

favourable results of previous studies comparing PTX with placebo<sup>[5,6]</sup>.

Specifically, up to now, the use of PTX in treatment of severe alcoholic hepatitis has been supported by two clinical studies<sup>[5,6]</sup>. The first one was conducted in 1991 by McHutchison *et al*<sup>[5]</sup>, in patients with severe alcoholic hepatitis (defined as DF score  $\geq 32$ ), which showed that PTX could reduce the development of hepatorenal syndrome and the mortality, in comparison to those receiving placebo. These findings were confirmed in 2000 by Akriavidis *et al*<sup>[6]</sup> in a double-blind placebo-controlled trial, which showed that 24% of PTX-treated patients and 46.1% of control patients died during hospitalization. The survival benefit of PTX was found to be related to a significant reduction in the development of hepatorenal syndrome. Among the patients who died, hepatorenal syndrome developed in 50% of PTX-treated patients and in 91.7% of placebo-treated patients. The study by De *et al*<sup>[1]</sup> is the first to compare PTX and corticosteroids head to head in a randomized controlled manner. The results of this study, demonstrating the advantage of PTX over corticosteroids, raise the question of the use of PTX as a standard treatment modality for severe alcoholic hepatitis.

A second issue we would like to comment on is the fact that the advantage of PTX in survival of patients with severe alcoholic hepatitis is clearly related to the prevention of hepatorenal syndrome<sup>[1,5,6]</sup>. The available data do not show the evident mechanism underlying this beneficial effect of PTX and only speculations could be made on this matter. The use of PTX in treatment of alcoholic hepatitis is based on its ability to inhibit the synthesis of tumor necrosis factor (TNF)- $\alpha$ , which is considered a pivotal mediator of alcohol-induced liver injury<sup>[6,7]</sup>. Although the authors did not assess in this study the immunological and inflammatory status (e.g. TNF- $\alpha$ ) of their patients, it has been previously reported that the prevention of hepatorenal syndrome and survival advantage in patients with severe alcoholic hepatitis after treatment with PTX are not associated with decreased circulating TNF- $\alpha$  levels or improved

liver function<sup>[6]</sup>. These findings give an alternative explanation for the positive effect of PTX on renal function of alcoholic hepatitis patients, which is the beneficial action of PTX on renal microcirculation and hemodynamics. This potential primary protective effect of PTX on renal function and its repeatedly confirmed efficacy on prevention of hepatorenal syndrome in severe alcoholic hepatitis patients (in this study, PTX prevented the development of hepatorenal syndrome) tempt us to speculate that it could potentially be used in the prevention and/or treatment of hepatorenal syndrome complicating other forms of advanced liver disease. To the best of our knowledge, this possibility has not been investigated up to now and appears to be an attractive new field for experimental and clinical research, given the current difficulties in the management of patients with hepatorenal syndrome.

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## Meetings

### Events Calendar 2009

January 12-15, 2009  
Hyatt Regency San Francisco, San Francisco, CA  
Mouse Models of Cancer

January 21-24, 2009  
Westin San Diego Hotel, San Diego, CA  
Advances in Prostate Cancer Research

February 3-6, 2009  
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)  
Second AACR Conference  
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009  
Hyatt Regency Boston, Boston, MA  
Translation of the Cancer Genome

February 8-11, 2009  
Westin New Orleans Canal Place, New Orleans, LA  
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009  
Hong Kong Convention and Exhibition Centre, Hong Kong, China  
19th Conference of the APASL  
<http://www.apasl2009hongkong.org/en/home.aspx>

February 27-28, 2009  
Orlando, Florida  
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009  
Vienna, Austria  
EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease  
[www.easl.ch/vienna2009](http://www.easl.ch/vienna2009)

March 13-14, 2009  
Phoenix, Arizona  
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009  
Marriott Wardman Park Hotel  
Washington, DC  
13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009  
Glasgow, Scotland  
British Society of Gastroenterology (BSC) Annual Meeting  
Email: bsg@mailbox.ulcc.ac.uk

April 8-9, 2009  
Silver Spring, Maryland  
2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009  
Colorado Convention Center, Denver, CO  
AACR 100th Annual Meeting 2009

April 22-26, 2009  
Copenhagen, Denmark  
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)  
<http://www.easl.ch/>

May 17-20, 2009  
Denver, Colorado, USA  
Digestive Disease Week 2009

May 29-June 2, 2009  
Orange County Convention Center Orlando, Florida  
45th ASCO Annual Meeting  
[www.asco.org/annualmeeting](http://www.asco.org/annualmeeting)

May 30, 2009  
Chicago, Illinois  
Endpoints Workshop: NASH

May 30-June 4, 2009  
McCormick Place, Chicago, IL  
DDW 2009  
<http://www.ddw.org>

June 17-19, 2009  
North Bethesda, MD  
Accelerating Anticancer Agent Development

June 20-26, 2009  
Flims, Switzerland  
Methods in Clinical Cancer Research (Europe)

June 24-27 2009  
Barcelona, Spain  
ESMO Conference: 11th World Congress on Gastrointestinal Cancer  
[www.worldgicancer.com](http://www.worldgicancer.com)

June 25-28, 2009  
Beijing International Convention Center (BICC), Beijing, China  
World Conference on Interventional Oncology  
<http://www.chinamed.com.cn/wcio2009/>

July 5-12, 2009  
Snowmass, CO, United States  
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009  
Aspen, CO, United States  
Molecular Biology in Clinical Oncology

August 1-7, 2009  
Vail Marriott Mountain Resort, Vail, CO, United States  
Methods in Clinical Cancer Research

August 14-16, 2009  
Bell Harbor Conference Center, Seattle, Washington, United States  
Practical Solutions for Successful Management  
<http://www.asge.org/index.aspx?id=5040>

September 23-26, 2009  
Beijing International Convention Center (BICC), Beijing, China  
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists (IASGO)  
<http://iasgo2009.org/en/index.shtml>

September 27-30, 2009  
Taipei, China  
Asian Pacific Digestive Week  
<http://www.apdwcongress.org/2009/index.shtml>

October 7-11, 2009  
Boston Park Plaza Hotel and Towers, Boston, MA, United States  
Frontiers in Basic Cancer Research

October 13-16, 2009  
Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States  
Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009  
Versailles, France  
Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009  
Boston, MA, United States  
The Liver Meeting

November 15-19, 2009  
John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States  
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009  
London, UK  
Gastro 2009 UEGW/World Congress of Gastroenterology  
[www.gastro2009.org](http://www.gastro2009.org)



### Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.



## Instructions to authors

### GENERAL INFORMATION

*World Journal of Gastroenterology* (*World J Gastroenterol* ISSN 1007-9327 CN 14-1219/R) is a weekly open-access (OA) peer-reviewed journal supported by an editorial board consisting of 1179 experts in gastroenterology and hepatology from 60 countries.

The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

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- 2 Lin GZ, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

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- 3 Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 4 Diabetes Prevention Program Research Group. Hypertension,

insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

*Both personal authors and an organization as author*

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

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- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900 DOI:10.1097/00003086-20020800-00026]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

## Books

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

*Chapter in a book (list all authors)*

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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- 13 **Hardden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming-EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

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- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc, assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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[1]Passed away on October 20, 2007

[2]Passed away on June 11, 2007

[3]Passed away on June 14, 2008



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### INTRODUCTION

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# What every gastroenterologist needs to know about common anorectal disorders

Moonkyung Cho Schubert, Subbaramiah Sridhar, Robert R Schade, Steven D Wexner

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## INTRODUCTION

Anorectal complaints are very common and are caused by a variety of mostly benign anorectal disorders. Many anorectal conditions may be successfully treated by primary care physicians in the outpatient setting, but patients tend not to seek medical attention due to embarrassment or fear of cancer. As a result, patients frequently present with advanced disease after experiencing significant decreases in quality of life. A number of patients with anorectal complaints are referred to gastroenterologists. However, gastroenterologists' knowledge and experience in approaching these conditions may not be sufficient. This article can serve as a guide to gastroenterologists to recognize, evaluate, and manage medically or non-surgically common benign anorectal disorders, and to identify when surgical referrals are most prudent. A review of the current literature is performed to evaluate comprehensive clinical pearls and management guidelines for each topic. Topics reviewed include hemorrhoids, anal fissures, anorectal fistulas and abscesses, and pruritus ani.

## Abstract

Anorectal complaints are very common and are caused by a variety of mostly benign anorectal disorders. Many anorectal conditions may be successfully treated by primary care physicians in the outpatient setting, but patients tend not to seek medical attention due to embarrassment or fear of cancer. As a result, patients frequently present with advanced disease after experiencing significant decreases in quality of life. A number of patients with anorectal complaints are referred to gastroenterologists. However, gastroenterologists' knowledge and experience in approaching these conditions may not be sufficient. This article can serve as a guide to gastroenterologists to recognize, evaluate, and manage medically or non-surgically common benign anorectal disorders, and to identify when surgical referrals are most prudent. A review of the current literature is performed to evaluate comprehensive clinical pearls and management guidelines for each topic. Topics reviewed include hemorrhoids, anal fissures, anorectal fistulas and abscesses, and pruritus ani.

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**Key words:** Anal fissures; Anorectal disease; Anorectal fistulas and abscesses; Hemorrhoids; Pruritus ani

## HEMORRHOIDS

The estimated prevalence rate of symptomatic hemorrhoids in the United States is 4.4% of the adult population; more than one million individuals annually are affected by hemorrhoidal conditions<sup>[1,2]</sup>. Hemorrhoids are cushions of nonpathologic vascular tissue in the anal canal, which microscopically are sinusoids because they do not have any muscle as do veins<sup>[3]</sup>. Hemorrhoidal tissue is thought to contribute to anal continence because 15%-20% of resting anal pressure derives from these cushions. Hemorrhoids may protect the sphincter during defecation, and could operate as plugs to permit the anus to completely close while at rest. Three main cushions are found in the left lateral, the right anterolateral, and the right posterolateral portions of the anal canal. The symptoms of hemorrhoidal disease are caused by pathologic and dilated changes in hemorrhoidal tissue.

## Etiology

Proposed etiologic factors include vascular congestion and mucosal prolapse<sup>[4]</sup>. Vascular congestion could derive from prolonged straining or increased intra-abdominal pressure due to ascites, obesity, or pregnancy. Mucosal prolapse may develop secondary to derangement of the internal sphincter or through aging causing the anatomic structures supporting the muscularis submucosa to weaken, leading to prolapse of the hemorrhoidal tissue<sup>[5]</sup>. Multiple studies have shown elevated anal resting pressure in patients with hemorrhoids<sup>[1,6]</sup>. Whether the elevated resting pressure is caused by or is due to enlarged hemorrhoids is unknown, but resting tone does become normal after a hemorrhoidectomy.

## Symptoms

Patients often self-refer with symptoms of itching, pain, or bleeding per the rectum. To the general population, anything problematic around the anus is suspected to be hemorrhoids. Internal hemorrhoids may prolapse or bleed, but rarely become painful unless they develop thrombosis or necrosis. Thus, anal pain usually suggests other pathology and mandates closer investigation. As many as 20% of patients with hemorrhoids have concomitant anal fissure(s). Usually, painless bright red bleeding that stains the water in the toilet occurs from internal hemorrhoids. This bleeding is arterial, from presinusoidal arterioles, and is mostly associated with bowel movements where the stool is itself brown. If rectal bleeding is not typical for hemorrhoidal bleeding as described, a prompt and thorough medical evaluation is warranted. Thrombosed external hemorrhoids may cause significant pain because the anoderm is richly innervated which is exactly why external hemorrhoids should not be ligated or excised without adequate local anesthetics. Skin tags are often confused with symptomatic hemorrhoids. A skin tag is redundant fibrotic skin at the anal verge, often persisting as the residual of a thrombosed external hemorrhoid. It is important to note that there is no increased risk of cancer in hemorrhoids.

## Classification

Hemorrhoidal conditions are classified according to their location. External hemorrhoids are situated distal to the dentate line and are covered by anoderm that is sensitive to touch, temperature, and stretch because of innervation by somatic nerves. The dentate line is the junction of ectoderm and endoderm, and therefore represents an important mark between two distinct origins of venous and lymphatic drainage, nerve supply, and epithelial lining. Internal hemorrhoids are covered by columnar or transitional epithelium, are located proximal to the dentate line, and are graded based on the degree of the prolapse<sup>[7,8]</sup>. First-degree hemorrhoids may bleed and may bulge into the anal canal and may prolapse beyond the dentate line on straining. Second-degree hemorrhoids prolapse through the anus but spontaneously reduce. Third-degree hemorrhoids

prolapse through the anal canal and require manual reduction. Fourth-degree hemorrhoids prolapse, but are irreducible, and thus are at risk for strangulation. However, most hemorrhoids are a combined type of internal and external hemorrhoids. Prognosis and treatment are mostly based on the classification.

## Diagnosis

Patients who complain of hemorrhoids need a careful evaluation to exclude other conditions. Either the prone or the left lateral decubitus position can be used to evaluate the anal area, although the lateral position is easier for pregnant patients and those patients with severe chronic obstructive pulmonary disease. Digital, anoscopic, and sigmoidoscopic examination are important initial evaluations. A thorough examination of the anorectal area is required. Inspection is performed by gentle retraction of the buttocks. The color or condition of the skin should be examined for findings such as swelling, induration, fissure, draining sinuses, or mass. The sacrococcygeal region and the perianal skin should be examined. Palpation with a lubricated gloved index finger begins at the anal orifice, and then proceeds circumferentially around the anal canal through the lower rectum to identify any abscesses, tumors, or sphincter defects. Also, evaluating resting and/or squeezing anal pressures by asking the patient to squeeze will provide more information on the anal sphincter and the puborectalis muscle. Internal hemorrhoids are generally difficult to palpate unless thrombosed or very large. An anoscopy is done to visualize internal hemorrhoids, which bulge into the lumen of the anoscope when the patient strains. A full examination of the colon with a barium enema or colonoscopy is considered if there are no compatible findings of hemorrhoidal disease, especially in patients older than 40 years.

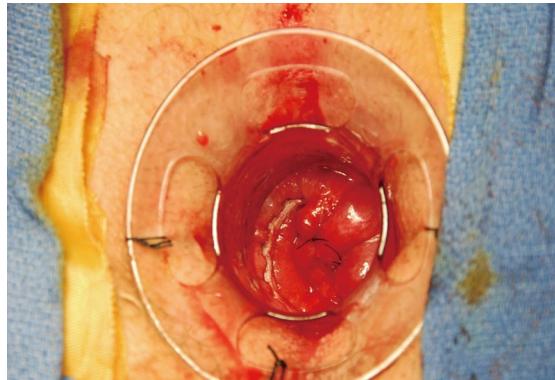
## Treatment

Because hemorrhoids are a normal part of anorectal anatomy, treatment is only indicated if they become symptomatic. However, in the general management of hemorrhoids, colorectal surgeons agree that all painful thrombosed hemorrhoids (Figure 1) should be excised. Some patients present at a time after thrombosis when symptoms have actually begun to subside. Excision is not mandatory in these cases, especially in the absence of erosion or significant tenderness to touch. Initial medical management is recommended for all but the most advanced cases. As a conservative treatment, the almost-universal recommendations are to add dietary fiber<sup>[9]</sup>, avoid straining during defecation, and to utilize sitz baths two to four times a day<sup>[10]</sup>. Although the available evidence to support the benefits of high fiber intake to manage and prevent hemorrhoids is limited, the use of high fiber is commonly recommended in clinical practice. Since fiber consumption can induce problems with abdominal bloating and pain, patients should start at a low dose of the fiber supplement and slowly increase the amount until reaching at least 20-30 g/d. Patients should be educated to



**Figure 1 Thrombosed hemorrhoids.**

drink plenty of water with fiber. Over-the-counter topical agents and suppositories have been used as the empirical treatment and can reduce some hemorrhoidal symptoms, but data supporting their effectiveness are lacking. Hemorrhoids that fail to respond to medical management may be treated with rubber band ligation, sclerosis, and thermotherapy by using infrared beam, electric current, CO<sub>2</sub> laser, or ultrasonic energy. These local techniques induce scarring and fixation of the hemorrhoids to the underlying tissues. These procedures are usually performed in the office setting, do not require anesthesia, and are mostly applied for second degree and some third-degree prolapsed hemorrhoids. Infrared photocoagulation works well on small bleeding hemorrhoids, but is less effective on large or bulky hemorrhoids. Infrared radiation, generated by a tungsten-halogen lamp, creates heat and thus leads to inflammation and later scarring of the tissue. Most authors report only infrequent complications to infrared treatments. Rubber band ligation has been demonstrated to be the most effective method to treat symptomatic internal hemorrhoids that have failed to respond to conservative management<sup>[11-14]</sup>. Controversy exists as to how many hemorrhoidal bundles can be effectively and safely banded at one time<sup>[15]</sup>. Complications associated with this procedure are not frequent (< 2%) and include vasovagal response, anal pain, bleeding from early dislodgment, and pelvic sepsis<sup>[16,17]</sup>. Operative hemorrhoidectomy is reserved for the large third- and fourth-degree hemorrhoids, mixed hemorrhoids with a prominent external component, and incarcerated internal hemorrhoids requiring urgent intervention. The hemorrhoidal tissue is excised, and the mucosal and skin defect may be left open as an open hemorrhoidectomy, may be partially closed, or may be closed with a running suture. Several randomized trials have compared different types of hemorrhoidectomies with a variety of open<sup>[18-20]</sup> and closed techniques with inconsistent results<sup>[20,21]</sup>. Similarly, a variety of techniques have been introduced to reduce postoperative pain. The stapled hemorrhoidopexy, also called Procedure for Prolapse and Hemorrhoids (PPH, Figure 2), is a technique that reduces the prolapse of hemorrhoidal tissue by using an intraluminal circular stapling device to remove a ring of redundant mucosa and submucosa from the upper anal canal, thereby reducing the prolapsing hemorrhoidal tissue back into the anal



**Figure 2 Procedure for prolapsing hemorrhoids (PPH).**

canal and fixing it into position. The hemorrhoidal inflow that transverses the excised segment is also interrupted, thus decreasing vascularity. Compared to conventional hemorrhoidectomy, PPH affects few nerve endings, which results in less post-operative pain. The ultrasonic scalpel hemorrhoidectomy and the bipolar sealing and cutting device have also been reported to produce less postoperative pain than conventional excisional hemorrhoidectomy<sup>[18,19,22,23]</sup>. However, long-term efficacy needs to be determined<sup>[21,24]</sup>.

## ANAL FISSURES

An anal fissure is a cut or split in the epithelial lining of the anal canal distal to the dentate line. A chronic anal fissure is usually categorized when the fissure fails to heal within 6-8 wk. Chronic fissures develop ulceration and heaped-up edges with exposure of the internal anal sphincter fiber at the base of the ulcer. There is often an associated external skin tag and/or an internal hypertrophied anal papilla. The vast majority of anal fissures occur in the posterior midline, while 10% to 15% occur in the anterior midline<sup>[25]</sup> and less than 1% of fissures occur in lateral positions.

### Differential diagnosis

If an anal fissure develops in atypical locations, one must consider other diseases. Crohn's disease is the most common cause of anal fissures associated with atypical locations, although other inflammatory bowel diseases, syphilis, tuberculosis, leukemia, cancer, and human immunodeficiency virus (HIV) are also known causes<sup>[26,27]</sup>.

### Symptoms

Anal fissures are the most common causes of severe anorectal pain. Characteristic symptoms include tearing pain with defecation and hematochezia that is usually present as blood on the toilet paper. Patients may also complain of a sensation of intensely painful anal spasms lasting for several hours after a bowel movement.

### Diagnosis

Anal fissures can be diagnosed through history and physical examination.



**Figure 3** Anal fissure.



**Figure 4** Anal fistula.

Gentle spreading of the buttocks to expose the perianal area may facilitate the examination. The fissure is easily seen in the anal canal (Figure 3). Some patients may experience extreme physical discomfort during examination and may require anesthesia. Digital or anoscopic examination may be poorly tolerated on the initial visit.

### **Pathophysiology**

Although the etiology of this condition is uncertain, the main hypothesis is that the posterior midline area may have decreased blood flow due to the configuration of the vessels of the anus<sup>[28]</sup>. Also, spasm of the internal anal sphincter may cause further reduction in blood flow to the posterior anal canal. Trauma from such factors as hard stools can aggravate the condition, and then eventually cause fissures. Once a tear occurs, it begins a cycle of pain, with increased contraction of the internal anal sphincter, thereby increasing pressure in the anal canal, which results in ischemia<sup>[29]</sup>. This cycle contributes to the development of a poorly healing wound that becomes a chronic fissure. Patients with chronic anal fissures also appear to have increased resting pressure in the anus per anal manometry<sup>[29,30]</sup>.

### **Treatment**

Medical therapy leads to healing in the vast majority of patients with acute anal fissures, and almost half of the patients with chronic fissures<sup>[31]</sup>. Therapy focuses on breaking the cycle of pain, spasm, and ischemia thought responsible for the development of the fissure. Initial conservative measures have consisted of three components: relaxation of the internal sphincter; institution and maintenance of atraumatic passage of stool; and pain relief. These goals can be accomplished with bulk agents and stool softeners, and warm sitz baths following bowel movements to relax the sphincter<sup>[32,33]</sup>. Warm sitz baths (not to exceed 120°F) may ease the acute pain in the anal area. The patient should undergo two to three sitz baths a day, especially after bowel movements, for about 10-15 min each session in a warm bath. After a sitz bath, the anal area should be carefully dried with a towel or a hair dryer with cool air. A significant reduction in anal pressure after soaking in warm water has been reported<sup>[34]</sup>. Based on the theory

that anal fissures are caused by ischemia through a spastic internal sphincter, pharmacological agents including glyceryl trinitrate (GTN)<sup>[35-37]</sup>, diltiazem<sup>[35,38-40]</sup>, and botulinum toxin may be useful. These agents have been employed to reduce the resting anal canal pressure and to improve blood flow, and as an alternative to surgical sphincterotomy for chronic fissures. GTN ointment applied two to four times per day to the anus has been the most extensively studied, resulting in various healing rates and the identification of a major side effect in dose-related headaches<sup>[36]</sup>. A randomized, controlled trial has shown that 0.2% nitroglycerin ointment applied twice daily healed chronic ulcers (68% compared with 8% in the placebo group) with evidence of a reduction in resting anal pressure and an increase in anoderm blood flow after eight weeks of treatment<sup>[37]</sup>. Diltiazem ointment (2%) appears to have similar efficacy to GTN, but may cause fewer side effects (headaches and gastrointestinal side effects) than GTN. Diltiazem maybe associated with the development of pruritus. Both diltiazem and GTN would be first-line therapies, while botulinum toxin is used as rescue treatment<sup>[41-44]</sup>. Botulinum toxin causes temporary muscle paralysis by preventing acetylcholine release from presynaptic nerve terminals, thereby decreasing the pressure in the internal sphincter<sup>[41,45]</sup>. The healing rate of anal fissures ranges from 60%-70% after a single injection of 15 or 20 U of the toxin<sup>[45,46]</sup>. One prospective randomized study showed that botulinum toxin had a better healing rate compared to 0.2% GTN ointment<sup>[47]</sup>. It may be beneficial to repeat the injection of botulinum toxin for patients with recurrent fissures<sup>[48,49]</sup>. There is no conclusion on the optimal dose of the toxin, the number of injections, or the precise site of injection. Despite relatively good efficacy, medical therapy has some limitations with poor compliance, unpleasant side effects, and recurrence of the fissure<sup>[35,43,50,51]</sup>. Surgical treatment is generally reserved for fissures that have failed medical therapy. A recent meta-analysis of four randomized, controlled trials revealed superior fissure healing rates with lateral internal sphincterotomy compared with topical nitroglycerin<sup>[52]</sup>. Lateral internal sphincterotomy is the procedure of choice for the majority of surgeons<sup>[53]</sup>. The aim of this procedure is to decrease spasms of the internal sphincter by dividing a portion of the

muscle. Depending on the preference of the surgeon, approximately 30% of the internal sphincter fibers are divided laterally by using either open or closed techniques. Although healing is achieved in more than 95% of patients and most patients experience immediate pain relief with overall satisfaction, most surgeons are reluctant to use this treatment as first-line therapy for chronic anal fissures due to possible postoperative incontinence. Persistent minor incontinence, which generally does not have a major impact on quality of life, has been reported as occurring in 1.2% to 35% in various studies<sup>[25,52,54-57]</sup>. However, several studies have also demonstrated no significant difference in minor fecal incontinence between sphincterotomy and topical nitroglycerin treatments<sup>[52,58,59]</sup>. A prospective randomized controlled trial with long-term follow-up and a large number of patients will be required to support lateral internal sphincterotomy as a first-line therapy.

## ANAL FISTULAS AND ANAL ABSCESES

“Fistula” means pipe or tube in Latin. An anal fistula is an abnormal connection between two epithelial-lined spaces of the anus and rectum, creating the appearance of a pipe or tube. Anal abscesses and fistulas are the acute and chronic manifestations of the same perirectal pathogenic process. The majority of these conditions originate from infected anal glands. Anal fistulas are classified by Parks and colleagues<sup>[60]</sup>, as intersphincteric, trans-sphincteric, suprasphincteric, and extrasphincteric fistula-in-ano. An in-depth understanding of anorectal anatomy is essential to successfully treat an anorectal abscess or fistula.

### Symptoms

The patient may complain of drainage, bleeding, pain with defecation or sexual activity, swelling, or diarrhea. Fistulas may be related to other diseases such as Crohn’s disease, proctitis, or anorectal cancer.

### Diagnosis

Physical examination may reveal the external opening as a protrusion or an induration, which drains pus. The importance of accurately characterizing the fistula tract (Figure 4) prior to therapy can not be overemphasized. The risk of incomplete healing, a recurrent fistula, or even inadvertent sphincter injury is increased if fistula anatomy is incorrectly delineated or an occult abscess is missed. Several imaging modalities are available to evaluate perianal fistulas and abscesses. Although correlation varies in the literature between 45%-95%, according to Goodsall’s rule, an imaginary transverse line should be drawn across the anus. External lesions seen anterior to this line run directly from the anal canal. If the external opening is detected posterior to this line, the fistula is more complex and tracks laterally around the anus prior to a midline posterior opening. Bidigital examination, with the index finger in the anal canal and the thumb exterior to it, may enable identification of the fistulous track as a cord-like lesion under the skin.

Endoscopy may detect the internal opening. Other methods include; passing a probe; injection of a dye such as hydrogen peroxide ( $H_2O_2$ ), milk, or methylene blue; fistulography; anal ultrasound with  $H_2O_2$  injection; and magnetic resonance imaging (MRI). Several studies have concluded that MRI and anorectal endosonography (EUS) are accurate means of delineating anatomy in relation to a fistula<sup>[61]</sup>. EUS is easily performed and less expensive than MRI, but it is not appropriate for the patient with severe anal pain or an anatomical stricture. Adopting endoanal coils and phased array imaging has contributed to the evolution of using MRI to evaluate anorectal disease<sup>[61-64]</sup>. The exact choice of which modality to use depends on local expertise, cost, and the available equipment.

### Management

The principal management is surgery. Anal abscesses should always be drained in a timely manner. Delayed or inadequate treatment may occasionally cause extensive or life-threatening suppuration with massive tissue necrosis and septicemia.

Thus, an early referral to a specialist is recommended. The goals of surgical therapy are to remove the fistula tract while preserving fecal continence. The surgical approach depends upon the type of fistula. Simple intersphincteric fistulas can be treated by fistulotomy (opening of the fistula tract). High transsphincteric and suprasphincteric fistulas are more safely treated by initial placement of a seton<sup>[65]</sup>. The seton is a foreign material placed through the fistula tract which is tightened at regular intervals until it eventually cuts through the sphincter. There are different types and seton techniques used in anorectal surgery<sup>[66-70]</sup>. The slow division of the anal sphincter with the simultaneous fibrosis allows the fistula tract to be more superficial and to re-establish continuity of the anorectal ring while preventing wide separation of the anal sphincter<sup>[66,71]</sup>. The literature has reported a 2%-8% recurrence rate following treatment with cutting setons. The rate of fecal incontinence following this procedure has been reported to be about 60%, but it is mostly minor incontinence to flatus; major incontinence to solid stool is 2%-3%. An anorectal advancement flap has been advocated for complicated and multiple fistulas including for patients with inflammatory disease and high transsphincteric or suprasphincteric fistulas<sup>[72,73]</sup>. Anal sphincter injury is the main morbidity after surgical management of anal fistulas. Fibrin glue has been used for the eradication of fistulas to reduce complications following surgical procedures<sup>[74-76]</sup>. However, fibrin glue should only be used as second or third line treatment because of the conflicting success rate of closing the fistulas (range = 30%-85%) and a high recurrence rate of up to 59%<sup>[77]</sup>. Its advantages as an acceptable alternative to conventional surgery are low morbidity, simplicity, and repeatability, especially in treating complex fistulas<sup>[78,79]</sup>. A relatively new treatment using a biodegradable “collagen plug” has been adopted to treat complex, high, and recurrent anorectal fistulas. Although the fistula

**Table 1 Etiologies of pruritus ani**

Anorectal disease	Anal fistula, fissure, skin tags, hemorrhoids, rectal prolapse, anal papillomas, rectal and anal carcinoma, fecal incontinence, hidradenitis
Systemic disease	Diabetes mellitus, chronic renal failure, iron deficiency, thyrotoxicosis, myxedema, Hodgkin's lymphoma, jaundice, leukemia, aplastic anemia
Dermatologic conditions	Psoriasis, erythrasma, seborrheic dermatitis, atopic dermatitis, intertrigo
Infections	Virus, bacteria, fungi, parasites
Gynecologic conditions	Vaginitis, endocervicitis
Neoplasms	Extramammary Paget's disease, squamous cell carcinoma, cloacogenic carcinoma, Bowen's disease
Hygiene	Poor cleansing or overaggressive cleansing with rubbing or excessive soap use
Diet	Coffee, chocolate, citrus, spicy foods, tea, beer, sodas containing caffeine, fat substitutes, milk and milk products
Local irritants	Obesity, excessive hair, tight-fitting clothing, anal creases, perfumed or dyed toilet paper, anal creams
Diarrheal states	Ulcerative colitis, crohn's disease, irritable bowel syndrome
Radiation	Postirradiation changes
Psychogenic	Anxiety, neuroses, psychoses
Drugs: Idiopathic	Quinidine, colchicine, antibiotics, ointments that may contain alcohol

**Table 2 Instructions for patients**

Do not scratch or rub the anal area
Wash the anal area with only water. Do not use soap or salt when you wash the anal area. Dry the area well after cleaning, by patting the skin with a soft towel or using a hair dryer with cool air
Make sure to clean the anal area after each bowel movement as instructed. Avoid the use of toilet paper that may be abrasive
When you shower or bathe, use unscented soap
Apply a thin cotton pledge directly in the anal crease in the morning and at bedtime, and change the pledge as needed if it becomes moist
Wear loose underwear
Soak the anal area in a warm sitz bath for 10 to 15 min two to three times a day. Do not add soaps, salt, oil, or skin softners to the water, and dry the anal area as above
Maintain a soft, large and nonirritating stool by having bulking agents such as psyllium or methylcellulose in 8-12 oz of water. Start at a low dose of the fiber supplement and slowly increase the amount of fiber until reaching at least 20-30 g/d
Eat a high fiber diet that includes 8-10 glasses of water a day
Avoid foods that include colas, spicy foods, citrus foods, coffee, beer, nuts, dairy products, tomatoes
You may apply 0.5% or 1% hydrocortisone ointment to the itch area, but only if directed by your doctor, and antihistamine tablets may be helpful for nighttime symptoms
Don't be despondent when recurrence occurs because it is common. Reconsult your doctor so that appropriate management can be applied

closure rate was inconsistent, the collagen plug might be a promising alternative for complex and high anorectal fistulas because it is minimally invasive and can be used repeatedly without damaging the anal sphincter<sup>[80,81]</sup>. However, large randomized controlled trials with long-term follow up are required to assess the value of this treatment.

Anal fistulas complicated by Crohn's disease are challenging because surgical treatment is associated with poor and delayed wound healing and the high risk of incontinence. Therefore, anal fistulas in this group should be initially managed with medical therapy that includes sitz baths, metronidazole, or IV infliximab, a tumor necrosis factor- $\alpha$  inhibitor<sup>[82]</sup>.

## PRURITUS ANI

Pruritus ani is a symptom complex, not a disease. It is a common but socially embarrassing condition. The word "pruritus" originated from the Latin word *prurire*, which means "to itch," and refers to an unpleasant cutaneous sensation. Thus, patients develop a nearly uncontrollable desire to scratch<sup>[83]</sup>. Excessive rubbing or scratching of the skin results in maceration, superinfection, and a decrease in thickness of the fatty skin layer, which

exacerbates the problem and leads to hypertrophy of the epidermis and lichenification. Pruritus ani affects 1%-5% of the adult population<sup>[83]</sup>, not uncommonly occurs in adolescents and the elderly, and is more prevalent in men than women<sup>[84]</sup>.

### Etiology

Pruritus ani is classified when it has an identifiable etiology that may include numerous anorectal diseases, other systemic diseases, transient internal sphincter relaxation, an exaggerated rectoanal inhibitory reflex, poor hygiene, overaggressive cleansing, and local irritants (Table 1)<sup>[85-90]</sup>. However, in more than one half of patients with pruritus ani the cause is categorized as idiopathic.

### Diagnosis

A variety of possible etiologic factors challenge the physician to approach the correct diagnosis and institute the appropriate management. A detailed history and close physical examination can often help in the identification of pruritus. Inspection, palpation, and anoscopic examination should be performed on the initial visit. Scrapings to exclude fungal and yeast infection may be helpful. Perianal skin biopsy may be useful in

suspicious skin lesions or severe cases. Some publications recommend sigmoidoscopy or colonoscopy to evaluate inflammatory bowel disease and colorectal neoplasms<sup>[88]</sup>.

### Treatment

Management is nonsurgical, thus should be aimed at the underlying cause; there is very rarely any reason for surgical referral. Appropriate follow-up care is necessary for therapeutic success. The goal to achieve success with patients who have no identifiable etiology is to maintain the perianal skin clean, dry, and slightly acidic. However, aggressive cleaning of the perianal area with alkaline soaps leads to chronic pruritus<sup>[88]</sup>. The vast majority of patients with pruritus ani can be treated by conservative management<sup>[89,91]</sup>. Instructions for patients, well described by Hicks and colleagues (Table 2)<sup>[88]</sup>, are removal of irritants, improving perianal hygiene<sup>[92]</sup>, avoiding scratching, wearing loose cotton underwear, adjusting the diet by adding a bulking agent to induce soft and non-irritating stools, and discontinuing offending agents, such as coffee, tea, cola, beer, chocolate, and tomatoes<sup>[88]</sup>. Hydrocortisone ointment 0.5 to 1.0% can provide symptomatic relief of idiopathic pruritus ani, but should not be used for prolonged periods of time because of dermal atrophy. Skin barriers such as topical zinc oxide, can also provide some relief. Nighttime sedating antihistamines or tricyclic antidepressants may be helpful. Refractory patients with intractable symptoms should be referred to a dermatologist<sup>[91]</sup>. Various therapies have been used with inconsistent results or insufficient data to judge efficacy, such as injection of methylene blue, surgical debridement, radiation therapy, ultraviolet phototherapy, cryotherapy, and intralesional corticosteroids<sup>[93-96]</sup>.

### CONCLUSION

Although most anorectal conditions are benign, knowledgeable and skilled physician intervention is often required. Understanding the pathophysiology of anorectal disease guides treatment selection. Initiating early appropriate treatments should lead to prompt symptomatic resolution in a cost-effective manner. A subgroup of patients who persistently present with symptoms despite applicable conservative or non-surgical management should be referred to a colorectal surgeon.

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EDITORIAL

## Current management strategy of hepatocellular carcinoma

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### Abstract

Hepatocellular carcinoma (HCC) still remains a considerable challenge for surgeons. Surgery, including liver transplantation, is the most important therapeutic approach for patients with this disease. HCC is frequently diagnosed at advanced stages and has a poor prognosis with a high mortality rate even when surgical resection has been considered potentially curative. This brief report summarizes the current status of the management of this malignancy and includes a short description of new pharmacological approaches in HCC treatment.

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**Key words:** Hepatocellular carcinoma; Locoregional procedures; Liver transplantation; Surgical resection; Systemic treatment

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### INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death and accounts for as many as 500 000 deaths annually being a considerable challenge for surgeons<sup>[1]</sup>. The incidence of HCC varies by geographic location from a relatively rare tumour, like those found in North America and Europe, to a very common and highly malignant tumour as occurs in sub-Saharan Africa and Southeast Asia. HCC has been found to account for 80% of all primary liver cancers, being the fifth most common cancer worldwide<sup>[1,2]</sup>. Most patients with HCC also suffer from coexisting cirrhosis, which is the major clinical risk factor for hepatic cancer and is correlated to hepatitis B virus or hepatitis C virus (HCV) infection<sup>[3]</sup>. However, cirrhosis from non-viral causes such as alcoholism, hemochromatosis and primary biliary cirrhosis are also associated with an elevated risk of HCC. Furthermore, concomitant risk factors such as HCV infection in addition to alcoholism, tobacco use, diabetes or obesity increase the relative risk of HCC development, as numerous studies in humans and animal models have shown<sup>[4-10]</sup>. The incidence of HCC varies by geographic area worldwide. Research has shown that Southeast Asia and sub-Saharan Africa have an incidence rate of HCC that ranges from 150 to 500 per 100 000 population, primarily because of the endemic nature of hepatitis B and C in those regions<sup>[11-13]</sup>.

HCV accounts for almost 90% of all cases of HCC in Japan, and in China, hepatitis B infection is diagnosed in about 80% of patients with HCC<sup>[12-14]</sup>. In Europe and North America, however, despite a significantly lower incidence rate of 3 to 4 per 100 000 population, a distinct increase in cases of HCC has been reported as a result of intravenous drug use, unsafe sexual practices, and other causes<sup>[15-17]</sup>. Because of a lack of effective HCV vaccination, underlying HCV infection is largely responsible for that increase. As a result of the interval between the onset of infection and the development of liver cirrhosis, the incidence of HCV-related HCC will continue to increase over the next few years<sup>[18]</sup>. In contrast to Asian populations, the percentage of Western patients with HCC but without underlying cirrhosis is considerable, and the development of HCC in cirrhotic individuals in the West is associated with a wider spectrum of underlying diseases. In the West, the percentage of virally engendered cirrhosis is lower than that in Asian regions, but alcohol-toxic or cryptogenic hepatic dam-

age is observed more frequently in Western countries<sup>[14]</sup>. Thus, the etiologic pattern of HCC in Western regions of low risk for that disease differs appreciably from that in Southeast Asia and sub-Saharan Africa.

Surgery, including liver transplantation (LT), still remains the most important therapeutic approach for patients with HCC. Over the past few decades, considerable progress has been made in the diagnosis and surgical treatment of HCC. In fact, tumors are now often identified at an early stage<sup>[19,20]</sup>, surgery is safer with an acceptable overall mortality rate (in cirrhotic patients < 5%), being characterized by a good long-term survival<sup>[21,22]</sup> and results of LT have steadily improved because of careful patient selection<sup>[23]</sup>.

However, HCC is still associated with a high rate of mortality and prognosis of this tumour is poor even when treatment has been considered potentially curative<sup>[24,25]</sup>.

This brief report summarizes the current status of the management of this disease and includes a short description of a new pharmacological approach in HCC treatment.

## STAGING

Various classification systems are available for HCC. The Barcelona Clinic Liver Cancer (BCLC) classification has emerged during recent years as the standard classification that is used for trial design and clinical management of patients with HCC<sup>[26,27]</sup>. This classification has been approved by EASL and the AASLD<sup>[24,28]</sup> and has subsequently been corroborated in clinical studies<sup>[29,30]</sup>. The BCLC staging system was constructed on the basis of the results obtained in the setting of several cohort studies and randomized controlled trials by the Barcelona group.

The main prognostic factors of this staging system are related to tumour status (defined by the number and size of nodules, the presence or absence of vascular invasion, and the presence or absence of extrahepatic spread), liver function (defined by the Child-Pugh score system, serum bilirubin and albumin levels, and portal hypertension), and general health status [defined by the Eastern Cooperative Oncology Group (ECOG), classification and presence of symptoms]. On the other hand, aetiology is not an independent prognostic factor. The BCLC classification links stage stratification with a recommended treatment strategy and defines standard of care for each tumour stage (Figure 1).

Patients with very early HCC (stage 0) are optimal candidates for resection. Patients with early HCC (stage A) should be considered for radical therapy [resection, LT, or local ablation *via* percutaneous ethanol injection (PEI) or radiofrequency (RF) ablation]. Patients with intermediate HCC (stage B) have been found to benefit from transarterial chemoembolization (TACE). Patients with advanced HCC, defined as the presence of macroscopic vascular invasion, extrahepatic spread, or cancer-related symptoms (ECOG performance status 1 or 2) (stage C), have recently been found to benefit from Sorafenib

treatment<sup>[31,32]</sup>. Patients with end-stage disease (stage D) will receive symptomatic treatment.

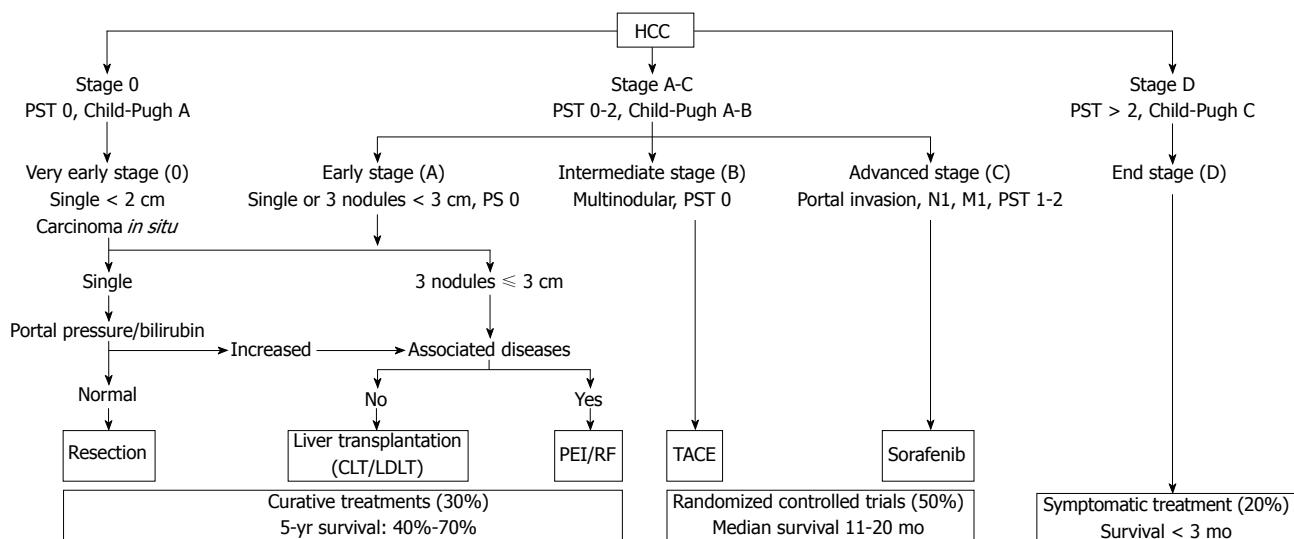
Treatment strategy will transition from one stage to another on treatment failure or contraindications for the procedures.

## HCC TREATMENT OPTIONS

### Liver resection

Pre-treatment imaging studies such as computed tomography and magnetic resonance imaging, either with or without angiography, can be used to match patients with their most appropriate treatment. Positron emission tomography is also useful in the identification of extrahepatic metastases which considerably influence clinical decision-making. These types of studies aid in detecting intrahepatic and extrahepatic disease as well as vascular invasion. Knowledge about the relation of the tumour to regional anatomic structures such as large vessels is crucial because it provides valuable information about resectability. Furthermore, volumetric studies can be used to define the residual parenchyma exactly. The determination of hepatic reserve is also significant when resection is considered. The healthy liver has a great capacity for regeneration and adjusts to the metabolic requirements of the host after liver resection due to hypertrophy of the residual liver. Partial hepatectomy usually ensures a safety margin of at least one centimeter and is associated with an operative mortality rate of less than 5%<sup>[33,34]</sup>. For patients with inadequate or borderline remnant parenchyma, hypertrophy of the prospective liver remnant can be induced by preoperative portal vein embolization<sup>[35]</sup>. However, the use of portal vein embolization to induce compensatory hypertrophy of healthy liver before major resection is controversial. Uncontrolled tumour progression as a result of the proliferation of malignant cells stimulated by this method and the risk of variceal bleeding resulting from acute portal hypertension should be carefully considered and for that reason this procedure raises some concerns<sup>[36]</sup>. In certain circumstances, an unfavourable location of the tumour and involvement of the confluence of the three hepatic veins and either the caval vein or the retrohepatic caval vein can render resection by conventional techniques impossible. In these rare cases, special techniques such as *in situ* or *ante situm* resection can be used<sup>[37]</sup>.

The overall long-term results after resection are favourable. However, only 20% to 30% of patients with HCC are eligible for resection because of advanced or multifocal disease or inadequate functional hepatic reserve<sup>[38]</sup>. In patients with solitary lesions of less than 5 cm, no vascular invasion, and a negative surgical margin of at least 1 cm, the 5-year survival rate after resection has been reported to be greater than 70%<sup>[39]</sup>. In a series consisting of 68 patients with HCC and non-cirrhotic liver, an overall 5-year survival rate of 40% was achieved even when extensive resection had been performed<sup>[40]</sup>. Similar results were observed in a large series in which patients with HCC and non-cirrhotic liver demonstrated a survival rate of 58% after 3 years and 42% after 5 years<sup>[41]</sup>.



**Figure 1** Barcelona Clinic Liver Cancer staging classification and treatment schedule<sup>[27]</sup>.

Despite earlier detection, safer surgical procedures and more aggressive treatment of HCC, recurrence, as a result of multicentric carcinogenesis or intrahepatic metastases from the primary tumour may occur. In selected patients, repeat resection provides good long-term benefits and is an option for those with solitary peripheral tumors that can be treated with segmental or atypical resection. In patients with adequate functional reserve capacity and no extrahepatic tumour growth, the 5-year survival rate after repeat resection has been reported to be as high as 86%<sup>[42]</sup>.

## LT

LT is one of the best treatment approaches for HCC in patients who fulfil the selection criteria (solitary tumors of less than 5 cm and up to three tumour nodules, each of which is smaller than 3 cm)<sup>[23]</sup>. First, it removes the tumour with the widest margin together with any intrahepatic metastasis. Second, it cures the underlying cirrhosis that is responsible for both hepatic decompensation and metachronous tumour after partial hepatectomy. Finally, it allows the histologic examination of the entire liver explant for the most accurate pathologic staging.

Study results have generally shown a significantly higher probability of survival in patients with incidentally discovered tumors, no vascular invasion, a negative nodal status, a tumour size of less than 5 cm, and a tumour of lower histologic grade<sup>[43-48]</sup>. The 5-year reported survival rate is around 26% after resection and 69% after transplantation in cirrhotic patients with HCC<sup>[43]</sup>. The decisive prognostic factor for patients with HCC is vascular invasion, which no system of medical imaging can accurately demonstrate at this time<sup>[49]</sup>. Therefore, the pre-operative prognosis is still based on the number and size of tumour nodes demonstrated, due to the evidence that vascular invasion has been found to be correlated to tumour size and number<sup>[44]</sup>. Because of the present lack of organs available, an accurate estimation of the patient's prognosis is important and not every patient with HCC

and cirrhosis can be treated with LT. Thus the need to obtain the optimal benefit from the limited number of organs available has prompted adherence to strict selection criteria, so that only those patients with early HCC and the highest likelihood of survival after transplantation are listed to undergo that procedure (Figure 1).

Excellent 5-year post-transplant patient survival of at least 70% has been reported from many centres<sup>[50,51]</sup>. In another study, excellent results were achieved in patients with solitary lesions of a maximum diameter of 6.5 cm, patients with a maximum of three lesions (the largest of which was no larger than 4.5 cm), and those who had no more than 8 cm in diameter in all tumour nodes. The 1-year and 5-year survival rates of these patients were as high as 90% and 72%, respectively<sup>[52]</sup>.

Even if the selection criteria for LT to treat HCC could be expanded, the current shortage of liver grafts and the lack of data defining the new limits for LT in patients with HCC makes the attempt to expand the listing criteria a very controversial issue. As a result of expanded listing criteria, the inclusion of patients with more advanced cancer may result in a higher dropout rate that in turn leads to poor survival rates in an intent-to-treat analysis<sup>[53,54]</sup>.

In their recent publication, Mazzaferro and colleagues<sup>[55]</sup> developed a new prognostic model that could be used to expand the Milan criteria and help identify patients whose odds of survival could be improved by having a liver transplant. Using the "up-to-seven" criteria, where seven is the maximum number obtained by adding the size of the largest tumour (in centimeter) to the total number of tumors, the report indicates that the 5-year survival in patients without microvascular invasion was 71.2%, similar to patients who met the Milan criteria. The researchers conclude: more patients with HCC could be candidates for transplantation if the current dual (yes/no) approach to candidacy, based on the strict Milan criteria, was replaced with a more precise estimation of survival contouring individual tumour characteristics and use of the up-to-seven criteria.

Therefore, the ultimate therapeutic choice should always result from the analysis of each individual case and should be based on the experience and judgment of the transplant team and not just on the statistical results derived from the literature.

The wait for a graft to become available still presents the greatest challenge. Methods of neoadjuvant therapy (PEI, RF, TACE) may be used to provide tumour control in patients on a waiting list for LT.

Patients can reach a prognostically unfavourable stage because of tumour progression with subsequent deterioration of their clinical profile while waiting and may no longer fulfil the criteria for LT<sup>[52]</sup>. As a result, these patients should be removed from the waiting list, indeed about 50% of HCC patients who were initially candidates for LT will become ineligible for transplantation if the median waiting period exceeds 1 year<sup>[53,56]</sup>.

Strategies to increase the donor pool and diminish the tumour progression rate have become a priority in many centres. In the United States, the United Network for Organ Sharing proposed a new system to allocate patients on the list according to the Model for End-Stage Liver Disease score<sup>[57]</sup>. This change gives priority to patients to minimize drop-out rates and has improved the access to timely liver transplant for patients with HCC<sup>[58]</sup>.

Recent advances in adult living-donor liver transplantation (LDLT) may produce a drastic change in the role of transplantation surgery for HCC. This option enables patients to avoid the long waiting time before transplantation and consequently to reduce the dropout rate. The keynote of living donation in patients with HCC and cirrhosis is supported by these factors: (1) Better graft function, because each graft is obtained from a healthy person; (2) Better clinical condition of the patient at the time of transplantation; (3) Optimal organ harvest, conservation, and reduced cold ischemia time; (4) A significantly reduced waiting time.

Whether LDLT is indicated in patients with HCC that exceeds the Milan criteria remains controversial<sup>[59,60]</sup>. A recent survey of transplant surgeons from North America, Europe, and Asia revealed that 41% of the respondents favoured LDLT for use in patients with HCC that exceeds the Milan criteria<sup>[61]</sup>. Patients who no longer fulfil the criteria because of tumour size or the number of tumour nodes may still retain the option of LDLT. Because a potential 5-year survival rate of around 50% in patients whose LT is justified by extended criteria has been described<sup>[53]</sup>, transplantation would offer a better chance of survival than would all other therapeutic options.

However, evidence-based universal guidelines for this important issue have not been established. Primary graft failure and the risk to the donor present ethical concerns that cannot be disregarded. Donor deaths and other complications, such as insufficiency of the donor's remaining liver (which required subsequent transplantation), have been reported<sup>[62]</sup>. Furthermore, some data also suggest the need to have a better evaluation of the donor, including liver histology<sup>[63]</sup>. The complication rate for LT in North America and Europe ranges between

9.2% and 40% for the donor, and the mortality risk in donors is still 0.3% to 0.61%<sup>[64,65]</sup>. In contrast, data in Japan showed a complication rate of 12% with no perioperative deaths in living liver donors<sup>[66]</sup>. Meticulous surgical techniques and perioperative management, lean body mass in individual Japanese donors, and genetic factors were given as possible explanations for the zero transplant-related mortality rate in Japan. In general, however, morbidity after living liver donation strongly correlates with the expertise of the staff of the transplant centre. Therefore, a combination of surgical expertise and thorough, individualized medical and psychological evaluations is vital to ensure the lowest morbidity rate and best outcome, not only in the recipient, but also in the donor.

### **Locoregional procedures and chemotherapy**

**Percutaneous ablation:** For patients who are not candidates for liver resection or transplantation, percutaneous ablation offers the best treatment option. However, to our knowledge, there are no randomized controlled clinical trials that have compared the results of this treatment option with those of surgical therapy for HCC, and none of the ablation techniques have been shown to offer a definitive survival advantage. The principle of ablation is based on the destruction of tumour cells by the application of chemical substances, such as ethanol, or by using RF or laser to modify the temperature in the tumour *via* the delivery of heat. Of all those techniques, PEI has been the most investigated<sup>[67]</sup>. In individuals who do not fit the optimal surgical profile, PEI is as effective as surgery and is associated with a 5-year survival rate as high as 72% if the accurate selection of patients is performed<sup>[68,69]</sup>. The low rate of procedure-related complications and the low cost of PEI are additional advantages. The main drawback of this technique is the need for repeated injections in separate sessions and the inability to achieve complete necrosis in larger tumors. In that regard, RF ablation has been shown to be more effective in achieving complete necrosis in tumors larger than 2 cm and to require fewer treatment sessions<sup>[70]</sup>. RF ablation involves the delivery of energy created by RF waves to tumors to induce thermal damage and coagulative necrosis. Study results have shown that RF ablation is superior to PEI in terms of causing complete tumour necrosis (90% *vs* 80%) and in the number of required treatments (1.2 *vs* 4.8)<sup>[71]</sup>. However, RF ablation causes more complications such as pleural effusion, bleeding, and tumour seeding than does PEI<sup>[71,72]</sup>. In addition, the effectiveness of RFA decreases as the tumour size exceeds 3 cm.

**Chemoembolization:** This approach can be used before liver resection to improve resectability, as a bridge to LT while awaiting organ availability, or as a palliative treatment, and it may offer patients with preserved liver function and no evidence of ascites a survival advantage<sup>[73]</sup>. Chemoembolization is based on the principle of arterial obstruction (obstruction of the hepatic artery during angiography *via* the use of agents such as an ab-

sorbable gelatin sponge, alcohol, *etc*; to induce ischemic tumour necrosis). This technique is effective because the growth of HCC depends primarily on the hepatic artery blood supply, but the healthy hepatic parenchyma has a dual blood supply (85% is supplied by the portal vein, and the remainder is supplied by the hepatic artery). The injection of a chemotherapeutic agent [usually cisplatin, doxorubicin hydrochloride (Adriamycin), or mitomycin C] before arterial obstruction (transcatheter arterial embolization) results in TACE, a method by which regionally elevated levels of these agents in the liver can be achieved while concomitant systemic toxicity is avoided. When compared with controls, patients treated with TACE exhibited a decrease in tumour size of 16% to 61% and a 1-year survival advantage as high as 82%<sup>[74-77]</sup>.

### **Systemic treatment**

A number of systemic chemotherapies have been evaluated in many clinical trials. Unfortunately, no single agent or combination of agents given systemically leads to reproducible response rates that show beneficial effect of systemic chemotherapy on survival rates<sup>[78]</sup>. Tamoxifen, octreotide, interferon, and interleukin-2 have not been shown to be effective in treating HCC in randomized controlled clinical trials<sup>[79,80]</sup>. The increasing knowledge in the molecular structure of HCC as well as the introduction of molecular targeted therapies in oncology have created an encouraging trend in the management of this disease<sup>[81]</sup>. These targeted molecular therapies are aimed at growth factors and their receptors, intracellular signal transduction and cell cycle control. Recent positive results from a preliminary study of the receptor tyrosine kinase inhibitor, sorafenib, have been reported in the treatment of HCC<sup>[31,52]</sup>. This oral multikinase inhibitor action of several kinases (VEGF, PDGF, c-kit receptor, Raf) involved in both tumour cell proliferation (tumour growth) and angiogenesis (tumour blood supply).

### **CONCLUSION**

Novel treatment options based on an improved understanding of the molecular pathogenesis of HCC have been proposed. Nonetheless a substantial improvement in the outcomes of intermediate and advanced stage HCC is expected with the advent of these targeted therapies, used in combination with surgical or locoregional therapies.

LT still remains an important treatment approach for HCC and is a well-documented and proven treatment modality for this disease. However, early unsatisfactory results have emphasized that only a highly selected patient population would benefit from transplantation. Pretransplantation therapies and the development of novel agents to prevent progression of HCC in patients awaiting LT are needed. Promotion of organ donation is necessary, the role of new systemic therapy is now becoming a new and interesting option in the treatment of patients with HCC, but LDLT as the dominant strategy will continue to escalate.

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# Characteristics of common solid liver lesions and recommendations for diagnostic workup

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## INTRODUCTION

Liver masses are increasingly being identified due to the widespread use of imaging modalities such as ultrasonography (US), computed tomography (CT), and magnetic resonance imaging. The majority of these lesions are detected incidentally in asymptomatic patients. An accurate history and physical examination are essential to the diagnosis and treatment of solid liver masses. For example, the use of oral contraceptives or anabolic steroids might be related to hepatic adenoma (HA)<sup>[1]</sup>; alcohol use and occupational exposure are associated with angiosarcoma<sup>[2]</sup> and primary sclerosing cholangitis, liver fluke, Caroli's disease, and choledochal cysts are associated with cholangiocarcinoma<sup>[3]</sup>. Physical examination should look for liver tenderness, stigmata of chronic liver disease, or general deterioration signs (fever, weight loss). High alkaline phosphatase, high lactate dehydrogenase (LDH), low albumin, high prothrombin time, and iron overload are non-specific but might suggest an underlying chronic hepatitis, cirrhosis or an infiltrative process<sup>[4]</sup>. A history of hepatitis B, C or liver cirrhosis might point to hepatocellular carcinoma (HCC). A previous neoplasm or history of chemotherapy increases the suspicion of metastatic liver disease.

## CURRENT KNOWLEDGE

In the majority of patients, a proper diagnosis can be

**Table 1 Clinical differential diagnosis of the most common liver masses**

	<b>Cirrhotic liver</b>	<b>Common lesions</b>	<b>Non-cirrhotic liver</b>	<b>Common lesions</b>
Malignant mass	Hepatocellular carcinoma	a,d	Metastasis	a,b
	Cholangiocarcinoma		Well differentiated HCC	
	High grade dysplastic nodule		Fibro lamellar HCC	a,b,c,g
	Lymphoma		Cholangiocarcinoma	
	Metastasis (exceptional)		Hemangio-Endothelioma	
Benign mass	Low grade dysplasia	d	Lymphoma	g
	Focal fatty liver		Melanoma	
	Hemangioma		Neuroendocrine tumor	
	Hepatic adenoma	g	Sarcoma (angiosarcoma,leiomyosarcoma)	
			Hemangioma	
			Focal nodular hyperplasia (FNH)	a,b
			Hepatic adenoma (HA)	a,b
			Nodular regenerative hyperplasia	b,f
			Partial nodular transformation	e,f
			Focal fatty infiltration	c,e
			Bile duct adenoma	

a: Hyper vascular liver tumor; b: Tumors that are extremely rare in cirrhosis but relatively frequent in healthy normal liver; c: Tumors frequent in the left lobe; d: Mainly in cirrhosis; e: Equally found in cirrhotic and non cirrhotic; f: Clinically mimics cirrhosis; g: Extremely rare tumors.

made based on the characteristics on imaging modalities (Lesions size < 1.0 cm are usually benign). For diagnostic purposes, liver masses should be divided into those occurring with and without cirrhosis. A liver mass in a cirrhotic liver should be viewed as HCC until proven otherwise. Multiple liver masses in a cirrhotic liver indicate diffuse HCC, high-grade dysplastic nodules or, on rare occasions, hepatic lymphoma<sup>[5]</sup>. Benign liver lesions are found in more than 20% of the general population<sup>[6]</sup>, including haemangioma (4%), focal nodular hyperplasia (FNH, 0.4%) and hepatic adenomas (0.004%). Multiple liver lesions in a normal liver usually indicate liver metastasis (most commonly from adenocarcinoma of the colon, stomach, lung or prostate), but could be cysts or hemangiomas<sup>[7]</sup>. Liver metastasis is a rare finding in a cirrhotic liver<sup>[8]</sup> (Table 1). Multiple liver lesions of a benign nature such as hemangiomas or focal nodular hyperplasia are not uncommon in a normal liver.

Calcifications have no diagnostic utility but might suggest fibro-lamellar carcinoma or colorectal adenocarcinoma, whereas hemorrhage within the lesion suggests adenoma<sup>[9]</sup>. The initial strategy in cirrhosis should be the measurement of  $\alpha$ -fetoprotein (AFP) followed by ultrasound, contrast CT or magnetic resonance imaging (MRI)<sup>[10,11]</sup>. Fine needle core biopsy (FNCB) might be required, but biopsy of potentially operable lesions should be avoided. This review discusses the various characteristics of the most common solid liver lesions and recommends a practical approach for diagnostic workup.

## DIAGNOSTIC VALUE OF TUMOR MARKERS

AFP, PIVKA 2 (< 0.1 U/mL), desgamma-carboxy prothrombin and CA 19-9 (< 37 U/mL) are tumor markers for HCC. AFP is the first choice when diagnosing HCC and 10 ng/mL is the cut-off level. The formula (Ca 19-9 + CEA × 40) provides an index accuracy of 86%

in diagnosis of cholangiocarcinoma<sup>[12]</sup>. AFP values > 400 ng/mL are indicative of HCC. 30% of patients with HCC < 2 cm have normal AFP, 20% of HCCs produce no AFP, and levels from 20-250 are frequently seen in regenerating nodules or viral cirrhosis. An increase in AFP over time is virtually diagnostic of HCC<sup>[13]</sup>. Globally, the serum level of at least one of the tumor markers was elevated in 88% of patients with proven malignancy. Elevation was marked in 57%. No tumor marker alterations were detected in patients with benign lesions. Early lesions may have elevated tumor markers in fewer than 30% of cases<sup>[12]</sup>.

## IMAGING TECHNIQUES (TABLE 2)

A single imaging modality might suffice in cases that show interval development or progression, such as metastasis. Hemangiomas are often diagnosed by a single dynamic contrast enhanced imaging modality (MDCT). When further imaging techniques are necessary, CT angiography, MRI, and contrast enhanced-CT are performed to plan a surgical strategy. If this is not available on site, the patient and physician should decide whether the patient should undergo a biopsy or be referred for additional imaging. The key features of imaging techniques in the diagnosis of liver mass are shown in Table 3. Imaging tools for tumor assessment include: (1) Angiogram, RBC scintigraphy, contrast-enhanced CT, Porto-angiography CT, color Doppler ultrasound, contrast ultrasound, and gadopentetate dimeglumine-enhanced MRI; (2) MD-DPD enhanced MRI imaging, Gd-Balta-enhanced MRI imaging, EOB DTPA (to assess hepatocyte function and biliary excretion); (3) Plain film, US, CT scan (to assess tumor calcifications); and (4) US, contrast US, enhanced CT, MRI (utilized to assess capsule formation). US and CT are indicated for diagnosis of biliary obstruction or gallbladder diseases and for differentiation of cysts from solid liver lesions. Intraoperative ultrasound detects small liver lesions (< 5 mm). Endoscopic US assesses the left

**Table 2 Accuracy and key features of imaging techniques in the diagnosis of most common liver masses**

	<b>US-US doppler, contrast ultrasound</b>	<b>Triphasic CT</b>	<b>MRI</b>	<b>PET SCAN</b>	<b>CT-angiography</b>
Hemangioma (1-10 cm)	++ Hyperechoic Doppler: low flow, low index, absence of spectral broadening	+++ Peripheral puddles, fill in from periphery, enhancement on delayed scan	++++ Peripheral enhancement centripetal progression Hyperintense on T2, hypo intense on T1 SS > 95%, SP 95%	No uptake	+++ Cotton wool pooling of contrast, normal vessels without AV shunt, persistent enhancement Normal finding
Focal fatty liver	++ Hyper echoic, no mass effect, no vessel displacement	++ Sharp interface Low density (< 40 u)	+++	No uptake	
FNH (< 3 cm)	+ Homogenous iso, hypo, or hyper echoic, central hyper echoic area Central arterial signal Doppler: high flow, spectral broadening	++ Homogeneous enhance strongly with hepatic arterial phase Isodense with liver; Central low density scar	++++ Hyper vascular +Gd Isodense T1 Hyper intense scar T2 SS > 95%; SP > 95%	No uptake	+++ Hyper vascular 70% centrifugal supply
Adenoma (5-10 cm)	++ Heterogeneous Hyper echoic If haemorrhage: anechoic center In doppler: variable flow, spectral broadening	++ Homogenous > Heterogeneous, Peripheral feeders filling in from periphery	++ Capsule, Hyper intense in T1 (intra lesional fat)	No uptake uptake if degeneration to HCC	++ Hyper vascular Large peripheral Vessel Central scar if haemorrhage
HCC	++ Hypo or hyper echoic Doppler: hyper vascular Doppler: index and flow high, spectral broadening	+++ Hyper vascular, often irregular borders Heterogeneous > Homogeneous abnormal internal vessel Hallmark is venous washout SS 52%-54%	+++ Hyper vascular Poor different: Hypo intense T-1, Hyper intense T2 Well different: Hyper intense T-1, Iso intense T-2 SS 53%-78%	++ Increased uptake, but many HCCs show no uptake at PET	++++ Hyper vascular Av shunting Angiogenesis
Cholangiocarcinoma	Bile duct dilatation if major ducts are involved. Intra-hepatic CCC: no bile dilatation	Hypo dense lesion. Delayed enhancement	Hyper intense T1 Hyper intense T2 MRCP is useful	Uptake ++	Hypervascular
Metastasis	+ SS 40%-70% hypo to hyper echoic; doppler: low index and flow; presence of spectral broadening	+++ SS 49%-74 % complete ring enhancement	+++ SS 68%-90 % Low intensity T-1 High intensity T-2	+++++ SS 90%-100%	++++ SS 88%-95% hyper vascular

<sup>1</sup>Intraoperative ultrasound, contrast ultrasound and EUS are highly sensitive to detect liver mass; +: Degree of accuracy; SS: Sensitivity; SP: Specificity; MRI: Magnetic resonance imaging; CT: Computed tomography; HCC: Hepatocellular carcinoma.

**Table 3 Immunohistochemical staining in the evaluation of hepatic tumors**

<b>Tumor</b>	<b>Recommended immunostaining</b>
HCC	Polyclonal CEA Cytokeratin 8/18 pair (+/+ staining) Cytokeratin 7/20 pair (-/- staining) Hep Par 1, AFP
Cholangiocarcinoma	Cytokeratin 7/19 pair (+/+ staining) Cytokeratin 7/20 pair (+/- staining) B-HCG, CEA, Mucin-1
Epithelioid hemangioendothelioma	CD34 CD31 Factor VIII
Angiomyolipoma	HMB-45, smooth muscle actin
Metastatic carcinoma	
Neuroendocrine	Chromagrin, synaptophysin, neural enolase
Pancreas	Cytokeratin 7/20 pair (+/+ staining)
Colorectal	Cytokeratin 7/20 pair (-/+ staining)
Breast	Cytokeratin 7/20 pair (+/- staining)
Lung	Cytokeratin 7/20 pair (+/- staining)

liver lobe and the gastrohepatic ligament lymph nodes, and can help perform FNA. Doppler US evaluates blood

vessels patency and portal hypertension<sup>[14]</sup>.

The gold standard for detection and location of focal lesions is MRI or triple phase dynamic spiral CT<sup>[15]</sup>. Conventionally, a triple phase CT scan includes unenhanced, arterial and venous phases. The fourth phase is a delayed venous scan quadruple phase MDCT<sup>[16]</sup>. This is required only for small lesions thought to be HCC or cysts and hemangiomas. CT portography is one of the most sensitive imaging modalities for secondary lesions, but it is an examination that is performed in highly selected cases only, in few institutions and not for all types of liver lesions<sup>[16,17]</sup>. FDG PET CT scan is not very useful for HCC and therefore is not the best imaging modality to distinguish benign from malignant lesions<sup>[18]</sup>. A nuclear scan with Tc-99m-sulfur colloid shows increased uptake in FNH. MIBG and octreotide scintigraphy detect neuroendocrine tumors<sup>[19]</sup>. Hepatic Tc-99m red blood cell scan diagnoses hemangiomas > 2.5 cm (most university centers do not use this method and prefer contrast enhanced US, CT, and MRI)<sup>[20]</sup>. Ultrasound contrast agents and MRI using iron or gadolinium contrast better detect smaller lesions, satellite lesions or distant metastasis<sup>[21-23]</sup>.

Radiographic characteristics favoring HCC include the presence of a capsule bulging beyond the normal hepatic contour or a lesion with different densities. Contrast injection produces an immediate enhancement of most hepatomas.

## FINE NEEDLE ASPIRATION AND CORE BIOPSY (FNAB)

FNAB is safe, accurate and cost effective. Its specificity approaches 100% and its sensitivity is 67%-100%<sup>[24,16]</sup>. FNAB under CT or US in an appropriate location is the method of choice. FNAB is superior to FNCB; however, the methods are complementary, i.e. FNAB and FNCB have an accuracies of 78% separately and 88% when considered in combination<sup>[25,26]</sup>. However, many pathologists state that core biopsies are much preferred over needle biopsies for diagnosis of hepatic masses, because well differentiated HCCs cannot be separated from normal liver. Complications (mostly hemorrhages) are rare, with 0.5% minor complications and 0.05% major complications<sup>[27-30]</sup>. Another concern is the possible seeding of a tumor. Blind FNAB is diagnostic in > 50% of cases<sup>[31]</sup>, which increases to 65% when performing a second pass. An additional 5%-10% of tumors will be recognized if a cell block is obtained. Cohn's cytological criteria help to distinguish HCC from non-neoplastic lesions (81% of HCC show at least two criteria)<sup>[32]</sup>, e.g. increased nuclear/cytoplasmic ratio, trabecular pattern, and atypical naked hepatocyte nuclei. Some key features of immunohistochemical staining in the evaluation of hepatic tumors are shown in Table 3.

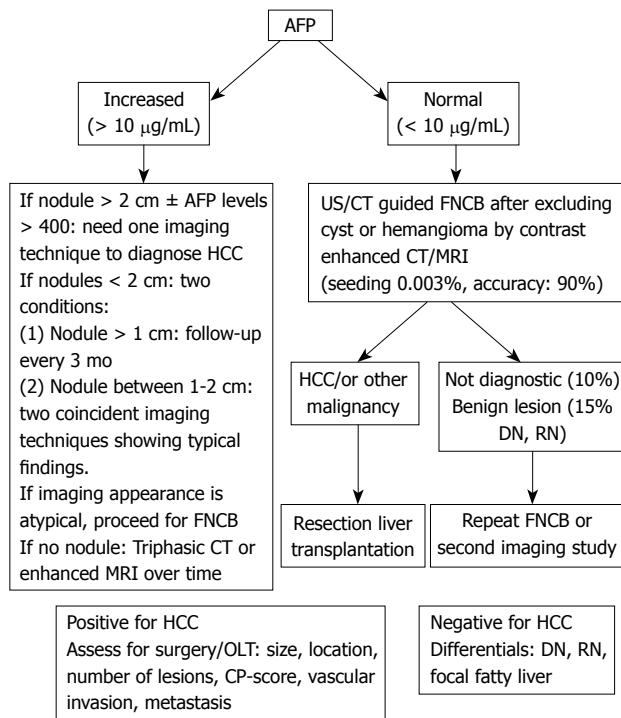
## GENERAL APPROACH TO LIVER MASSES IN CIRRHOTIC LIVERS (TABLE 1, FIGURE 1)

### Mass > 2 cm

Enhancement in the arterial phase and washout in the portal venous phase is essential for the diagnosis of a liver lesion > 2 cm in a cirrhotic liver. More than 80% of masses > 2 cm in a cirrhotic liver are HCC<sup>[33,34]</sup>. An elevated AFP confirms the diagnosis. If AFP is normal, further imaging will be diagnostic (triphasic CT, MRI)<sup>[13]</sup>. If there is still doubt, FNCB might be indicated (Figure 1).

### Mass < 2 cm

Seventy-five percent of masses < 2 cm in a cirrhotic liver are HCCs<sup>[35]</sup>. AFP levels and imaging might secure the diagnosis. If still in doubt, repeated imaging that detects enlargement of the lesion, or FNAB/FNCB might be indicated. Due to the risk of tumor seeding, biopsy should be avoided if surgical resection is possible<sup>[36]</sup>. A small nodule can be preneoplastic or benign. The American Association for the Study of Liver Diseases (AASLD) distinguishes lesions < 1 cm from those > 1 cm but < 2 cm. They suggest performing two imaging techniques from among US, CT, and MRI with IV contrast injection. If two techniques display typical



**Figure 1 Algorithm for the investigation of a liver mass in a cirrhotic liver.** Some hepatologists consider biopsy to be unnecessary for a mass in a cirrhotic liver even if the  $\alpha$ -fetoprotein (AFP) < 10; FNCB: Fine needle core biopsy; MRI: Magnetic resonance imaging.

imaging criteria, it is possible to diagnose HCC. Larger nodules should be diagnosed and small lesions should be surveyed every three months<sup>[37]</sup>. Caturelli showed that 69% of new nodules in a cirrhotic liver are malignant. Moreover, liver cell dysplasia is found in 60% of cirrhotic livers containing HCC and in only 10% of non-cirrhotic livers<sup>[38]</sup>.

### AFP is increased without a liver mass

In this case, repeated dynamic CT or MRI every three months is the rule<sup>[39]</sup>. An elevated AFP does not necessarily diagnose HCC, especially in patients with HCV who commonly have modest elevation of AFP without HCC. A marked AFP is helpful, but modest elevations would certainly not be an indication for OLT in the absence of a liver mass.

**HCC:** HCC is a common malignancy with an incidence of 1%-6% among cirrhotic patients<sup>[13,40-42]</sup>. Risk factors include cirrhosis, alcohol, HBV, HCV, metabolic liver diseases, environmental carcinogens, hormonal treatments and smoking<sup>[43,44]</sup>. Ninety to ninety five percent of HCCs arise in cirrhotic livers. Autopsy studies indicate that 20%-40% of patients with cirrhosis have HCC. Tumor size and severity of liver disease influence the survival rate. Patients with tumors < 5 cm have a survival of 80% at one year and 20% at three years. New abdominal pain, recent hepatomegaly, hemoperitoneum, persistent fever or weight loss in a cirrhotic patient should raise suspicion of HCC. Laboratory results that characterize HCC include a sudden increase in alkaline phosphatases, an increased ratio AST/ALT, an erythrocytosis, persistent leukocytosis,

recurrent hypoglycemia, hypercholesterolemia and hypercalcemia. The last four findings are paraneoplastic manifestations<sup>[45]</sup> together with ectopic hormonal syndrome, hypertrophic osteoarthropathy and porphyria cutana tarda<sup>[46]</sup>. Complications of HCC include obstructive jaundice<sup>[47]</sup>, and rupture of HCC (60%-90% mortality).

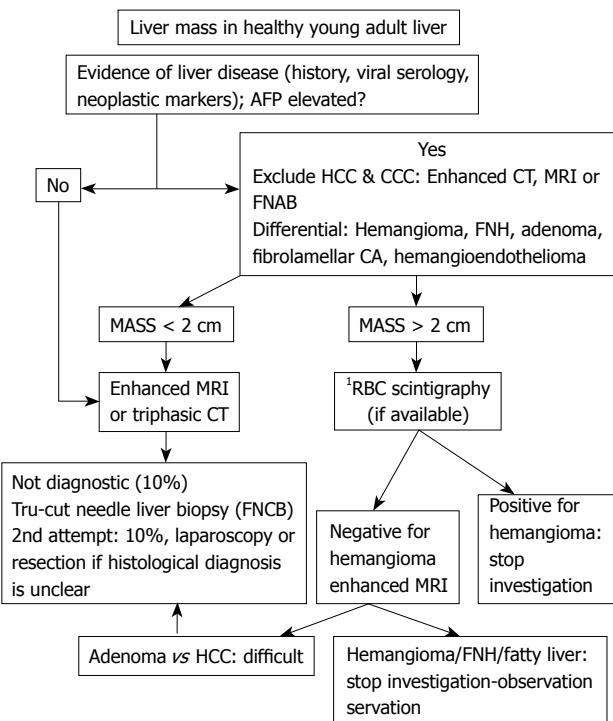
Screening for HCC includes US + AFP levels every six months. The AASLD guidelines recommend US only. AFP is of little additional value. Lesions > 2 cm need just one imaging technique showing typical findings (arterial hypervascularization) or one imaging technique and AFP levels > 400 in order to make a non-invasive diagnosis of HCC<sup>[13]</sup>. Lesions < 2 cm are divided into larger and smaller than 1 cm. Nodules > 1 cm but < 2 cm (1-2 cm) need diagnostic workup with two coincident or serial imaging techniques, rather than just proceeding with a biopsy. Nodules < 1 cm need screening follow up every three months. Nine to thirty seven percent of HCC are resectable at diagnosis<sup>[48]</sup>. Contraindications to resection include decompensated cirrhosis, extra-hepatic metastases, involvement of hepatic nodes or inferior vena cava (IVC), or bilobar extension<sup>[45]</sup>. The histological variants in cirrhotic livers include trabecular (65%), mixed (15%), compact (12%), pseudo glandular (5%) fibro lamellar (1.5%), and scirrhouous 0.5%<sup>[49]</sup>.

**Regenerative nodules:** Dysplastic nodules often occur within regenerative cirrhotic nodules. They can show low- or high-grade dysplasia. A progression from regenerative nodule with low-grade dysplasia to high-grade dysplasia, in well differentiated and poorly differentiated HCC, is possible<sup>[50-51]</sup>. MRI best differentiates this iso- or hypo-intense lesion from hyper intense HCC. In difficult cases, histology is required after liver resection or liver transplant. If HCC cannot be confirmed, the investigation must be repeated later. Over time, high-grade dysplastic nodules can become malignant, suggesting preventive ablation by ethanol<sup>[7]</sup>.

## GENERAL APPROACH TO LIVER MASSES IN NON-CIRRHOTIC LIVERS (TABLE 1, FIGURE 2)

Liver masses present with fever, pain, abdominal discomfort, or accidentally without overt symptomatology. Benign masses include hemangioma, FNH, nodular regenerative hyperplasia (NRH), and HA. The most frequent malignancy is gastrointestinal, urogenital, lung or breast carcinoma metastasis. Liver primary malignancy includes fibrolamellar carcinoma, cholangiocarcinoma, hepatic lymphoma and angiosarcoma<sup>[52,53]</sup>.

Elevated AFP diagnoses HCC<sup>[54]</sup>, otherwise, further imaging is required. US or CT classifies cysts, metastases and hemangioma. MRI identifies focal fatty liver, FNH, HA and hemangioma<sup>[55]</sup>. When the diagnosis remains uncertain, FNAB or follow up imaging is considered. Resection is indicated for large (> 5 cm) or growing adenomas.



**Figure 2 Algorithm for the management of a liver mass in a non-cirrhotic liver.** <sup>1</sup>Most centers do not use RBC scintigraphy to diagnose hemangioma due to their use of cross sectional imaging such as contrast enhanced ultrasonography (US)/CT/MRI.

### Benign lesions

**Hemangiomas:** Hemangiomas are found in 20% of the general population, more commonly in women<sup>[56,57]</sup>. The majority are asymptomatic. Giant hemangiomas (> 4 cm) are symptomatic in 40% of cases. Symptoms are rare and can include abdominal pain, early satiety, anorexia, and nausea<sup>[58]</sup>. Tc-99m labelled RBC Spect is the best and least expensive modality (specificity 100%) for lesions > 2.5 cm and MRI for lesions < 2 cm<sup>[59,60]</sup>. University centers rarely require RBC Spect for diagnosis of hemangiomas due to the use of cross sectional imaging. Histology of liver hemangiomas is blood-filled vascular sinusoids separated by connective tissue septa (peliosis lacks endothelial layer and fibrous trabeculae)<sup>[61]</sup>. The risk of rupture is minimal and does not justify resection. Other complications include thrombosis, sclerosis, and calcification. Liver hemangiomas can grow during pregnancy or oral contraception. Kasabach-Merritt syndrome (consumption coagulopathy) and Bornman-Terblanche-Blumgart syndrome (fever and abdominal pain) constitute uncommon complications<sup>[61]</sup>.

**FNH:** Focal nodular hyperplasia is found more often in women 20-50 years of age (80%). The pathogenesis is unknown, but includes vascular injury. The lesion is usually < 3 cm, asymptomatic and discovered accidentally. The main difficulty for the physician is differentiating FNH from adenoma or fibrolamellar carcinoma by imaging techniques. However, fibrolamellar carcinomas enhance heterogeneously, wash out, and have central calcifications and enlarged lymph nodes. These features are very rarely

found in FNH. Lack of symptoms, normal liver enzymes and no use of oral contraceptives argue in favour of FNH. Recent literature suggests that MRI has 70% sensitivity and 98% specificity for FNH (homogeneous hypervascular lesion with central scar)<sup>[62,63]</sup>. If radiology is unequivocal, most hepatologists advocate a "wait and see" attitude. Otherwise, image guided percutaneous biopsies are performed, one in the tumoral tissue and the other in the normal liver tissue. The histology resembles inactive cirrhosis with proliferating hepatocytes around a normal prominent central artery with a central fibrous scar<sup>[62]</sup>. The natural history of FNH is variable (stable, regressive or progressive over time), but resection is not necessary because it does not progress to malignancy. Complications are rare and include hepatic vein thrombosis or Kasabach-Merritt syndrome<sup>[63,64]</sup>.

**HA:** Adenoma occurs in women with oral contraception use > 5 years<sup>[65]</sup> or in diabetic patients. Multiple adenomas are associated with glycogen storage disease type I and type III<sup>[66]</sup>. Adenomatosis (> 10 adenomas) is observed with anabolic or androgenic steroids consumption<sup>[67]</sup>. Abdominal discomfort is common<sup>[68]</sup>. The lesion is hypo- to hyper-echoic on US and hypo- to hyper-dense on CT. MRI is not specific<sup>[69,70]</sup>. The lesions are often < 8 cm but can be > 15 cm microscopically; they appear as monotonous sheets of normal or small hepatocytes with no bile duct, portal tract or central vein. Five percent of hepatic adenomas transform to HCC<sup>[71]</sup>.  $\beta$ -catenin immunostaining might be useful for diagnosis. Spontaneous rupture and hemoperitoneum occur in 10% of cases, especially during menstruation, pregnancy or post partum. Most hepatologists advocate resection and discontinuation of oral contraception<sup>[72,73]</sup>.

**NRH and partial nodular transformation (PNT):** NRH (large regenerative nodule) is associated with Behcet's disease, rheumatic diseases, myeloproliferative disorders, chronic venous congestion, metastatic neuroendocrine tumors, Budd-Chiari syndrome, and various drugs (steroids, contraceptives, antineoplastics, anticonvulsives, and immunosuppressives)<sup>[74]</sup>. Some lesions present with portal hypertension and cholestasis. A diagnostic open liver biopsy is rarely required<sup>[75]</sup>. NRH shows diffuse hyperplastic nodules with thickened liver cell plates without fibrosis. Reticulin changes are characteristic. Portal vein thrombosis could lead to NRH by parenchymal atrophy and compensatory hyperplasia. Portal vein thrombosis has also been invoked in the pathogenesis of PNT<sup>[76]</sup>.

**Focal fatty infiltration of the liver:** In 10% of patients with fatty liver, fat accumulates focally or shows focal sparing, usually in the anteromedial segment of the left lobe. These patients usually have diabetes, hyperlipidemia, obesity, drink alcohol or take steroids<sup>[77]</sup>. On US, fat is hyper echoic. On CT, it has low attenuation. Focal fatty liver does not displace intrahepatic vessels. The gold standard imaging technique is MRI with increased signal on T1 sequence<sup>[78]</sup>. Fat suppression techniques are also very promising.

### Other rare benign tumors

**Hepatobiliary cyst adenoma:** This lesion is rare, occurs predominantly in middle-aged women and causes abdominal pain. It differs from benign cyst by having thick septated wall. Up to 25% become malignant (cystadenocarcinoma), therefore surgical excision is indicated<sup>[79]</sup>.

**Bile duct adenoma (cholangioma):** Bile duct adenomas are solitary sub capsular nodules measuring 1-20 mm described in patients with  $\alpha$ -1-antitrypsin deficiency. The bile ducts are smaller and do not contain bile.

**Biliary papillomatosis:** Only 50 cases have been described worldwide<sup>[80]</sup>. It is a tumor of middle age, leading to biliary obstruction by tumor shedding, mucus and lithiasis, cholangitis and hemobilia. Diagnosis is made by endoscopic retrograde cholangiopancreatography (ERCP), which shows typical mucinous discharge from a dilated ampulla, multiple filling defects and stenosis. Hepatectomy can effect a cure.

**Mesenchymal and neural tumors:** This category includes lipomas, myolipomas, angiomyolipomas, schwannomas, neurofibromas and chondromas.

**Inflammatory pseudotumor:** This is a rare, benign inflammatory condition of middle-aged men. Patients might present fever, weight loss, leukocytosis and elevated ESR. If the diagnosis can be made on biopsy, there should be no need for resection; rather the primary source should be treated<sup>[81]</sup>.

**Pseudo-lesions:** A pseudo-lesion is a non-diseased area of different signal intensity, attributable to focal alteration in hemodynamics or parenchymal metabolism<sup>[82]</sup>. Pseudo-lesions seen in cirrhotic and non-cirrhotic livers include arterio-portal shunts, regenerating nodules, confluent fibrosis, and abnormal blood inflow.

### Malignant lesions

**Liver metastasis:** The liver is the most common site of metastasis from the gastrointestinal tract, pancreas, breast, and lung<sup>[41]</sup>. Multiple defects in the liver imaging suggest a metastatic process. Only 20% of liver metastases present as solitary lesions. Generally, both hepatic lobes are involved. On CT-scan, colorectal metastases appear as low attenuation lesions, often with irregular margins and necrotic centres<sup>[14]</sup>. During the early vascular phase of dynamic CT, metastasis appears with increased enhancement. The sensitivity of CT (85%) can be augmented by CT arterial portography<sup>[83]</sup>. Intraoperative US has excellent sensitivity and specificity for colorectal adenocarcinoma metastasis<sup>[84]</sup>. The most promising imaging modality is PET CT with FDG that accumulates in cells with hypermetabolism. Colon, lung and breast cancers can be staged with PET CT with sensitivity of 92%-100% and specificity of 85%-100%<sup>[85]</sup>. The hypervascularity of neuroendocrine tumors is often evident on dynamic CT<sup>[86]</sup>. Somatostatin receptor

scintigraphy can localize 90% of neuroendocrine tumors (gastrinoma)<sup>[87]</sup>.

In metastatic colorectal carcinoma, the prognosis is improved following surgical resection. Contraindications to resection include: N > 4 liver metastases, extrahepatic spread and involvement of hepatic lymph nodes. Metastatic liver tumors that might calcify include colon, leiomyosarcoma, osteogenic sarcoma, rhabdomyosarcoma, chondrosarcoma, ovarian cystadenocarcinoma, melanoma, pleural mesothelioma, neuroblastoma, and testis tumors. Calcified metastases from stomach, pancreas, lung and breast to the liver are extremely rare. Guided FNA will help identify the primary lesion<sup>[88]</sup>.

**HCC (see previous section on HCC):** Almost all patients with HCV related HCC have cirrhosis, whereas patients with HCC related to HBV are less likely to have cirrhosis. The absence of cirrhosis makes this tumor more amenable to surgical resection<sup>[34]</sup>.

**Fibrolamellar carcinoma:** The fibrolamellar variant is a distinctive subtype of HCC but is not associated with classic risk factors for HCC. It occurs at a mean age of 26 years, presenting as a large, solitary painful mass usually located in the left lobe. The AFP level is normal<sup>[89]</sup>. The term "fibrolamellar" characterizes the microscopic appearance of the lesion: thin layers of fibrosis separate the neoplastic hepatocytes<sup>[90]</sup>. A fibrous central scar may be seen on imaging studies<sup>[91]</sup>. 50% of lesions are resectable at the time of diagnosis<sup>[90]</sup>.

**Intrahepatic cholangiocarcinoma:** Cholangiocarcinoma accounts for 20% of primary liver tumors and arises as adenocarcinoma or papillary or mucinous carcinomas<sup>[92]</sup>. Risk factors include cirrhosis, primary sclerosing cholangitis (PSC, 10%), bile duct adenoma, choledochal cysts, biliary papillomatosis, Caroli's disease, and liver fluke<sup>[93-95]</sup>. Jaundice is the most common clinical presentation<sup>[96]</sup>, and rapidly increasing bilirubin associated with weight loss predicts cholangiocarcinoma. Tumor markers CEA, CA-19-9 or AFP might be elevated. CA 19-9 level > 100 has 89% sensitivity and 86% specificity<sup>[97]</sup>. There are three anatomic subtypes: Peripheral intrahepatic 15%, perihilar central (Klatskin tumor) 60%, and distal common bile duct 25%. Peripheral cholangiocarcinoma resembles HCC without cirrhosis. The central hilar and distal types are associated to sclerosing cholangitis, inflammatory bowel disease, or other chronic biliary disease. US and CT show marked intrahepatic duct dilatation<sup>[98]</sup>. An abrupt change in the calibre of the bile duct suggests malignancy<sup>[99]</sup>. Digital image analysis and fluorescent in situ hybridization are more sensitive than routine standard brush cytology in the diagnosis of cholangiocarcinoma. ERCP, percutaneous transhepatic cholangiography (PTC) and magnetic resonance cholangiopancreatography (MRCP) assess the resectability of the tumor.

PET CT stages these tumors with a sensitivity of 93%. The suggested screening includes US, CEA and CA 19-9 every six months, ERCP and brush cytology if there

is biliary stenosis. Combined HCC-cholangiocarcinoma shows features of both hepatocellular and biliary epithelial differentiation<sup>[100]</sup>.

**Epithelioid hemangioendothelioma:** This low-grade malignancy affects individuals between 20-80 years of age. It is associated with oral contraception and exposure to polyvinyl<sup>[101]</sup>. Epithelioid hemangioendothelioma presents with abdominal pain, hepatomegaly, low fever and normal liver enzymes. Endothelial cells stain for CD34, CD31 and factor VIII. The prognosis is good with surgical resection or liver transplantation<sup>[102,103]</sup>.

**Cystadenocarcinoma:** Usually in the right lobe, cystadenocarcinoma is multicytic and contains bile-stained material. It presents as abdominal pain with weight loss<sup>[104]</sup> and prognosis is good<sup>[105]</sup>.

**Lymphomas and leukemia:** Liver involvement is common in Hodgkin's disease including lymphoma infiltration (diffuse small nodules or large masses), drugs, viral hepatitis, and sepsis. Cholestasis is uncommon and vanishing bile duct syndrome has been described<sup>[106]</sup>. The differential diagnosis includes reactive infiltrate and T-cell lymphomas.

Primary hepatic lymphoma is rare and can present as solitary or multiple masses, as a diffuse hepatic involvement with hepatomegaly, or as hepatic failure with elevated LDH<sup>[107]</sup>. Peripheral gamma delta T cell lymphomas with massive hepatic sinusoidal infiltration and splenic involvement have also been described<sup>[108]</sup>. The liver might be diffusely or locally infiltrated by multiple myelomas or leukemia (chronic lymphoid leukemia, hairy cell leukemia)<sup>[109-113]</sup>.

**Neuroendocrine tumors:** Neuroendocrine tumors originating in the gastrointestinal tract frequently metastasize to the liver<sup>[114]</sup>. Liver metastases can be resected<sup>[115]</sup>. Traditional chemotherapy is not effective.  $\alpha$ -interferon has been associated with tumor response. The use of somatostatin analogs in the carcinoid syndrome improves symptoms. Liver transplant remains an option<sup>[116]</sup>.

**Angiosarcoma:** Angiosarcoma is associated with exposure to vinyl chloride<sup>[117]</sup>. Most patients are not amenable to surgery. Usually both hepatic lobes are involved and rapid tumor growth and tendency to metastasize contribute to its dismal prognosis. Chemotherapy and radiotherapy have no role, and ligation of the hepatic artery might permit palliation.

**Undifferentiated sarcoma of the liver:** This rare tumor mainly affects children. The clinical features are fever and a liver mass with recurrent hypoglycemia. The median survival is two months. Imaging shows a solid and cystic lesion with multiple loculi<sup>[118]</sup>.

**Other mesenchymal cell malignancies:** Rhabdomyosarcoma is the most common tumor of the biliary tree in young children. The tumor can mimic a choledocal

cyst<sup>[119]</sup>.

Fibrosarcoma presents as a hepatic mass with recurrent hypoglycemia that will resolve after resection. Serum IGF-2 is elevated<sup>[120]</sup> and Leiomyosarcoma presents with general deterioration and right upper quadrant pain. Angiograms or CT-angiography show a hypervascular tumor. Liver transplantation is possible<sup>[121]</sup>.

## LIVER BIOPSY VERSUS LIVER MASS RESECTION

Before hepatic resection, lesions should be measured, counted and localized to the Couinaud segments. Their relationship to major anatomical structures (portal vein, hepatic artery, inferior vena cava, and hepatic vein) should be detailed<sup>[122-125]</sup>. If malignancy is obvious, biopsy should be avoided because of possible dissemination<sup>[30,44,126]</sup>. Liver histology by true cut needle biopsy is much more profitable than fine needle aspiration and cytological examination but has several disadvantages. If the tumor is small (< 3 cm), a second attempt should be made in 20% of cases<sup>[127]</sup>, bleeding is mild in 1% and severe in 0.1%. In 10% of cases, a firm diagnosis is not established and resection should be performed.

The Child Pugh score helps select which patients should undergo hepatic resection<sup>[128]</sup>. Survival depends on the regenerative potential and the presence of cirrhosis<sup>[129]</sup>. Traditionally, cirrhosis is a contraindication to hepatic resection because of the high mortality rate (20%). A dilemma arises when patients with cirrhosis require a hepatic resection. The problem is that 10%-20% of patients with cirrhosis have primary hepatic malignancy. Moreover, 80%-90% of patients with HCC and 10%-20% of patients with cholangiocarcinoma have cirrhosis. The operative mortality of extensive hepatic resection in patients without cirrhosis is 10%<sup>[130]</sup>.

Treatment modalities include radiofrequency ablation (RFA), percutaneous ethanol injection, cryoablation, hepatic arterial chemoembolisation (TACE), and laparoscopic liver resection. Patients with compensated cirrhosis might benefit from liver resection, RFA or TACE, but patients with decompensated cirrhosis would probably experience no survival benefit<sup>[131]</sup>. In highly selected patients with incidental, central or multifocal tumors, hepatic transplantation might be more beneficial.

## CONCLUSION

In the diagnostic strategy of liver masses, two scenarios are examined: (1) incidentally discovered solid lesions or masses in a cirrhotic patient. The most likely diagnosis is HCC, followed by high and low-grade dysplastic nodule. Lesions > 2 cm are diagnosed by imaging techniques, lesions of 1-2 cm require histology if imaging modalities are atypical, and lesions < 1 cm require US follow-up every three months; (2) incidentally discovered solid lesions or masses in a non cirrhotic patient. The most prevalent lesion is hemangioma. FNH and adenoma should be ruled out in young women with contraceptive treatment. If the lesion is found at

staging or follow up of a known primary malignancy, histology is required when the lesion is doubtful. The most common liver metastases are from adenocarcinoma of colon, stomach, lung, prostate, or breast.

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REVIEW

## Perfusion computed tomography in colorectal cancer: Protocols, clinical applications and emerging trends

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### INTRODUCTION

Colorectal cancer (CRC) is the second most common cause of cancer mortality in the developed countries with increasing prevalence in developing countries due to the changing life style and eating habits<sup>[1]</sup>. More than 940 000 new cases of CRC and nearly 500 000 related deaths are reported each year worldwide.

CRCs are almost primary adenocarcinomas. Neovascularization is considered as an initial triggering event in colorectal tumor growth and dissemination<sup>[2]</sup>. The main mechanisms inciting neovascularization are angiogenesis, the growth of new blood vessels by budding or sprouting from the existing vasculature and vasculogenesis which is the de novo vascular formation from precursor endothelial cells known as angioblasts. Neovascularization arises early in the adenoma-carcinoma sequence via upregulation of various angiogenic factors including vascular endothelial growth factor<sup>[3]</sup>. Thus, the *in vivo* imaging techniques that assess the microvasculature play an immense role in the management of patients with CRC.

Functional imaging techniques including perfusion computed tomography (CT) have emerged as new dimensional tools in the evaluation of blood supply and kinetics in the oncological as well as non oncological aspect of medical fields. A definite radiological preoperative staging is mandatory for adequate management because the treatment protocols need to be standardized based on tumor invasion depth, status of the regional lymph nodes, and distant metastases. Although perfusion CT has been used for a while in the arena of neurological science, it has gained momentum recently in cancer management. In the field of oncology, perfusion CT is emerging as a novel technique applied in the diagnosis, staging, grading, prognostic evaluation based on tumor vascularity, and therapeutic response monitoring. Although many other promising imaging techniques allow for tissue perfusion measurement, CT is particularly ideal for various reasons such as its

### Abstract

Perfusion computed tomography (CT) has emerged as a novel functional imaging technique with gradually increasing importance in the management of colorectal cancer (CRC). By providing the functional tumor microvasculature, it also helps the assessment of therapeutic response of anti-angiogenic drugs as it may reflect tumor angiogenesis. Perfusion CT has been applied in clinical practice to delineate inflammatory or neoplastic lymph nodes irrespective of their size, identify micro-metastases and to predict metastases in advance of their development. It is of increasing significance for preoperative adjuvant therapies and avoidance of unnecessary interventions. Despite controversies regarding the techniques employed, its validity and reproducibility, it can be advantageous in the management of CRCs in which the prognosis is dependent on preoperative staging. With recent advances in the perfusion CT techniques, and incorporation to other modalities like positron emission tomography, perfusion CT will be a novel tool in the overall management of CRCs. This article aims at reviewing the existing clinical applications and recent advances of perfusion CT with a reference to future development in the management of CRCs.

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widespread availability, affordability, prevalence of better experience, routine availability of commercial software and readily incorporation of perfusion software for vascular measurements into existing CT protocols<sup>[4,5]</sup>.

## PERFUSION CT PRINCIPLES AND PROTOCOLS IN CRCs

The basic principle governing perfusion CT is the temporal changes in the tissue enhancement following intravenous administration of iodinated contrast media<sup>[6,7]</sup>. Compartmental and deconvolution analyses are the two commonly used analytical methods to quantify vascular physiology from the data acquired from the dynamic CT. In the compartmental modeling technique, analysis can be done by one compartment method which assumes the intravascular and extravascular compartment as a single compartment and allows measurement of tissue perfusion based on Fick's principle during the first pass of contrast, and by double compartment method which assumes the intravascular spaces as two compartments and estimates capillary permeability and blood volume (BV) by Patlak analysis which denotes the passage of contrast into the extravascular space from the intravascular space<sup>[6]</sup>. The deconvolution model on the other hand is based on determining the impulse reside function for the targeted tissue which applies to the arterial and tissue concentration-time curves.

A general perfusion CT technique typically requires a baseline unenhanced image acquisition, followed by a series of images acquired over a time period after an intravenous bolus injection of iodinated contrast media<sup>[8,9]</sup>. The unenhanced CT acquisition primarily provides a wide coverage to the targeted organ, thus serving as a localizer to select the appropriate tissue area to be covered in the following dynamic CT acquisition range. A large coverage area of about 8-16 cm in the dynamic acquisition scan providing increased sample volume has become possible with the recent advances in the scanner technology, although a 2-cm or 4-cm coverage area is still in practice in majority of the institutions. For perfusion measurement, the first pass study includes the images acquired during the initial phase for a total of approximately 40-60 s. By the compartment method, the acquisition of images is done every 3-5 s whereas by deconvolution method, images are acquired every 1 s<sup>[8,9]</sup>. For permeability measurement, second-phase images are acquired, ranging 2-10 min after the first pass study. By compartmental method, the images are obtained every 10-20 s whereas by deconvolution method every 10 s for 2 min after the first pass study<sup>[9,10]</sup>.

Special considerations have to be taken while performing the perfusion studies in CRC. Patients are usually kept on 4-6 h of fasting before water soluble contrast material is given for ingestion to assist in opacifying the small intestine. Image misregistration and errors in estimation of perfusion values due to the respiratory as well as abdominal motions during image acquisition must be avoided. Although respiratory

gating may not be so practicable, it is worthwhile instructing patients for minimal respiratory efforts. Abdominal gating is useful for proper assessment of the tumor physiology. Use of abdominal straps to curtail anterior abdominal wall excursion has been routinely performed. To overcome the bowel peristalsis during perfusion examination of colorectal region, spasmolytic drugs such as hyoscine butyl bromide or glucagon are recommended<sup>[11-14]</sup>. Besides, the use of water or saline to distend the lumen of colorectal region is advantageous for the optimal delineation of the tumor and accurate assessment of the bowel wall thickness<sup>[15]</sup>. Various fallacies do exist while selecting the region of interest, thus mandating careful judgment to avoid attenuation artifacts from various prosthesis as well as misregistration in areas prone to motion. Besides, the proper selection of arterial input is necessary to avoid errors in perfusion value measurement<sup>[7]</sup>.

## APPLICATIONS OF PERFUSION CT IN CRC

Perfusion CT has been applied increasingly in the diagnosis, differentiation, staging, grading and prognosis of CRC and for identification of occult malignancies and local or regional metastases and relapse, and prediction of tumor activity and monitor of treatment response to antiangiogenic drugs and chemoradiotherapy as well.

Henderson *et al*<sup>[16]</sup> noted that perfusion CT enables characterization of the tumor status based on extrapolating the physiological vascular parameters, thus allowing *in vivo* quantification of the microvasculature of the tumor. It has been considered that the gross morphological changes in tumor pathology occur at a later time period than the associated functional vascular profile alteration, precluding accurate and early quantification. Perfusion CT has therefore of a substantial value in assessing the tumor physiology, which is superior to other earlier performed methods<sup>[17-19]</sup>.

### **Validation and correlation with microvessel density (MVD) in CRC**

Perfusion CT exploits the enhancement characteristics of the tissue following contrast which heralds the vascular physiologic changes. Thus, perfusion CT which reflects the tumor physiology can be regarded as an indirect imaging biomarker for the *in vivo* evaluation of angiogenesis. MVD on the other hand represents the number of tumor blood vessels, varied in different tumor types, which may not reflect the angiogenic activity or angiogenic dependence of the tumor.

Dynamic contrast enhanced CT has been regarded as an appropriate non-invasive technique for assessment of angiogenesis, and positive correlations between CT parameters and immunohistologic measures, such as MVD, have also been noted<sup>[20]</sup>. Although Li *et al*<sup>[21]</sup> in their study revealed that there was no significant correlation in perfusion parameters between CRC and MVD, later observations by Goh *et al*<sup>[22,23]</sup> suggested

that BV and permeability surface area product (PS) measurement reflects angiogenesis and that perfusion CT is an appropriate technique for assessing tumor vasculature. CT perfusion imaging may be considered more valid for assessing tumorigenesis in CRC compared with the histological MVD<sup>[21]</sup>.

### **Value in diagnosis of CRC**

White *et al*<sup>[24]</sup> reported that MVD progressively increases from normal colonic mucosa through adenomas to CRC. However, MVD was not related to the Dukes' stage and appeared to decrease slightly with increasing Dukes' stage or tumor differentiation<sup>[21]</sup>. Bossi *et al*<sup>[25]</sup> reported that angiogenesis is an initial prerequisite in colorectal tumorigenesis. Goh *et al*<sup>[22]</sup> observed that CRC has high blood flow (BF), BV, PS and low mean transit time (MTT) values as compared with diverticular disease, which helps differentiate cancer from colonic thickness due to diverticulitis. Li *et al*<sup>[21]</sup> found that superficial invasive carcinomas are capable of eliciting neovascularization comparable to those with distant metastases. Rectal cancer demonstrated higher BF and shorter MIT than normal rectum. In their initial study, Bellomi *et al*<sup>[26]</sup> also found that BF, BV and PS are significantly higher in rectal cancer than in the normal rectal wall. Sahani *et al*<sup>[15]</sup> observed a significant difference in BF and MTT between rectal tumor and normal rectum. However, there was no significant difference in the BV and PS. Perfusion CT has not been found to be correlated with the tumor stages, depth of invasion, and lymph node metastasis in various studies in CRC. However, Li *et al*<sup>[21]</sup> observed that perfusion measurement had a negative correlation with increasing Duke's stage.

### **Value in prognosis of CRC**

Besides a diagnostic value in CRC, perfusion CT has also been employed to assess various treatment modalities and predict the tumor response to those therapies<sup>[15,20]</sup>. Controversies exist while taking MVD as prognostic indicator because of incoherent results and various practical limitations. Bossi *et al*<sup>[25]</sup> indicated that MVD did not provide significant prognostic information in CRC. Pietra *et al*<sup>[27]</sup> observed that MVD in CRC without lymph nodes involvement has no correlation with conventional prognostic factors and provides no significant prognostic information. Sahani *et al*<sup>[15]</sup> observed that poor prognosis was more likely in tumors with high BF and low MIT possibly due to the increased angiogenic activity of the tumor cells. Bellomi *et al*<sup>[26]</sup>, however, found that rectal cancers with high baseline BF and BV showed good response to chemoradiotherapy.

### **Value in metastases and treatment monitoring in CRC**

Leggett *et al*<sup>[28]</sup> observed that increased arterial perfusion appeared to be an indicator of liver metastases in patients with CRC. It was also noted that progressive disease may be indicated by a reduction in the portal perfusion. Irrespective of the presence of focal liver lesions, there are alterations in the microvasculature and perfusion values

in CRC, it is not ideal to employ the same enhancement protocols in patients with liver metastases<sup>[27]</sup>.

Perfusion CT has played an increasing role in monitoring therapeutic response to antiangiogenic drugs and chemoradiation as it may reflect tumor angiogenesis and provide insight of the functional tumor microvasculature<sup>[15,20,26]</sup>. However, there have been few studies to predict subsequent relapse in CRC. Goh *et al*<sup>[29]</sup> prospectively studied the vascular measurements in patients with rectal cancer to determine if it can predict subsequent metastatic relapse. It was observed that there was a significant difference in the vascularity of the tumors in patients who will subsequently develop metastases despite of curative surgery. Vascular parameter such as MTT was significantly high whereas tumor BF and permeability surface area product were low in patients who developed metastases. It was also noted that PS measurements were significantly higher in patients who already had evidence of metastatic disease compared with patients who subsequently developed metastatic disease<sup>[29]</sup>.

Perfusion CT definitely has an implied role in the monitoring and prediction of treatment response to chemotherapy and radiation therapy in rectal cancers<sup>[26]</sup>. Sahani *et al*<sup>[15]</sup> observed that after completion of chemoradiotherapy, rectal cancer showed a consistent decrease of BF and an increase of MIT. Bellomi *et al*<sup>[26]</sup> observed similar results with decrease in BF, BV and PS after chemoradiation in rectal cancer. In a clinical trial of patients with rectal cancer treated with antiangiogenic therapy (bevacizumab), Willett *et al*<sup>[20]</sup> observed that CT perfusion helped monitor antiangiogenic changes within 2 wk of treatment and its characteristics are correlated with tumor MVD, which presented with decrease in MVD and other surrogate markers of angiogenesis.

## **CONCLUSION**

Perfusion CT is strengthening its role as a preferred functional imaging technique in the management of CRC. Despite various limitations, perfusion CT has substantially influenced the imaging and treatment aspect in the management of CRC patients, particularly as a biomarker for monitoring the response of various treatment modalities, which may have further clinical usefulness in the staging of the disease and prognostic value as well. With the advancement in perfusion software and the development of new generation of scanners having a wider cine acquisition, it has allowed single or even multiple organ perfusions with the advantage of decreased radiation exposure and motion artifacts. Moreover, the integration of positron emission tomography (PET)-CT systems with FDG-PET data represents an exciting new innovative technology that has a wide range of clinical application as a single examination while minimizing the image misregistration. Development of new contrast agents which retain longer in intravascular compartment may also overcome some of the complexities of physiological modeling

required for conventional contrast agents that exhibit two-compartment pharmacokinetics. Perfusion CT which was primarily introduced as a research tool has now emerged as a definitive functional technique in the management of CRC.

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ORIGINAL ARTICLES

# Cystic echinococcosis of the liver and lung treated by radiofrequency thermal ablation: An *ex-vivo* pilot experimental study in animal models

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## Abstract

**AIM:** To evaluate radiofrequency thermal ablation (RTA) for treatment of cystic echinococcosis in animal models (explanted organs).

**METHODS:** Infected livers and lungs from slaughtered animals, 10 bovine and two ovine, were collected. Cysts were photographed, and their volume, cyst content, germinal layer adhesion status, wall calcification and presence of daughter or adjacent cysts were evaluated by ultrasound. Some cysts were treated with RTA at 150 W, 80°C, 7 min. Temperature was monitored inside and outside the cyst. A second needle was placed inside the cyst for pressure stabilization. After treatment, all cysts were sectioned and examined by

histology. Cysts were defined as alive if a preserved germinal layer at histology was evident, and as successfully treated if the germinal layer was necrotic.

**RESULTS:** The subjects of the study were 17 cysts (nine hepatic and eight pulmonary), who were treated with RTA. Pathology showed 100% success rate in both hepatic (9/9) and lung cysts (8/8); immediate volume reduction of at least 65%; layer of host tissue necrosis outside the cyst, with average extension of 0.64 cm for liver and 1.57 cm for lung; and endocyst attached to the pericystium both in hepatic and lung cysts with small and focal *de novo* endocyst detachment in just 3/9 hepatic cysts.

**CONCLUSION:** RTA appears to be very effective in killing hydatid cysts of explanted liver and lung. Bile duct and bronchial wall necrosis, persistence of endocyst attached to pericystium, should help avoid or greatly decrease *in vivo* post-treatment fistula occurrence and consequent overlapping complications that are common after surgery or percutaneous aspiration, injection and repiration. *In vivo* studies are required to confirm and validate this new therapeutic approach.

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**Key words:** Cystic echinococcosis; Hydatid cyst; Radiofrequency thermal ablation; Hepatic hydatidosis; Pulmonary hydatidosis

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## INTRODUCTION

Cystic echinococcosis is a helminthic zoonosis that can occasionally affect human beings. The organs most frequently involved are the liver and the lungs<sup>[1,2]</sup>. Viability and pathogenicity of the cyst are conditioned by the integrity of its germinal membrane. For many years elective treatment has been surgery (organ resection, cysto-pericystectomy)<sup>[3,4]</sup>. Recently percutaneous aspiration, injection and reaspiration (PAIR), using hypertonic saline or ethanol, has become a valid alternative option, principally for the liver<sup>[5,6]</sup>. This is now widely advocated in the treatment of uncomplicated fluid-filled liver cysts because of lower recurrence and complication rates<sup>[3,7,8]</sup>. There are, however, few reports in the literature on PAIR treatment of pulmonary echinococcosis<sup>[9,10]</sup>. Chemotherapy with albendazole can be used in combination with interventional procedures, but is seldom effective alone<sup>[3,11-13]</sup>. Both surgery and PAIR can result in post-procedure complications, namely, biliary or bronchial fistulae promoted by endocyst detachment<sup>[14-16]</sup>; chemical cholangitis or pneumonia due to passing of hypertonic saline or ethanol into the biliary or bronchial tree through a pre-existing communication<sup>[9,10,17,18]</sup>; and infection or abscess on the cyst cavity<sup>[3,7,10]</sup>. Chemical cholangitis is a frightful complication since it invariably requires liver transplantation<sup>[17]</sup>. Radiofrequency thermal ablation (RTA) is currently used for treatment of neoplasms, primarily hepatocellular carcinoma<sup>[19-21]</sup>.

To our knowledge, there is no controlled study in the literature on possible treatment of hydatid cyst with RTA. The aim of our study was to evaluate RTA for treatment of cystic echinococcosis of liver and lung in animal models (explantated organs). The primary end point was to evaluate efficacy in killing the parasite. The secondary end-points were to evaluate germinal layer adhesion status after the procedure and the effect of radiofrequency (RF) on host organ tissue outside the cyst.

## MATERIALS AND METHODS

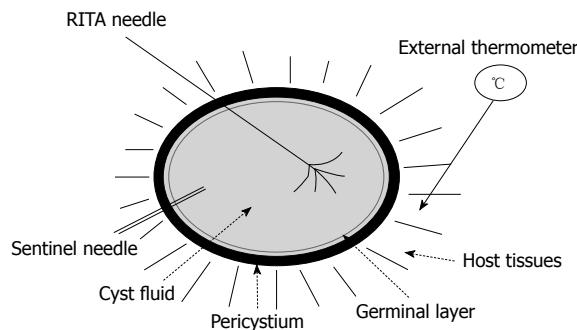
### Material

Cystic echinococcosis is an endemic zoonosis in Sicily<sup>[22,23]</sup>. To perform our study we used infected livers and lungs from slaughtered animals, 10 bovine and two ovine. Samples were provided by the Veterinary Service of the Region of Sicily within 12 h of their collection. By using slaughtered animals with endemic zoonosis, our study had minimal economic costs and raised no ethical concerns.

### Procedure

Each cyst was photographed and evaluated by ultrasound (US). Cyst volume was calculated with the ellipsoid volume formula ( $4/3 \pi abc$ ;  $abc$  = cyst hemidiameters). Cyst content, germinal layer adhesion status, presence of wall calcification and presence of daughter or adjacent cysts were also evaluated by US.

The *ex-vivo* approach, large size and superficial place



**Figure 1** Schematic description of radiofrequency thermal ablation (RTA) on echinococcal cyst.

of the cysts made US evaluation easy in the lung as well as the liver.

Each cyst was placed on a RITA® dispersive electrode and, under US guidance, a 9-tip expandable needle (RITA® StarBurst TM XL, RITA Medical Systems) was inserted into the cyst, expanded and connected to a RF generator (RITA Medical System 1500 X). Temperature inside the cyst was monitored by RITA needle tip temperature transducers. Under US guidance a thermometer (bimetal thermometer 0 + 120°C, probe length: 110 mm - Ø 4 mm, dial Ø 45 mm) was also placed outside the cyst, up to 1 cm from its wall, for external temperature monitoring. Both internal and external temperature was monitored throughout the procedure and for the following 20 min.

In addition to the RITA needle, a second needle, which we named "sentinel needle," was also placed inside the cyst in order to stabilize internal cyst pressure during the procedure (Figure 1). In fact, during pre-study sessions of the treatment that we preliminarily performed in order to optimize the technical aspects of the procedure, we observed that while reaching target temperature, endocytic pressure quickly increases and invariably causes cyst rupture unless pressure stabilization is obtained. The increase in endocytic pressure is likely due to gas formation (water vapor, CO<sub>2</sub>) at the interface of the fluid/needle because of the high local temperature.

The RTA parameters we used were: 150 watts, 80°C, 7 min. Treated cysts were then sectioned and a pathology examination was performed. Histology on the samples evaluated the germinal layer, the presence of scolices, the pericystium, parenchymal cells, blood vessels, biliary ducts (liver) and the bronchial walls (lung).

All the data were stored in an Excel database and used for statistics. In addition to the treated cysts, there were also some untreated parasitic cysts randomly examined with US and histology, using the same criteria as that used for treated cysts, as an addendum to the study.

### Viability definition

Alive cyst: histological evidence of preserved germinal layer; killed cyst: necrotic endocyst at histology.

### Statistical analysis

By definition, pilot studies are conducted on a small

**Table 1 Baseline characteristics of liver and lung cysts and results of treatment with RTA**

	Liver (n = 9)		Lung (n = 8)	
	Baseline	Post-RTA	Baseline	Post-RTA
Cyst volume (mL) <sup>1</sup>				
Average	40.5	14.9	158.7	57.1
Range	8.3-61.6	2.0-28.3	4.2-471	1.3-287.8
Fluid echo pattern <sup>2</sup>				
Anechoic	9	0	6	0
Anechoic + ground	0	0	2	0
Disomogeneous	0	9	0	8
Prolifer status <sup>1</sup>				
Adhered	9	6	6	6
Focally detached	0	3	2	2
Detached	0	0	0	0
Wall calcification (any) <sup>1</sup>	0	0	0	0
Daughter/adjacent cysts <sup>1</sup>	1	1	1	1
Internal	0	0	0	0
External	1	1	1	1
Killing rate (%) <sup>3</sup>	-	100	-	100
Pericyst necrosis (cm) <sup>3</sup>				
Mean (range)	-	0.64 (0-2)	-	1.57 (0.8-2.5)
External temperature (°C) <sup>4</sup>				
Average of peaks	42.9		52.6	
Range of peaks	23-69		39-64	
Gradient T (range) <sup>5</sup>	11.9-58.3		16.9-38.7	

<sup>1</sup>Ultrasound and pathology assessment; <sup>2</sup>Ultrasound assessment;<sup>3</sup>Pathology assessment; <sup>4</sup>During the procedure; <sup>5</sup>Gradient T = T<sub>max</sub> internal - T<sub>max</sub> external. RTA: Radiofrequency thermal ablation

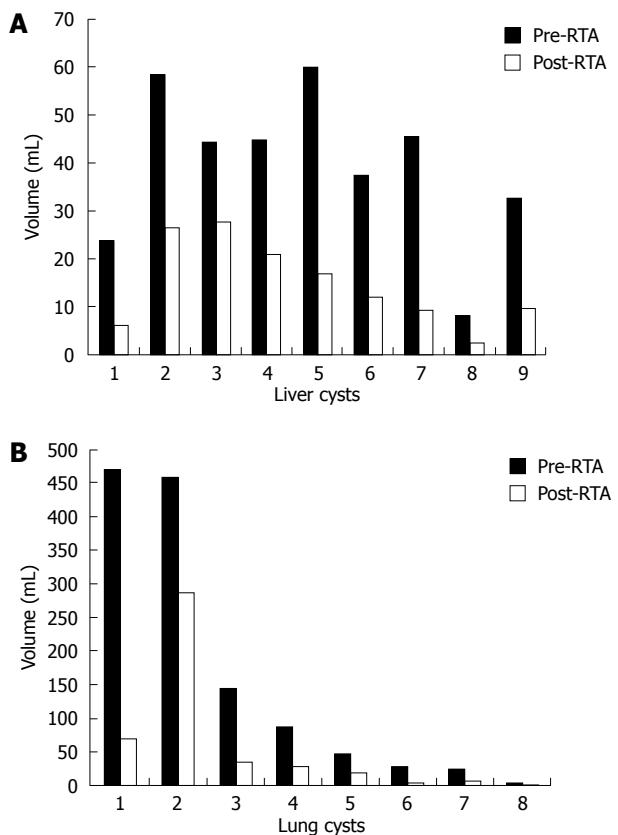
number of cases, which makes reliable statistical correlations difficult to establish. Nevertheless, we performed a statistical analysis of our data in an attempt to identify significant trends. All values were expressed as a mean, and the Mann-Whitney *U*-test was used for comparison among groups. All *P* values were two-sided, and *P* < 0.05 was considered statistically significant. The Pearson test was used to investigate the linear relationship between two groups of data.

## RESULTS

A total of 28 cysts, 16 hepatic and 12 pulmonary, were evaluated. Seventeen, treated with RTA, were the object of our study (Table 1). In addition, 11 untreated cysts, seven hepatic and four pulmonary, were randomly evaluated. All of them were unilocular with anechoic content, no wall calcification, germinal membrane adherence were observed at US, and all were alive at pathology.

### Treated cysts

Seventeen cysts were treated with RTA: nine hepatic, average volume 40.5 mL (range, 8.3-61.6 mL), and eight pulmonary, average volume 158.7 mL (range, 4.2-471 mL). Baseline US showed the endocyst was attached to the pericystium in 100% (9/9) of liver and 75% (6/8) of lung cysts, and focally detached in 25% (2/8) of lung cysts. Average time to reach the target temperature inside the cyst was 6.3 min for the liver (range, 3-14 min) and 9.5 min for the lung (range, 3-19 min). During RF administration the internal temperature remained stable at target temperature



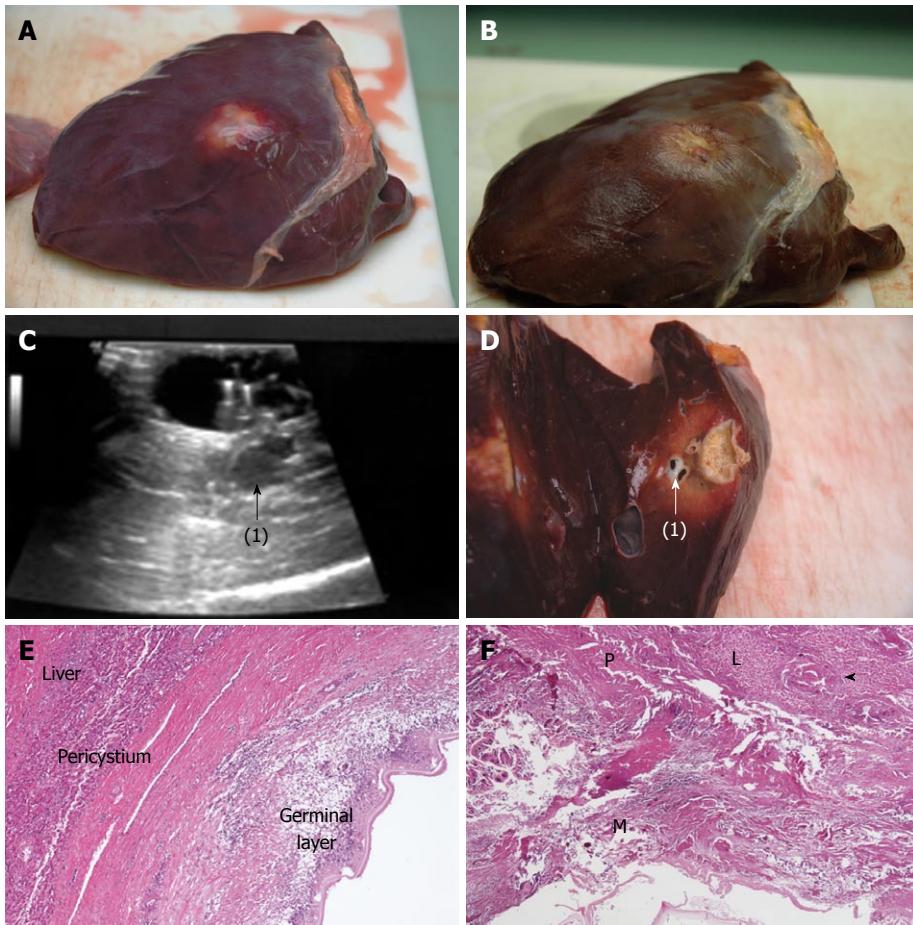
**Figure 2 Cyst volume before and after RTA in liver (A) and lung (B).** All treated cysts showed a strong and immediate reduction in volume.

in all treated cysts.

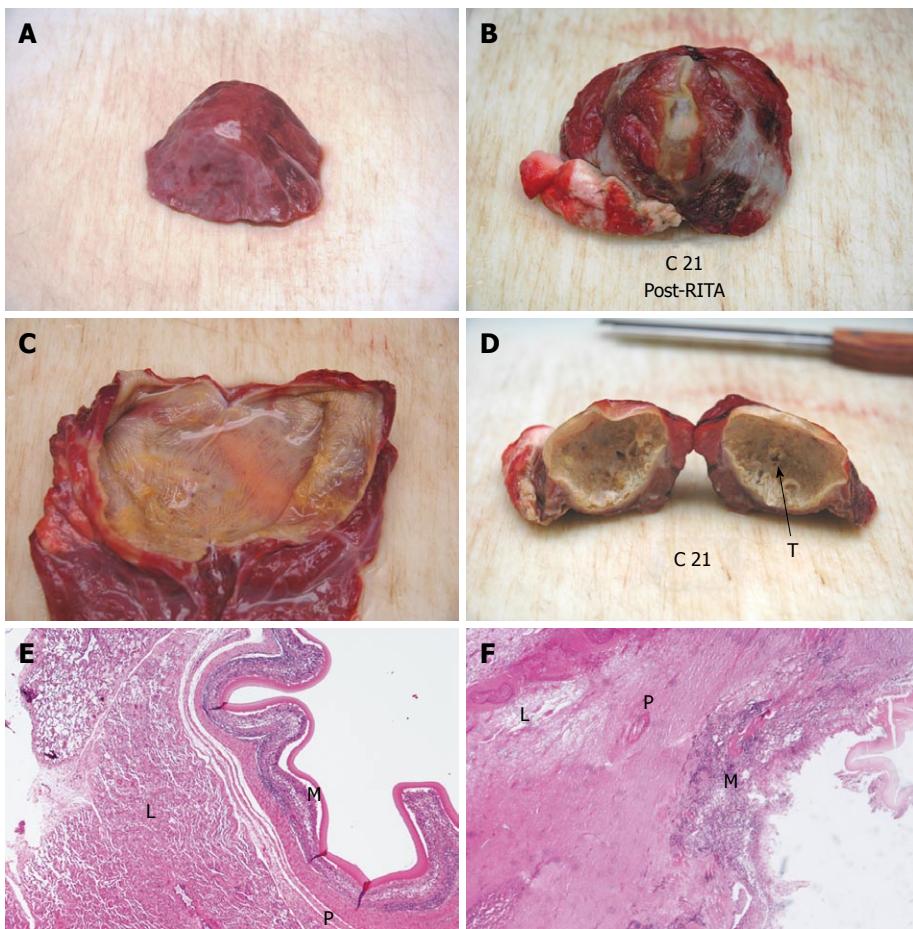
After RTA, 100% of the cysts had volume reduction. For the liver, the cyst volume average decreased from 40.5 mL before to 14.9 mL after RTA (*P* = 0.004). For the lung, the cyst mean volume decreased from 158.7 to 57.1 mL (*P* = 0.13). Hence, immediate reduction of mean volume for liver and lung cysts was 65% and 69%, respectively (Figure 2).

Compared with baseline, the endocyst remained attached to the pericystium after RTA in hepatic and lung cysts. We observed just a small and focal (i.e. few millimeters) *de novo* endocyst detachment in three hepatic cysts. Histology showed a 100% success rate in both hepatic (9/9) and lung cysts (8/8). A coagulative necrosis of the endocyst and the pericystium was observed, as well as a concentric area of host organ tissue necrosis, variable in size, with an average extension of 0.64 cm (range, 0-2 cm) for liver, and 1.57 cm (range, 0.8-2.5 cm) for lung (Figures 3-6). Possible correlation between cyst volume and size of pericyst necrosis was evaluated. For the liver, an inverse correlation of 68% was observed between pre-RTA volume and pericyst necrosis extension (*P* = 0.044). For lung cysts this correlation was 30% (*P* = 0.471). It is worth noting that within the necrotic area, parenchymal cells, blood vessels, bile ducts (liver) and bronchial walls (lung) were all necrotic in all treated cysts. Organ samples taken from areas far from the treated cysts showed normal histology in all cases.

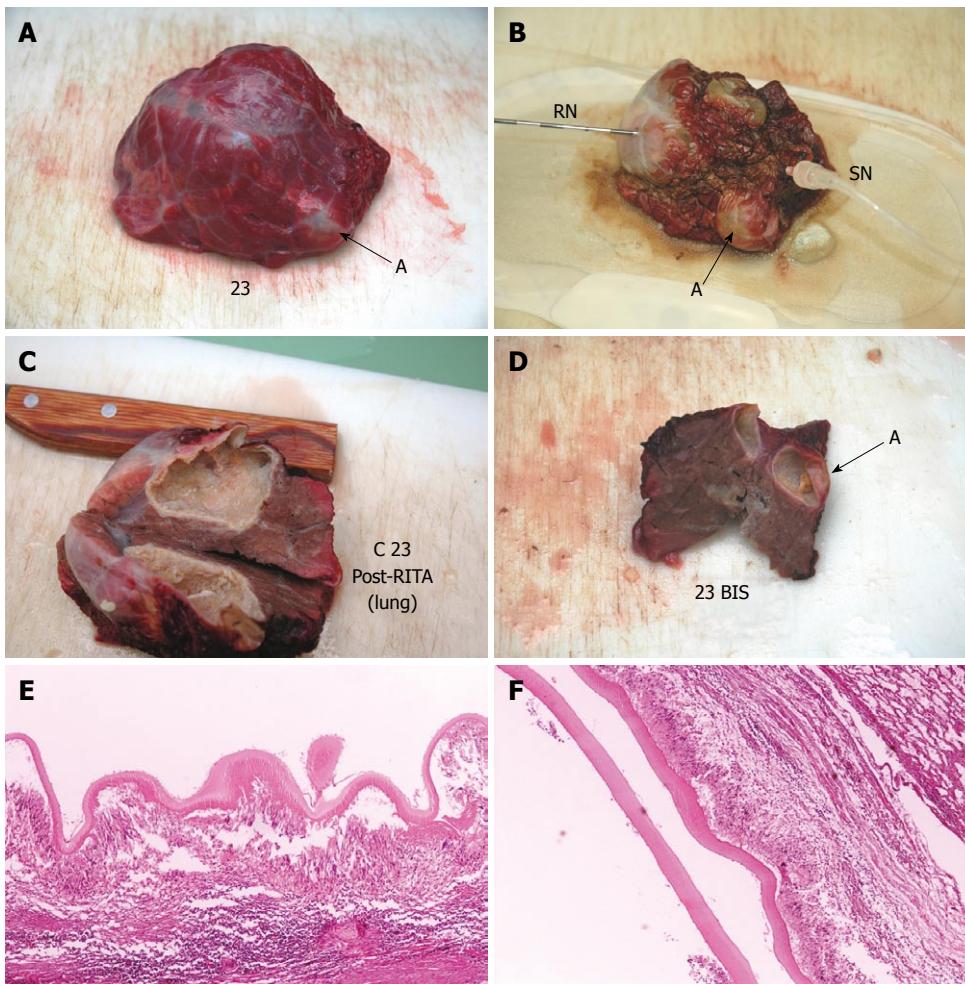
Two cysts, hepatic and pulmonary, respectively,



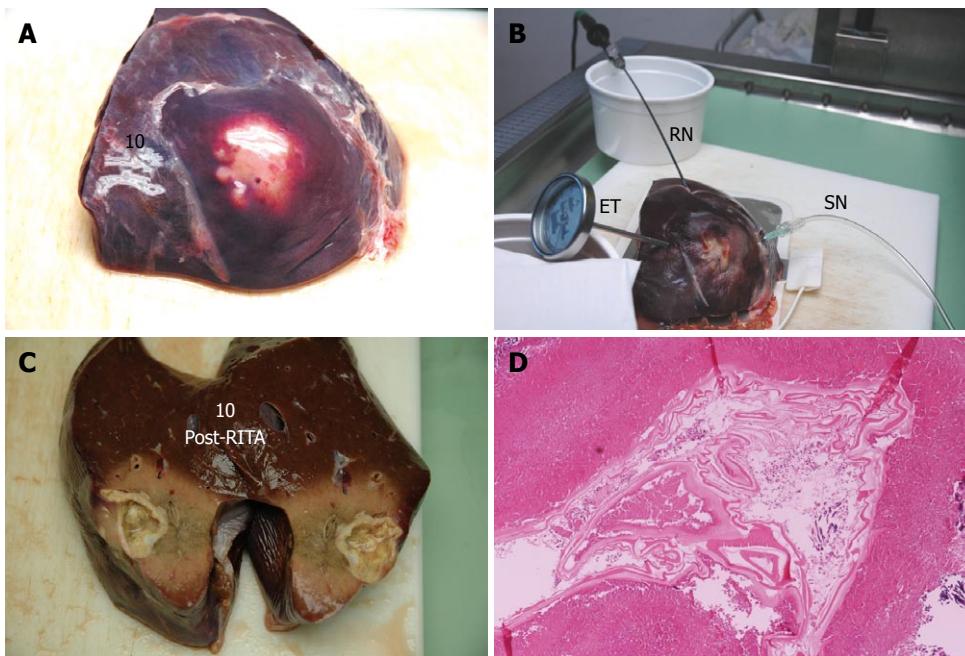
**Figure 3 Hydatid cyst of the liver treated with RITA.** The upper panel shows the surface of the liver before (A) and after (B) RITA. The procedure caused cyst retraction. The middle panel shows pre-procedure ultrasound (C) and post-RTA section (D) of the cyst. A transverse US scan shows the RITA needle expanded inside the cyst and a smaller adjacent cyst (1) situated within the necrotic area surrounding the treated cyst. Histology showed the smaller cyst was necrotic and killed despite no direct treatment. In the lower panel, histology (HE stain,  $\times 4$ ) of an untreated cyst (E) is compared with that of the treated one (F). In the former, all structures are well recognizable, while in the latter the cyst layers are totally necrotic. M: Germinal membrane; P: Pericystium; L: Liver parenchyma. Arrow tips indicate necrotic portal tracts. 1: Adjacent cyst.



**Figure 4 Hydatid cyst of the lung.** Untreated (left) and treated (right) cysts are compared. External surface (upper panel), findings after sectioning (middle panel), and  $4 \times$  HE stained histology (lower panel) are shown. Untreated cyst shows normal surface, flaccidity after sectioning, thin and translucent endocyst and well preserved cyst layers at histology. Treated cyst looks dehydrated, rigid, has a thick and papyraceous-like endocyst and is totally necrotic at histology. T: RITA-needle tract; M: Germinal membrane; P: Pericystium; L: Lung parenchyma.



**Figure 5** *Echinococcus* cyst of the lung before (A) and after (B-D) RITA. External surface of the cyst (upper panel), findings after sectioning (middle panel), and histology (lower panel). After treatment (B) the cyst looks shrunken, rigid and dehydrated. Post-RITA section of the cyst (C) shows the endocyst is thick, papyraceous-like and attached to pericystium. Histology (HE stain,  $\times 4$ ) shows complete necrosis of the cyst wall (E). An adjacent cyst (indicated by arrow A in slides A, B, D) was necrotic and killed at histology (F) despite no direct treatment. RN: RITA needle; SN: Sentinel needle.



**Figure 6** *Echinococcus* cyst of the liver treated with RITA. The upper panel shows the surface of the liver before (A) and after (B) RITA. Procedure caused cyst retraction. RITA needle, sentinel needle and external thermometer, still in place, can be seen (B). The left lower panel shows post-RITA section (C) of the cyst. Histology of a paramedian section (right lower panel, HE stain,  $\times 2$ ) shows cyst walls and surrounding liver closest to the cyst are totally necrotic and with granular amorphous aspect (D). RN: RITA needle; SN: Sentinel needle; ET: External thermometer.

adjacent ( $< 1$  cm) to the ones treated were killed despite no direct treatment (Figures 3 and 5). Another cyst, an external daughter lung cyst not directly treated, was partially alive at histology, though the procedure on the mother cyst was non-optimal because the cyst ruptured early.

Temperature curves detected inside and outside the cysts during the entire procedure had similar courses, but with clearly lower values in the host tissue near the cysts (data not shown). The highest temperature gradient ( $T_{\max}$  internal -  $T_{\max}$  external) ranged between  $11.9^{\circ}\text{C}$  and  $58.3^{\circ}\text{C}$  (mean,  $38.0^{\circ}\text{C}$ ) in liver cysts and between  $16.9^{\circ}\text{C}$

and 38.7°C (mean, 27.7°C) in the lung. The external temperature peak was never above 70°C, and in 88% of the samples was below 55°C.

## DISCUSSION

Our primary goal was to investigate whether RTA can kill *Echinococcus granulosus* cysts in explanted livers and lungs. We observed that RF (1) warmed the hydatid cyst fluid and caused endocyst necrosis in both hepatic and lung cysts in 100% of cases; (2) maintained endocyst attachment to the pericystium; (3) caused a layer of host tissue damage outside the cyst; (4) gave lower temperature values outside the cyst than inside it; and (5) resulted in a post-procedure volume reduction average of more than 65%.

From a review of the literature we found only two case reports on using RTA for treatment of cystic lesions in humans. No controlled study on RTA treatment of hydatid cysts is reported. Rhim *et al*<sup>[24]</sup> successfully treated a simple cyst of the liver, though this differs from the hydatid cyst from biological and pathological points of view. Brunetti *et al*<sup>[25]</sup> reported treatment with RTA on a single patient with cystic echinococcosis of the liver. However, here, the assessment of treatment efficacy was based solely on disappearance of scolices upon post-procedure microscopic examination of cyst fluid. This is not sufficient for assessing the definitive killing of the cyst if long term clinical follow-up is lacking, though the ideal procedure is microscopic verification of permanent damage to the germinal membrane, as mentioned by Brunetti *et al*<sup>[25]</sup> themselves in their comments on Khuroo's paper<sup>[26]</sup>. In this regard, we would like to underscore that in our study a microscopic examination with methylene blue stain on cyst fluid collected through the sentinel needle just before, during, and soon after the RF administration was initially performed on a limited number of cysts, looking for scolices and their viability. This was discontinued in the study because of its total lack of sensitivity (no scolex was seen in the centrifuged fluid, even though they were present later at histology). Finally, Brunetti *et al*<sup>[25]</sup> in their case report say nothing of pressure stabilization to avoid cyst rupture.

Actually, in our pre-study sessions of treatment, all the cysts we treated invariably "exploded" a few minutes after the start of the procedure, independently of their baseline volume. That no longer happened after we started placing a "sentinel needle" inside the cyst. We also observed that ruptured cysts, despite reaching the target internal temperature faster, were still alive and often contained well preserved scolices on pathology examination. This suggests that RTA kills the parasite by using its own fluid, which assures homogeneous and sustained values of high temperature inside the whole cyst. If there is a rupture of the cyst and its fluid is released, RF causes only focal damage, probably where the needle tips are in contact with the cyst wall.

It has been demonstrated that during PAIR on cystic echinococcosis, the germinal membrane usually detaches entirely and collapses inside the cyst<sup>[3,9,14-16]</sup>. This always

occurs, by definition, during peri-cystectomy. Endocyst detachment can cause the appearance of biliary or bronchial fistulae inside the cyst cavity. This is more likely if a pre-existing virtual communication between the parasitic cyst and the biliary or bronchial tree is present<sup>[9,10,27,28]</sup>. The biliary fistula is one of the most frequent complications after surgery or percutaneous treatment of liver hydatid cysts<sup>[15,29-31]</sup>, though at lower rates after PAIR<sup>[3,32]</sup>. It often evolves into infected collection and abscess, thus increasing hospital length of stay, economic costs and patient discomfort<sup>[32]</sup>. The heat-related coagulative necrosis we obtained with RTA can explain both the killing effect on the parasite and also why the endocyst remained attached to the pericystium. Based on that, it seems reasonable to suppose a low (if not lack of) incidence of fistula occurrence after RTA *in vivo*. Furthermore, the layer of host tissue necrosis we observed outside the treated cysts, whether thinner or thicker, could further increase the killing effect (by stopping *in vivo* blood vessel supply) and decrease the incidence of post-procedure fistula occurrence (by causing bile duct or bronchial wall necrosis). This, of course, must still be proven by controlled *in vivo* studies. Nevertheless, an indication of this possibility emerged in a recently published case report by Thanos *et al*<sup>[33]</sup>, who successfully treated a post-surgical biliocystic communication and cystocutaneous fistula with RF.

Based on our results we expect that *in vivo* blood flow would have no significant effect on the intra-cystic killing power of RTA. In fact, data on treatment of hepatocellular carcinoma and other hepatic tumors show how RTA can cause *in vivo* necrosis of vascularized nodules up to 5 cm in size<sup>[19,20]</sup>, and up to 8.5 cm in size if associated with occlusion of tumor blood supply<sup>[21]</sup>. This should occur even more on a hydatid cyst that has no internal blood flow (and therefore no internal heat sink effect), that is full of fluid and that has a relatively thick wall that creates a physical barrier from the outside, as also demonstrated by the gradient we calculated between the temperature inside the cysts and outside. In any case, only *in vivo* controlled studies can definitively answer this question.

Several studies report a volume reduction of the cyst of 65% or more after PAIR or pericystectomy, and such rates are described usually 6-19 mo after the treatment<sup>[16,31,34,35]</sup>. In our study, compared with baseline, we observed this level of volume reduction immediately after treatment. This is probably due to necrosis and dehydration of the cyst wall and the surrounding host tissues (Figures 5 and 6). The volume decrease after RTA and correlation of cyst volume with extension of pericyst necrosis were significant for the liver but not for the lung. This is probably due to different tissue characteristics (liver is solid, while lung has some air inside); to the small number of cases analyzed; and, lastly, to the fact that the lung cysts provided were larger and mainly exophytic compared with the hepatic cysts. An optimal statistical investigation of such correlations would require larger numbers.

With respect to our goals, we believe the results of

our pilot study are very interesting, particularly the 100% success rate we observed despite different baseline cyst volumes and types of organs treated.

Potential advantages expected with *in vivo* RTA on cystic echinococcosis as follows: (1) While PAIR sometimes requires more sections to assure definitive killing of the cyst, RTA can kill a hydatid cyst in a single section, since the entire cyst wall is made necrotic by a heat-related mechanism; (2) Given that RTA avoids the use of chemical media there is no risk of chemical cholangitis related to its accidental passing into the biliary tree. Hence, pre-existence of cysto-biliary communication is no longer a contraindication to percutaneous treatment; (3) Compared with PAIR or surgery, persistence of endocyst attachment to pericystium in a high percentage of cases should help avoid or greatly decrease post-RTA fistula occurrence, and consequent overlapping complications.

The results, and the experimental hypothesis raised by our study, must be confirmed by *in vivo* study.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Treatment of cystic echinococcosis is surgery or percutaneous aspiration, injection and reaspiration (PAIR) using hypertonic saline or ethanol, and it is aimed to cause permanent damage to the endocyst. Surgery and PAIR can result in biliary or bronchial fistulae, prompted by endocyst detachment; chemical cholangitis or pneumonia, due to passing of hypertonic saline or ethanol into the biliary or bronchial tree; and infection or abscess on residual cyst cavity. Radiofrequency thermal ablation (RTA) is currently used for treatment of neoplasms, primarily hepatocellular carcinoma.

### Research frontiers

Features of an ideal therapy for hydatid cysts should include the following: (1) certainty of killing the parasite; (2) avoidance of post-procedure fistulae and consequent overlapping complications; (3) mini-invasive approach. The authors experimented with the use of RTA trusting in its heat-related killing power as well as on the possibility that, after heat-related coagulative necrosis of cyst wall, the parasitic endocyst could have remained attached to the pericystium. This approach is unlike surgery or PAIR, which invariably cause endocyst detachment.

### Innovations and breakthroughs

The research showed that radiofrequency is able to warm and irreversibly damage hydatid cysts in a single section, since the entire cyst wall is made necrotic by a heat-related mechanism. Avoiding the use of chemical media, there is no risk of chemical cholangitis. Unlike PAIR, the existence of cysto-biliary communication would no longer be a contraindication to percutaneous treatment of the hydatid cyst. The persistence of endocyst attachment to pericystium should help avoid or greatly decrease post-RTA fistula occurrence and consequent overlapping complications.

### Applications

This pilot study suggests that a new therapeutic approach in treatment of hydatid cyst is possible and, indeed, very effective. The *ex vivo* model is expected to be predictive of what would be the *in vivo* effect of RTA, since cystic lesions have no internal blood vessels. *In vivo* blood flow would not have a significant impact, particularly in view of the fact that HCCs of several cm can be currently ablated with RTA despite being vascularized (heat sink effect), and that RTA power is increased by occlusion of tumor blood supply. The study also seems to suggest that RTA could potentially be used to attempt treatment of other types of cystic lesions (symptomatic treatment in polycystic disease,

cystic neoplasms, pancreatic pseudocysts, etc).

### Terminology

*Cystic echinococcosis* is a helminthic zoonosis that can affect human beings, most frequently involving the liver or the lungs, where it causes expansive cystic lesions. Endocyst or *prolifera* membrane is the thin, delicate, and translucent inner membrane that produces the cyst fluid and generates new larval elements able to expand the infestation.

### Peer review

This is an *ex vivo* pilot study on RFA in cystic echinococcosis in a relatively small animal sample size. The authors report 100% success rate in treating cysts and several innovative technical aspects. Results and literature data reported in the paper discussion suggest that this technique is very promising.

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ORIGINAL ARTICLES

# Prophylaxis with carnosol attenuates liver injury induced by intestinal ischemia/reperfusion

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## Abstract

**AIM:** To investigate the possible protective effects of carnosol on liver injury induced by intestinal ischemia reperfusion (I/R).

**METHODS:** Rats were divided randomly into three experimental groups: sham, intestinal I/R and carnosol treatment ( $n = 18$  each). The intestinal I/R model was established by clamping the superior mesenteric artery for 1 h. In the carnosol treatment group, surgery was performed as in the intestinal I/R group, with intraperitoneal administration of 3 mg/kg carnosol 1 h before the operation. At 2, 4 and 6 h after reperfusion, rats were killed and blood, intestine and liver tissue samples were obtained. Intestine and liver histology was investigated. Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and interleukin (IL)-6 were measured. Liver tissue superoxide dismutase (SOD) and myeloperoxidase (MPO) activity were assayed. The liver intercellular adhesion molecule-1 (ICAM-1)

and nuclear factor  $\kappa$ B (NF- $\kappa$ B) were determined by immunohistochemical analysis and western blot analysis.

**RESULTS:** Intestinal I/R induced intestine and liver injury, characterized by histological changes, as well as a significant increase in serum AST and ALT levels. The activity of SOD in the liver tissue decreased after I/R, which was enhanced by carnosol pretreatment. In addition, compared with the control group, carnosol markedly reduced liver tissue MPO activity and serum IL-6 level, which was in parallel with the decreased level of liver ICAM-1 and NF- $\kappa$ B expression.

**CONCLUSION:** Our results indicate that carnosol pretreatment attenuates liver injury induced by intestinal I/R, attributable to the antioxidant effect and inhibition of the NF- $\kappa$ B pathway.

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**Key words:** Carnosol; Liver injury; Ischemia reperfusion; Nuclear factor  $\kappa$ B

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Yao JH, Zhang XS, Zheng SS, Li YH, Wang LM, Wang ZZ, Chu L, Hu XW, Liu KX, Tian XF. Prophylaxis with carnosol attenuates liver injury induced by intestinal ischemia/reperfusion. *World J Gastroenterol* 2009; 15(26): 3240-3245 Available from: URL: <http://www.wjgnet.com/1007-9327/15/3240.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.3240>

## INTRODUCTION

Intestinal ischemia reperfusion (I/R) is one of the most common types of cell injury, which is caused by many factors, such as acute blood loss, shock, ileus and multiple trauma. Intestinal I/R not only leads to intestinal damage itself, but also causes severe destruction of remote organs<sup>[1-3]</sup>. The liver is the first distant organ involved after intestinal I/R<sup>[4,5]</sup>. Although the detailed mechanism of liver injury induced by intestinal I/R remains to be elucidated, a variety

of inflammatory mediators including intracellular adhesion molecule-1 (ICAM-1) and cytokines, as well as infiltration of neutrophils have been implicated in this process<sup>[6-8]</sup>.

Nuclear factor κB (NF-κB) is one of the most important transcriptional factors, which consists of p50 and p65 subunits. In quiescent cells, it exists as a latent cytoplasmic complex bound to an inhibitor protein, I-κB. In response to an activation signal, I-κB is phosphorylated and degraded through the proteosomal pathway. The free NF-κB complex is then able to translocate into the nucleus and induce expression of various target genes that are critical for cell survival, inflammation and immunity<sup>[9,10]</sup>. NF-κB is also activated and plays a major role during the local and systemic inflammatory response following intestinal I/R<sup>[11]</sup>. Thus, modulating the NF-κB pathway is a new concept and therapeutic option to attenuate intestinal I/R-induced local and remote organ injury<sup>[11-13]</sup>.

Carnosol, a major component of rosemary, is a phenolic diterpene that has potent antioxidant and anti-inflammatory activities<sup>[14-16]</sup>. Carnosol has been found to suppress NO production and inducible nitric oxide synthase gene expression by inhibiting NF-κB activation<sup>[17]</sup>.

In this study, we investigated the effects of carnosol on liver injury following intestinal I/R and explored the mechanism of its protective action.

## MATERIALS AND METHODS

### Animals

Male Wistar rats (Animal Center of Dalian Medical University, Dalian, China) weighing 190-220 g were used in this study, which was approved by the Institutional Ethics Committee. All rats were fed with standard laboratory chow and water, and housed in accordance with institutional animal care guidelines.

### Experimental design

Rats were assigned randomly into three experimental groups: control, intestinal I/R and carnosol pretreatment groups ( $n = 18$  in each group). The rats in the control group underwent surgical preparation including isolation of the superior mesenteric artery (SMA) without occlusion; the intestinal I/R group was subjected to 1 h intestinal ischemia and various times of reperfusion after the SMA was isolated and occluded<sup>[18]</sup>. In the carnosol pretreatment group, surgery was performed as in the intestinal I/R group with intraperitoneal administration of 3 mg/kg carnosol (Cayman Chemical Company, Ann Arbor, MI, USA) 1 h before the operation. Carnosol was dissolved in 3% DMSO before administration. The dose of carnosol administered was determined according to a previous study<sup>[19]</sup>, with modification from our preliminary experiments. The rats in the control and intestinal I/R group were treated with an equal volume of 3% DMSO when needed. At each of the indicated time points (2, 4 and 6 h after reperfusion), six rats were

killed randomly from each group, and blood, intestine and liver tissue samples were obtained for further analysis.

### Intestine and liver morphological assessment

The isolated intestine and liver tissues were harvested and fixed in 10% formalin. After being embedded in paraffin, the tissues were stained with hematoxylin and eosin for light microscopy.

### Measurement of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and interleukin (IL)-6 levels

The serum levels of AST and ALT were measured with an OLYMPUS AU1000 automatic analyzer (AusBio Laboratories Co., Ltd. Beijing, China). The serum levels of IL-6 were determined using a radioimmunoassay kit (Radioimmunity Institute of PLA General Hospital, Beijing, China) following the manufacturer's instructions.

### Liver superoxide dismutase (SOD) and myeloperoxidase (MPO) activity assay

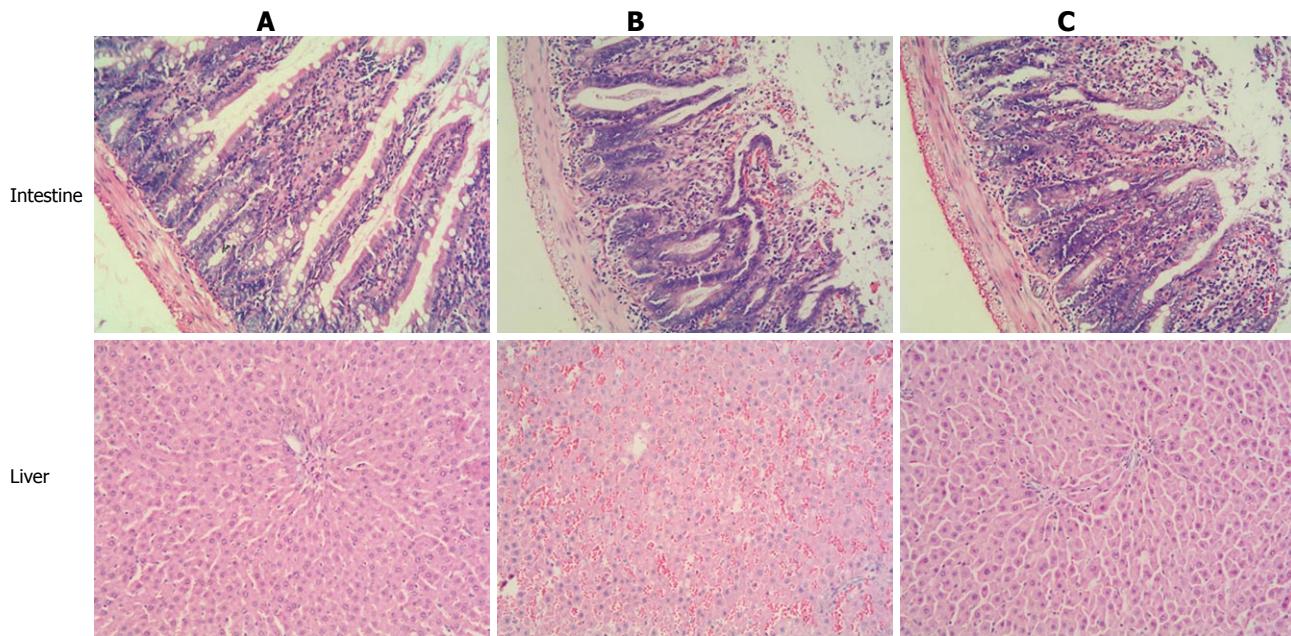
The liver tissues were harvested and homogenized immediately on ice in five volumes of normal saline. The homogenates were centrifuged at 1200 r/min for 10 min. The SOD and MPO activity in the supernatant was determined using an assay kit (Nanjing Jincheng Corp., China), according to the manufacturer's recommendations.

### Liver NF-κB immunohistochemical analysis

Formalin-fixed, paraffin-embedded liver tissue sections of 4-μm thickness were stained with SP immunohistochemistry technique for NF-κB. After being dewaxed or washed in PBS, tissue sections were cultured in 3% hydrogen peroxide to eliminate intrinsic peroxidase, and quenched in normal goat serum for 30 min. The sections were then incubated overnight at 4°C with polyclonal rabbit anti-rat NF-κB antibody (NeoMarkers Corp, Jingmei Biotech, Shenzhen, China), followed by addition of anti-rabbit immunoglobulin and streptavidin conjugated to horseradish peroxidase (HRP). Finally, slides were stained with 3,3'-diaminobenzidine (DAB), and hematoxylin was used for counter staining. Brown staining in cytoplasm and nucleus were considered as positive.

### Liver ICAM-1 and NF-κB western blotting

Cellular plasma and nuclear protein were extracted from frozen live tissue with a protein extraction kit (Pierce, Rockford, IL, USA). The protein was separated by 10% SDS-PAGE and then electroblotted onto nitrocellulose membranes (Millipore, Bedford, MA, USA) for 30 min. The membranes were then incubated overnight at 4°C with rabbit polyclonal antibody ICAM-1 (Boster Corp, Ltd. Wuhan, China) and NF-κB against rat. The membranes were incubated for 1 h at 37°C with an anti-rabbit IgG conjugated with HRP. The signals were visualized by a DAB assay kit (Fuzhou Maixin Biological



**Figure 1 Changes in histology of intestine and liver tissues ( $\times 200$ ) in the control (A), intestinal I/R (B) (1 h ischemia and 4 h reperfusion) and carnosol pretreatment (C) groups.** A: Normal structure of intestine and liver; B: Edema, hemorrhage and neutrophil infiltration were observed in intestinal mucosa and liver tissue; C: Relatively normal histology of intestine and liver with less inflammatory cell infiltration.

Technology Co., Ltd, Fuzhou, China) and analyzed with a gel imaging system (Kodak System EDAS120, Japan).

#### Statistical analysis

All data were expressed as mean  $\pm$  SD. The *F* test was used to evaluate statistical significance using SPSS 10.0 statistical software (Chicago, IL, USA).  $P < 0.05$  was considered statistically significant.

## RESULTS

#### Effect of carnosol on intestinal I/R-induced liver injury

Intestinal I/R induced apparent intestine and liver injury at all the time points (2, 4 and 6 h) after reperfusion, manifested as histological changes in the intestine and liver with edema, hemorrhage and neutrophil infiltration, as well as a significant increase in serum AST and ALT level ( $P < 0.01$ ,  $P < 0.05$ ) when compared with the control group. Compared with the I/R group, the intestine and liver pathological damage was improved in the carnosol pretreatment group (Figure 1). In addition, there was a significant difference in liver function between the intestinal I/R and carnosol pretreatment groups ( $P < 0.05$ , Figure 2), which indicates that carnosol significantly attenuated the intestinal I/R-induced liver injury.

#### Effect of carnosol on liver SOD activity

Compared with the control group, liver tissue SOD level in the I/R group reduced significantly ( $P < 0.01$ ). SOD level was elevated markedly in carnosol pretreatment ( $P < 0.05$ , Figure 2).

#### Effect of carnosol on liver neutrophil infiltration

Liver neutrophil infiltration was determined by MPO

activity. Compared with the control group, liver tissue MPO activity increased significantly after intestinal I/R ( $P < 0.01$ ). Administration of carnosol reduced MPO activity in liver tissue significantly ( $P < 0.05$ , Figure 2).

#### Effect of carnosol on serum IL-6 level

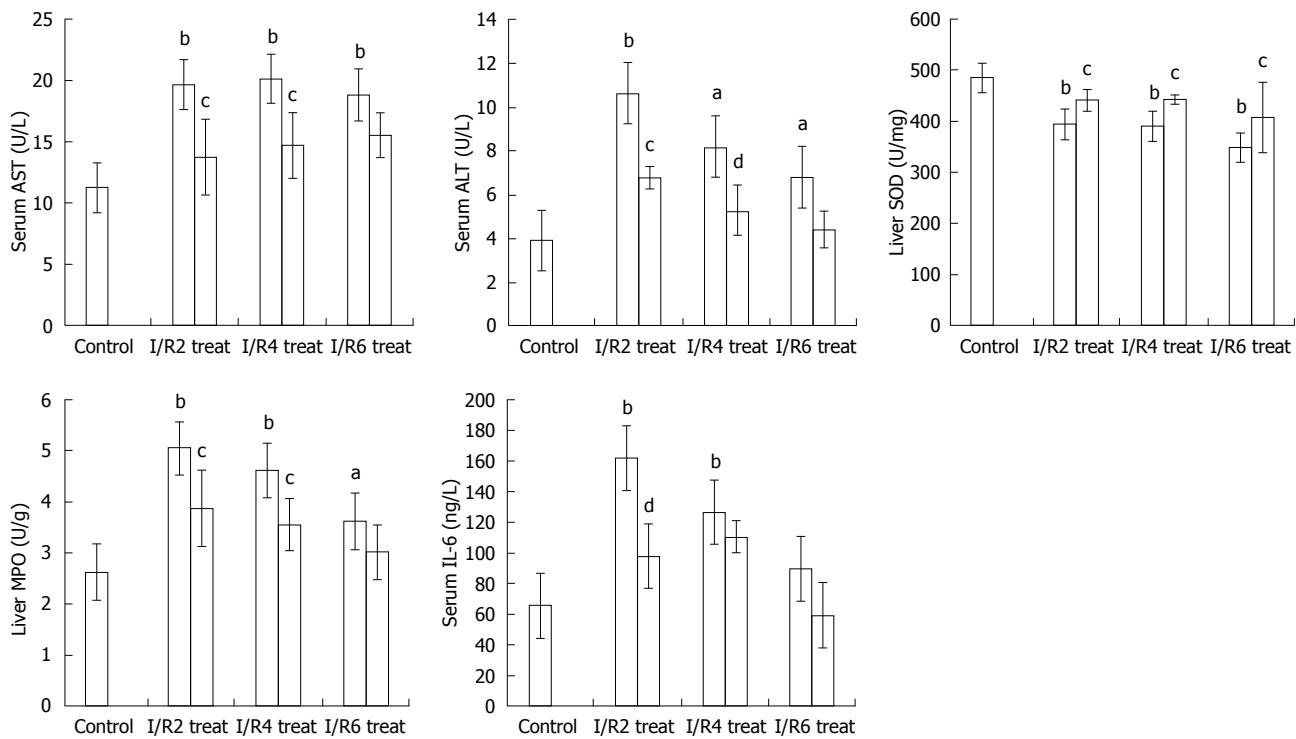
Compared with the control group, there was a marked increase in serum IL-6 level in the intestinal I/R group at 2 and 4 h of reperfusion ( $P < 0.01$ ), however carnosol pretreatment decreased serum level of IL-6 significantly at 2 h of reperfusion when compared with the I/R group ( $P < 0.01$ , Figure 2). This indicated that carnosol was sufficiently effective to suppress the increase of IL-6 level at early times after reperfusion.

#### Effect of carnosol on liver NF- $\kappa$ B p65 expression

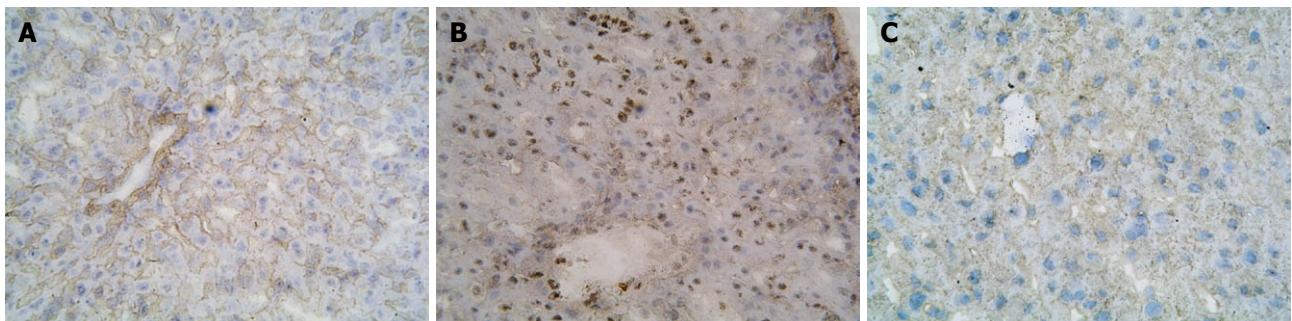
The immunohistochemical study showed that there was little staining of NF- $\kappa$ B p65 in the control group. In comparison, the strong positive expression of NF- $\kappa$ B p65 as brown staining was observed in the nucleus and cytoplasm in the intestinal I/R group. While the positive staining of NF- $\kappa$ B p65 expression was weakened markedly in the carnosol pretreatment group (Figure 3), western blotting showed weak staining for liver NF- $\kappa$ B p65 in the control group. Compared with the control group, IOD level of NF- $\kappa$ B p65 increased markedly in the intestinal I/R group ( $P < 0.05$ ). After pretreatment with carnosol, the IOD level of NF- $\kappa$ B p65 decreased significantly ( $P < 0.05$ , Figure 4A).

#### Effect of carnosol on liver ICAM-1 expression

Western blotting showed weak positive staining for ICAM-1 in the liver in the control group. However, a significant ICAM-1 protein signal was observed in the



**Figure 2 Activity of serum AST (U/L), serum ALT (U/L), liver tissue SOD (U/mg), liver tissue MPO (U/g) and serum IL-6 (ng/L) in different groups.** After 1 h intestinal ischemia and 2, 4 and 6 h reperfusion, the serum was assayed for AST, ALT and IL-6 activity. The liver tissue was assayed for SOD and MPO level. Data are expressed as mean  $\pm$  SD. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs control group; <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$  vs I/R group.



**Figure 3 Immunohistochemical staining of liver NF-κB p65 ( $\times 400$ ) in the control (A), I/R (B) (1 h ischemia and 4 h reperfusion) and carnosol pretreatment (C) groups (carnosol 3 mg/kg intraperitoneal administration).**

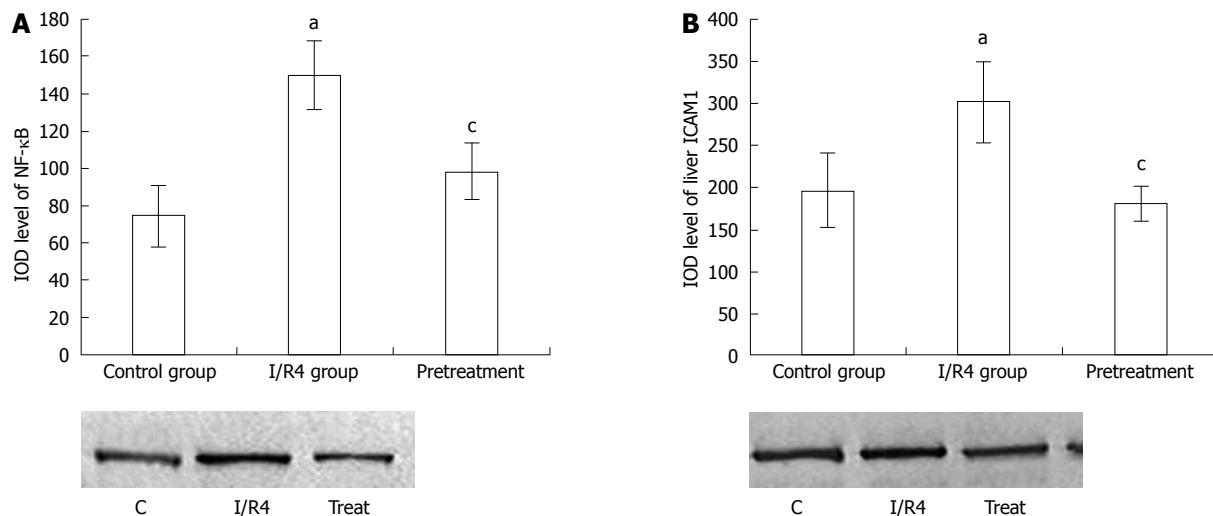
intestinal I/R group. Compared with the control group, IOD level of ICAM-1 increased markedly ( $P < 0.05$ ). This decreased markedly after pretreatment with carnosol ( $P < 0.05$ , Figure 4B).

## DISCUSSION

Intestinal I/R is not only necessary to the intestine itself, but involves severe distant tissue dysfunction. Liver is particularly vulnerable to the negative consequences of intestinal I/R because its vasculature is coupled in series with that of the intestine<sup>[20]</sup>. Although the precise mechanisms of intestinal I/R-induced liver injury have not been elucidated fully, previous research has shown that liver injury associated with intestinal I/R appear to be dependent on leukocyte adhesion and activation. Intestine- and/or liver-derived mediators, such as oxygen

radical species, IL-6 and tumor necrosis factor- $\alpha$ , have been suggested as participants in the I/R-induced, leukocyte-mediated liver responses<sup>[5,7,21]</sup>.

Recent studies have implied that the NF-κB pathway is involved in this process. A multitude of signaling factors, including oxidants, inflammatory cytokines, immune stimuli and viruses, can activate the transcriptional factor NF-κB<sup>[11,12,21]</sup>. In the early stage of intestinal I/R, the gut barrier function is damaged progressively, and bacteria, endogenous endotoxin, bacteriotoxin and reactive oxygen species invade the circulation and induce expression of NF-κB<sup>[10]</sup>. Since NF-κB activation requires nuclear translocation of the Rel/p65 subunit of NF-κB, nuclear NF-κB p65 level was examined to assess the activation of NF-κB. In the present study, intestinal I/R-induced liver injury manifested as pathological liver injury, significantly



**Figure 4** IOD level of western blotting analysis of liver NF-κB p65 (A) and ICAM-1 (B) in the control, intestinal I/R (1 h ischemia and 4 h reperfusion) and carnosol pretreatment groups (carnosol 3 mg/kg intraperitoneal administration). Data are expressed as mean  $\pm$  SD. <sup>a</sup> $P < 0.05$  vs control group; <sup>c</sup> $P < 0.05$  vs I/R group.

increased serum ALT and AST levels, and alterations in the biochemical indicators of oxidative stress in the liver. These changes were parallel to the increased level of serum IL-6 and liver NF-κB p65 expression, which suggests that activation of the NF-κB pathway plays an important part in the pathogenesis of intestinal I/R-induced liver injury. The above observation is also consistent with our previous study<sup>[12,13]</sup>.

Extracts of *Rosmarinus officinalis* L. have been used widely as a preservative in the food industry because of the antioxidant activity of their constituents such as carnosol and carnosic acid<sup>[22-24]</sup>. Carnosol and carnosic acid are good scavengers of peroxyl radicals and are able to block the formation of the hydroxyl radical generated in non-lipid systems<sup>[25]</sup>. They have been shown to inhibit non-enzymatic-induced lipid peroxidation in liver microsomes when incubated in the presence of FeCl<sub>3</sub><sup>[25]</sup> or Fe(NO<sub>3</sub>)<sub>3</sub><sup>[23]</sup>. Carnosol has also been shown to suppress the formation of proinflammatory leukotrienes in rat leukocytes<sup>[26]</sup>. In addition, inhibition of activation of transcription factor NF-κB has been proposed as one of the important underlying mechanisms of action of carnosol<sup>[16,17]</sup>. Therefore, it is reasonable to postulate that carnosol may exert protective effects in intestinal I/R-induced liver injury.

As expected, the present study showed that pretreatment with carnosol considerably attenuated intestinal I/R-induced liver injury, including reduced liver morphological injury and oxidative stress, as well as decreased serum ALT and AST activity. In addition, carnosol pretreatment resulted in significant down-regulation of the proinflammatory factor IL-6, which in turn, limited activation of circulation leukocytes in the microcirculation of the liver and other tissues. This led to reduced inflammation-mediated tissue injury. Moreover, endothelial adhesion molecules expressed on the surface of endothelial cells, such as ICAM-1, which play an important role in mediating firm adhesion and emigration of activated leukocytes in postcapillary

venules, were attenuated after carnosol treatment. The down-regulation of IL-6 and ICAM-1 was consistent with the decreased expression of NF-κB, which indicated that the protective effect of carnosol on intestinal I/R-induced liver injury may be related partly to the inhibition of NF-κB activation.

In conclusion, we showed pretreatment with the natural antioxidant carnosol attenuated liver injury induced by intestinal I/R, attributable to the antioxidant effect and inhibition of the NF-κB pathway.

## COMMENTS

### Background

Carnosol is a phenolic diterpene that has potent antioxidant and anti-inflammatory activities. There have been no reports about its effect on liver injury induced by intestinal ischemia/reperfusion (I/R). The present study investigated the preconditioning effects of carnosol on liver injury induced by intestinal I/R and confirmed the hypothesis that the protective effect of carnosol is mediated via inhibition of nuclear factor κB (NF-κB) activity.

### Research frontiers

Recently, it has been found that NF-κB plays a major role during the local and systemic inflammatory response following I/R. This study attempted to confirm that modulating the NF-κB pathway is a novel concept and therapeutic strategy for attenuating liver injury caused by intestinal I/R.

### Innovations and breakthroughs

The study showed pretreatment with the natural antioxidant carnosol attenuated liver injury induced by intestinal I/R, attributable to the antioxidant effect and inhibition of the NF-κB pathway.

### Applications

This study indicated that carnosol pretreatment protects liver injury induced by intestinal I/R. The protective effects may be associated with inhibition of NF-κB activation. This may represent a novel and attractive approach to prevent intestinal I/R injury.

### Terminology

NF-κB proteins are a family of transcriptional factors that control the expressions of many genes in immune and acute phase inflammatory responses, cell adhesion, differentiation, oxidative stress responses and apoptosis. Recently, it has been found that NF-κB plays an important role during intestinal I/R.

### Peer review

The authors demonstrated that carnosol attenuated liver injury induced by intestinal I/R in rats. The study is very interesting and the results are sound.

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ORIGINAL ARTICLES

# Insulin-like growth factor binding protein-7 induces activation and transdifferentiation of hepatic stellate cells *in vitro*

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and the level of fibronectin, collagen I and  $\alpha$ -smooth muscle actin (SMA). The pro-apoptotic effect of anti-IGFBP-7 antibody was determined by flow cytometry.

**RESULTS:** Immunocytochemistry and Western blotting revealed that the expression of IGFBP-7 in TGF- $\beta_1$  treated HSC was significantly up-regulated compared to that in the control group. In addition, fibronectin, collagen I and  $\alpha$ -SMA also showed enhanced expression in accordance with the transdifferentiation process in a dose-dependent manner to some extent. Moreover, flow cytometry suggested that anti-IGFBP-7 antibody induced apoptosis of activated HSC, which is responsible for the development of liver fibrosis, and may represent a novel pathway and target for therapeutic intervention.

**CONCLUSION:** IGFBP-7 showed increased expression in activated HSC and played an important role in the activation and transdifferentiation process of HSC. Anti-IGFBP-7 antibody may ameliorate liver fibrogenesis.

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**Key words:** Insulin-like growth factor-binding protein-7; Smooth muscle actin; Fibronectins; Collagen type I ; Hepatic stellate cells

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## Abstract

**AIM:** To investigate the role of insulin-like growth factor binding protein-7 (IGFBP-7) in the activation and transdifferentiation of hepatic stellate cells (HSC) *in vitro*.

**METHODS:** Rat HSC-T6 cells were cultured in separate dishes and treated with various concentration of transforming growth factor (TGF)- $\beta_1$ , IGFBP-7 or anti-IGFBP-7 antibody for 24 h. The supernatant or a cytoplasm suspension was obtained from cultured HSC, followed by transfer of cells to form cell-coated dishes. Immunocytochemistry and Western blotting were used to analyze the expression of IGFBP-7 induced by TGF- $\beta_1$

## INTRODUCTION

Hepatic fibrosis and cirrhosis are characterized by excessive fibrosis attributable to hepatic stellate cell (HSC) proliferation and activation<sup>[1]</sup>, resulting in excessive production of extracellular matrix (ECM), including fibrin-forming collagens I and III, proteoglycans, fibronectins

and hyaluronic acid<sup>[2]</sup>. The occurrence of hepatic fibrosis is also a common response to most chronic liver injuries such as viral hepatitis, parasitic infection, metabolic or autoimmune diseases, congenital abnormalities, and alcohol abuse<sup>[1,3]</sup>. However, the pathogenesis of hepatic fibrosis is still unknown<sup>[4]</sup>. In recent studies, HSC were the primary source of excess ECM assembly in liver fibrosis<sup>[5]</sup>. During the development of liver fibrosis, HSC undergo a process of activation, resulting in the expression of ECM, reduced retinoid storage capability and transdifferentiation to a myofibroblast-like phenotype which is characterized by expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA)<sup>[6]</sup>. Fibrotic changes result in structural changes and lead to reconstruction of hepatic lobules, dysfunction of liver bioactivity, and even death. At present, there is no effective treatment to halt the progression of liver fibrosis and cirrhosis. The only current life-prolonging intervention is liver transplantation. Thus, HSC play an important role in the pathogenesis of liver fibrosis.

HSC activation process is very complicated and is the result of a complex interplay between different hepatic cell types such as inflammatory cells and secreted cytokines, chemokines and growth factors<sup>[7]</sup>. The cellular transformation that develops gradually *in vitro* can be mimicked *in vitro* by short term culture of HSC on plastic, providing a model to study the intra- and extra-cellular determinants that regulate the transformation/activation process. Excessive cellular proliferation and an abundant ECM protein production that is not counteracted by increased ECM protein degradation have been shown to be the specific hallmark of HSC activation<sup>[8]</sup>. Another major feature of the activation process is the responsiveness of HSC to cytokines, resulting in the up-regulation of the platelet-derived-growth factor (PDGF) and members of the transforming growth factor (TGF)- $\beta$  family. PDGF appears to a major mitogen for stellate cells, and members of the TGF- $\beta$  family are the primary fibrogenic cytokines<sup>[9,10]</sup>. Boers *et al*<sup>[11]</sup> have detected a significant change in expression of insulin-like growth factor binding protein (IGFBP)-7 as a novel marker for hepatic fibrosis.

IGFs play an important role in the regulation of metabolism, development and growth of HSC<sup>[12]</sup>. The capacity of IGFs to exert their biological effects *via* interactions with specific cell surface receptors is modulated by the presence of a family of structurally related IGF-binding proteins. So far, 6 distinct IGFBPs have been identified and differ in molecular mass, binding properties for IGFs and posttranslational modifications as well as tissue and development regulated expression<sup>[13]</sup>. In addition, low affinity binders termed IGFBP-rPs have been found. IGFBP-7, the first protein proven to be functionally related to IGFBPs, called IGBP-rP1, is one of these low affinity binders to IGF-I and is expressed relatively more highly upon HSC activation, especially in the end stage<sup>[11]</sup>.

The aim of the present study was to investigate whether IGFBP-7, at different concentrations, may induce the activation and perpetuation of HSC. Our initial experiments demonstrated that expression of IGFBP-7 was up-regulated in patients with liver fibrosis

and cirrhosis. We examined roles of IGFBP-7 in different stages of HSC by exposure of HSC to exogenous cytokines. The involvement of IGFBP-7 was evaluated in activated HSC and increased ECM was detected. IGFBP-7 also induced the differentiation of HSC from a quiescent to an activated phase, whereas the specific antibody of IGFBP-7 can induce apoptosis of activated HSC. The data suggest that IGFBP-7 is sufficient to initiate the activation of HSC in conjunction with or prior to observation, which strongly implicates IGFBP-7 in the pathogenesis of hepatic fibrosis. In addition, overexpression of IGFBP-7 provides a novel cellular model to study the pathogenesis of human hepatic fibrosis.

## MATERIALS AND METHODS

HSC-T6 lines were a generous gift from Scott L Friedman of the Mount Sinai School of Medicine (NY, USA). Briefly, samples were washed and transferred into 25 cm<sup>2</sup> culture dishes (Orange Scientific, Belgium) for culture under standard conditions in a normoxic atmosphere of 16% O<sub>2</sub>, 5% CO<sub>2</sub>, and 79% N<sub>2</sub> (by volume) in RPMI1640 medium (Gibco, USA) containing 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 g/mL streptomycin and 3.57 g/L HEPES at 37°C. HSC were kept in secondary culture (partially “activated” HSC) and were passaged once every 2 to 3 d. Briefly, for cells in secondary culture, 10<sup>5</sup> cells/well were seeded into 12-well plates supplemented with RPMI1640 (Gibco) without FCS, or 10 cells/well were transferred to 96-well plates. After allowing HSC to attach overnight, cultures were incubated with TGF- $\beta$ 1 (Peprotech, UK) or IGFBP-7 (R&D, US), or anti-IGFBP-7 antibody (R&D, US), in different concentrations or in combination for 24 h before each experimental manipulation.

### Immunocytochemistry

After 24 h in cell culture to allow cells to attach, cell-coated dishes were obtained, fixed with 1% paraformaldehyde for 10 min, and washed by PBS. Endogenous peroxidases and biotins were then quenched using an endogenous peroxidase blocking kit and biotin blocking kit, respectively. The sections were blocked with 3% FCS (Roche, US) and incubated with one of the following antibodies: polyclonal anti- $\alpha$ -SMA/monoclonal anti-collagen I (Abcam, UK), and polyclonal anti-fibronectin (Santa Cruz, US). Sections were washed and incubated with biotinylated secondary antibody (Santa Cruz, US and Jackson, US). Bound secondary antibody was detected using the UltraSensitive™ SP kit (DAKO, US) according to the manufacturer's instructions. For modeling negative controls, the primary antibodies were substituted with PBS. The reaction products were visualized by diaminobenzidine tetrahydrochloride (DAB) (DAKO, US). Stained sections were viewed under a Nikon Eclipse 800 microscope and IOD (integrated optical density) or IA (integrated absorption) of the positive brown particles determined semiquantitatively by examining 5 fields randomly at  $\times 200$  magnification in each slice.

**Table 1 Immunocytochemistry staining and Western blotting analysis of IGFBP-7 (mean  $\pm$  SD)**

	Immunohistochemistry		Western blotting	
	n	(The value of IOD $\times 10^5$ )	n	(The value of A $\times 10^5$ )
Control	6	3.70 $\pm$ 0.32	6	0.03 $\pm$ 0.01
TGF $\beta$ 1 2 ng/mL	6	4.86 $\pm$ 0.27 <sup>a</sup>	6	1.05 $\pm$ 0.03 <sup>a</sup>
TGF $\beta$ 1 4 ng/mL	6	6.45 $\pm$ 0.32 <sup>a</sup>	6	1.16 $\pm$ 0.02 <sup>a</sup>
TGF $\beta$ 1 8 ng/mL	6	4.91 $\pm$ 0.30 <sup>a</sup>	6	1.01 $\pm$ 0.02 <sup>a</sup>
TGF $\beta$ 1 16 ng/mL	6	4.31 $\pm$ 0.26 <sup>a</sup>	6	1.06 $\pm$ 0.02 <sup>a</sup>

IOD: Integrated optical density; A: Absorbance; IGFBP: Insulin-like growth factor binding protein; <sup>a</sup>P < 0.05 vs control group.

### Western blotting

Culture supernatant and cellular lysates were obtained from a cultured HSC-T6 line according to the standard protocol. In brief,  $2 \times 10^5$ /mL active cells were cultured in 12-well plates in the absence or presence of TGF- $\beta$ 1 at different concentrations. Cultures were harvested after 12 h of incubation, supernatants were extensively dialyzed, and the remaining cells were trypsinized, then centrifuged, lysed, and finally cellular lysates were attained. Thirty microliter of cellular lysates were separated on 15% sodium dodecyl polyacrylamide gels (SDS-PAGE) under reducing conditions and then transferred onto PVDF membranes (SABC, US). After blocking with TBST (TBS and 0.1% Tween) for 3 h, the membranes were incubated with anti-IGFBP-7 antibody as the primary antibody overnight then washed with PBS. Secondary antibodies were conjugated with horseradish peroxidase for 3 h. The signals were visualized by DAB. The relative signal intensities of the bands were quantitated using Molecular Analyst software, and the results were normalized to levels of  $\beta$ -actins (Santa Cruz, US) in each sample. In selected experiments, the secretion of collagen I to the media was determined by ELISA (R&D, US) following incubation of confluent cultures of HSC-T6 with TGF- $\beta$ 1 (Peprotech, UK) or anti-IGFBP-7 antibody in different concentrations and combinations for up to 24 h, in accordance with the manufacturer's instructions.

### Flow cytometry

HSC-T6 lines were maintained under standard conditions as mentioned before, and then were transferred to 12-well dishes. After 24 h in culture, serum-free media was added containing anti-IGFBP-7 antibody in concentrations of 0.25 mg/L, 0.50 mg/L and 1.00 mg/L. Following another 14 h in culture, supernatants were discarded, and 500 g of cells were centrifuged. The remaining cells were washed with PBS, followed by centrifugation again for 5 min at 500 g at 4°C. Then, cell pellets were resuspended in ice-cold binding buffer to  $1 \times 10^6$  cells/mL, with tubes on ice. Both 5  $\mu$ L of annexin-V and 2.5  $\mu$ L of propidium iodide (PI) (Beckman Coulter, US) were added to 100  $\mu$ L of cell suspensions. Tubes were kept on ice for 10 min in the dark, and then another 100  $\mu$ L of binding buffer was added to the preparations and mixed gently. Quantitative analysis of the preparations was performed after

**Table 2 ELISA determination of collagen I (mean  $\pm$  SD)**

	n	Collagen I (ng/mL)
Control	6	36.61 $\pm$ 1.28
TGF $\beta$ 1 2 ng/mL	6	41.93 $\pm$ 2.95 <sup>c</sup>
TGF $\beta$ 1 4 ng/mL	6	49.44 $\pm$ 3.21 <sup>c</sup>
Anti-IGFBP-Pr1 antibody 0.1 $\mu$ g/mL	6	35.46 $\pm$ 5.80 <sup>a</sup>
Anti-IGFBP-Pr1 antibody 1 $\mu$ g/mL	6	36.38 $\pm$ 2.59 <sup>a</sup>
Anti-IGFBP-Pr1 antibody 2 $\mu$ g/mL	6	37.26 $\pm$ 2.12 <sup>a</sup>
TGF $\beta$ 1 2 ng/mL + Anti-IGFBP-Pr1 antibody 0.1 $\mu$ g/mL	6	38.24 $\pm$ 2.76 <sup>c</sup>
TGF $\beta$ 1 2 ng/mL + Anti-IGFBP-Pr1 antibody 1 $\mu$ g/mL	6	33.95 $\pm$ 3.27 <sup>c</sup>
TGF $\beta$ 1 2 ng/mL + Anti-IGFBP-Pr1 antibody 2 $\mu$ g/mL	6	34.59 $\pm$ 3.97 <sup>c</sup>
TGF $\beta$ 1 4 ng/mL + Anti-IGFBP-Pr1 antibody 0.1 $\mu$ g/mL	6	40.24 $\pm$ 3.46 <sup>e</sup>
TGF $\beta$ 1 4 ng/mL + Anti-IGFBP-Pr1 antibody 1 $\mu$ g/mL	6	32.61 $\pm$ 4.86 <sup>e</sup>

Relative expression of collagen I was measured by ELISA; after HSC incubated for 24 h, supernatant was obtained and analyzed. Statistical analysis was done by the SNK-q test. <sup>a</sup>P < 0.05, compared with the control group; <sup>c</sup>P < 0.05, compared with the TGF $\beta$ 1 2 ng/mL; <sup>e</sup>P < 0.05, compared with TGF $\beta$ 1 4 ng/mL.

30 min by flow cytometry. Meanwhile, following the 14 h culture, 20  $\mu$ L MTT and 100  $\mu$ L PBS were added to each well at 37°C in 5% CO<sub>2</sub> atmosphere (by volume) for 4 h. Supernatants were discarded, and 150  $\mu$ L DMSO was added and mixed gently. After incubation with chromogenic substance for 10 min, absorbance (A) at 490 nm was measured in samples to measure the value of A under different concentrations of anti-IGFBP-7 antibody.

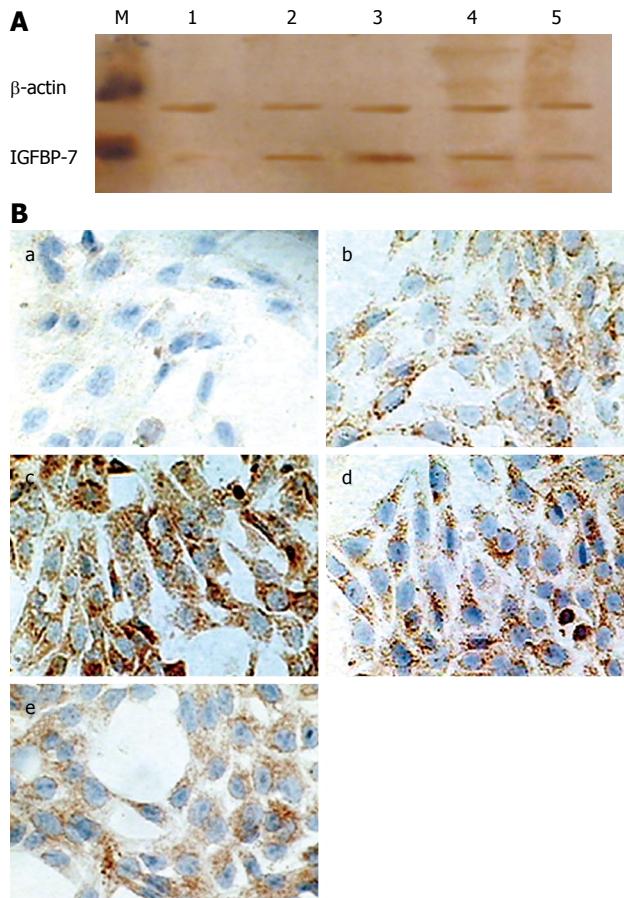
### Statistical analysis

Data are presented as the mean  $\pm$  SD, and all calculations were made using SPSS11.5. Statistical comparisons were performed using an ANOVA correlation analysis. Paired comparisons were done using the SNK-q test. Significance was set at a value of P < 0.05.

## RESULTS

### In vitro expression of IGFBP-7 in HSC

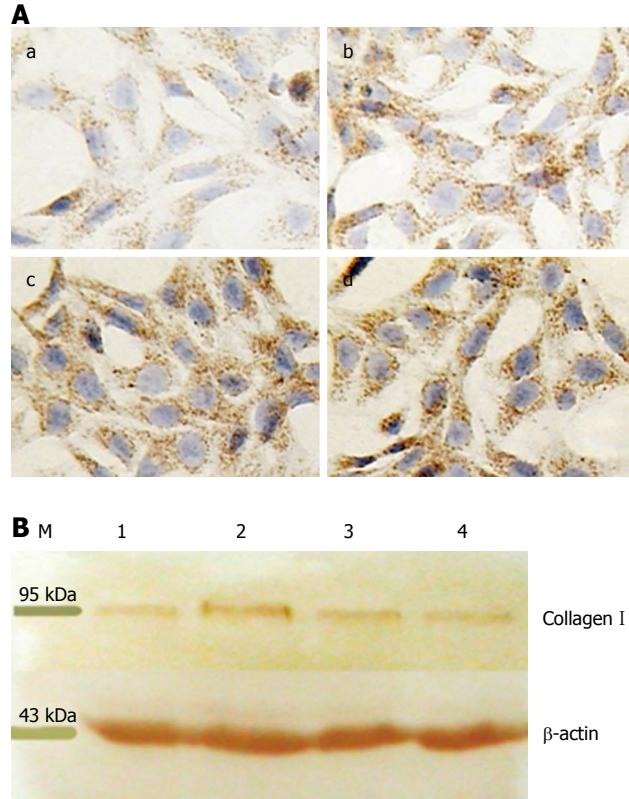
Differences in absorbance between TGF- $\beta$ 1-treated cells and controls are shown in Table 1. Expression of IGFBP-7 was up-regulated by TGF- $\beta$ 1 of 2, 4, 8 and 16  $\mu$ L/L, as detected in the cytoplasm of HSC by Western blotting analysis of cellular lysates (Figure 1A). Immunohistochemistry also demonstrated an increased expression of IGFBP-7 in TGF- $\beta$ 1-treated HSC on cell-coated dishes. As shown in Figure 1B, in the TGF- $\beta$ 1-treated groups, the amount of positive brown staining was abundant and the value of IOD of positively stained particles increased significantly (Table 1), as compared with the control group in which only a few cells were positively stained. The expression in cells treated with 4  $\mu$ L/L of TGF- $\beta$ 1 was stronger than most other groups. Perhaps this effect is the reason that the autocrine expression of TGF- $\beta$ 1 is strengthened, and exogenous TGF- $\beta$ 1 expression decreases the secretion of IGFBP-7.



**Figure 1** *In vitro* expression of IGFBP-7 in HSC. A: Detection of IGFBP-7 by Western blotting. Cultured HSC were incubated with TGF- $\beta$ 1; lysates were harvested after 24 h, and analyzed by Western blotting for IGFBP-7.  $\beta$ -actin was managed as a loading control. Each experiment was replicated 6 times. M: Marker; 1: Control group; 2, 3, 4, 5 represent 2  $\mu$ g/L, 4  $\mu$ g/L, 8  $\mu$ g/L and 16  $\mu$ g/L TGF- $\beta$ 1, respectively; B: Expression of IGFBP-7 in activated HSC. Expression of IGFBP-7 was examined on HSC-coated dishes after treatment with TGF- $\beta$ 1 at different concentrations for 24 h (a-d indicated control, 2  $\mu$ g/L, 4  $\mu$ g/L, 8  $\mu$ g/L and 16  $\mu$ g/L TGF- $\beta$ 1, respectively). IGFBP-7 was detected by immunocytochemistry, and the level of the expression of IGFBP-7 was enhanced in a dose-dependent manner to some extent compared with the control group. Original magnification:  $\times 200$ .

#### Quantitation of collagen I deposition in HSC

To analyze the *in vitro* effects of IGFBP-7 on HSC secretion of collagen I, supernatant was collected and the amount of collagen I was analyzed by ELISA following a standard protocol according to the manufacturer's instructions. As shown in Table 2, the secretion of collagen I was prominent in the TGF- $\beta$ 1-treated groups, compared with the control group and the anti-IGFBP-7-treated groups. However in the wells treated with 2 or 4 ng/mL TGF- $\beta$ 1 and 0.1, 1 or 2  $\mu$ g/mL anti-IGFBP-7 antibody, the amount of collagen I was significantly decreased, as compared with the groups treated with TGF- $\beta$ 1 alone. We further performed immunohistochemistry to determine the expression of collagen I in HSC. As shown in Figure 2A, positive brown collagen I staining in IGFBP-7-treated HSC obviously increased compared with the controls in a dose-dependent manner to some extent. Western blotting analysis was also used to characterize the amount of collagen I protein in the cytoplasm of HSC. As shown

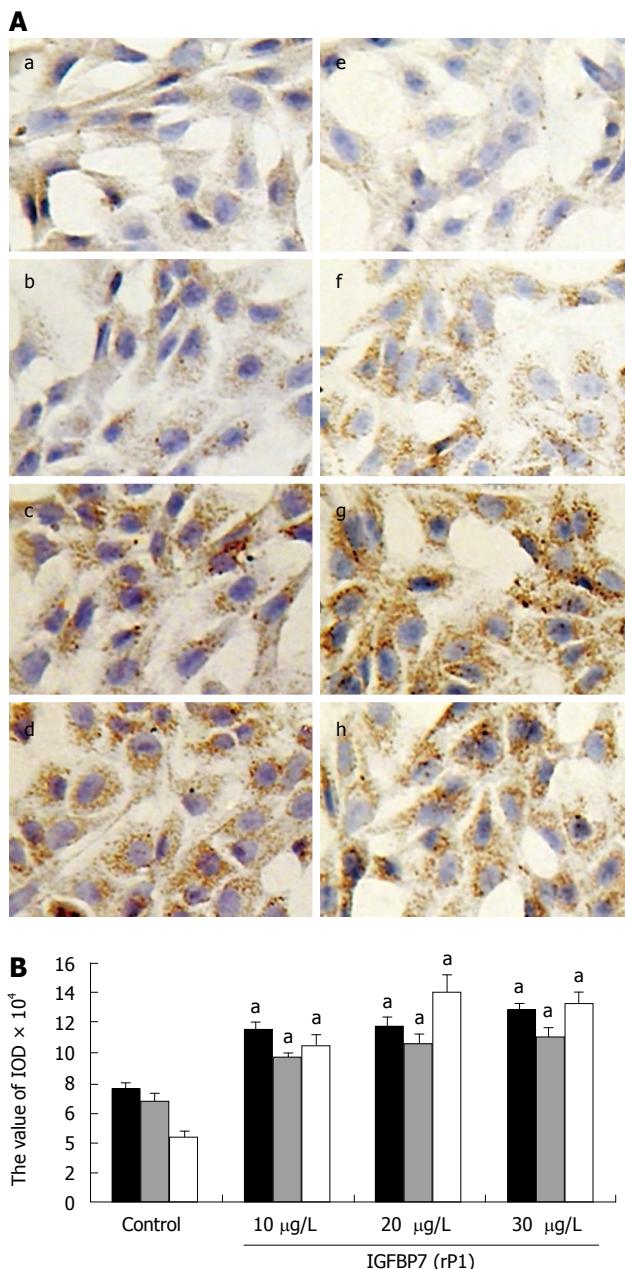


**Figure 2** Quantitation of collagen I deposition in HSC. A: Immunocytochemical detection of collagen I. Expression of collagen I was examined on HSC-coated dishes after treatment with IGFBP-7 at different concentrations for 24 h (a-d indicated control, 10  $\mu$ g/L, 20  $\mu$ g/L, and 30  $\mu$ g/L IGFBP-7, respectively). Collagen I was examined by immunocytochemistry and the level of the expression of collagen I was enhanced in a dose-dependent manner in sequence compared with the control group. Original magnification:  $\times 200$ ; B: Detection of collagen I by Western blotting. Cultured HSC were incubated with IGFBP-7 for 24 h, and then lysates of HSC were harvested and analyzed by Western blotting for collagen I.  $\beta$ -actin was used as a loading control. Each experiment was replicated 6 times. M: Marker; 1: Control group; 2, 3, 4 represent 10  $\mu$ g/L, 20  $\mu$ g/L, 30  $\mu$ g/L.

in Figure 2B, the protein was prominently synthesized in accordance with HSC activation. The differences in absorbance between IGFBP-7-treated groups at different concentrations and the control group in sequence were  $0.7663 \pm 0.0412$ ,  $0.7439 \pm 0.0720$ ,  $0.7039 \pm 0.0889$ , and  $0.6402 \pm 0.0475$ , respectively ( $P < 0.05$ ).

#### Comparison of fibronectin levels and fibroblast phenotype

The deposition of fibronectin was examined by immunohistochemistry. As shown in Figure 3A (a-d), fibronectin was detected in the cytoplasm of HSC. In the control group, only a few brown particles were detected although the amount of staining was higher in the IGFBP-7-treated groups, also in a dose-dependent manner. To characterize the HSC phenotype,  $\alpha$ -SMA expression was examined. As shown in Figure 3A (e-h),  $\alpha$ -SMA-positive HSC were more abundant in IGFBP-7-treated cells than in the control group, and the increase was in a dose-dependent manner. The comparison and analysis of IOD of fibronectin and  $\alpha$ -SMA was performed and presented in Figure 3B. A significant difference was seen between each IGFBP-7-treated group at different concentrations and the control group. Thus,



**Figure 3 Comparison of fibronectin levels and fibroblast phenotype.** A: Characterization of  $\alpha$ -SMA and fibronectin. Immunocytochemistry was performed on HSC after incubation with IGFBP-7 at different concentrations (0, 10  $\mu$ g/L, 20  $\mu$ g/L and 30  $\mu$ g/L) for 24 h.  $\alpha$ -SMA (a-e) and fibronectin (f-h) were examined by immunocytochemistry. The amount of the expression of  $\alpha$ -SMA (b-d) and fibronectin (f-h) were enhanced in a dose-dependent manner in sequence compared with the control group (a, e). Original magnification:  $\times 200$ . B: Comparison of the expression of collagen I, fibronectin and  $\alpha$ -SMA at different concentrations. The expression of collagen I, fibronectin and  $\alpha$ -SMA was detected through immunocytochemistry, and the value of IOD of the positive-brown particles was calculated. The black, gray and white bars represents the IOD value of  $\alpha$ -SMA, collagen I and fibronectin, respectively. Statistical analysis was done using the SNK-q test.  $^aP < 0.05$ .

IGFBP-7 induced an increased expression and deposition of fibronectin, which led to the transformation of HSC into a myofibroblastic phenotype.

#### Anti-IGFBP-7 antibody-induced HSC apoptosis

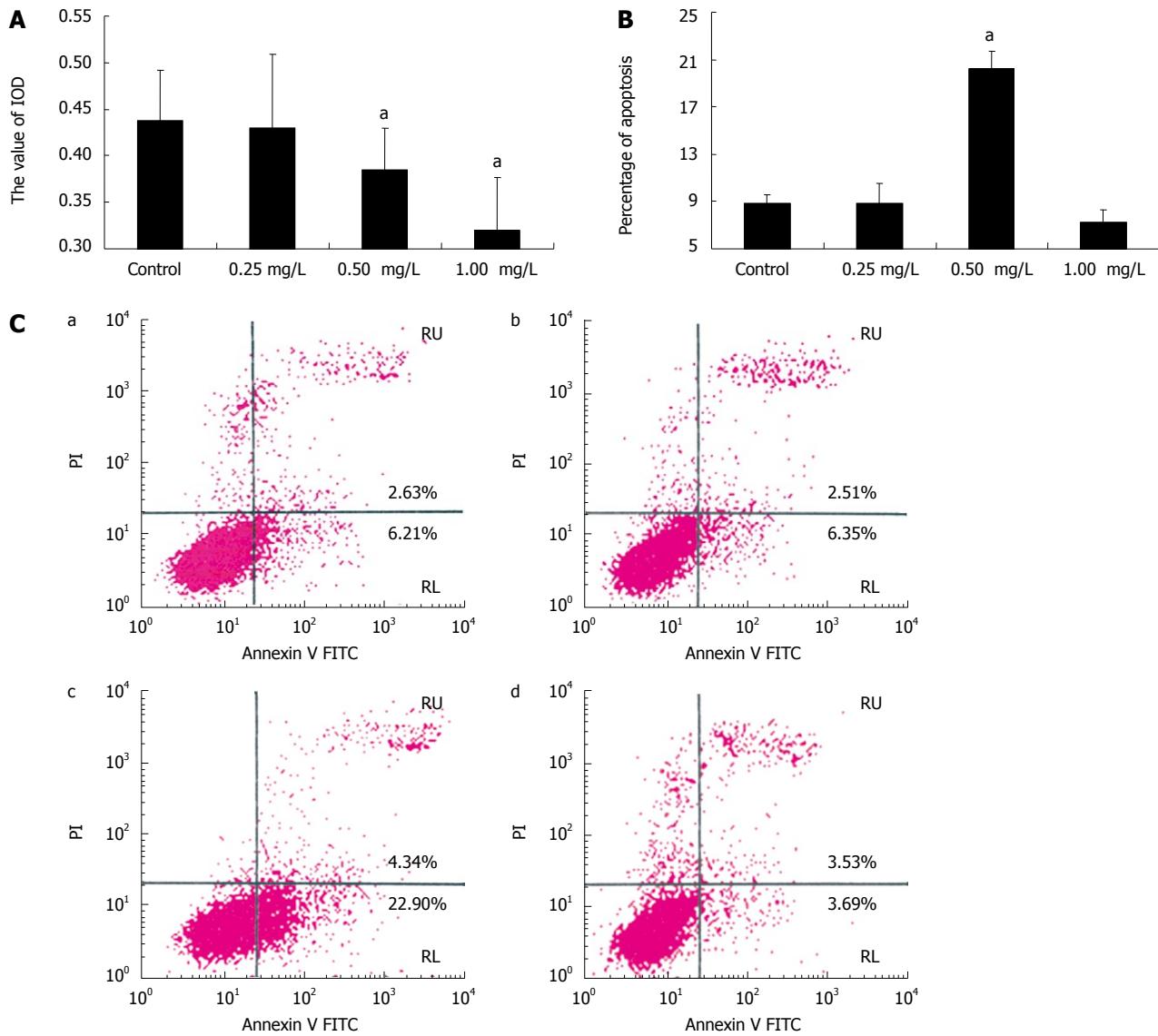
We further examined the effect of anti-IGFBP-7 antibody on activated HSC. To characterize the inhibitory effect of

anti-IGFBP-7 antibody on activated HSC, MTT assays were performed. As shown in Figure 4A,  $A$  decreased after anti-IGFBP-7 antibody treatment, compared with the control group. Also, treatment with 0.50 mg/L and 1.00 mg/L of antibody could significantly reduce the value of  $A$ . To determine the proportion of apoptotic cells to all activated cells, flow cytometry with annexin/PI was used. As shown in Figure 4B, the ratio of apoptotic cells was prominent in the 0.50 mg/L anti-IGFBP-7 antibody treatment group, as compared with the control group. The difference between 0.25 mg/L, 1.00 mg/L and the control was not significant (Figure 4). Statistical analysis of these results is shown in Figure 4C.

## DISCUSSION

HSC are the main source of ECM, which contributes to the occurrence and development of liver fibrosis<sup>[14]</sup>. We previously reported increased IGFBP-7 protein levels in patients with liver fibrosis and cirrhosis. In this study, expression and function of IGFBP-7 were studied in cultured HSC which were administered different reagents. The object was to define the bioactivity and function of IGFBP-7 in such a model of HSC in different stages of activation and transdifferentiation. For this purpose, we used TGF- $\beta$ 1 as the profibrotic cytokine to activate HSC from quiescent to partially activated and ultimately completely transdifferentiated HSC. Then, the expression of IGFBP-7 was analyzed in cell-coated dishes. The resulting stained-cell dishes revealed that the expression of IGFBP-7 was significantly increased in TGF- $\beta$ 1-treated HSC ("activated" HSC). It has been demonstrated that the IGFBP-7 showed enhanced expression and could work as a novel marker in the pathogenesis of HSC<sup>[11]</sup>. Our observations further confirmed this statement. HSC could be considered to be one of the principal effectors involved in fibrogenesis. In response to liver injury as well as other cytokines, HSC are considered to undergo a phenotypic transformation from a quiescent to a myofibroblast-like phenotype, characterized by DNA synthesis, motility, contractility and synthesis of ECM<sup>[15,16]</sup>. TGF- $\beta$ 1 is the best-known primary fibrogenic cytokine<sup>[9,10]</sup>, whereas it is not clear which specific gene is responsible for the fibrotic response.

IGFBPs showed high expression together with other markers of HSC activation, especially in the end stage. The liver is the major producer of IGFBPs, mainly IGFBP-1 and -3<sup>[17]</sup>. The present study mainly focused on the function and distribution of IGFBP superfamily members among different organs and tissues. IGFBPs are the binding proteins of IGF-1 and -2; they regulate the interaction of IGF-1 and -2 with their receptors, and modulate the growth-promoting and metabolic activities of IGF-1 and -2 by affecting the downstream signal transduction of IGFR<sup>[18]</sup>. *In vitro*, the proliferation of HSC is enhanced by IGF-1<sup>[19,20]</sup>. *In vivo*, however, production of IGF-1 by HSC has recently been reported to attenuate hepatic cirrhosis<sup>[17]</sup>. Furthermore, IGF-1 replacement therapy seems to benefit patients with hepatic cirrhosis<sup>[21]</sup>.



**Figure 4** Anti-IGFBP-7 antibody induces HSC apoptosis. **A:** HSC viability assay. The activated HSCs had anti-IGFBP-7 antibody of different concentrations added, and the absorbance (A) of each treated group was analyzed by the MTT cell viability assay to investigate the bioactivity of cultured cells. Statistical analysis was done using the SNK-q test.  $^aP < 0.05$ ; **B:** Percentage of apoptosis in all treated cells. Activated HSCs were divided into 4 groups, and then each group was treated with anti-IGFBP-7 antibody at different concentrations of 0 mg/L, 0.25 mg/L, 0.50 mg/L and 1.00 mg/L, and analyzed after 24 h culture, using flow cytometric assay. Statistical analysis was done using the SNK-q test.  $^aP < 0.05$ ; **C:** Flow cytometry assay. Cultured cells were treated with anti-IGFBP-7 body at different concentrations of 0 mg/L, 0.25 mg/L, 0.50 mg/L and 1.00 mg/L. After 24 h incubation, flow cytometry was used to analyze the pro-apoptotic effect of anti-IGFBP-7 antibody.

Because of the binding activity of IGFBPs, the induction of these binding proteins may affect hepatic cirrhosis. An *in vitro* study revealed that IGFBPs could display intrinsic bioactivities that are dependent on or independent of binding of IGF-1 and -2, in the process of hepatocyte growth, differentiation and activity<sup>[22]</sup>. So far, 6 distinct IGFBPs have been identified, which differ in molecular mass and binding affinities for IGF-1 and -2<sup>[13]</sup>. Moreover, low affinity IGF binders termed IGFBP-rPs have been found. IGFBP-7, also called IGFBP-rP1, is one of these low affinity binders of IGF-1. In the transcription of activated human HSC, expression of 5 IGFBP family members was detected. The most prominent was the extremely high expression of IGFBP-5, which is known to be the most abundantly expressed protein in activated HSC. In addition, IGFBP-7 is obviously detected in these cells, whereas a smaller induction is seen for IGFBP-4,

-6 and -3<sup>[6]</sup>. The growth-promoting and metabolic activities of IGF-1 and -2 are modulated by the family of IGFBPs<sup>[23]</sup>. In certain pathological conditions, IGFBP-1 through -6 all may inhibit the growth of cells. IGFBP-1 and -3 may destroy glucose equilibrium in humans; IGFBP-1, -5, and -6 may also disturb this capability<sup>[24]</sup>, whereas IGFBP-7 (rP1) could modulate proliferation, cohesion of cells, the regeneration of blood vessel and biosynthesis of prostacyclin<sup>[25]</sup>. IGFBP-8 (rP2) is considered to be a key cytokine in the fibrogenesis of tissues and organs<sup>[13]</sup>. Since modulation of IGF activity is very complex and varied, their differences may be important factors in determining the local autocrine and paracrine activities of IGFs. The striking up-regulation of IGFBP-7 predicted that it is a good marker of HSC activation and is also a promising target for therapeutic intervention in hepatic fibrosis.

Increased collagen and fibronectin deposition were detected in IGFBP-7-treated HSC, demonstrating that IGFBP-7 can activate and induce ECM production and fibrosis *in vitro*. Moreover,  $\alpha$ -SMA-expressing cells increased among TGF- $\beta$ 1-treated cells, suggesting that they acquired a myofibroblastic phenotype<sup>[26]</sup>. Taken together, our findings indicate that superexpression of IGFBP-7 induces the deposition of ECM through the transformation of HSC into myofibroblastic cells which then contribute to the resulting fibrosis. We have shown that myofibroblastic changes are induced *in vitro* by IGFBP-7. In patients with liver fibrosis and cirrhosis, HSC are mainly responsible for the excessive deposition of ECM, many of which may have the phenotypic characteristics of myofibroblasts. A myofibroblast is strictly defined by electromicroscopic findings as a cell that is vimentin- and/or  $\alpha$ -SMA-positive, with prominent rough endoplasmic reticulum, a modestly developed periphery, with focal densities, and producing collagen, granules, gap, and actin-filament-based junctions<sup>[27]</sup>. However, most of the present studies are restricted to an immunohistochemical determination without electron microscopy, such as spindle-shaped cells expressing  $\alpha$ -SMA<sup>[28]</sup>. Thus,  $\alpha$ -SMA-expressing cells in our model undergo a myofibroblastic change, and this *in vitro* phenomenon can also be triggered by exogenous IGFBP-7 applied to human stellate cells. In humans, HSC are mainly located at the sinusoid within the subendothelial space of Disse in close contact with the hepatocytes. They comprise roughly one-third of the nonparenchymal cell population or about 5%-8% of total liver cells<sup>[29]</sup>. During the development of liver fibrosis, HSC undergo a process of activation, resulting in a reduced retinoid storage capability and transformation to a myofibroblastic phenotype that is characterized by expression of  $\alpha$ -SMA. In fact, the myofibroblast-like phenotype is not limited to liver, but also is a prominent feature of fibrosis in other tissues including pancreas, kidney, lung and skin<sup>[30]</sup>.

Apoptosis of activated HSC is significantly detected when anti-IGFBP-7 antibody is administered to the medium. Flow cytometric analysis is a novel, specific and exact way to detect and measure apoptosis, based on the new parameters appearing on the surface of cells such as phospholipids that become exposed at the cell surface and form one of the specific signals for recognition and removal of apoptotic cells by macrophages. The anti-IGFBP-7 antibody acts as a proapoptotic factor, having great impact on the survival and proliferation of HSC. We predict that the anti-IGFBP-7 antibody also performs this function *in vivo*, which will be investigated in subsequent studies.

In summary, we have shown that IGFBP-7 plays a pivotal role in the pathogenesis of HSC activation and has profibrotic activities *in vitro*. IGFBP-7 induces the production of ECM via activation of HSC and the development of a myofibroblastic phenotype in HSC. The over-expression of IGFBP-7 *in vitro* also provides a new model to study the pathogenesis of HSC activation. The inhibitory effect of anti-IGFBP-7 antibody on

activated HSC provides a promising intervention for liver fibrosis.

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## COMMENTS

### Background

Hepatic stellate cells (HSC) are the main source of extracellular matrix (ECM), and thus play an important role in the occurrence and development of liver fibrosis. Insulin-like growth factor binding protein (IGFBP)-7 is a novel protein, which may be relevant to the activation of HSC.

### Research frontiers

IGFBP-7 expression has been detected significantly during fibrogenesis, but how this protein is related to liver fibrosis is largely unknown. In this study, the authors demonstrated that IGFBP-7 could activate HSC in accordance with the transdifferentiation process in a dose-dependent manner to some extent.

### Innovations and breakthroughs

Recent studies have highlighted the importance of IGF-I and IGFBP-3 in the liver tissues and the roles they play in fibrogenesis. This is the first study to report that expression of IGFBP-7 is also enhanced in this process and IGFBP-7 could activate HSC *in vitro*. Furthermore, this protein may be the main mediator of HSC bioactivity.

### Applications

By understanding how IGFBP-7 is relevant to liver fibrosis, this study may represent a new strategy for therapeutic intervention in the treatment of patients with liver fibrosis.

### Terminology

HSC are specific cells located in the hepatic lobules, and are the primary source of excess ECM assembly during liver fibrosis. The activation of HSC is regulated by known and unknown cytokines. IGFBP-7 is one member of the IGFBP superfamily, which is thought to work as a potential activator of HSC.

### Peer review

The paper by Liu *et al* is an interesting one, not only because of its findings, but also because of the quality of the presented data.

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BRIEF ARTICLES

## Thermal hypersensitivity in a subset of irritable bowel syndrome patients

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Thermal hyperalgesia was present in both groups of IBS patients relative to controls, with IBS patients reporting significantly lower pain threshold and pain tolerance at both test sites. Cluster analysis revealed the presence of subgroups of IBS patients based on thermal hyperalgesia. One cluster (17% of the sample) showed a profile of heat pain sensitivity very similar to that of healthy controls; a second cluster (47% of the sample) showed moderate heat pain sensitivity; and a third cluster (36% of the sample) showed a very high degree of thermal hyperalgesia.

**CONCLUSION:** A subset of IBS patients had thermal hypersensitivity compared to controls, who reported significantly lower HPTh and HPTo. All IBS patients had a higher score on the FBDSI than controls. Interestingly, the subset of IBS patients with high thermal sensitivity (36%) had the highest FBDSI score compared to the other two groups of IBS patients.

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**Key words:** Irritable bowel syndrome; Visceral pain; thermal hypersensitivity; Heat pain threshold; Heat pain tolerance

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### Abstract

**AIM:** To characterize thermal hypersensitivity in patients with constipation- and diarrhea-predominant irritable bowel syndrome (IBS).

**METHODS:** Thermal pain sensitivity was tested among patients with diarrhea-predominant IBS (D-IBS) and constipation-predominant IBS (C-IBS) compared to healthy subjects. A total of 42 patients (29 female and 13 male; mean age  $27.0 \pm 6.4$  years) with D-IBS; 24 patients (16 female and eight male; mean age  $32.5 \pm 8.8$  years) with C-IBS; and 52 control subjects (34 female and 18 male; mean age  $27.3 \pm 8.0$  years) participated in the study. Thermal stimuli were delivered using a Medoc Thermal Sensory Analyzer with a 3 cm  $\times$  3 cm surface area. Heat pain threshold (HPTh) and heat pain tolerance (HPTo) were assessed on the left ventral forearm and left calf using an ascending method of limits. The Functional Bowel Disease Severity Index (FBDSI) was also obtained for all subjects.

**RESULTS:** Controls were less sensitive than C-IBS and D-IBS (both at  $P < 0.001$ ) with no differences between C-IBS and D-IBS for HPTh and HPTo.

### INTRODUCTION

Irritable bowel syndrome (IBS) is one of the most common gastrointestinal disorders encountered by gastroenterologists. Patients classically present with chronic abdominal pain associated with an alteration in bowel habits. Even though the pathophysiology of the IBS is unclear, visceral hypersensitivity is a common clinical marker of the disorder<sup>[1,2]</sup>. Visceral hypersensitivity may account for symptoms of

abdominal pain, urgency, and bloating experienced by many patients with this disorder.

Although visceral hypersensitivity is considered a hallmark feature of IBS, conflicting evidence exists regarding somatic hypersensitivity in this patient population. Somatic pain conditions, such as fibromyalgia and migraine headaches show significant comorbidity with IBS, which suggests that somatic hypersensitivity characterizes at least a subpopulation of IBS patients<sup>[2,3]</sup>. Several investigators have found no evidence for heightened somatic pain sensitivity in IBS patients. For example, two studies reported that IBS patients showed lower sensitivity to painful electrocutaneous stimuli compared to healthy controls<sup>[4,5]</sup>. Also, others have reported similar cold pressor pain tolerance in IBS patients and controls<sup>[6,7]</sup>. In contrast, our recent studies using hot water immersion have shown widespread somatic hyperalgesia associated with IBS<sup>[2,8,9]</sup>, and others using the cold pressor test have demonstrated somatic hypersensitivity in IBS patients compared with healthy controls<sup>[3,10]</sup>.

These conflicting findings may result from differing somatic pain testing procedures. Alternatively, patient sampling may be a contributing factor, given that there may be subgroups of IBS patients who differ in their somatic sensitivity. For example, somatic hypersensitivity may be present only in a subset of patients based on IBS subtype [i.e. diarrhea-predominant IBS (D-IBS) vs constipation-predominant IBS (C-IBS)], symptom severity and/or psychological profile.

Previous studies have explored the correlates of visceral hypersensitivity among patients with IBS<sup>[11-16]</sup>. For example, depression is correlated with rectal pain thresholds only in patients who alternate between constipation and diarrhea. Others have reported no association of psychological factors with rectal sensitivity; however, hypersensitivity to rectal distention is associated with increased IBS symptom severity assessed by daily diary<sup>[11]</sup>. Similarly, other investigators have found that rectal pain sensitivity is correlated positively with clinical symptoms among IBS patients<sup>[12-14]</sup>, while others have reported no such association<sup>[15,16]</sup>. However, the association of somatic hypersensitivity with clinical symptoms in IBS has not been evaluated.

To further evaluate somatic hyperalgesia among patients with IBS, we evaluated thermal pain sensitivity among patients with D-IBS and C-IBS compared with healthy subjects. The aims of the present study were: (a) to compare the spatial distribution and magnitude of thermal hyperalgesia between D-IBS and C-IBS patients and controls; and (b) to compare the spatial distribution and magnitude of thermal hyperalgesia among IBS patients as a function of symptom severity.

## MATERIALS AND METHODS

### Participants

A total of 42 patients (29 female and 13 male; mean age  $27.0 \pm 6.4$  years) with D-IBS; 24 patients (16 female and eight male; mean age  $32.5 \pm 8.8$  years) with C-IBS; and 52

**Table 1 Demographic variables for IBS patients and controls**

	D-IBS (n = 42)	C-IBS (n = 24)	Controls (n = 52)
Age (mean $\pm$ SD)	$27.0 \pm 6.4$	$32.5 \pm 8.8$	$27.3 \pm 8.0$
Sex (female), n (%)	29 (69)	16 (67)	34 (64)
FBDSI	62.8 (23.2)	68.4 (25.3)	2.1 (4.9)
Self-reported race/ethnicity n (%)			
White	33 (79)	15 (63)	43 (83)
Black	3 (7)	6 (25)	3 (6)
Hispanic	3 (7)	1 (4)	3 (6)
Asian	3 (7)	2 (8)	3 (6)

D-IBS: Diarrhea-predominant irritable bowel syndrome; C-IBS: Constipation-predominant irritable bowel syndrome. Columns may not sum to 100%, because values were rounded to the nearest 1%. Scores on the Functional Bowel Disorder Severity Index (FBDSI) that share a superscript are different at  $P < 0.01$ .

control subjects (34 females and 18 male; mean age  $27.3 \pm 8.0$  years) participated in the study. The demographics of the participating subjects are presented in Table 1. IBS subjects and healthy controls were recruited via advertisements posted at the University of Florida and the Ohio State University. The study was approved by the University of Florida, the North Florida/South Georgia Veterans Health System, and the Ohio State University Institutional Review Boards. All subjects signed informed consent prior to the start of the study.

None of the control subjects had any evidence of acute or chronic somatic/abdominal pain or IBS based on a questionnaire and complete physical examination by an experienced gastroenterologist. Also, controls were free of any systemic medical disease or psychological conditions that could affect sensory responses. All IBS subjects had symptoms for at least 5 years. The diagnosis of IBS was made by the same gastroenterologist who examined patients based on the ROME III criteria and exclusion of organic disease<sup>[17]</sup>. All subjects with IBS were examined for fibromyalgia (FM) using the 1990 American College of Rheumatology criteria for FM<sup>[18]</sup>. None of the patients were diagnosed as having FM. None of the IBS or control subjects were taking analgesics, serotonin uptake inhibitors, serotonin antagonists, or tricyclic antidepressants for a period of at least 3 wk prior to the study.

All subjects underwent experimental psychophysical testing during a single session. All sessions were conducted between 9 AM and 6 PM to control for circadian rhythm effects. Subjects were instructed to refrain from the use of any analgesic medication for 48 h and from caffeine for 4 h before their sessions. Prior to each session, participants received a reminder concerning the restrictions on analgesic medication and caffeine use.

Female subjects participated during the follicular phase of their cycles (i.e. 4-9 d post onset of menses). This cycle phase was chosen because it is characterized generally by the least sensitivity to pain and by minimal menstrual cycle related symptoms<sup>[19]</sup>. The menstrual cycle has been reported to alter pain perception in women, and IBS symptoms have also been reported to fluctuate across the menstrual cycle<sup>[20]</sup>. In addition, the follicular phase was chosen because some female

subjects would have been using oral contraceptives (OCs), and the follicular phase is the cycle phase during which women who use OCs and normal cycling women are the most similar in their responses to experimental pain<sup>[21]</sup>.

### **Psychophysical measures**

Multiple psychological factors have been related to pain responses in a number of studies, and these factors may mediate partially group differences in pain sensitivity. Therefore, all participants completed psychological questionnaires that assessed coping style, anxiety, depression, and hyper-vigilance prior to the first experimental session. In addition, a measure of current affective state was administered prior to each experimental session. These measures were used as control variables (as further described in the data analysis section) to determine whether group differences in pain sensitivity remain significant after controlling for the influence of psychological variables.

The Coping Strategies Questionnaire (CSQ) consists of 44 items that are related to how individuals cope with pain<sup>[22]</sup>. It yields seven subscales based on the pain coping strategies: diverting attention, catastrophizing, praying and hoping, ignoring pain sensations, reinterpreting pain sensations, increasing behavioral activity, and coping self-statements. The CSQ also provides measures of subjects' perceived ability to control and decrease pain. It has been used widely with various pain populations, and has been modified for use with healthy pain-free subjects, by having individuals respond to the instrument based on how they cope typically with day-to-day aches and pains<sup>[23]</sup>. Responses on the CSQ have previously been related to experimental pain responses<sup>[24]</sup>, as well as clinical pain, including IBS<sup>[25-27]</sup>. We have used this scale in previous psychophysical and clinical research<sup>[28-30]</sup>.

The Kohn reactivity scale consists of 24 items that assess an individual's level of reactivity or central nervous system arousability. It has been used recently as a measure of the construct of hypervigilance<sup>[31]</sup>. This measure has been shown to correlate negatively with pain tolerance<sup>[31,32]</sup> and has been reported to have adequate internal consistency, ranging from an  $\alpha$  value of 0.73 to 0.83<sup>[33]</sup>.

The State-Trait Anxiety Inventory (STAXI) consists of 20 items that assess dispositional (i.e. trait) anxiety<sup>[34]</sup>. This is a well-validated and widely used instrument for assessing general anxiety.

The Beck Depression Inventory (BDI) is a widely used, 21-item, self-report measure that assesses common cognitive, affective and vegetative symptoms of depression. Research that has evaluated the psychometric properties of the BDI has suggested that it shows excellent reliability and validity as an index of depression<sup>[35]</sup>. Since chronic pain patients often endorse somatic symptoms assessed by the BDI, which may artificially inflate their scores<sup>[36]</sup>, the BDI has been separated into a 13-item cognitive affective subscale and an eight-item somatic-performance subscale<sup>[37]</sup>.

The Profile of Mood States-bipolar Form (POMS-BI) consists of 72 mood-related items, and subjects

indicate the extent to which each item describes their current mood<sup>[38]</sup>. This questionnaire assesses positive and negative affective dimensions. The POMS-BI has been well validated with other mood measures and is sensitive to subtle differences in affective state. We have used this measure in previous psychophysical studies<sup>[28]</sup>. This mood measure was administered to determine the current affective state at the beginning of each sensory testing session.

Functional Bowel Disorder Severity Index (FBDSI) comprises three variables: current pain (by visual analog scale), diagnosis of chronic abdominal pain, and number of physician visits in the past 6 mo<sup>[39]</sup>. The FBDSI is sufficiently sensitive to distinguish among the different groups, from healthy controls, through non-IBS patients, to patients with IBS only and, finally, IBS patients with concomitant FM. Severity is rated as none (0 points, controls), mild (1-36 points, non-IBS patients), moderate (37-110 points, IBS patients), and severe ( $> 110$  points, IBS and FM). All IBS patients that participated in the study were characterized with this index.

### **Thermal procedures**

Thermal stimuli were delivered using a computer-controlled Medoc Thermal Sensory Analyzer (TSA-2001; Ramat Yishai, Israel). This is peltier-element-based stimulator with a 3 cm  $\times$  3 cm surface area. Temperature levels were monitored by a contactor-contained thermistor, and returned to a preset baseline of 32°C by active cooling at a rate of 10°C/s. Heat pain threshold (HPT<sub>h</sub>) and heat pain tolerance (HPT<sub>o</sub>) were assessed on the left ventral forearm and left calf using an ascending method of limits. The cutoff temperature (to avoid tissue damage) for all trials was 52°C. Subjects were assigned randomly within each group (controls, C-IBS, D-IBS) to receive thermal nociceptive stimulation to the left ventral forearm versus the left calf in a random order that was counterbalanced across all groups.

From a baseline of 32°C, probe temperature increased at a rate of 0.5°C/s until the subject responded by pressing a button on a handheld device. This slow rate of rise preferentially activates C-fibers and diminishes artifacts associated with reaction time.

### **HPT<sub>h</sub>**

For HPT<sub>h</sub>, subjects were informed via digitally recorded instructions to press the button when the sensation first became painful. Four trials of HPT<sub>h</sub> were performed at each site (ventral forearm and calf). The average of the four trials at each site was computed for HPT<sub>h</sub>. The position of the thermode was altered slightly between trials in order to avoid either sensitization or habituation of cutaneous receptors. In addition, interstimulus intervals of at least 30 s were maintained between successive stimuli. Four trials of HPT<sub>h</sub> were presented followed by a 15-min rest period, and then four trials of HPT<sub>o</sub> were performed.

### **HPT<sub>o</sub>**

For HPT<sub>o</sub>, subjects were instructed to press the button

when the sensation was no longer tolerable. Four trials of HPTo were performed at each site (ventral forearm and calf). The average of the four trials at each site was computed for HPTo. The position of the thermode was altered slightly between trials in order to avoid either sensitization or habituation of cutaneous receptors. In addition, interstimulus intervals of at least 30 s were maintained between successive stimuli.

### Statistical analysis

The primary analyses involved one between- and two within-subject variables, and consequently, the repeated measures command within the General Linear model module of SPSS was used. In the first primary analysis, differences between controls and D-IBS and C-IBS patients for HPTh and HPTo at the forearm and calf were tested. In the second primary analysis, associations between IBS symptom severity and HPTh and HPTo at the forearm and calf were tested. A series of one-way ANOVAs were used to test for group differences in psychological inventories. As a result of the number of statistical tests performed, a Bonferroni correction was used to maintain family-wise type 1 error rate at  $P < 0.05$ .

## RESULTS

### Samples

A total of 118 participants were studied, which included 42 patients (29 female and 13 males; mean age  $27.0 \pm 6.4$  years) with D-IBS; 24 patients (16 female and eight male; mean age  $32.5 \pm 8.8$  years) with C-IBS; and 52 control subjects (34 female and 18 males; mean age  $27.3 \pm 8.0$  years) (Table 1). There was no difference in age or sex between the groups (controls, D-IBS and C-IBS). FBDSI score was higher in the D-IBS ( $62.8 \pm 23.2$ ) and C-IBS ( $68.4 \pm 25.3$ ) patients than the controls ( $2.1 \pm 4.9$ ), but did not differ between the patient groups.

### HPTh and HPTo

HPTh and HPTo (mean  $\pm$  SD) at both sites for C-IBS, D-IBS, and controls are presented in Table 2. We found significant effects for site, pain measure, and group (all at  $P < 0.001$ ). Pair-wise comparisons supported the hypothesis that controls were less sensitive than C-IBS and D-IBS patients (both at  $P < 0.001$ ), with no differences between C-IBS and D-IBS. Two-way interactions for pain measure  $\times$  group ( $P = 0.002$ ) and site  $\times$  pain measure ( $P < 0.001$ ) were also significant. Pair-wise comparisons revealed that controls had higher HPTh and HPTo than C-IBS and D-IBS (both at  $P < 0.001$ ) on the forearm and the calf. There was no difference in HPTh or HPTo between C-IBS and D-IBS patients.

A group effect emerged for Kohn scores ( $P = 0.004$ ), with C-IBS ( $78.8 \pm 9.9$ ) having higher scores than D-IBS ( $70.8 \pm 10.8$ ) and controls ( $70.1 \pm 11.1$ ) (collapsed across groups,  $72.1 \pm 11.2$ ). There were no differences in the BDI ( $3.4 \pm 3.7$ ), STAXI ( $28.0 \pm 4.8$ ), POMS positive ( $53.2 \pm 15.7$ ), POMS negative ( $29.8 \pm 16.3$ ) or any of the subscales of the CSQ (distracting attention,  $10.0 \pm 6.1$ ; cognitive

**Table 2** HPTh and HPTo for the arm and leg

Groups (n)	Threshold (HPTh)		Tolerance (HPTo)	
	Forearm	Calf	Forearm	Calf
Controls (n = 52)	43.2 $\pm$ 1.8	45.1 $\pm$ 1.7	48.0 $\pm$ 1.8	48.1 $\pm$ 1.4
Diarrhea (n = 42)	39.1 $\pm$ 3.3	41.1 $\pm$ 4.0	44.7 $\pm$ 3.8	44.9 $\pm$ 2.1
Constipation (n = 24)	39.6 $\pm$ 3.3	41.3 $\pm$ 3.5	45.0 $\pm$ 3.7	46.0 $\pm$ 3.1
IBS symptom groups				
Moderate symptoms (n = 42)	41.6 $\pm$ 1.7	43.6 $\pm$ 1.9	47.2 $\pm$ 1.6	47.0 $\pm$ 1.3
Severe symptoms (n = 24)	35.6 $\pm$ 1.5	36.9 $\pm$ 1.6	40.2 $\pm$ 2.1	42.1 $\pm$ 1.6

Values represent temperature mean  $\pm$  SD.

**Table 3** Demographic variable for IBS pain subgroups

	Moderate sensitivity (n = 42)	High sensitivity (n = 24)
Age (SD)	28.7 (7.0)	29.1 (7.3)
Sex (female) n (%)	30 (71)	15 (63)
FBDSI	49.3 (9.1)	96.4 (8.7)
	Range 38–72	Range 82–110
Diarrhea subtype n (%)	25 (60)	17 (41)
Constipation subtype n (%)	17 (40)	7 (29)
Self-reported race/ethnicity n (%)		
White	30 (71)	18 (37)
Black	9 (21)	0 (0)
Hispanic	1 (2)	3 (75)
Asian	2 (5)	3 (60)

Columns may not sum to 100% because values were rounded to the nearest 1%.

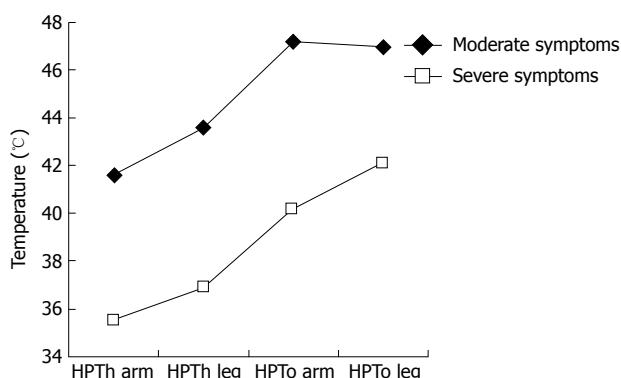
self-statements,  $13.3 \pm 5.3$ ; ignoring pain sensations,  $11.0 \pm 6.1$ ; praying and hoping,  $5.4 \pm 4.9$ ; reinterpreting pain sensations,  $4.9 \pm 4.8$ ; catastrophizing,  $5.9 \pm 6.1$ .

### Differences as a function of IBS symptoms

We found significant effects for site, pain measure, and symptom severity (all at  $P < 0.001$ ). The site by symptom severity interaction was not significant; however, the 3-way interaction (site  $\times$  pain  $\times$  symptoms) was significant ( $P < 0.01$ ). To interpret this interaction involving an internal level variable (IBS symptom severity) and two nominal variables, we examined scatter plots for HPTh and HPTo at the leg and arm by FBDSI scores. The results suggest a naturally occurring gap between scores of 72 and 82 that was associated with pain sensitivity; therefore, two groups were formed based on FBDSI scores, with a cutoff at 80. Demographic variables for the two symptom severity groups are presented in Table 3. HPTh and HPTo at both sites for the two IBS symptom severity groups are presented in Table 2 and Figure 1. The IBS symptom subgroups did not differ for Kohn, BDI, STAXI, POMS, or CSQ scores.

## DISCUSSION

Overall, our findings indicate thermal hyperalgesia for IBS-C and IBS-D patients relative to controls, with IBS patients reporting significantly lower pain threshold and pain tolerance at both test sites. These findings add further



**Figure 1** HPT and HPTo for IBS pain subgroups.

support to the notion that IBS patients show somatic hyperalgesia. A unique finding of our study is that we detected a strong relationship between heat pain measures and FBDSI scores. IBS patients with high FBDSI scores had the highest thermal pain sensitivity compared to IBS patients with low to moderate FBDSI scores.

In contrast to our current findings, several previous studies have indicated lack of somatic hyperalgesia among IBS patients relative to controls<sup>[6,7]</sup>, with some studies actually showing higher pain threshold among IBS patients relative to controls<sup>[4,5]</sup>. One possible explanation for this is differences in the painful stimulus, as previous studies have used electrical stimuli, mechanical pressure, and cold immersion, but none has used contact heat. Also, Chang and colleagues<sup>[40]</sup> have reported that female IBS patients showed significantly higher pressure pain threshold than female controls in response to a randomly administered series of fixed stimuli, but no group differences emerged for threshold assessed using ascending stimuli. Randomly administered stimuli are thought to reduce response bias, which is likely driven by psychological factors such as hypervigilance. Since we used ascending stimuli in the present study, it is possible that response bias contributed to our results. However, this seems unlikely given the lack of correlations between psychological factors, including anxiety and hypervigilance, and heat pain responses. Differences in the nature of the patient population may present another explanation for the differences between our findings and those of previous studies. Most prior investigations<sup>[4-7]</sup> recruited IBS patients from clinical settings, typically in tertiary care centers, whereas our IBS sample was recruited from the community, using print and postal advertisements. This community-based recruitment approach yielded a psychologically healthy IBS population, which was similar to the controls for most psychological measures. Moreover, most previous studies have included a female-only population, while we included women and men in both the IBS and control samples. Thus, differences in the experimental pain stimulus and patient population may have contributed to the differing pattern of results.

Consistent with the present findings, other investigators have reported somatic hyperalgesia in IBS patients, using cold pain<sup>[3,10]</sup>, and we have shown similar results

with heat immersion<sup>[2,8]</sup>. However, to the best of our knowledge, this is the first report of an association between somatic pain sensitivity and clinical symptoms among patients with IBS. Wilder-Smith and colleagues<sup>[10]</sup> have reported a strong association between somatic and visceral hypersensitivity. However, there is mixed evidence regarding the association between visceral hypersensitivity and clinical symptoms in IBS<sup>[11,13,15,16,41]</sup>. In our study, the association of somatic hyperalgesia with FBDSI score suggests that central mechanisms contribute to the severity of patients' clinical symptoms. This has potentially important treatment implications, as one might speculate that patients exhibiting somatic hypersensitivity may benefit from treatments that alter central processes, rather than those that are restricted to peripheral targets.

Several limitations of this study should be mentioned. First, somatic pain testing was limited to HPT and HPTo and did not include other stimuli. Thus, it is not possible to determine whether these findings are specific to heat pain. Second, we did not assess sensitivity to visceral stimuli, and it would be interesting to know whether heat pain sensitivity is associated with visceral sensitivity. However, given our relatively large sample size, visceral testing was not feasible. Finally, based on the current design, we were unable to identify the mechanisms that underlie heat hyperalgesia and its association with clinical symptoms. Despite these limitations, the relatively large sample size, the use of heat pain measures designed to activate C-fibers, and the strong association between heat pain sensitivity and IBS symptom severity represent unique and valuable features of our study.

## CONCLUSION

Our study indicates somatic hypersensitivity for both groups of IBS patients relative to controls, with IBS patients reporting significantly lower thermal pain threshold and tolerance. Moreover, somatic pain sensitivity was associated with IBS symptoms, such that patients with high FBDSI scores showed significantly greater pain sensitivity compared to those with low to moderate FBDSI scores. Further studies are warranted to evaluate somatic hypersensitivity as a predictor of clinical symptoms in IBS.

## COMMENTS

### Background

Irritable bowel syndrome (IBS) is one of the most common gastrointestinal disorders encountered by gastroenterologists. Patients present classically with chronic abdominal pain associated with an alteration in bowel habits. Even though the pathophysiology of IBS is unclear, visceral hypersensitivity is a common clinical marker of the disorder. Visceral hypersensitivity may account for symptoms of abdominal pain, urgency, and bloating experienced by many patients with this disorder.

### Research frontiers

Although visceral hypersensitivity is considered a hallmark feature of IBS, conflicting evidence exists regarding somatic hypersensitivity in this patient population. Somatic pain conditions, such as fibromyalgia and migraine headaches show significant comorbidity with IBS, which suggests that somatic hypersensitivity characterizes at least a subpopulation of IBS patients. Several

recent studies using hot water immersion have shown widespread somatic hyperalgesia associated with IBS, and others using the cold pressor test have demonstrated somatic hypersensitivity in IBS patients compared with healthy controls.

### Innovations and breakthroughs

Overall, the findings indicate thermal hyperalgesia for constipation- and diarrhea-dependent IBS relative to controls, with IBS patients reporting significantly lower pain threshold and tolerance at both test sites. These findings add further support to the notion that IBS patients show somatic hyperalgesia. A unique finding of this study is that the authors detected a strong relationship between heat pain measures and Functional Bowel Disease Severity Index (FBDSI) scores. IBS patients with high FBDSI scores had the highest thermal pain sensitivity compared to those IBS patients with low to moderate FBDSI scores.

### Applications

This study suggests that a subset of IBS patients has evidence of somatic hypersensitivity that may relate to extra-intestinal symptoms.

### Peer review

This is an elegant study that evaluated systematically thermal hypersensitivity in a large number of IBS patients. The results are novel and interesting and may lead to new therapy in a subset of IBS patients with somatic hypersensitivity.

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BRIEF ARTICLES

## Specific probiotics alleviate allergic rhinitis during the birch pollen season

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During May, there was a tendency for fewer subjects, (76.2% vs 95.2%,  $P = 0.078$ ) to report runny nose, while during June, fewer subjects, 11.1% vs 33.3%, reported nasal blocking in the probiotics group ( $P = 0.101$ ). Concomitantly, fewer subjects in the probiotic group had infiltration of eosinophils in the nasal mucosa compared to the placebo group, 57.1% vs 95% ( $P = 0.013$ ). Eye symptoms tended to be slightly more frequent in the probiotic group, 12.5 d [interquartile range (IQR) 6-18] vs 7.5 d (IQR 0-11.5) ( $P = 0.066$ ) during May. Fecal IgA was increased in the placebo group during the pollen season; this increase was prevented by the probiotics ( $P = 0.028$ ).

**CONCLUSION:** Birch pollen allergy was shown to be associated with changes in fecal microbiota composition. The specific combination of probiotics used was shown to prevent the pollen-induced infiltration of eosinophils into the nasal mucosa, and indicated a trend for reduced nasal symptoms.

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**Key words:** Probiotics; Pollen allergy; Intestines; Microbiology; Immunity; Eosinophilia; *Lactobacillus acidophilus*; *Bifidobacterium lactis*

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### Abstract

**AIM:** To investigate whether birch pollen allergy symptoms are linked with gut microbiota changes and whether probiotics have an effect on these.

**METHODS:** Forty seven children with confirmed birch pollen allergy were randomized to receive either a probiotic combination of *Lactobacillus acidophilus* (*L. acidophilus*) NCFM™ (ATCC 700396) and *Bifidobacterium lactis* (*B. lactis*) BI-04 (ATCC SD5219) or placebo in a double-blind manner for 4 mo, starting prior to onset of the birch pollen season. Symptoms were recorded in a diary. Blood samples were taken for analysis of cytokines and eosinophils. Fecal samples were analysed for microbiota components, calprotectin and IgA. Nasal swabs were taken for analysis of eosinophils.

**RESULTS:** The pollen season induced a reduction in *Bifidobacterium*, *Clostridium* and *Bacteroides* which could not be prevented by the probiotic intervention. During the intervention, significantly higher numbers of *B. lactis*  $11.2 \times 10^7 \pm 4.2 \times 10^7$  vs  $0.1 \times 10^7 \pm 0.1 \times 10^7$  bacteria/g feces ( $P < 0.0001$ ) and *L. acidophilus* NCFM™  $3.5 \times 10^6 \pm 1.3 \times 10^6$  vs  $0.2 \times 10^6 \pm 0.1 \times 10^6$  bacteria/g feces ( $P < 0.0001$ ) were observed in the probiotic group compared to the placebo group.

### INTRODUCTION

Allergic rhinitis and asthma are common chronic conditions, with a recently reported annual symptom prevalence of 15% for both diseases in young teenagers in Western Europe<sup>[1]</sup>. Allergic rhinitis is often seasonal and caused by exposure to environmental allergens. It has been shown that, in allergic rhinitis, nasal allergen

provocation induces an inflammatory response not only in nasal but also in bronchial mucosa<sup>[2]</sup>, and *vice versa*<sup>[3]</sup>, emphasizing the importance of the airways as a single anatomic-functional unit. Furthermore, chronic allergic rhinitis is known to be an independent risk factor of asthma<sup>[4]</sup>. Allergic rhinitis is frequently accompanied by allergic conjunctivitis, characterized by decreased conjunctival epithelial integrity and repair mechanisms even outside the pollen season<sup>[5]</sup>.

Probiotics are live microorganisms which, when administered in adequate amounts confer a health benefit on the host<sup>[6]</sup>. Specific probiotic strains have been shown to be effective in the prevention<sup>[7-9]</sup> and treatment<sup>[10]</sup> of atopic eczema, alleviating allergic inflammation both locally and systemically. The evidence of probiotic efficacy against allergic rhinitis and immunological sensitization predisposing to asthma is insufficient and contradictory at present. The previously studied probiotic strains or combinations of these may not have targeted airway allergies, or the populations studied may not have been responsive to immune modulation<sup>[9]</sup>.

We hypothesized that allergic rhinitis can be alleviated by the use of specific probiotics selected to both modify the intestinal microbiota and to confer immune effects, which can be demonstrated in the intestinal tract prior to an allergic reaction. We assumed that the nasal mucosa is the most vulnerable structure of the airways, allowing the greatest inhalational allergen penetration. Thus we chose to study the effects of selected probiotic bacteria in allergic rhinitis in children during the birch pollen season. The specific strains used in this study were selected based on their anti-inflammatory properties and an expected effect in promoting a Th1 type of immune response<sup>[11,12]</sup>. The study population was chosen to cover subjects with confirmed birch pollen allergy. By selecting children, we hypothesized that there would be more opportunity to modify the immune responses.

## MATERIALS AND METHODS

### Patients

Forty-seven children with clinically and immunologically documented and physician-verified birch pollen allergy were enrolled in the study. All had previously had symptoms of allergic rhinitis confined to the birch pollen season, and the specific test result to birch pollen had been positive, either with skin prick testing (positive result if the mean wheal diameter for birch pollen allergen was greater than 3 mm) or demonstration of specific IgE antibody in serum (according to the manufacturer's reference values, the level for positivity was a specific IgE more than 0.35 kU/L). The symptoms of the patients during the previous pollen season included sneezing and runny or blocked nose, and the nasal symptoms were often associated with allergic eye symptoms, such as itching and redness of the conjunctiva. Patients with diagnosed asthma, habitual use of probiotics and/or prebiotics and recent use of antibiotics were excluded from the study.

The study was approved by the Ethics Committee

of the Hospital District of South-Western Finland and informed, written consent was received from the participants and their parents. The study was registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) under the identifier NCT00746226.

### Study material

The study material consisted of capsules containing  $5 \times 10^9$  CFU of a combination of 25% *Lactobacillus acidophilus* (*L. acidophilus*) NCFM™ (ATCC 700396) and 75% *Bifidobacterium lactis* (*B. lactis*) Bl-04 (ATCC SD5219), Danisco Cultures, Madison, USA. The strains were selected based on their anti-inflammatory properties and Th1-type immune stimulating effects and their ratio was chosen to optimize these functions. The viability of the study material was determined at monthly intervals during the study by Danisco Cultures, Madison. This laboratory was not involved in the study, and no significant reduction in viability was observed (results not shown). The placebo consisted of identical capsules containing microcrystalline cellulose. The study material was randomized at the site of production by a person not involved in the study. The volunteers, researchers and biostatisticians remained blinded for the duration of the study and the analyses. The code was broken after completion of the statistical analyses.

### Study design

Eligible subjects were assigned to receive one of the individually coded test products. The subjects or their parents were instructed to consume one capsule daily or to suspend its contents in a suitable liquid.

The patients attended the study unit 3 times during spring 2006 (Figure 1). The first visit was in March before the birch pollen season, and consumption of the study material started after this visit. The second visit was at the end of April-beginning of May during the birch pollen season and the third visit was in June at the end of the season, when consumption of the study product was finished.

Clinical examination of the patients was carried out at each study visit. At each visit, nasal smears were taken from both nostrils by gently rubbing the middle or inferior turbinate with a cotton covered stick, followed by fixing and staining with eosin and methylene blue. Eosinophil counts were determined semiquantitatively by microscope as follows: -, no eosinophils in any of the fields of view; +, a few scattered eosinophils at least in some fields of view; ++, eosinophils easily found in every field of view; and +++, every field of view abundant in eosinophils. Venous blood samples were drawn at each study visit. At the first and third visit, the blood eosinophil count was determined by automated blood cell counter (Advia 120, Bayer Health Care, Germany), and allergen-specific IgE antibodies in serum were measured by an immunofluorometric assay (ImmunoCAP™, Phadia AB, Uppsala, Sweden). All samples were initially screened using a mix of inhalation allergens (Phadiatop ImmunoCAP). The samples which were positive at screening were subsequently tested for

birch pollen-specific IgE.

The concentrations of the cytokines interleukin (IL)-4, IL-5, IL-6, IL-10 and tumor necrosis factor (TNF)- $\alpha$  were measured by multiplex flow cytometric assay using a commercial Human Th1/Th2 Cytokine Kit (BD Immunocytometry Systems and BD Biosciences Pharmingen). The concentrations of transforming growth factor (TGF)- $\beta$ 2 were measured at the first and second visit and soluble CD-14 (sCD-14) at the first and third visit using commercial sandwich ELISA specific for these molecules (R&D Systems Europe Ltd., Abingdon, UK).

The presence of blocked or runny nose, respiratory difficulty, coughing, eye symptoms or eczema was recorded by the parents on the diary cards throughout the study. The number of days with each symptom during each of the study months was reported.

#### **Analysis of gut microbiota by fluorescent in situ hybridization (FISH)**

The major groups of fecal bacteria were analyzed using the FISH method as previously described<sup>[13]</sup>. In brief, fecal samples were suspended in PBS and homogenized. Bacteria were fixed with paraformaldehyde and hybridized with Cy3 indocarbocyanin-labeled oligonucleotide probe. Probes included Bac303 (5'-CCAATGTGGGGACCTT-3') for the *Bacteroides-Prevotella* group, Bif164 (5'-CAT CGGCATTACCACCC-3') for bifidobacteria, His150 [5'-TTATGCGGTATTAATCT (C/T) CCTTT-3'] for clostridia of the *C. histolyticum* group and MUC-1437 (5'-CCTTGCAGGTGGCTTCAGAT-3') for *Akkermansia muciniphila*-like bacteria<sup>[14]</sup>, total cells were enumerated using an EUB338 (5'-GCTGCCTCCGTAGGAGT-3')-fluorescein (FITC)-labelled probe<sup>[15,16]</sup>.

Flow-cytometric analyses were performed using a BD<sup>TM</sup> LSR II flow cytometer (Becton, Dickinson and Co., USA) equipped with 4 lasers (355, 405, 488 and 635 nm). We used the 488 nm laser at 15 mW. This standard instrument is equipped with 2 light scatter detectors which measure forward (FSC) and side scatter (SSC) and 4 fluorescence detectors detecting appropriately filtered light at green (FL1, 525 nm), yellow (FL2, 575 nm), orange (FL3, 620 nm), and red (FL4, 675 nm) wavelengths. To avoid cell coincidence, the flow rate was kept at the lowest setting (data rate 200-300 events per second). At least 30 000 events were recorded for each sample and all experiments were conducted in duplicate. Data were stored as list-mode files and analyzed off-line using the BD FACSDiva<sup>TM</sup> software version 4.1.1 (Becton, Dickinson and Co., USA).

Immediately prior to analysis, Flow-Count fluorospheres (Beckman Coulter, USA) were added to each sample. Absolute bacterial cell counts were determined following the manufacturer's instructions, using the ratio of positive bacteria to fluorospheres counted using the following formula: cells/ $\mu$ L = [(cells counted)/(fluorospheres counted)]  $\times$  fluorospheres/ $\mu$ L. To avoid loss of the signal intensity of hybridized cells, they were kept in the dark on ice at 4°C until the flow

cytometry assay. Results were expressed as the numbers of cells hybridizing with the specific group-Cy3 probe and total bacteria EUB 338-FITC probe.

#### **Analysis of microbiota by quantitative real-time PCR (qRT-PCR)**

DNA extractions from pure cultures of the different microorganisms and fecal samples were extracted using the QIAamp DNA stool Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. qPCRs were conducted as previously described<sup>[10,11]</sup>. For characterization of the fecal microbiota PCR primers were designed targeting *B. lactis* according to Gueimonde and co-workers<sup>[17]</sup>. *L. acidophilus* NCFM<sup>TM</sup> was quantified using primers as described by Ouwehand and co-workers<sup>[18]</sup>. The oligonucleotides were purchased from the Thermo Electron Corporation (Thermo Biosciences, Ulm, Germany). Briefly, PCR amplification and detection were performed with an ABI PRISM 7300-PCR sequence detection system (Applied Biosystems, UK). Each reaction mixture of 25  $\mu$ L was composed of SYBR<sup>®</sup> Green PCR Master Mix (Applied Biosystems, UK.), 1  $\mu$ L of each of the specific primers at a concentration of 0.25  $\mu$ mol/L, and 1  $\mu$ L of template DNA. The fluorescent products were detected at the last step of each cycle. A melting curve analysis was made after amplification to distinguish the targeted PCR product from the non-targeted PCR product.

#### **Microbial activity**

Microbial activity was determined by measurement of lactic acid, short chain fatty acids: acetic, butyric, lactic and propionic acids, and branched chain fatty acids: valeric, isobutyric, 2-methylbutyric and isovaleric acid. These were determined by gas chromatography as described previously by Holben *et al*<sup>[19]</sup>. The results were expressed in mmol/kg.

#### **Determination of intestinal immune responses**

Changes in the immunological status of the intestine were monitored by measuring the concentrations of IgA and calprotectin from the soluble fraction of feces. For IgA measurements the frozen samples were thawed and extracted with BSA as described previously and stored at -20°C before analysis<sup>[20]</sup>. Concentrations of IgA were then determined with the ELISA according to the manufacturer's instructions (E80-102, Bethyl Laboratories, Inc., Montgomery, TX, USA). The concentrations of calprotectin were determined with ELISA following the manufacturer's instructions (Calpro AS, Oslo, Norway). The results were expressed as  $\mu$ g per g fresh weight.

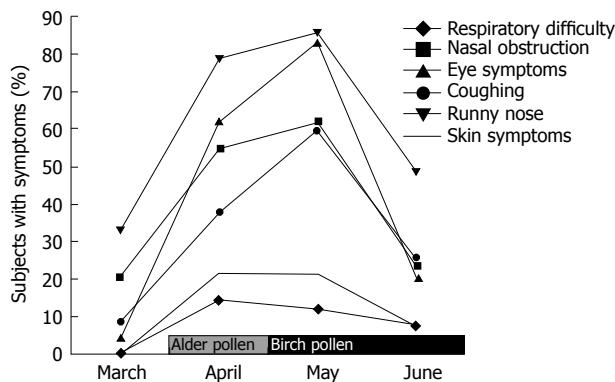
#### **Statistical analysis**

Differences between the treatment groups were analysed using the Mann-Whitney U test. Differences within the treatment groups between the different time points were analyzed using the Wilcoxon signed rank test. Correlations between the level of fecal colonization of *B. lactis* or *L. acidophilus* NCFM<sup>TM</sup> and immune or

**Table 1** The clinical characteristics of the study subjects

	Probiotic	Placebo	P-value
N	24	23	
Drop-outs	2	1	$P > 0.99$
Age (yr)	9.0 (4.2-11.9)	8.9 (6.1-12.9)	$P = 0.845$
Gender (M/F)	11 (13)	8 (15)	$P = 0.24$
Length (cm)	135.2 (107.3-156.5)	133.6 (113.8-152.5)	$P = 0.50$
Weight (kg)	31.0 (11.9-47.4)	32.4 (20.2-45.3)	$P = 0.80$

Data are expressed as mean (range).



**Figure 1** Percentage of subjects with specific symptoms of birch pollen allergy among allergic children. The gray bar represents the alder pollen season and the black bar the birch pollen season in Turku (Finland) during the study period ( $> 100$  pollen/m $^3$ ).

microbiological variables in the probiotic group during the second visit were determined with the Spearman rank correlation test. To determine differences between the groups and over time, the data was analyzed with a linear mixed effects model, having effects for time, group, and their joint effect. Individual baseline differences were taken into account by including the first time point as a covariate in the model. P-values below 0.05 were considered significant.

## RESULTS

### Clinical characteristics

Baseline clinical characteristics of the 2 groups are presented in Table 1. Twenty patients in the probiotic and 21 in the placebo group completed the study. Three patients, 2 in the probiotic group and 1 in the placebo group, were excluded during the study because of newly developed asthma. Three patients, 2 in the probiotic group and 1 in the placebo group, decided to drop out of the study. The mean duration of breast-feeding was 7.5 mo (range 0-18 mo), there being 2 patients whom were not breast-fed.

### Impact of the birch pollen season on allergy

The impact of the onset of the birch pollen season on allergy symptoms in the two study groups is depicted in Figure 1. The peak period of birch pollen exposure, April and May, resulted in specific symptoms such as nasal obstruction and runny nose. All the recorded

**Table 2** Number of subjects with nasal eosinophilia n (%)

Month	Placebo	Probiotic
March	14 (63.6)	17 (70.8)
April/May	19 (95.0)	12 (57.1) <sup>1</sup>
June	12 (60.0)	12 (60.0)

<sup>1</sup>P = 0.013 compared to placebo group.

symptoms increased during April and May (the height of the pollen season in Turku, Finland), and decreased again in June ( $P < 0.001$ ).

At the start of the study, there was no difference in the observed symptoms between the 2 study groups. Runny nose tended ( $P = 0.078$ ) to be reported in May by fewer subjects in the probiotic group (76.2%) than in the placebo group (95.2%). During June, fewer subjects reported nasal blocking in the probiotics group (11.1%) than in the placebo group (33.3%). This did not, however, reach statistical significance ( $P = 0.101$ ). Concomitantly with the differences in nasal symptoms, the number of subjects with infiltration of eosinophils in the nasal mucosa increased in the placebo group during the April/May visit but an increase was not observed in the probiotic group ( $P = 0.013$ ), where the fraction of subjects with nasal eosinophil infiltration remained unchanged (Table 2).

During May, the subjects in the probiotic group reported more days with eye symptoms, 12.5 d (interquartile range (IQR) 6-18 d), than subjects in the placebo group, 7.5 d (IQR 0-11.5 d),  $P = 0.066$ . In June, this difference was no longer detectable.

### Impact of intervention on immunological parameters

In addition to the increase in symptoms over the season, concentrations of birch pollen-specific IgE also increased in both groups from March to June,  $P = 0.0015$  and  $P = 0.0002$  for placebo and probiotic respectively (Table 3). Likewise, blood eosinophil numbers increased in both placebo ( $P = 0.03$ ) and probiotic groups ( $P = 0.002$ ), from March to June (Table 3). Concentrations of IL-6 decreased in both groups from March to April/May,  $P = 0.0004$  and  $P = 0.0002$  in placebo and probiotic groups, respectively (Table 3). Concentrations of IL-10 were reduced in the placebo group from March to April/May,  $P = 0.0495$ . For both groups, concentrations of TNF- $\alpha$  decreased from March to April/May,  $P = 0.0007$  and  $P = 0.0005$  in placebo and probiotic groups, respectively. Concentrations of TNF- $\alpha$  were higher in the placebo group at the start of the study,  $P = 0.0062$ , however, at the April/May visit no difference was observed,  $P = 0.17$ . Concentrations of interferon- $\gamma$  and IL-2 were below the detection limit for both groups at all 3 time points tested. No differences in the concentrations of TGF- $\beta$ 2, IL-4, IL-5 and CD14 were detected (Table 3).

Concentrations of fecal IgA and calprotectin exhibited great individual variance ( $P < 0.0001$  and  $P = 0.015$ , respectively). Because of this significant variation, the data were analyzed taking the baseline

**Table 3** Serum immune markers; before, during and after the birch pollen season, median

	March		April/May		June	
	Placebo	Probiotic	Placebo	Probiotic	Placebo	Probiotic
Specific IgE birch pollen (kU/L)	26.0 (18.0-45.8)	25.5 (15.5-54.0)	-	-	150.0 (36.0-150.0) <sup>1</sup>	150.0 (150.0-150.0) <sup>1</sup>
Blood eosinophils (10 <sup>9</sup> /L)	0.30 (0.24-0.44)	0.18 (0.12-0.34)	-	-	0.40 (0.25-0.72) <sup>1</sup>	0.42 (0.28-0.69) <sup>1</sup>
IL-4 (pg/mL)	2.20 (1.5-2.8)	1.70 (0.0-2.2)	1.70 (0.8-2.2)	1.7 (1.3-2.1)	-	-
IL-5 (pg/mL)	19.6 (0.0-69.7)	9.10 (0.0-24.2)	14.70 (0.0-28.3)	18.4 (0.0-60.2)	-	-
IL-6 (pg/mL)	16.6 (5.7-38.3)	4.70 (3.0-11.4) <sup>3</sup>	4.20 (2.3-7.3) <sup>1</sup>	3.0 (2.0-3.9) <sup>1</sup>	-	-
IL-10 (pg/mL)	1.60 (1.4-1.9)	1.50 (0.0-2.1)	1.40 (0.0-1.8) <sup>2</sup>	0.0 (0.0-1.5)	-	-
TNF-α (pg/mL)	7.70 (3.9-13.5)	3.50 (2.5-5.2) <sup>3</sup>	2.90 (2.3-4.8) <sup>1</sup>	2.5 (2.1-3.2) <sup>1</sup>	-	-
TGF-β2 (pg/mL)	242 (0.0-342)	247 (0.0-369)	213.6 (0.0-314.3)	267.9 (0.0-445.9)	-	-
CD-14 (μg/mL)	1.20 (1.1-1.3)	1.20 (1.1-1.3)	-	-	1.20 (1.1-1.3)	1.10 (1.1-1.3)

<sup>1</sup>Significantly different from previous time point;  $P < 0.01$ . <sup>2</sup>Significantly different from previous time point;  $P < 0.05$ . <sup>3</sup>Significantly different between groups;  $P < 0.05$ . -: No sample collected. Data are expressed as median (interquartile range).

**Table 4** Fecal immune markers; before, during and after the birch pollen season

	Visit 1 (March)		Visit 2 (April/May)		Visit 3 (June)	
	Placebo	Probiotic	Placebo	Probiotic	Placebo	Probiotic
Fecal IgA (μg/g)	157.0 (105.0-278.8)	157.0 (114.0-371.0)	233.0 (136.0-288.8) <sup>1</sup>	155.0 (44.5-336.5) <sup>1</sup>	203.0 (99.0-388.0) <sup>1</sup>	135.0 (63.4-325.3) <sup>1</sup>
Fecal calprotectin (μg/g)	21.6 (9.5-58.6)	14.3 (10.7-30.1)	21.1 (12.6-46.7)	16.2 (13.1-31.8)	22.1 (10.9-53.2)	15.5 (12.0-31.5)

<sup>1</sup>Significantly different between groups;  $P < 0.05$ . Data are expressed as median (interquartile range).

**Table 5** Alteration of the fecal microbiota composition during the birch pollen season

	March		April/May		June	
	Placebo	Probiotic	Placebo	Probiotic	Placebo	Probiotic
<i>Eubacterium</i> <sup>4</sup>	$2.7 \times 10^9$ (0.3 × 10 <sup>9</sup> )	$2.5 \times 10^9$ (0.4 × 10 <sup>9</sup> )	$1.8 \times 10^9$ (1.0 × 10 <sup>9</sup> )	$1.6 \times 10^9$ (0.2 × 10 <sup>9</sup> ) <sup>2</sup>	$2.00 \times 10^9$ (0.2 × 10 <sup>9</sup> )	$1.9 \times 10^9$ (0.3 × 10 <sup>9</sup> )
<i>Bifidobacterium</i> <sup>4</sup>	$5.0 \times 10^8$ (5.0 × 10 <sup>8</sup> )	$3.5 \times 10^8$ (3.3 × 10 <sup>8</sup> )	$2.1 \times 10^8$ (0.4 × 10 <sup>8</sup> ) <sup>2</sup>	$1.6 \times 10^8$ (0.3 × 10 <sup>8</sup> ) <sup>1</sup>	$2.50 \times 10^8$ (0.4 × 10 <sup>8</sup> )	$2.0 \times 10^8$ (0.2 × 10 <sup>8</sup> )
<i>Bacteroides</i> <sup>4</sup>	$4.9 \times 10^8$ (3.5 × 10 <sup>8</sup> )	$5.4 \times 10^8$ (3.2 × 10 <sup>8</sup> )	$2.3 \times 10^8$ (0.4 × 10 <sup>8</sup> ) <sup>2</sup>	$1.7 \times 10^8$ (0.3 × 10 <sup>8</sup> ) <sup>1</sup>	$1.30 \times 10^8$ (0.2 × 10 <sup>8</sup> )	$1.5 \times 10^8$ (0.4 × 10 <sup>8</sup> )
<i>Clostridium</i> <sup>4</sup>	$1.4 \times 10^8$ (1.0 × 10 <sup>8</sup> )	$1.5 \times 10^8$ (0.3 × 10 <sup>8</sup> )	$0.7 \times 10^8$ (0.2 × 10 <sup>8</sup> ) <sup>2</sup>	$0.8 \times 10^8$ (0.2 × 10 <sup>8</sup> ) <sup>1</sup>	$1.30 \times 10^8$ (0.3 × 10 <sup>8</sup> ) <sup>2</sup>	$1.6 \times 10^8$ (0.2 × 10 <sup>8</sup> ) <sup>1</sup>
<i>Akkermansia</i> <sup>4</sup>	$1.3 \times 10^8$ (0.2 × 10 <sup>8</sup> )	$1.3 \times 10^8$ (0.2 × 10 <sup>8</sup> )	$1.2 \times 10^8$ (0.2 × 10 <sup>8</sup> )	$1.1 \times 10^8$ (0.1 × 10 <sup>8</sup> )	$0.90 \times 10^8$ (0.2 × 10 <sup>8</sup> )	$1.1 \times 10^8$ (0.2 × 10 <sup>8</sup> )
<i>B. lactis</i> <sup>5</sup>	$2.6 \times 10^7$ (1.7 × 10 <sup>7</sup> )	$1.5 \times 10^7$ (1.3 × 10 <sup>7</sup> )	$0.1 \times 10^7$ (0.1 × 10 <sup>7</sup> )	$11.2 \times 10^7$ (4.2 × 10 <sup>7</sup> ) <sup>1,3</sup>	$0.01 \times 10^7$ (0.05 × 10 <sup>7</sup> )	$2.6 \times 10^7$ (1.0 × 10 <sup>7</sup> ) <sup>3</sup>
<i>L. acidophilus</i> NCFM <sup>5</sup>	$1.0 \times 10^6$ (0.8 × 10 <sup>6</sup> )	$0.7 \times 10^6$ (0.5 × 10 <sup>6</sup> )	$0.2 \times 10^6$ (0.1 × 10 <sup>6</sup> ) <sup>1</sup>	$3.5 \times 10^6$ (1.3 × 10 <sup>6</sup> ) <sup>3</sup>	$0.02 \times 10^6$ (0.1 × 10 <sup>6</sup> )	$4.2 \times 10^6$ (2.4 × 10 <sup>6</sup> ) <sup>3</sup>

<sup>1</sup>Significantly different from previous time point;  $P < 0.01$ . <sup>2</sup>Significantly different from previous time point;  $P < 0.05$ . <sup>3</sup>Significantly different from placebo;  $P < 0.01$ . <sup>4</sup>Determined by fluorescent *in situ* hybridization (FISH). <sup>5</sup>Determined by quantitative real-time PCR. Data are expressed as mean (SE), microbes/g feces.

level into account. Neither the probiotic treatment, nor pollen season had any significant effects on the fecal calprotectin concentrations. However, fecal IgA concentrations were increased in the placebo group during April/May ( $P = 0.028$ ), which was prevented by the probiotic intervention. In the latter group, the concentration remained stable during the entire study period, Table 4.

#### Impact of probiotics on the gut microbiota

Administration of *B. lactis* Bl-04 and *L. acidophilus* NCFM™ led to a significant increase in the fecal numbers of *B. lactis* ( $P = 0.0032$ ) and *L. acidophilus* NCFM™ ( $P = 0.0036$ ), from March to April/May and numbers remained high until the end of the intervention in June, indicating good compliance by the subjects (Table 5). The differences were also significant compared to the placebo group during April/May; *B. lactis* ( $P < 0.0001$ ) and *L. acidophilus* NCFM™ ( $P = 0.0002$ ), and June; *B. lactis* ( $P < 0.0001$ ) and *L. acidophilus* NCFM™ ( $P <$

0.0001), Table 5.

A general decrease in numbers of the major bacterial groups *Eubacterium*, *Bifidobacterium*, *Bacteroides* and *Clostridium* accompanied the birch pollen season in both study groups (Table 5). This may not be explained by the increase in fecal numbers of *B. lactis* and *L. acidophilus* as the decrease in fecal numbers was manifested in both probiotic and placebo groups. In the placebo group, concentrations of 2-methylbutyric acid were reduced from April/May to June,  $P = 0.0501$ . However, no differences in concentrations of other measured fecal organic acids were observed between the 2 groups for any of the time points (Table 6).

#### DISCUSSION

Probiotics have been used to reduce the risk of atopic eczema, frequently the first allergic symptom to manifest itself. Previous intervention studies showing risk reduction for eczema have failed to reduce the risk of

**Table 6** Concentrations of fecal organic acids (mmol/kg)

	March		April/May		June	
	Placebo	Probiotic	Placebo	Probiotic	Placebo	Probiotic
Acetic acid	64.6 (6.0)	74.50 (7.2)	59.20 (5.6)	65.50 (5.8)	66.90 (5.1)	58.20 (4.5)
Propionic acid	15.5 (1.3)	17.10 (1.6)	15.20 (1.2)	16.10 (2.1)	16.20 (1.9)	14.60 (1.5)
Isobutyric acid	2.30 (0.3)	2.20 (0.2)	2.00 (0.2)	1.90 (0.1)	1.80 (0.2)	1.90 (0.2)
Butyric acid	11.9 (2.0)	13.40 (2.5)	12.50 (1.2)	11.70 (1.7)	14.00 (1.8)	11.50 (1.7)
2-methylbutyric acid	1.60 (0.2)	1.30 (0.2)	1.60 (0.2)	1.20 (0.1)	1.10 (0.1) <sup>1</sup>	1.20 (0.2)
Isovaleric acid	1.80 (0.2)	1.70 (0.1)	1.60 (0.2)	1.50 (0.1)	1.50 (0.2)	1.50 (0.2)
Lactic acid	0.14 (0.14)	0.83 (0.56)	0.00 (0.00)	0.14 (0.14)	0.36 (0.25)	0.50 (0.38)
Valeric acid	2.70 (0.3)	2.60 (0.3)	2.70 (0.2)	2.50 (0.2)	2.60 (0.3)	2.50 (0.3)
Caproic acid	0.06 (0.06)	0.31 (0.20)	0.17 (0.12)	0.27 (0.16)	0.03 (0.03)	0.22 (0.09)

<sup>1</sup>Significantly different from previous time point;  $P = 0.05$ . Data are expressed as mean  $\pm$  SE.

respiratory allergies. This may be due to strain and host specific characteristics. However, a recent study with a combination of *Lactobacillus rhamnosus* (*L. rhamnosus*) GG and *B. lactis* Bb-12, achieved for the first time, a reduction in risk of both eczema and later sensitization, but only in a high-risk population<sup>[21]</sup>. Nevertheless, we need to acknowledge that a more profound understanding of the complex nature of atopy and atopic disease is needed, as it is likely that there are distinct etiological factors and pathogenetic mechanisms underlying the heterogeneous manifestations of the disorder. Thus, one mode of prevention or treatment may not suffice to target the plethora of allergic disease.

In the present study, we observed in the probiotic group a reduction of nasal eosinophil infiltration, an objective marker of allergic rhinitis. The degree of eosinophil infiltration of the respiratory mucosa is known to directly correlate with the intensity of the disease<sup>[22]</sup>. Indeed, trends for less runny nose during the peak of the pollen season and reduced nasal blocking towards the end of the season were observed in the current study. Earlier trials with probiotics and pollen allergy have shown that *L. rhamnosus* GG was not effective in relieving birch pollen allergy symptoms<sup>[23]</sup>. Likewise, *Lactobacillus casei* Shirota, was not found to be effective in reducing symptoms of Japanese cedar pollen allergy<sup>[24]</sup>, although the strain did reduce serum concentrations of IL-5, IL-6, interferon- $\gamma$  and specific IgE in subjects with allergic rhinitis<sup>[25]</sup>. A recent study showed, however, that *B. longum* BB536 was able to relieve eye symptoms in subjects suffering from Japanese cedar pollen allergy<sup>[26,27]</sup>, likewise *L. rhamnosus* GG and *L. gasseri* TMC0356 reduced nasal symptoms of Japanese cedar pollen allergy<sup>[28]</sup>.

Interestingly, our results indicate that gut microbiota are involved in regulating the inflammatory processes also in airway allergies. The fecal levels of bifidobacteria, clostridia and *Bacteroides* were reduced at the peak of the birch pollen season. As the change occurred in both treatment groups, it is not likely to be a consequence of the intervention, but may rather relate to the birch pollen season or possibly, but unlikely, to the concomitant antihistamine medication. Changes in fecal microbiota composition, a decrease in *Bacteroides fragilis* levels, in response to cedar pollen challenge have previously been

reported<sup>[29]</sup>. The underlying reasons for the temporary reduction of the numbers of the major phyla of the microbiota can only be speculated. Despite the change in the composition of the fecal microbiota, only a small change in 2-methylbutyric acid concentrations was observed, and no other changes in the determined intestinal microbial metabolites were detected.

The probiotic strains used in the current study were selected on the basis that they had either anti-inflammatory properties or could be expected to have a Th1 response-promoting effect<sup>[11,12]</sup>. Their ratio was chosen to optimize these 2 effects. The probiotic intake was reflected in temporary colonization of the gut by the study probiotics and in their enhanced numbers in fecal samples from the study children. This indicates that the study subjects had good compliance to the treatment and the study procedure. Furthermore, fecal *L. acidophilus* NCFM™ numbers correlated positively with fecal acetic acid concentrations ( $R = 0.612$ ,  $P = 0.0116$ ), fecal propionic acid concentrations ( $R = 0.449$ ,  $P = 0.0642$ ) and fecal butyric acid concentrations ( $R = 0.519$ ,  $P = 0.0323$ ). These observations may suggest that the presence of, in particular, *L. acidophilus* NCFM™ increases microbial fermentation in the colon. However, the consumption of the probiotic strains did not prevent changes in the colonization pattern when compared to the placebo group.

The study population was chosen to cover subjects with confirmed birch pollen allergy. The subjects selected suffered from significant symptoms of birch pollen allergy; this may have exceeded the potential effect of the intervention. Furthermore, all subjects were allowed to use oral antihistamines prophylactically. Also this has undoubtedly reduced the possibility of observing a treatment effect. Notwithstanding these limitations, positive influences could be observed on established clinical markers of pollen allergy, particularly in mucosal responses, where the increases in nasal eosinophil infiltration and increases in fecal IgA were prevented. Inhibition of increased Th2 type reactivity in the gut during the birch pollen season in the probiotic group may indicate that the shift to Th2 responses which has been associated with the development of allergy can be relieved by supplementation with this particular probiotic combination.

In conclusion, our study showed that consumption of a combination of *L. acidophilus* NCFM™ and *B. lactis* Bl-04 could positively influence markers of respiratory allergy, especially in the mucosae, and also resulted in a tendency for a reduction in reported nasal symptoms.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Pollen allergies are increasing in affluent societies. This is thought to be related to a reduced microbial exposure. Probiotics could provide a safe form of microbial exposure possibly through modulation of the intestinal microbiota.

### Research frontiers

Studies have shown that selected probiotics may modulate the immune response and contribute to the relief or primary prevention of atopic dermatitis. Few studies have, however, investigated the potential of probiotics on pollen allergies. The present study indicates a potential for the specific combination of *Lactobacillus acidophilus* (*L. acidophilus*) NCFM and *Bifidobacterium lactis* (*B. lactis*) Bl-04 in the alleviation of birch pollen allergy in children.

### Innovations and breakthroughs

The present randomized, placebo-controlled, double-blind study showed that the intestinal microbiota changes during the birch pollen season, and the combination of *L. acidophilus* NCFM and *B. lactis* Bl-04 was not able to counteract this change. Despite the prophylactic use of antihistamines, the probiotic combination did reduce an objective marker of pollen allergy (nasal eosinophilia) and indicated a trend for a reduction in subjective markers, nasal blocking and runny nose.

### Applications

Probiotics may provide an alternative or complementary treatment for pollen allergies. A future study could investigate whether this would lead to a reduced use of antihistamines.

### Terminology

Probiotics are live microorganisms which, when administered in adequate amounts confer a health benefit on the host. Specific strains of probiotics have been observed to modulate the immune system and the composition and activity of the intestinal microbiota.

### Peer review

The study addresses an interesting and relevant issue on which data is scarce, and the findings may contribute to our understanding of the role of probiotics in allergic rhinitis.

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BRIEF ARTICLES

## Color Doppler sonography and angioscintigraphy in hepatic Hodgkin's lymphoma

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was observed. Lesions were mostly hypoechoic and mixed, solitary or multiple. Some of the patients presented with dilated splenic veins and hepatofugal blood flow. A pulse wave was registered in the centre and at the margins of lymphoma. The average velocity of the pulse wave was higher at the margins ( $P > 0.05$ ). A continuous venous wave was found only at the margins of lymphoma. There was no linear correlation between lymphoma size and velocity of pulse and continuous wave ( $r = 390$ ,  $P < 0.01$ ). HPI was significantly lower in patients with lymphomas than in controls ( $P < 0.05$ ), pointing out increased arterial perfusion in comparison to portal perfusion.

**CONCLUSION:** Color Doppler ultrasonography is a sensitive method for the detection of neovascularization in Hodgkin's hepatic lymphoma and estimation of its intensity. Hepatic radionuclide angiography can additionally help in the assessment of vascularisation of liver lesions.

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**Key words:** Color Doppler ultrasonography; Hodgkin's disease; Lymphoma; Liver; Radionuclide angiography; Velocity; Hepatic artery; Portal vein; Blood flow; Splenomegaly

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### Abstract

**AIM:** To estimate the characteristics of Color Doppler findings and the results of hepatic radionuclide angiography (HRA) in secondary Hodgkin's hepatic lymphoma.

**METHODS:** The research included patients with a diagnosis of Hodgkin's lymphoma with metastatic focal lesions in the liver and controls. Morphologic characteristics of focal liver lesions and hemodynamic parameters were examined by pulsed and Color Doppler in the portal, hepatic and splenic veins were examined. Hepatic perfusion index (HPI) estimated by HRA was calculated.

**RESULTS:** In the majority of patients, hepatomegaly

### INTRODUCTION

The first choice technique for the investigation of

liver lesions, including Hodgkin's hepatic lymphoma, is ultrasound<sup>[1]</sup>. The diagnosis of this type of lymphoma can be made by non-specific sonographic signs, such as hepatomegaly, irregularity of liver margins, number and appearance of the lesions, echogenicity, enlarged retroperitoneal lymph nodes<sup>[2]</sup>, presence of pleural effusion or ascites, etc. Biopsy of focal lesions of the liver controlled by ultrasound in patients with malignant lymphomas is a reliable technique which can replace lymphography and other imaging procedures such as CT and nuclear magnetic resonance. Thrombosis of the portal and splenic vein has been described as a real rare complication of secondary hepatic lymphoma. The use of Color Doppler (CD) in the investigation of vascularisation of focal lesions in the liver brings about new possibilities in differential diagnostics of focal hepatic lesions<sup>[2]</sup>.

In the pre-evaluation and diagnosis of patients with liver tumors, after ultrasonography and Doppler-US and contrast enhanced ultrasonography (CEUS), significant nuclear medicine methods are used: radiocolloids, blood pool, hepatobiliary scintigraphy, angioscintigraphy with radiolabeled microspheres and "first pass" radionuclide angiography<sup>[3-6]</sup>. These methods precede selective angiography and other invasive methods. CT and nuclear magnetic resonance, although used mainly for the morphological examination of the liver, can be also performed in hepatic blood flow studies<sup>[7,8]</sup>. Recently, positron emission tomography has also been included, in combination with other imaging modalities. Nuclear medicine methods are basically founded on the analysis of hepatic radionuclide angiography (HRA), obtained after the "first pass" of the radioactive bolus and registered activity over the liver and other abdominal organs after intravenous injection of <sup>99m</sup>Tc. As well as methods based on the assessment of total liver blood flow and assessment of shunts the technique of radionuclide angiography and determination of the hepatic perfusion index (HPI) proposed by Sarper<sup>[9]</sup> is a noninvasive method providing additional information on portal blood flow.

The aim of our examination within this study was the evaluation of characteristics of Color Doppler findings and the results of HRA in secondary Hodgkin's hepatic lymphoma. Another aim was the estimation of reliability of CD ultrasound (US) in respect of neovascularization and level of intensity of vascularisation of the focal liver change.

## MATERIALS AND METHODS

The study included 25 patients with a diagnosis of Hodgkin's lymphoma who had secondary focal lesions in the liver and 30 controls. Controls were patients with different complaints, examined in the Clinical Center of Serbia, who underwent, among other investigations, nuclear medicine examinations (radionuclide ventriculography or another study which required iv injection of <sup>99m</sup>Tc pertechnetate) and abdominal and cardiology US. All the results, including laboratory analysis were physiological, and showed that they did not

have any disease or disorders. All controls were informed that their results would be included in the study.

Focal hepatic lesions had histopathological findings that correlated with our ultrasonographic studies. The ATL Ultramark9 system, and a convex probe with pulsed Doppler and CD of 2.5 MHz (WF50 Hz) were used for ultrasonographic examination. Hemodynamic parameters examined by pulsed Doppler and CD in the portal, hepatic and splenic vein were: diameter, flow, respiratory dependence of flow and collaterals. Examination of vascularisation of the focal hepatic lesion involved detection and measurement of blood flow in the centre of the focal lesion, as well as at its margin and periphery. Counting and recording for each specific focal change was performed by detection of the CD signal at a certain depth of scanning per cm<sup>2</sup>. The CD signal was created by reflection of an ultrasonic beam from the small blood vessels. Examination was repeated several times for every focal lesion, since the number of CD signals varied while breathing or changing the angle of the ultrasonic beam. An average of 5 planes of scanning were used for every hepatic change. Flow at the margins and in the center of every focal lesion was measured by combination of Color and pulsed Doppler. The results were statistically analyzed using Wilcoxon's, Kruskal-Wallis and  $\chi^2$  tests.

HRA was performed in patients with Hodgkin's hepatic lymphoma and controls after bolus injection of 740 MBq <sup>99m</sup>Tc-pertechnetate, (1 min, 1f/s), using a ROTA scintillation camera and MicroDelta computer. The arterial-hepatic and portal-venous phase of HRA were separated at the moment when maximal activity over the left kidney region of interest (ROI) was registered. The HPI was calculated according to Sarper's method<sup>[9]</sup>. Thus, the HPI reflects the value of the relative portal contribution to the liver blood flow. As well as the mean values (mean) and standard deviation (SD), statistical analyses included *t* tests, *U* tests, one and two way analysis of variance and a multiple range test.

The final diagnoses were based on the clinical findings, results of the functional and laboratory analysis, US and Doppler US, angiography, biopsy with histopathology and other clinical examinations. Consent was obtained from each patient, and the study protocol conformed to the established ethical guidelines. Written consent was obtained from all patients, according to the regulations of the School of Medicine Ethical Committee.

## RESULTS

Hepatomegaly existed in 23 patients (91%). Hyperechoic appearance of node was present in 11% of cases, hypoechoic in 55% and heterogeneous mixed echoic structure in 34%. Solitary tumor nodes were found in the liver in 19 patients (48%) and multiple nodes existed in 16 patients (52%). The smallest tumor node was 1.5 cm, and the biggest was 14 cm. There was a diffuse infiltration of the liver in 2 patients. Solitary tumor knots were most frequently localized in the right lobe (36%),

**Table 1** The values of hemodynamic parameters (Doppler-US)

Hemodynamic parameters	Statistical parameters					
	Unit	n	Mean	SD	CV	Med
Patients with secondary Hodgkin's hepatic lymphoma						
Diameter portal vein	cm	25	1.7	0.4	26	1.7
Velocity portal vein	cm/s	25	18.0	22.0	18	13.0
Volume of blood flow portal vein	mL/min	25	859.0	574.0	69	643.0
Velocity hepatic artery	cm/s	25	40.0	16.0	38	40.0
Control group						
Diameter portal vein	cm	30	1.0	0.1	10	1.0
Velocity portal vein	cm/s	30	17.0	2.0	13	17.0
Volume of blood flow portal vein	mL/min	30	781.0	164.0	23	763.0
Velocity hepatic artery	cm/s	30	38.0	5.0	14	36.0

US: Ultrasound; CV: Coefficient of variation; Med: Median.

between right and left lobe (53%) and in the left lobe of liver (11%). The position at the right liver lobe was frequently followed by irregular margins of the liver and pleural effusion at the right side. Ascites was present in 4 patients (16%). Retroperitoneal lymph nodes were found in 16 patients (64%). Splenomegaly was detected in 16 (64%). The spleen consisted of heterogeneous echoes with bigger or smaller knots in 10 patients (40%).

Dilated splenic veins were present in 40% of cases and hepatofugal blood flow in 32%. Hepatofugal flow in the splenic vein was observed in 16% of the patients, while the others had normal hepatopetal flow. A spontaneous splenorenal shunt and collateral blood flow was detected in 12% of patients with mass splenomegaly. A hepatofugal direction of flow existed in the portal vein in 50% of patients. Hemodynamic parameters in patients with lymphomas and controls are presented in Table 1. Thus, the diameter of the portal vein was significantly ( $P < 0.05$ ) bigger than standard values and ranged from 1.1-2.4 cm. However, the average flow velocity in the portal vein was not significantly different ( $P > 0.05$ ) from that in the controls. The volume of blood flow through the portal vein was 600-1200 mL/min which was not significantly different from normal values ( $P > 0.05$ ). Flow velocity in the hepatic artery was 15-55 cm/s, which was significantly different from the values in the control group ( $P < 0.05$ ).

In 3 patients without nodules in the liver visible on US examination, slight perfusion disorders were detected by Doppler-US. The volume of portal blood flow was slightly increased (1000-1200 mL/min), without any impact on the statistical calculation.

Two types of spectral curved lines were found: pulse-arterial wave and continuous venous wave, and velocities are presented in Table 2. A pulse wave was registered in the centre and at the margins of lymphoma and its velocity ranged from 80-140 cm/s. The average velocity of the pulse wave at the margins was a little higher ( $P > 0.05$ ) than in the center of the lesion. A continuous venous wave was found only at the margins of the lymphoma and its maximum velocity was 35 cm/s. There was no linear correlation between lymphoma size

**Table 2** Average velocities of the pulse and continuous waves in the center and at the margins of lymphoma

Wave	Tumor center			Tumor margin		
	Mean	SD	Med	Mean	SD	Med
Pulse (cm/s)	80	23	84	84	25	88
Continuous wave (cm/s)				25	9	24

**Table 3** The values of hemodynamic parameters in the control group and in the patients with lymphomas

Hemodynamic parameter	Statistical parameters				
	n	Mean	SD	CV	Med
Controls	30	0.66	0.07	8	0.66
Lymphoma patients	25	0.27	0.22	73	0.35

and velocity of pulse and continuous waves ( $r = 390$ ,  $P < 0.01$ ).

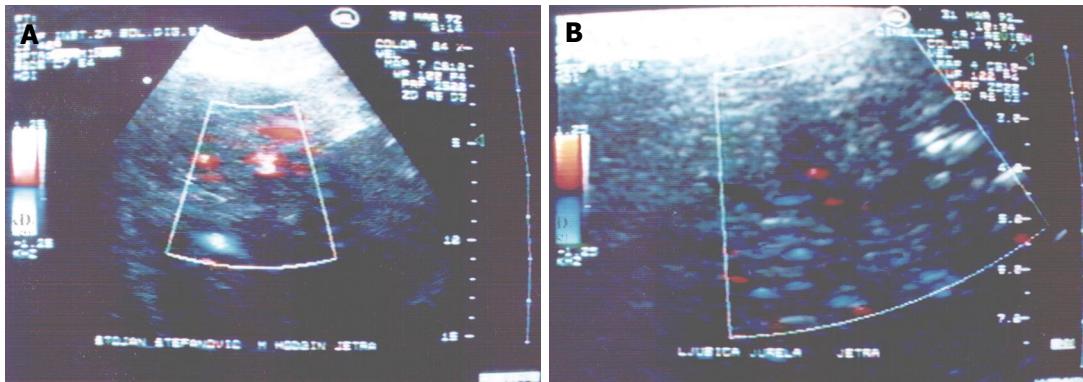
The CD signal could not be found within the parts of the focal lesion where there was no flow in small blood vessels or where the flow could not be detected by the CD system. The CD signal in hypoechoic lesions varied from 0.5-0.9 of blood vessels/cm<sup>2</sup>. Maximum values of the number of CD signals were noticed in lesions 3-5 cm in size and they ranged from 1-3 blood vessels/cm<sup>2</sup> of area in hypoechoic zones. In the case of hyperechoic lesions the result was 0.5-0.6 vessels/cm<sup>2</sup>. Comparing number of CD signals *in vivo* to number of blood vessels in histological tissue, we could not find any relevant differences between these two methods ( $P > 0.05$ ) (Figure 1).

Results of HRA in patients with secondary Hodgkin's hepatic lymphoma (Table 3), showed that HPI was significantly lower in patients with lymphomas than in controls ( $P < 0.05$ ). Thus, arterial hepatic perfusion was increased in patients with secondary hepatic lymphomas, while the venous component of hepatic perfusion was decreased.

## DISCUSSION

Most of the patients with Hodgkin's hepatic lymphoma had hepatomegaly, mainly with hypoechoic or heterogenous mixed echoic structures, while in a small number of patients it was hyperechoic. The lesions were solitary or multiple, of various sizes. Solitary tumor knots were most frequently localized between the lobes or in the right lobe and the rarest lesions were in the left lobe. Some patients had irregular liver margins with pleural effusion at the right side, while some had ascites. Most of them had enlarged retroperitoneal lymph nodes.

Our results regarding the morphology of lesions, are in accordance with the literature data<sup>[1,2]</sup>, where the non-specific sonographic sign in the majority of secondary hepatic lymphomas is hepatomegaly with irregular, cogged like liver margins. According to our results, secondary hepatic lymphomas appear in solitary or multinodular shapes or as diffuse hepatic infiltration. A rarer echoic structure is the shape of the target lesion, or



**Figure 1** Color doppler ultrasonography. A: Intense-central neovascularization in the focal liver lesion; B: Intense peripheral neovascularization in the focal liver lesion.

mixed echoic structure<sup>[2]</sup>. The appearance of secondary hepatic lymphoma is associated with enlarged spleen, or with retroperitoneal lymphadenopathy or pleural effusion in a great number of cases<sup>[1]</sup>. Ascites is also very often a finding in patients with secondary hepatic lymphoma<sup>[1]</sup>.

Splenomegaly was detected in half of the patients. Splenic lesions were heterogeneous with bigger or smaller knots in nearly half of the patients. Because of the higher vascular pressure, dilated splenic veins were often present with hepatofugal blood flow in a third of the patients. In some patients, blood flow was in 2 directions. A spontaneous splenorenal shunt and collateral blood flow could be detected in patients with mass splenomegaly. In patients with liver Hodgkin's lymphomas, changes in the diameter of the splenic vein were due to changes in resistance in the portal vascular system, hilar adenopathy and splenomegaly.

Changes in venous vessels of the portal system in patients with liver Hodgkin's lymphomas included dilated portal veins ( $P < 0.05$ ) and changes in flow direction (hepatofugal in 50%) ( $P < 0.05$ ). However, no significant changes in volume of blood flow or blood flow velocity through the portal vein ( $P > 0.05$ ) were observed. However, significant differences in blood flow velocity through the hepatic artery were seen ( $P < 0.05$ ). In 3 patients without nodules in the liver visible on US examination, slight perfusion disorders were detected by Doppler-US. The volume of portal blood flow was slightly increased because of the increased inflow through the portal vein due to splenomegaly. This fact could enable early further investigation and detection of lymphomas in the liver, before the obvious changes in liver tissue occur. These values were not statistically significant.

A pulse wave was registered in the center and a little higher ( $P > 0.05$ ) at the margins of lymphoma. A continuous venous wave was found at the margins of lymphoma. There was no linear correlation between lymphoma size and velocity of pulse and continuous waves ( $r = 390$ ,  $P < 0.01$ ). Our results show intense neovascularization of the focal lesions in the center, as well as on the periphery, which differentiate those tumors from other secondary deposits. The highest pulse wave velocities correspond to arteriovenous shunts.

The CD signal could not be found within the parts

of focal lesions with no flow in small blood vessels or where flow could not be detected by the current CD system. The CD signal in hypoechoic lesions varied from 0.5-0.9 of blood vessels/cm<sup>2</sup>. Estimation of CD signals *in vivo* in these tumors corresponds to the number of blood vessels in histological tissue. HPI values were significantly lower in patients with lymphomas than in controls ( $P < 0.05$ ). Thus, arterial hepatic perfusion was increased in patients with secondary hepatic lymphomas, while the venous component of hepatic perfusion was decreased which is usual for malignant lesions.

Various literature results prove the value in Doppler-US in the estimation of characteristics of liver tumors that could help in the early diagnosis, differential diagnosis and appropriate choice of therapy. Although there is much literature data about Doppler-US characteristics and possibilities of differential diagnosis of malignant and benign liver tumors, not many of them deal with hepatic metastases of Hodgkin's lymphoma. According to Tcelepi *et al*<sup>[10]</sup>, detecting and characterizing focal liver lesions is one of the most difficult challenges in imaging emphasizing the main strengths being in its ability to definitively characterize common benign lesions (cysts and hemangiomas), safety, low cost, and its ability to guide biopsy. Disadvantages include its inability to image the entire liver in many patients and its inferiority to CT as a means of detecting extrahepatic malignant disease. Sonography is less sensitive than CT or MRI in detecting focal lesions. However, US contrast agents can improve liver lesion detection and characterization. Tscelepi indicated that intraoperative US is the most sensitive imaging modality in detecting focal liver lesions. However, some authors obtained better results with CEUS. Thus, Ernst *et al*<sup>[11]</sup> with a new US contrast agent showed that metastatic liver lesions had previously undetected blood flow in the rim of the tumor; hepatocellular carcinoma displayed enhanced signal intensity in the vessels of the rim and in the center of the tumor, while adenoma and focal nodular hyperplasia showed signal enhancement in the central area of the tumor. No signal enhancement was observed in hemangiomas, a focal fatty lesion, or in a carcinoid metastatic lesion. Thus, enhanced CD flow study may aid in the detection of flow signals or sonographic differentiation of hepatic tumors. Also, some authors, after the study of patients with malignant

diseases (hepatocellular carcinoma, cholangiocellular carcinoma, metastasis and lymphoma) and those with benign lesions, with color stimulated acoustic emission in the late phase of Levovist enhancement showed a high specificity and sensitivity for differentiation between benign and malignant focal liver lesions<sup>[12]</sup>.

Results of HPI show that arterial hepatic perfusion in Hodgkin's lymphoma is dominant and that venous-portal inflow is reduced in comparison with standard values. Although there are no precise data from the literature concerning metastatic lymphoma in the liver, the results of other authors regarding the flow in malignant lesions in general are in accordance with our findings. Thus, according to Petrović *et al*<sup>[13]</sup> HPI is a sensitive indicator of the presence of malignant liver tumors, but is within normal range in patients with hepatic hemangioma. The results of Dragoteanu *et al*<sup>[14]</sup> showed that malignant tumors (primitive or metastases) increase the arterial supply of the liver and decrease the portal flow. However, benign tumors do not change the portal/arterial liver blood flow ratio. However, recently, according to Robinson<sup>[15]</sup> the sensitivity of US, CT and magnetic resonance techniques for detecting liver metastases assessed in comparison with surgical inspection, intraoperative US and pathological examination, are of uncertain accuracy in detecting very small lesions. With current imaging technology, even with optimum imaging, it is possible to detect only about one-half of metastatic nodules < 1 cm in patients undergoing liver resection and pathological correlation. Robinson emphasized that micrometastases produce alterations in blood flow that may be recognized by radionuclide or Doppler perfusion methods in the very early phase.

Currently, use of CT and MRI to detect hepatic lymphoma as well as its perfusion has been employed. Generally speaking, CT reveals Hodgkin's lymphoma as homogeneously hypodense clearly delineated nodules. After contrast application the lesions appear hypodense, although a weak enhancement may be detected. However, in the case of the infiltrative form, a diffusely decreased attenuation may be seen, without the possibility of distinguishing it from fatty infiltration. MRI reveals focal hepatic lymphomas are homogeneously hypointense in comparison to the normal liver parenchyma on unenhanced T1-weighted images. However, dynamic imaging after gadolinium contrast application reveals hypointensity on arterial phase images followed by homogeneous delayed enhancement on portal venous phase imaging. However, it is still difficult to differentiate lymphoma, metastases and hepatocellular carcinoma.

Thus, according to Liu *et al*<sup>[16]</sup>, a plain CT scan identified hypodense lesions which did not display marked enhancement on arterial phase and portal venous phase scans. On delayed phase scan, the border of the lesions became clear, and slight enhancement was observed in the periphery and some partitions of the lesions. According to Vinnicombe *et al*<sup>[17]</sup>, CT accurately depicts nodal enlargement above and below the

diaphragm, has variable sensitivity for intra-abdominal visceral involvement and is generally outstanding in depicting the extent of disease, especially extranodal extension. Despite the advances in CT technology, there are still areas where CT performs less well, even when using an intravenous contrast medium. The results of Earl *et al*<sup>[18]</sup> show that although CT scan could occasionally demonstrate disease in nodes, its value was limited by its inability to detect involvement of nodes which were not significantly enlarged. However, CT scan would appear to be the investigation of choice in patients with suspected abdominal relapse because of the more frequent presence of disease in sites not seen on lymphography. Gossman *et al*<sup>[19]</sup> emphasized that fast MRI has considerably reduced imaging time, and it is now considered to be as diagnostic as CT for staging Hodgkin's disease. The excellent soft-tissue contrast and the lack of exposure to ionizing radiation are the main advantages of MRI imaging, especially with the application of newly developed lymphotropic contrast agents. In addition, Hoane *et al*<sup>[20]</sup> concluded that MRI and CT may be equivalent imaging modalities in the detection of nodal involvement, and that MRI has an advantage in its ability to diagnose marrow involvement. According to MRI findings in primary lymphoma of the liver, on T1-weighted imaging the tumor was isointense in one and homogeneously hypointense to the liver parenchyma in the other. On T2-weighted imaging both tumors were homogeneously hyperintense. In one case the margin of the tumor was poorly defined, and portal branches were identified within the tumor, an unusual finding in other liver neoplasms. Additionally, according to Hori *et al*<sup>[21]</sup> it is possible to investigate the morphological, hemodynamical and functional nature of focal hepatic lesions and correctly establish a diagnosis of liver tumors based on those findings using dynamic CT and MRI with extracellular contrast material or MRI with liver-specific contrast material.

Some recent papers employ positron emission tomography for the assessment of tumor perfusion. Growth of malignant tumors is dependent on sufficient blood supply, an enhanced microvessel density is seen as part of these reactions and this is associated with increased perfusion as measured by PET<sup>[22]</sup>. Some authors used PET and CT in the investigation of liver tumor blood flow and metabolism<sup>[23]</sup>.

In comparison to other metastatic liver tumors, Hodgkin's lymphoma has increased vascularization which we have seen only in primary hepatocellular carcinoma, which can help in differentiating it from other metastatic tumors. The significant impact on assessment of microvascularization can be obtained using contrast enhanced US. The absence of splenomegaly and retroperitoneal lymph nodes can further help in differentiation of hepatocellular carcinoma from lymphoma. In that case, further investigation is needed. Firstly, laboratory analysis can be employed (tumor marker levels, the absence of hepatotropic viruses B and C), as well as estimation of the age group, and anamnestic data (alcohol abuse and former cirrhosis).

For an exact diagnosis, radionuclide methods, as well as MDCT and MRI, can be also used.

Liver metastases of different tumors have different flow patterns. According to our results, a special characteristic of Hodgkin's disease is the demonstration of continuous venous flow at the periphery and pulsed arterial flow both at the margins and at the center of the lesion. Increased vascularization has also been obtained in metastases of carcinomas of the kidneys and suprarenal gland. In the center of the lesion, pulse waves did not register at all, while those registered at the good vascularized tumor margin had slightly lower velocities (68-78 cm/s) in comparison to Hodgkin's lymphoma. In metastases of colorectal adenocarcinomas, there was no pulse wave registered in the center, while it was registered at the margin in only 50% of patients, with obviously decreased velocities in comparison to Hodgkin's lymphoma (27 cm/s). A continuous wave registered on the edges in all the patients, which showed decreased velocities (19 cm/s). Metastases of pancreatic and gallbladder adenocarcinomas showed pulse wave velocity on the tumor margin was slightly decreased in comparison to Hodgkin's lymphoma (65-75 cm/s), while continuous wave velocity in the tumor margins registered in all patients had a similar velocity (28-40 cm/s) to that in Hodgkin's lymphoma<sup>[24]</sup>. Another type of lymphoma (secondary non-Hodgkin's) shows a similar echo type, but different vascular characteristics. Arteriovenous shunts in this type of lymphoma are localized on the edges of the tumor, and thus, the pulse waves registered in this area with CD showed lower values (50-60 cm/s). No continuous wave on the periphery was registered in any patient with non-Hodgkin's lymphoma, and when registered, velocities were also lower (10-26 cm/s)<sup>[24]</sup>.

However, in order to perform prompt and adequate therapy, diagnosis and monitoring of malignant liver disease should be improved. According to our results, as well as the above mentioned data of other authors, contemporary diagnostic imaging methods mainly provide morphological data about the tissue while perfusion imaging of the liver can improve this limitation. According to some authors<sup>[7]</sup> liver flow scintigraphy and flow quantification at Doppler US have focused on characterization of global abnormalities. CT and MRI imaging can provide regional and global parameters. However, some shortcomings of these methods need to be overcome (by reduction of radiation doses associated with CT perfusion imaging, improvement of spatial and temporal resolution at MR imaging, accurate quantification of tissue contrast material at MR imaging, and validation of parameters obtained from fitting enhancement curves to biokinetic models, applicable to all perfusion methods).

In conclusion, CD US is a useful method for detection of vascularization in Hodgkin's hepatic lymphoma and estimation of its intensity. Thus, it could help in the early diagnosis and differential diagnosis of malignant liver lesions, including hepatic Hodgkin's lymphoma. In comparison to other metastatic liver tumors, Hodgkin's lymphoma has

increased vascularization which we have seen only in primary hepatocellular carcinoma, which can help in differentiating it from other metastatic tumors. A significant impact on assessment of microvascularization can be obtained using contrast enhanced US. The presence of splenomegaly and retroperitoneal lymph nodes can further help in differential diagnosis. However, in most cases, further investigation (such as laboratory analysis, estimation of the age group and anamnestic data) is needed. For a more reliable diagnosis, radionuclide methods, MDCT and MRI can be also used.

## COMMENTS

### Background

The aim of the paper was to estimate the characteristics of Color Doppler findings and the results of hepatic radionuclide angiography (HRA) in secondary Hodgkin's hepatic lymphoma, in order to try to distinguish these entities from other hepatic tumors without using more advanced diagnostic methods.

### Research frontiers

Possibility of distinguishing liver involvement by Hodgkin's hepatic lymphoma by estimation of the morphology of the liver by US, as well as estimation of typical flow patterns (pulse and continuous flow) at the center and margins of the lymphoma.

### Innovations and breakthroughs

"It seems that a peculiar characteristic of Hodgkin's disease is the demonstration of continuous venous flow at the periphery and pulsed arterial flow both at the margins and at the center of the lesions. This is interesting and merits further investigation (reviewer's opinion)".

### Applications

By carefully examining the liver, one can, using easily available methods, differentiate benign from malignant lesions, as well as suspect Hodgkin's hepatic lymphoma.

### Peer review

This paper evaluated of characteristics of Color Doppler finding and the results of radionuclide angiography in secondary Hodgkin's hepatic lymphoma. This is interesting and merits further investigation.

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BRIEF ARTICLES

## Impact of fecal incontinence on quality of life

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### Abstract

**AIM:** To explore the impact of fecal incontinence (FI) on quality of life (QOL) of patients attending urogynecology and colorectal clinics (CCs).

**METHODS:** Cross-sectional study of 154 patients (27 male) with FI, who attended the clinics at a regional hospital in North Queensland, Australia in 2003 and 2004, and completed the Fecal Incontinence Quality of Life Scale (FIQL: 1 = very affected; 4 = not affected).

**RESULTS:** More than 22% of patients had their QOL affected severely by FI. Patients reported that they had not previously been asked about FI by a medical practitioner nor did they voluntarily disclose its presence. The median FIQL scores for all participants were: lifestyle = 3.24; coping = 2.23; depression = 2.42; and embarrassment = 2.33. Increasing frequency of soiling had a negative effect on all four FIQL scales ( $P < 0.001$ ) as did the quantity of soiling ( $P < 0.01$ ). Female CC patients had poorer FIQL scores than urogynecology clinic patients for lifestyle ( $P = 0.015$ ), coping ( $P = 0.004$ ) and embarrassment ( $P = 0.009$ ), but not depression ( $P = 0.062$ ), despite having experienced FI for a shorter period.

**CONCLUSION:** Failure to seek treatment for FI degrades the quality of patients' lives over time. FI assessment tools should incorporate the quantity of fecal loss.

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**Key words:** Quality of life; Fecal incontinence; Rural health; Colorectal cancer; Urogynecology

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### INTRODUCTION

Fecal incontinence (FI) is the involuntary discharge of liquid or solid stools. FI severity has been described as a combination of the frequency and type of stools, the severity of urgency, and frequency of pad usage<sup>[1,2]</sup>. This problem affects both men and women, irrespective of social, employment or financial status<sup>[3,4]</sup>. The prevalence of FI increases with age<sup>[5,6]</sup> and Australian studies have reported some of the highest rates in the world<sup>[5,7,8]</sup>.

People with mild fecal loss such as staining are unwilling to admit to themselves that they have an FI problem<sup>[9]</sup>. As the problem worsens and patients inevitably accept that they have FI, they are reluctant to disclose the problem to others<sup>[9-15]</sup>, with only 5%-27% seeking help from their doctors<sup>[15]</sup>. Lack of disclosure may be to the result of embarrassment, the erroneous belief that FI is a normal part of aging, or the perception that no treatment is available. Doctors may fail to comprehend patient hints about diarrhea and FI<sup>[15]</sup> or may be reluctant to ask about fecal leakage, perhaps because of their own embarrassment or the perception that FI is a trivial concern<sup>[16]</sup>.

FI can range from causing mild embarrassment to becoming an insidious burden on the person's quality of life (QOL)<sup>[17-21]</sup>. Tools used to measure the impact of FI on QOL have been under development for 20 years and include lifestyle components in summary scales, generic measures, disease-specific measures, utility measures, and more recently, direct questioning of objective measures<sup>[1]</sup>. The Fecal Incontinence Quality Of Life (FIQL) questionnaire, a disease-specific tool, was designed to

evaluate the impact of FI on four aspects of patients' QOL: lifestyle; coping behavior; depression or self perception; and level of embarrassment. Each aspect is described as a score measured on a scale between 1 and 4, where 1 is very affected and 4 is not affected<sup>[22]</sup>. Validity and reliability of the FIQL have been established and it has been recommended as a useful tool to assess FI<sup>[23]</sup>.

This study was designed to explore the impact of FI on the QOL of patients attending urogynecology and colorectal surgical outpatient clinics at a publicly funded regional hospital with a large rural catchment<sup>[24]</sup>.

## MATERIALS AND METHODS

### Participants

Participants were consecutive patients attending the colorectal clinic (CC) and urogynecology clinic (UC) at The Townsville Hospital, in North Queensland Australia, between January and June 2003 and August and November 2004. Ethical approval was obtained from the ethics committees of Townsville Hospital and James Cook University.

### Study procedure

The study procedure has been described previously<sup>[5,9]</sup>. Briefly, a cross-sectional survey was conducted among patients at the CC and UC. On arrival, all patients attending these clinics were invited to participate in the study. Exclusion criteria included children (under 18 years), pregnancy, terminal illness, mental illness, or gastrointestinal stoma. Eligible subjects completed a patient consent form. Participants were then given a self-administered questionnaire that contained questions about patient demographics, alcohol consumption, preexisting medical conditions, and prior surgical history; all known risk factors for FI. Patients who answered "yes" to the question "do you ever accidentally soil your clothes or underclothes with feces?" proceeded to questions relating to the frequency, severity and management of FI, and the 29 question FIQL survey tool<sup>[22]</sup>. The physicians of participating patients also surveyed them using the Cleveland Clinic Florida Fecal Incontinence Score<sup>[25]</sup>, which was compared with the self-administered survey tool results to investigate disclosure problems<sup>[9]</sup>. Here, we report information about the QOL questions from the self-administered questionnaire.

### Statistical analysis

Mean and SD were used to describe age. Medians and interquartile ranges were used as measures of central tendency and estimates for dispersion for duration of FI and FIQL scales. FIQL scales were calculated in accordance with the developers recommendations<sup>[26]</sup>. For bivariate testing of categorical variables, exact versions of  $\chi^2$  tests were conducted for nominal items, while exact trend tests were used for ordinal variables. Multiple linear regressions were used to investigate relationships between FIQL scales and clinics, basic severity (type  $\times$  frequency) and duration of FI among

female participants, and FIQL scales and components of severity<sup>[2]</sup>, with and without quantity of fecal loss, in all participants. Potential components were initially considered separately and were then combined. As FIQL scales were not distributed normally, they were transformed by taking the square root. As no major differences between transformed and untransformed regression results were found, the untransformed regression data have been presented. Results of linear regression analyses are presented by regression coefficients ( $\beta$ ) with 95% CIs and/or  $P$  values. A significance level of 0.05 was adopted *a priori*.

## RESULTS

The recruitment methodology and tools used were the same in 2003 and 2004. There were no differences in age ( $P = 0.603$ ), sex ( $P = 0.149$ ) or prevalence of FI ( $P = 0.076$ ) in participants between the two recruitment periods, thus the data were combined for analysis.

### Response rate

A total of 769 patients (451 in 2003 and 318 in 2004) were invited to participate. Of these, 423 women attended the UC and 146 men and 200 women attended the CC, with 93.4% ( $n = 718$ ) of the eligible patients completing the self-administered FI questionnaire. As 43 patients completed the survey more than once, the initial responses were used and duplicate data were removed from the combined database, which left a total of 675 unique entries.

### Prevalence

Of the 675 patients in the study 154 (22.8%; 95% CI: 19.6%-26.0%) reported having accidentally soiled their clothes or underclothes with feces and answered the FIQL questions. There were 27 men from the CC, and 127 women, 52 from the CC and 75 from the UC, with FI. The mean age (SD) of the participants with FI was 56.2 (14.3) years. There was no age difference between sexes ( $P = 0.281$ ) or clinics ( $P = 0.82$ ), or the women attending the two clinics ( $P = 0.87$ ).

### Etiology

Patients reported the following etiological risk factors for FI. There were 27 participants who reported having bowel disease (25 from CC, nine male), with 14 of them (all from CC, two male) having been diagnosed with colorectal cancer. Twenty seven women (eight from CC) had undergone vaginal repair surgery, whilst 51 women (16 from CC) reported difficult vaginal births using forceps, vacuum extraction or long second-stage labor. Twenty-one participants (11 from CC, two male) reported rectal prolapse; 23 (20 from CC, nine male) had undergone surgery for hemorrhoids, anal fissures or fistulas; 101 (39 from CC, 10 male) had urinary incontinence; 11 (six from CC, four male) had anal injuries; 18 (10 from CC, four male) had diabetes; six (four from CC, two male) reported spinal cord disease; three (one from CC, one male) had neurological disease; 39 (21

from CC, seven male) reported chronic constipation; and 17 women (five from CC) had psychiatric problems that required medication. Some patients had multiple factors.

### Duration

Patients attending the UC documented having FI for a significantly longer period (median: 24 mo, IQR: 12-60), than those attending the CC [12 (6-24),  $P = 0.001$ ]. Similarly, women in the UC clinic had FI for longer than those in the CC clinic [UC = 24 (12-60); CC = 12 (6-24),  $P = 0.002$ ].

### Frequency

More than half (55.8%) of the participants admitted to soiling at least once per month: 17% (25) of patients reported soiling daily, 24% (35) weekly, 15% (22) monthly, and 44% (65) less than once a month. There was no difference in the frequency of incontinent episodes between sexes ( $P = 0.678$ ). However, women who attended the CC reported more frequent leakage, with 27.5% soiling daily compared with 11.0% of those in the UC ( $P = 0.037$ ), possibly as a result of their bowel condition.

### Type

In the CC, significantly more men (79%) disclosed liquid bowel leakage than women (46%), whilst fewer men reported solid (8%) and combined solid/liquid (13%) leakage than women (solid = 18%, combined = 36%;  $P = 0.008$ ). There was no significant difference between women attending the CC or UC with regard to type of leakage (UC: liquid bowel leakage, 46%; solid bowel leakage, 28%; combined solid/liquid bowel leakage, 25%,  $P > 0.05$ ). More women attending the CC reported passive leakage (80%) than those attending the UC (62.5%,  $P = 0.037$ ) or men (48%,  $P = 0.026$ ). There were no differences between sexes or clinics for fecal urgency or quantity of fecal soiling.

### QOL impact

The median (IQR) results of the FIQL scales (range: 1-4; 4 = not affected) for all participants were: lifestyle = 3.24 (2.22-3.80); coping = 2.23 (1.60-3.00); depression = 2.42 (1.95-3.33); and embarrassment = 2.33 (1.67-3.58) (Table 1). Participants who attended the CC had significantly poorer scores than those who attended the UC for lifestyle ( $P = 0.005$ ), coping ( $P = 0.003$ ) and embarrassment ( $P = 0.024$ ) but not depression ( $P = 0.056$ ).

There were no significant differences in any of the FIQL scales between sexes when compared in total or within the CC (Figure 1). Women who attended the CC had lower scores on all scales compared with those who attended the UC (lifestyle:  $P = 0.015$ ; coping:  $P = 0.004$ ; depression:  $P = 0.062$ ; embarrassment:  $P = 0.009$ ; Figure 1). The four multiple linear regression analyses [FIQL = clinic + duration + basic FI severity (type  $\times$  frequency) + error] used to investigate the relationships between QOL of women by clinic, FI type/frequency and duration determined that the poorer QOL results in the CC with regard to clinic and type/frequency

**Table 1** FIQL of patients attending CCs and UCs in North Queensland

Scale <sup>3</sup>	n <sup>1</sup>	Missing	Mean (range) <sup>2</sup>	SD	Median (IQR)
Scale 1: lifestyle	119	35	2.99 (1.0-4.0)	0.899	3.24 (2.22-3.80)
Scale 2: coping	130	24	2.36 (1.0-4.0)	0.884	2.23 (1.60-3.00)
Scale 3: depression	128	26	2.57 (1.0-4.0)	0.806	2.42 (1.95-3.33)
Scale 4: embarrassment	124	30	2.53 (1.0-4.0)	0.990	2.33 (1.67-3.58)

FIQL, Rockwood *et al*<sup>[22]</sup>; <sup>1</sup>Patients who consented to participate in the 2003 and 2004 studies and answered yes to ever soiling with feces,  $n = 154$ ; <sup>2</sup>QOL scale of 1 represents very low functional status and 4 is not affected by FI; <sup>3</sup>Scales calculated as per letter to the editor, Rockwood<sup>[26]</sup>.

remained significant (all  $P < 0.05$ ), whilst duration of soiling remained significant for the lifestyle, depression and embarrassment scales (all  $P < 0.05$ ) but not for the coping scale ( $P = 0.103$ , Table 2).

Increasing frequency and quantity of soiling had a significant negative effect across all four QOL scales (frequency,  $P < 0.001$ ; quantity,  $P < 0.01$ ). QOL was poorer in participants with greater urgency and passive soiling, whilst those participants who documented both solid and liquid bowel leakage had poorer QOL than those with either alone. Not all scales reached significance (Table 3). When comparing QOL between female participants who attended the CC and UC, the scores for frequency, quantity, type and fecal urgency were significantly poorer across all FIQL scales for female participants in the CC ( $P < 0.05$ ). Women who reported passive FI in the CC had poorer QOL scores than those in the UC for lifestyle ( $P = 0.040$ ), coping ( $P = 0.020$ ), depression ( $P = 0.181$ ), and embarrassment ( $P = 0.019$ ).

The model (FIQL = frequency + type + urgency + pad use + error) used to investigate the relationships between the four FIQL scales and the components of severity showed that the significant components that affected QOL were frequency and fecal urgency with regard to lifestyle and coping (all  $P < 0.05$ ); and frequency and type with regard to depression and embarrassment (all  $P < 0.05$ ). As a result of the low rate of pad usage (36% wore pads), passive leakage (68% reported) was included in the model, and the quantity of fecal loss was also investigated. This amended model (Table 4) showed: daily, solid, and large losses of stool to be significant factors for lifestyle ( $P < 0.05$ ); monthly and daily leakage, urgency, pad wearing and large quantities of fecal loss to be significant factors for coping ( $P < 0.05$ ); frequent and major leakage of both solid and liquid stool were significant factors for depression ( $P < 0.05$ ); whilst frequent, solid and liquid, and passive stool loss significantly affected embarrassment ( $P < 0.05$ ).

## DISCUSSION

The major findings of this study were that more than 22% of patients who attended the UC and CC in 2003 and 2004 for matters other than FI had their QOL severely affected by FI, with the QOL of participants who

**Table 2** Multiple linear regression identifying relationship between FIQL scales and clinic, duration, FI severity<sup>1</sup> in females [regression coefficient (95% CI)]

	<b>Lifestyle</b>	<b>P</b>	<b>Coping</b>	<b>P</b>	<b>Depression</b>	<b>P</b>	<b>Embarrassment</b>	<b>P</b>
CC	-0.582 (-0.938, -0.225)	0.002	-0.499 (-0.835, -0.162)	0.004	-0.365 (-0.685, -0.045)	0.026	-0.568 (-0.923, -0.214)	0.002
Duration (mo)	-0.002 (-0.004, 0.000)	0.042	-0.002 (-0.004, 0.000)	0.103	-0.002 (-0.004, 0.000)	0.041	-0.003 (-0.005, 0.000)	0.020
Severity (1-8) <sup>1</sup>	-0.159 (-0.241, -0.076)	< 0.001	-0.176 (-0.255, -0.097)	< 0.001	-0.143 (-0.216, -0.069)	< 0.001	-0.207 (-0.290, -0.124)	< 0.001

<sup>1</sup>Fecal incontinence (FI) severity = soiling type × frequency, i.e. liquid (1), solid (1), both (2) × daily (4), weekly (3), monthly (2), less often (1). CC: Colorectal clinic.

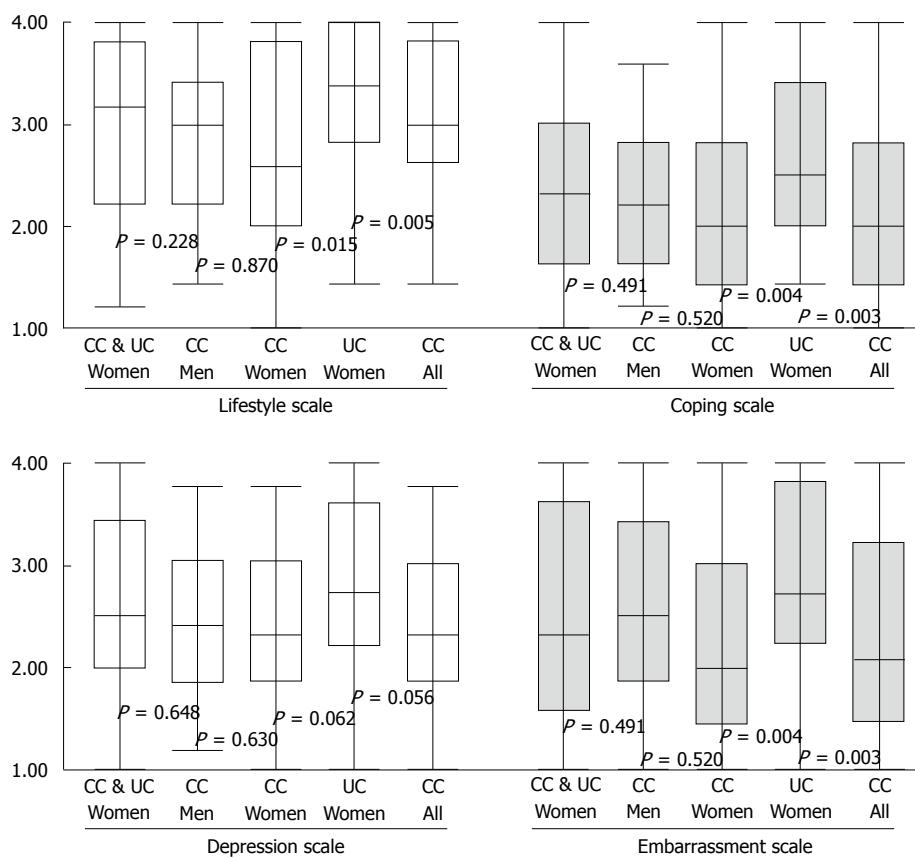
**Table 3** Association of descriptive FI with FIQL of patients attending CCs and UCs in North Queensland

<b>Categorical variables</b>	<b>Scale 1: lifestyle<sup>1</sup></b>			<b>Scale 2: coping<sup>1</sup></b>			<b>Scale 3: depression<sup>1</sup></b>			<b>Scale 4: embarrassment<sup>1</sup></b>		
	<b>n</b> (missing)	<b>Median</b> (IQR) <sup>2</sup>	<b>P value</b>	<b>n</b> (missing)	<b>Median</b> (IQR) <sup>2</sup>	<b>P value</b>	<b>n</b> (missing)	<b>Median</b> (IQR) <sup>2</sup>	<b>P value</b>	<b>n</b> (missing)	<b>Median</b> (IQR) <sup>2</sup>	<b>P value</b>
Frequency of fecal soiling (seven cases not stated)												
Daily	24 (1)	2.10 (1.33-2.91)	< 0.001 <sup>a</sup>	24 (1)	1.33 (1.18-1.83)	< 0.001 <sup>a</sup>	23 (2)	1.71 (1.37-2.29)	< 0.001 <sup>a</sup>	23 (2)	1.46 (1.00-2.07)	< 0.001 <sup>a</sup>
Weekly	31 (4)	3.10 (2.40-3.60)		32 (3)	2.26 (1.47-2.82)		30 (5)	2.54 (2.06-3.08)		30 (5)	2.33 (1.33-3.00)	
Monthly	18 (4)	3.30 (2.60-3.70)		18 (4)	2.14 (1.94-2.82)		19 (3)	2.29 (1.77-3.38)		19 (3)	2.33 (2.00-3.33)	
Less often	48 (17)	3.70 (2.85-4.00)		54 (11)	2.88 (2.15-3.57)		52 (13)	3.02 (2.19-3.66)		50 (15)	3.00 (2.33-4.00)	
Type of fecal soiling (eight cases not stated)												
Liquid	59 (16)	3.26 (2.50-3.80)	0.077 <sup>a</sup>	64 (11)	2.44 (1.78-3.11)	0.024 <sup>a</sup>	62 (13)	2.78 (2.14-3.57)	0.005 <sup>a</sup>	62 (13)	2.67 (2.00-3.67)	0.003 <sup>a</sup>
Solid	24 (7)	3.37 (2.60-3.93)		27 (4)	2.33 (1.63-3.22)		27 (4)	2.64 (2.09-3.50)		25 (6)	2.33 (1.83-3.67)	
Both	35 (5)	3.00 (1.50-3.70)		38 (2)	2.00 (1.26-2.50)		37 (3)	2.19 (1.60-2.57)		36 (4)	2.00 (1.33-2.67)	
Quantity of fecal soiling (four cases not stated)												
Minimal soiling	65 (18)	3.60 (3.00-4.00)	< 0.001 <sup>a</sup>	71 (12)	2.67 (2.17-3.40)	< 0.001 <sup>a</sup>	69 (14)	2.80 (2.20-3.61)	0.001 <sup>a</sup>	69 (14)	2.67 (2.00-3.67)	0.010 <sup>a</sup>
Major soiling	41 (11)	3.00 (2.06-3.55)		46 (6)	2.06 (1.44-2.58)		45 (7)	2.27 (1.89-2.92)		43 (9)	2.32 (2.00-3.00)	
Soiling outer clothes	8 (1)	2.12 (1.63-2.45)		8 (1)	1.28 (1.14-1.46)		8 (1)	2.00 (1.72-2.59)		7 (2)	1.67 (1.33-2.33)	
Soiling furniture	5 (1)	1.30 (1.25-1.95)		5 (1)	1.00 (1.00-2.14)		5 (1)	1.37 (1.19-2.28)		5 (1)	1.00 (1.00-2.67)	
Fecal urgency (four cases not stated)												
Never	15 (10)	4.00 (3.30-4.00)	0.001 <sup>a</sup>	16 (9)	3.40 (2.24-3.76)	< 0.001 <sup>a</sup>	20 (5)	2.45 (2.19-3.40)	0.177 <sup>a</sup>	17 (8)	2.67 (1.56-3.83)	0.507 <sup>a</sup>
Sometimes	88 (21)	3.25 (2.22-3.70)		98 (11)	2.24 (1.60-2.96)		92 (17)	2.48 (1.88-3.37)		91 (18)	2.33 (2.00-3.33)	
Always	15 (1)	2.30 (1.80-2.60)		15 (1)	1.50 (1.33-2.06)		15 (1)	2.24 (1.77-2.64)		15 (1)	2.00 (1.33-3.33)	
Women only	47 (11)	3.30 (2.70-3.80)	0.005 <sup>b</sup>	52 (6)	2.44 (1.79-3.11)	0.002 <sup>b</sup>	50 (8)	2.72 (2.08-3.56)	0.034 <sup>b</sup>	49 (9)	2.67 (2.00-3.67)	0.003 <sup>b</sup>
UC-ever												
Women only	38 (7)	2.45 (1.72-3.60)		42 (3)	1.86 (1.28-2.44)		39 (6)	2.24 (1.65-2.86)		39 (6)	2.00 (1.33-2.67)	
CC-ever												
Passive fecal soiling (seven cases not stated)												
Never	38 (12)	3.40 (2.67-4.00)	0.086 <sup>a</sup>	41 (9)	2.56 (1.97-3.42)	0.049 <sup>a</sup>	43 (7)	3.05 (2.28-3.66)	0.008 <sup>a</sup>	40 (10)	3.33 (2.33-4.00)	< 0.001 <sup>a</sup>
Sometimes	63 (16)	3.10 (2.20-3.60)		69 (10)	2.17 (1.56-2.78)		65 (14)	2.23 (1.80-2.90)		64 (15)	2.00 (1.42-2.92)	
Always	15 (3)	2.50 (1.70-3.90)		17 (1)	2.06 (1.39-3.20)		17 (1)	2.27 (1.90-3.39)		17 (1)	2.00 (1.00-2.96)	
Women only	36 (9)	3.30 (2.76-3.82)	0.040 <sup>b</sup>	39 (6)	2.44 (1.78-3.22)	0.020 <sup>b</sup>	39 (6)	2.48 (1.94-3.27)	0.181 <sup>b</sup>	38 (7)	2.33 (1.92-3.00)	0.019 <sup>b</sup>
UC-ever												
Women only	33 (7)	2.50 (1.61-3.60)		37 (3)	1.91 (1.28-2.44)		34 (6)	2.20 (1.70-2.86)		34 (6)	1.83 (1.33-2.67)	
CC-ever												

FIQL, Rockwood *et al*<sup>[22]</sup>; *n*: Number of participants in each category. <sup>1</sup>Scales calculated as per letter to the editor. Rockwood<sup>[26]</sup>; <sup>2</sup>QOL score of 1 represents very low functional status and 4 is not affected by FI. <sup>a</sup>Kruskal-Wallis test; <sup>b</sup>Mann-Whitney *U* test.

attended the CC being poorer than that of those from the UC. Furthermore, the negative impact on participants'

lives worsened with the loss of both solid and liquid stool and the increased frequency and quantity of soiling.



**Figure 1** Association of FIQL scales with sex and CC and UC. FIQL, Rockwood *et al*<sup>[22]</sup>; Box and whisker demonstrates median, IQR, minimum and maximum. *P* values calculated by unpaired Wilcoxon test (Mann-Whitney); QOL score of 1 represents very low functional status and 4 is not affected by FI; FIQL scales calculated as per letter to the editor, Rockwood<sup>[26]</sup>.

**Table 4** Multiple linear regression identifying relationship between FIQL and components of FI severity

Components of FI severity	FIQL scales			
	Lifestyle	Coping	Depression	Embarrassment
Frequency				
Monthly	0.061	0.002	0.008	0.006
Weekly	0.241	0.846	0.531	0.595
Daily	< 0.001	0.012	0.023	0.021
Type				
Solid	0.010	0.192	0.515	0.633
Both solid/liquid	0.254	0.085	0.007	0.014
Urgency				
Sometimes	0.961	0.325	0.565	0.888
Always	0.106	0.023	0.554	0.636
Wears pads	0.209	0.022	0.488	0.107
Passive leakage				
Sometimes	0.659	0.169	0.103	0.787
Always	0.626	0.485	0.252	0.007
Quantity of leakage				
Major (Requires immediate underwear change)	0.001	< 0.001	0.004	0.096
Soiling of outer clothes	0.023	0.217	0.969	0.405
Soiling of furniture/bedding	0.578	0.781	0.381	0.662

In this study of the impact on QOL of FI in rural and regional north Queensland, our overall FIQL results for lifestyle (3.24), coping (2.23), depression (2.42) and embarrassment (2.33) were within the range of comparable clinic-based studies in other countries<sup>[21,27-31]</sup>, and closely reflected the scores found in a similar study

conducted at a Pelvic Floor Center in Minnesota, USA<sup>[21]</sup> and baseline scores from two Victorian (Australia) clinical studies that investigated injectable material for FI<sup>[32,33]</sup>.

The QOL of patients who attended the CC was more severely affected than that of participants from the UC, even though they had reported FI for a shorter duration. There were no significant differences between those who had a diagnosis of bowel disease and those who did not. Thus the poorer QOL in CC participants may be a direct result of recent colorectal surgery, in which the sudden and unexpected onslaught of FI was more devastating than for the UC participants who may have learnt to cope with their progressive FI over an extended time period. These CC FIQL results are comparable with previously published results from a pouch, non-pouch study<sup>[29]</sup>, but the participants in our study were less able to cope, and were more depressed and embarrassed, although this did not affect their lifestyle to the same degree.

The UC patients' QOL scores were lower than those in a similar study in Texas, USA<sup>[30]</sup> but higher than in the study in Minnesota, USA<sup>[21]</sup>, for lifestyle, coping and embarrassment, but not depression. This difference may have been caused by a longer duration with FI in the older cohorts in our study and the Minnesota study, (mean age 56 years), compared with the younger cohort in the Texas study (mean age 39 years). This suggests that older women with FI had poorer QOL than younger affected women, which implies that delaying treatment for this condition results in poorer QOL over time.

This study investigated the relationship between FI severity and the FIQL scales individually and collectively. A negative impact was found on patients' lives, which increased with frequency of soiling. This was evident on all scales of the FIQL, but there was little difference in each of the FIQL scales between weekly and monthly incontinent episodes. This lack of difference may be because an incontinent episode that occurs infrequently is unexpected, and hence, would be similarly distressing.

The data collected for pad wearing was dichotomous, which may explain why only significant results for the coping scale were obtained. If, in addition, the number of pads worn per day had been assessed, the embarrassment scale may also have reached significance.

The type of soiling affected participants' QOL differently. Patients with both solid and liquid soiling reported a poorer QOL than those with either solid or liquid only incontinent episodes. This is consistent with the Texas study in which a liquid component of anal incontinence was reported to have a greater impact upon QOL of participants than either flatus or non-liquid incontinence<sup>[30]</sup>.

The association between quantity of fecal loss and FIQL scales was found to be highly significant. Patients with the most soiling, i.e. those who soiled furniture, had the lowest possible FIQL score for coping and embarrassment, and performed only marginally better on the lifestyle and depression scales. There are few reports of the relationship between quantity of fecal loss and QOL, however, a Japanese study investigating the QOL of patients following total proctocolectomy and ileal J-pouch-anal anastomosis determined that greater soiling resulted in higher levels of frustration, which is an anxiety measure calculated using a Japanese translation of Cattell's anxiety scale<sup>[34]</sup>. When quantity was included in the FIQL/severity regression model, it was found to be significant ( $P < 0.05$ ) for the lifestyle, coping and depression scales, but did not reach significance for the embarrassment scale ( $P = 0.065$ ). Given this relationship between the FIQL scales and quantity of fecal soiling, it is suggested that the definition of FI severity should include quantity of fecal loss as well as frequency, type, urgency and pad wearing.

In conclusion, to the best of our knowledge, this is the first study to measure the effect of FI on QOL of people in rural and regional Australia. More than 22% of the patients attending the UC and CC in North Queensland, for matters other than FI, had their QOL severely affected by this condition. Patients reported that they had not been asked about FI by their general practitioners or hospital physicians, nor did they voluntarily disclose its presence<sup>[9]</sup>. Women who have obstetric-injury-related FI suffer in silence, and their QOL deteriorates as they age. The definition of FI severity should include quantity of fecal loss<sup>[35]</sup>. Further community-based research in Australia is warranted in regard to the impact of FI on QOL of people who suffer with this complaint.

## ACKNOWLEDGMENTS

We thank Dr. Rane AJ for facilitating access to the patients in his UCs.

## COMMENTS

### Background

As many as one in seven adults suffer with fecal incontinence (FI), which can have a devastating effect on the lifestyle of people with frequent, ad hoc or large amounts of fecal seepage. These people often fail to seek treatment due to embarrassment, believing the problem is uniquely theirs, or because they are unaware of the existence of available treatments.

### Research frontiers

Patients referred to urogynecology and colorectal surgical clinics, for other reasons, have a high incidence of FI. They do not seek assistance whilst attending these clinics from physicians who can recommend or refer for treatment. In this study, the authors demonstrated the impact that FI had on these patients' quality of life (QOL).

### Innovations and breakthroughs

Recent reports have highlighted the impact conservative treatments, such as biofeedback, and more invasive treatments, such as injectable bulking agents, sacral nerve stimulation, artificial bowel sphincter and dynamic graciloplasty, have had on the QOL of patients with FI. This study reports that despite these improvements many people continue to suffer unaware of such advances.

### Applications

By understanding more than one in five patients attending colorectal and urogynecological clinics have their QOL severely affected by FI, attending physicians can enable access to treatment by directly asking patients about this problem.

### Terminology

FI is the involuntary discharge of solid or liquid stools.

### Peer review

FI severity, including the quantity of stool loss, can have a severe negative impact on QOL. This is an interesting and relevant study that deals with a demanding subject.

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## Is ERCP really necessary in case of suspected spontaneous passage of bile duct stones?

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subjected to ERCP within 3 d of MRCP to check for the presence of stones.

**RESULTS:** No stones were detected by ERCP in any patient, confirming the results of MRCP.

**CONCLUSION:** When clinical symptoms improve, blood biochemical parameters have normalized, and MRCP shows there are no stones in the common bile duct, it can be considered the stone has spontaneously passed and thus ERCP is not necessary.

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**Key words:** Magnetic resonance cholangiopancreatography; Endoscopic retrograde cholangiopancreatography; Spontaneous passage of bile duct stones; Bile duct stones; Pancreatitis

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### Abstract

**AIM:** To investigate the usefulness of magnetic resonance cholangiopancreatography (MRCP) and the need for endoscopic retrograde cholangiopancreatography (ERCP) in cases of suspected spontaneous passage of stones into the common bile duct.

**METHODS:** Thirty-six patients with gallbladder stones were clinically suspected of spontaneous passage of stones into the common bile duct because they presented with clinical symptoms such as abdominal pain and fever, and showed signs of inflammatory reaction and marked rise of hepatobiliary enzymes. These symptoms resolved and they showed normalized values of blood biochemical parameters after conservative treatment without evidence of stones in the common bile duct on MRCP. All these patients were

### INTRODUCTION

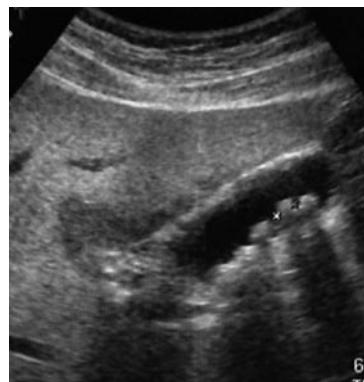
Magnetic resonance cholangiopancreatography (MRCP) is a relatively new imaging technique developed for the diagnosis of patients with pancreaticobiliary disease. The signal of structures filled with static or slowly moving fluid, such as the bile and pancreatic ducts, is greatly increased on T2-weighted images, resulting in an increased duct-to-background contrast. Recent studies have shown that MRCP is as useful as endoscopic retrograde cholangiopancreatography (ERCP) regarding the diagnosis of extrahepatic bile duct and pancreatic duct abnormalities such as common bile duct stones<sup>[1-4]</sup>,

malignant obstruction of the bile and pancreatic ducts<sup>[1,2,5]</sup>, congenital anomalies<sup>[1,6,7]</sup>, and chronic pancreatitis<sup>[8-10]</sup>. In some institutions, MRCP is becoming the first-choice technique for imaging the biliary system, with ERCP being reserved for therapeutic indications. In recent studies, MRCP showed extremely high performance in visualizing bile duct stones with both a sensitivity and a specificity of 90% or higher in most cases<sup>[11-17]</sup>. In the false negative cases, all stones were as small as 3-5 mm and were present near the duodenal papilla. Thus, MRCP is very useful for the diagnosis of bile duct stones and the determination of a therapeutic strategy. Since ERCP involves the risk of complications such as acute pancreatitis<sup>[18-21]</sup>, it should be performed only when absolutely necessary. In some patients clinically suspected of choledocholithiasis, ERCP shows a cleavage at the duodenal papilla, but does not detect the stones in the common bile duct. These findings suggested that stones in the common bile duct had probably passed spontaneously. In fact, it was reported that three-fourths of common bile duct stones passed spontaneously before cholecystectomy<sup>[22]</sup>, indicating that many patients with bile duct stones may not need ERCP. There are reports on the clinical course of patients suspected of spontaneous passage of bile duct stones after MRCP alone<sup>[23,24]</sup>. However, since ERCP was not performed, it was not clear whether the stones were actually present. Presence of bile duct stones could cause acute cholangitis, which, if aggravated, may be fatal. Consequently, we investigated the usefulness of MRCP and the need for diagnostic ERCP in patients suspected of spontaneous passage of stones.

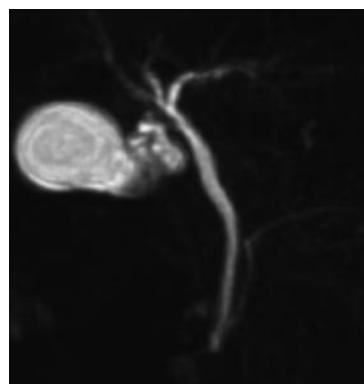
## MATERIALS AND METHODS

From April 2007 to January 2009, 36 patients suspected of spontaneous passage of bile duct stones were prospectively evaluated. There were 26 men and 10 women and the mean age of the patients was 62.1 years (range: 33-82 years). The mean observation period was 6.7 mo (1-9 mo). After blood sampling the patients were subjected to ultrasound examination (Power Vision 6000; TOSHIBA Tochigi Japan) to assess the presence of stones in the gallbladder and common bile duct. The patients had gallbladder stones and were clinically suspected of spontaneous passage because they presented with clinical symptoms that included abdominal pain and fever, and showed signs of inflammatory reaction as well as a marked rise of hepatobiliary enzymes. These symptoms resolved and patients showed normalized values of blood biochemical parameters after conservative treatment, without evidence of stones in the common bile duct on MRCP. Patients who had originally had intrahepatic cholestasis were considered as "improved" if the level of hepatobiliary enzymes had normalized. The US, MRCP and ERCP images of typical cases are shown in Figures 1-3, respectively.

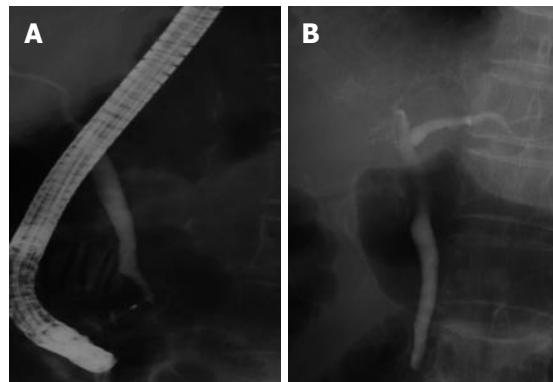
In this study, the need for urgent drainage was assessed first. Accordingly, blood was collected from



**Figure 1** A 56-year-old man. Several gallbladder stones measuring about 5-10 mm were detected by abdominal ultrasonography.

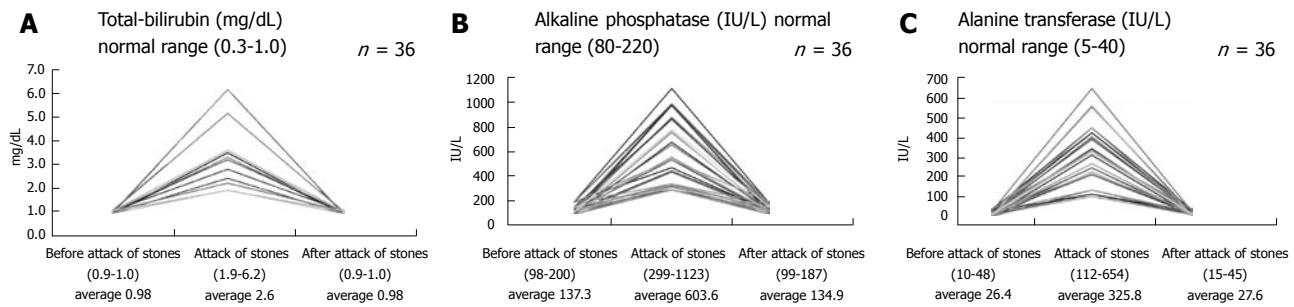


**Figure 2** No translucency indicative of stones in the bile duct was shown by magnetic resonance cholangiopancreatography.



**Figure 3** No translucency indicative of stones in the bile duct was shown by endoscopic retrograde cholangiopancreatography (A). After withdrawal of the fiber, the posture was changed and the bile duct was imaged again. However, no translucency was observed (B).

the patients on arrival at the hospital for the assessment of acute cholangitis. Patients with moderate to severe acute cholangitis<sup>[25]</sup> that did not respond to the initial medical treatment, were excluded from this study and were subjected to emergency ERCP with biliary drainage. When a patient presented symptoms of acute cholangitis during follow-up, ERCP was conducted provided the patient had given his/her informed consent prior to the examination. Patients with symptoms of acute pancreatitis were excluded from this study. Even



**Figure 4** Changes in serum. A: Total-bilirubin; B: Alkaline phosphatase; C: Alanine transferase.

if a marked increase in hepatobiliary enzymes was detected before arrival at the hospital, we waited for an improvement in clinical symptoms and blood test values if cholangitis was mild, before performing MRCP followed by ERCP.

MRCP was performed using a 1.5T GE CV/I scanner and a phased-array torso coil (General Electric Medical Systems, Milwaukee, WI). The radiographic conditions were as follows: thin-section MRCP images were obtained in the coronal plane using half Fourier single-shot fast spin echo (SSFSE) imaging at a slice thickness of 4.0 mm, 34FOV, 256 × 256 matrix, and a TE of 180. Thick-slab SSFSE MRCP images were obtained in the plane using a matrix of 256X256 and a maximum TE. Imaging in the coronal and axial planes was done using a fast 3D spoiled gradient echo sequence with a TR of 3.9 TE minutes, 2.4 mm slice thickness (interpolated to 1.2 mm), and a 256 × 192 matrix. Images were reviewed on an Advantage Windows workstation (General Electric Medical Systems) that allowed 3D reconstruction. MRCP images were read after evaluating clinical symptoms and hematological findings to examine the common bile duct for the presence of stones. First, a radiologist with more than 20 years of experience examined the MRCP images and if there were no bile duct stones, the same MRCP images were subsequently examined by a gastroenterologist with more than 15 years of experience. Only after the absence of stones was confirmed by these 2 specialists, was the case defined as spontaneous passage of stones. MRCP was conducted after the clinical symptoms and blood test findings had sufficiently improved. ERCP was conducted within 4 d of MRCP to check for the presence of stones. ERCP images were read only by the gastroenterologist who had examined the MRCP images. As a measure against complications, the duration of ERCP was strictly limited to within 15 min. This prospective study was conducted after obtaining informed consent from the patients. Furthermore, the protocol of this study was discussed and approved by the Institutional Review Board of Funabashi Central Hospital.

## RESULTS

ERCP was successfully conducted in all 36 patients after MRCP confirmed the absence of stones in each of them. The ERCP procedure was completed within 15 min in all these cases. Mild acute pancreatitis,

assessed according to Cotton's criteria<sup>[21]</sup>, occurred as a complication after ERCP in 1 (2.8%) of the 36 patients. Cannulation was difficult in this patient and pancreatitis was assumed to be caused by pancreatography, which was performed 3 times during ERCP. However, remission was attained after conservative treatment. No other complications occurred. Both the radiologist and the gastroenterologist who read MRCP images ruled out the presence of bile duct stones in the 36 patients ( $K = 1.0$ ) (Figures 2 and 3).

MRCP showed the bile duct had a mean diameter of 6.6 mm (range, 4.8-8.3 mm). Biochemical average (and range) data were total bilirubin 2.6 (1.9-6.2) mg/dL (Figure 4A), alkaline phosphatase (ALP) 603.6 (299-1123) IU/L (Figure 4B), and alanine transferase (ALT) 325.8 (112-654) IU/L (Figure 4C) at the time of common bile duct obstruction while the normal range values were total bilirubin 0.3-1.0 g/dL; ALP 80-220 IU/L and ALT 5-40 IU/L. Subsequently, 6 patients underwent cholecystectomy. During the observation period, none of the 36 patients experienced a second spontaneous passage of stones. At the time of registration for this study, the clinical symptoms of 6 patients suspected of spontaneous passage of stones were already showing a tendency towards remission. Yet these patients were not registered for the study because they had moderate cholangitis. Accordingly, urgent ERCP was conducted without MRCP. The result indicated no bile duct stone in any of the 6 cases.

## DISCUSSION

MRCP can detect bile duct stones with a high rate of accuracy and sensitivity. In the present study, ERCP detected no stones in any of the patients suspected of spontaneous passage of stones and confirmed by the MRCP conducted after the clinical symptoms had improved and blood test values had normalized or returned to baseline levels. Nevertheless, the possible presence of small stones undetectable by ERCP cannot be denied. Intraductal ultrasonography (IDUS), which can be used following ERCP, is a valuable tool for the imaging of tiny stones. IDUS is likely to detect very small bile duct stones more accurately than ERCP alone<sup>[26]</sup>. Bile duct stones have, however, been reported to pass spontaneously in most cases<sup>[22]</sup>. Although the aggressive use of IDUS may increase the detection of

bile duct stones, prolonged ERCP practice may result in accidental events. Thus, this procedure was not included in the study design.

The presence or absence of small stones is a matter of controversy. However, it is basically reasonable to assume a spontaneous passage of stones if MRCP has detected no stones in the patients whose clinical symptoms improved and whose blood test values normalized or returned to the baseline levels. Today, most reports conclude that MRCP is comparable to ERCP in the diagnosis of biliary stones. Institutions with high-magnetic-field MRI make it a rule not to perform diagnostic ERCP in patients who have no stones identified by MRCP. The possibility that the present study would be useful in considering indications for use of ERCP was suggested. The results of this study indicate the feasibility of diagnosis of spontaneous passage of stones in the common bile duct based on clinical symptoms, blood biochemistry, US, and MRCP without exposure to X-ray, thus reducing such exposure for medical personnel responsible for conducting ERCP<sup>[27]</sup>, and avoiding the potential complications of ERCP.

US is a simple examination tool for imaging bile duct stones but is less satisfactory for the detection of stones<sup>[26]</sup>. However, it provides information on the gallbladder and important information on the diameter of the bile duct considering the presence of bile duct stones and thus it is a very useful examination. Given the higher performance of MRCP, diagnosis employing US or MRCP rather than diagnostic imaging will become more common when bile duct stones are suspected. However, the patients with spontaneous passage of stones included in this study presented with mild cholangitis. In patients with moderate or severe cholangitis, drainage of the biliary tract that allows the detection of stones more rapidly than MRCP, may prove very useful<sup>[25]</sup>. When clinical symptoms improve, blood biochemical parameters have normalized, and MRCP shows there are no stones in the common bile duct, it can be considered that the stone has spontaneously passed and thus ERCP would not be necessary.

## COMMENTS

### Background

Magnetic resonance cholangiopancreatography (MRCP) is a relatively new imaging technique developed for the diagnosis of patients with pancreaticobiliary disease. Recent studies have shown that MRCP is as useful as endoscopic retrograde cholangiopancreatography (ERCP) for the diagnosis of extrahepatic bile duct and pancreatic duct abnormalities such as common bile duct stones.

### Innovations and breakthroughs

When clinical symptoms improve, blood biochemical parameters have normalized, and MRCP shows there are no stones in the common bile duct, it can be considered the stone has spontaneously passed and thus ERCP would not be necessary.

### Peer review

This is a prospective, non-controlled study from a single center. The observation is made that ERCP is not helpful when a passed stone is suspected and MRCP is negative.

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BRIEF ARTICLES

# Psychometrics of the chronic liver disease questionnaire for Southern Chinese patients with chronic hepatitis B virus infection

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**Author contributions:** All authors participated in the design of the study; Lam ETP, Lai CL and Yuen MF collected the data; Lam ETP, Lam CLK and Fong DYT were involved in data analysis and interpretation; Lam ETP and Lam CLK drafted the manuscript; All authors read and approved the final manuscript. **Supported by** Small Project Grant from the Committee of Research and Conference Grant, CRCG project, No. 10207293, the University of Hong Kong and the Health and Health Service Research Fund, HHSRF project, No. 05060741, Food and Health Bureau, Government of Hong Kong Special Administration Region, China

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**CONCLUSION:** The Chinese (HK) CLDQ is valid, reliable and sensitive for patients with CHB. Some modifications to the scaling structure might further improve its psychometric properties.

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**Key words:** Chronic liver disease; Health-related quality of life; Hepatitis B; Southern Chinese; Validity

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Lam ETP, Lam CLK, Lai CL, Yuen MF, Fong DYT. Psychometrics of the chronic liver disease questionnaire for Southern Chinese patients with chronic hepatitis B virus infection. *World J Gastroenterol* 2009; 15(26): 3288-3297 Available from: URL: <http://www.wjgnet.com/1007-9327/15/3288.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.3288>

## INTRODUCTION

Chronic hepatitis B (CHB) virus infection remains a major global health problem. It is estimated that 350 million people worldwide are chronically infected, of whom one third (120 million) are Chinese<sup>[1]</sup>. The prevalence is higher in southern China (> 10%) than Northern China (6%-10%)<sup>[2]</sup>. Up to 25% of patients may die from CHB complications, such as cirrhosis-related complications or hepatocellular carcinoma (HCC), posing a threat to both mental and physical health, leading to impairment of health-related quality of life (HRQOL).

HRQOL has become an important outcome measure in clinical and health policy settings in the last two decades. Disease-specific measures are often needed to complement generic measures to give a more comprehensive evaluation of the HRQOL of patients with specific diseases. Several HRQOL measures have been developed specifically for chronic liver disease (CLD), such as the Chronic Liver Disease Questionnaire (CLDQ)<sup>[3]</sup>, the Hepatitis Quality of Life (HQLQ)<sup>[4]</sup>, the Liver Disease Quality of Life<sup>[5]</sup> and the Liver Disease Symptom Index (LDSI)<sup>[6]</sup>. The CLDQ developed by Younossi *et al*<sup>[3]</sup> was the first and is the most widely used. The other liver disease-specific HRQOL measures are not commonly used because they are either too long, or the validity data are limited<sup>[4-8]</sup>.

## Abstract

**AIM:** To test the psychometric properties of a Chinese [(Hong Kong) HK] translation of the chronic liver disease questionnaire (CLDQ).

**METHODS:** A Chinese (HK) translation of the CLDQ was developed by iterative translation and cognitive debriefing. It was then administered to 72 uncomplicated and 78 complicated chronic hepatitis B (CHB) patients in Hong Kong together with a structured questionnaire on service utilization, and the Chinese (HK) SF-36 Health Survey Version 2 (SF-36v2).

**RESULTS:** Scaling success was ≥ 80% for all but three items. A new factor assessing sleep was found and items of two (Fatigue and Systemic Symptoms) subscales tended to load on the same factor. Internal consistency and test-retest reliabilities ranged from 0.58-0.90 for different subscales. Construct validity was confirmed by the expected correlations between the SF-36v2 Health Survey and CLDQ scores. Mean scores of CLDQ were significantly lower in complicated compared with uncomplicated CHB, supporting sensitivity in detecting differences between groups.

The CLDQ consists of 29 items which are grouped into 6 subscales: abdominal symptoms (AS), fatigue (FA), systemic symptoms (SS), activity (AC), emotional function (EF) and worry (WO). It is applicable to all types of liver diseases including CHB. It has been shown to have adequate internal reliability, validity and sensitivity. Test-retest reliability was more variable with intra-class correlation (ICC) ranging from 0.23 to 0.72 for different subscales<sup>[3]</sup>. Previous studies showed that the CLDQ is more responsive than a generic measure to detect a change in patients with CLD<sup>[3,9]</sup>. It has been translated and validated in different languages<sup>[9-13]</sup>, supporting its potential for cross-cultural adaptation. However, most of the psychometric data of the CLDQ have been derived from patients with hepatitis C virus (HCV) infection and Western populations. There are few data on its applicability for Southern Chinese CHB patients despite the fact that China has the world's largest population suffering from CLD.

Recently, the CLDQ has been translated into Mandarin Chinese but this Chinese (Mainland) version may not be applicable to Southern Chinese who speak Cantonese, a dialect that has significant differences in the usage of words and terms from Mandarin. In addition, information on the validity, reliability and other psychometric properties of the Chinese (Mainland) CLDQ version is limited. The aim of this study was to test the psychometric properties of a Chinese [Hong Kong (HK)] translation of the CLDQ for Southern Chinese CHB patients. This would enable the evaluation of the impact of CHB infection and assess the effect of anti-viral drug treatments on HRQOL in the world's largest population of CHB patients.

The objectives of this study were: (1) To develop a Chinese (HK) CLDQ that is semantically equivalent to the original; (2) To test the scaling assumptions and factor structure of the Chinese (HK) CLDQ; (3) To assess the psychometric properties in terms of reliability, construct validity, and sensitivity of the Chinese (HK) CLDQ; (4) To determine whether any modification of the CLDQ can improve its psychometric properties for Southern Chinese CHB patients.

## MATERIALS AND METHODS

### Ethics

This research project was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (IRB reference No., UW 06-089 T/1114 and trial registration No., HKC-TR-151).

### Development of the Chinese (HK) CLDQ

The Chinese (HK) translation of the CLDQ was developed by iterative translations, expert panel review and cognitive debriefing, as recommended guidelines by experts<sup>[14,15]</sup>. The original CLDQ was translated into Chinese by two independent professional translators. Reconciliation of the forward translations into a single forward translation was carried out by a bilingual expert in HRQOL measures (Lam CLK) and the translators. The reconciled Chinese translation was back-translated into English by another professional translator. The back

translation was reviewed by the original author and the bilingual expert to identify any non-equivalence in the Chinese translation, which was then revised. The first draft of the Chinese (HK) CLDQ was evaluated by cognitive debriefing interviews with six Southern Chinese patients with CHB infection and further revision was made to ensure item clarity and equivalence to become the final Chinese (HK) CLDQ (used in this study on psychometrics properties).

### Subjects

Patients with complicated CHB were recruited from out-patient hepatitis clinics of a regional hospital and patients with uncomplicated CHB were randomly selected from the computerized registers of three public primary care clinics serving over 100 000 people in one of five regions in Hong Kong. Patients aged 18 years or older who were hepatitis B surface antigen-positive for more than six months were included in the study. Patients were excluded if they could not communicate in Cantonese; had cognitive impairment shown by the patient's inability to understand the study to give consent; were co-infected with HIV, HCV or hepatitis D virus; had undergone liver transplantation or had end-stage non-hepatitis B-related illnesses; were currently taking excessive alcohol (> 30 U/wk) or illegal drugs; or refused to give consent. Each patient completed the Chinese (HK) CLDQ, the Chinese (HK) SF-36v2 Health Survey and a structured questionnaire on morbidity and socio-demographics, administered by a trained interviewer. Each patient was asked if he/she had ever been diagnosed by a registered practitioner for more than four weeks to have hypertension, diabetes mellitus, heart disease, stroke, chronic lung disease, arthritis, psychological illness (i.e. depression, anxiety, neurasthenia or psychosis) or any other chronic diseases. Chronic co-morbidity was measured by the total number of diseases (summation of positive responses to the questions) and the presence of a specific diagnosis. Clinical data related to the CHB infection including Child's staging for patients with cirrhosis and the biomarkers of liver disease (alanine aminotransferase, aspartate aminotransferase,  $\alpha$ -fetoprotein and total bilirubin) in each patient were retrieved from medical records. Socio-demographic data including age, gender, education, marital status, occupation, household income and family history of liver disease were also collected.

The Chinese (HK) CLDQ was re-administered to the 46 subjects with uncomplicated CHB, whose condition was expected to be stable, by telephone two weeks from the first administration, in order to assess the test-retest reliability of the Chinese (HK) CLDQ. Sixty one percent of the repeat interviews were carried out by the same interviewer.

### Instruments

The Chinese (HK) CLDQ consists of 29 items measuring six subscales as described above. Each item is rated on a 7-point (1 = all of the time to 7 = none of the time) Likert scale. Scores for each of the six domains are calculated by the mean of the item scores within the subscale. A summary score is calculated by the mean of all subscale scores. The scores range from 1 to 7 with a higher score indicating better HRQOL.

The Chinese (HK) SF-36v2 Health Survey is a generic HRQOL measure that has been translated, validated and normed on the general Chinese population in Hong Kong<sup>[16,17]</sup>. It measures eight domains of HRQOL on physical functioning (PF), role-physical (RP), bodily pain (BP), general health (GH), vitality (VT), social functioning (SF), role-motional (RE) and mental health (MH). Summations of item scores of the same domain give the domain scores, which are transformed into a range from 0 to 100. A higher score indicates better HRQOL. The eight domain scores are summarized to form the physical component (PCS) and mental component (MCS) summary scores.

### **Statistical analysis**

All data analysis was carried out in SPSS for Windows 15.0. Statistical significant levels were set at  $P$  values less than 0.05.

### **Scaling assumptions**

The CLDQ item and subscale scores were calculated and tested against the following scaling assumptions: (1) Items should be substantially linearly correlated to the hypothesized subscale score with a coefficient of 0.4 or above by Spearman rank correlation test, to show the item is a significant indicator for the subscale concept. (2) An item should have a stronger correlation with its hypothesized subscale than other subscales indicating scaling success<sup>[18]</sup>. This is a test of item discriminant validity. The difference between correlations is statistically significant if it is greater than two standard errors (1 divided by the square root of sample size).

### **Construct validity**

**Factor analysis:** Exploratory factor analysis using principal components with varimax rotation was performed to evaluate the factor structure of the Chinese (HK) CLDQ. The criterion for factor extraction was an eigenvalue greater than one. The highest factor loading was identified for each item. The scree plot was also used to determine the number of factors.

**Convergent validity:** Construct validity was also tested by convergent validity determined by Spearman correlations between corresponding CLDQ and SF-36v2 Health Survey domain scores. It was hypothesized that moderate ( $r = 0.4$  to  $0.7$ ) to strong ( $r > 0.7$ ) correlations should exist between CLDQ FA and SF-36v2 VT; between CLDQ SS and SF-36v2 BP; between CLDQ AC and SF-36v2 PF, RP and RE; and between CLDQ EF and SF-36v2 MH scores.

### **Sensitivity**

The mean CLDQ scores were compared between two CHB patient groups, and the difference was tested by independent  $t$  to evaluate its sensitivity in detecting a difference between patients with complicated and uncomplicated infections. The sensitivity of the CLDQ was also assessed by the effect size (difference between group mean scores/overall standard deviation). According to Cohen<sup>[19]</sup>, effect sizes of 0.3, 0.5 and 0.8 were considered small, medium and large differences, respectively. An effect size of less than 0.3 was considered not significant.

### **Reliability**

Different methods were used to assess reliability, including internal consistency and test-retest reliability. Internal consistency was measured by Cronbach's  $\alpha$ , which is a measure of the extent to which items in a questionnaire are homogeneous (correlated) in supporting the same concept<sup>[20]</sup>. Test-retest reliability refers to the stability of an instrument over time<sup>[21]</sup>, which was measured by the intra-class correlation (ICC) between the two-week test-retest results. Reliability coefficients  $\geq 0.7$  and 0.9 are usually expected for group comparisons and individual comparisons, respectively<sup>[21]</sup>.

## **RESULTS**

### **Translational equivalence of the Chinese (HK) CLDQ**

All items except item 11 (level of energy) were found to be understood by 6 patients. Three out of six patients did not understand item 11. Five patients (83%) interpreted the meaning of all except four items (11, 13, 19 and 28) correctly. Four out of six patients misinterpreted the meaning of item 11 with three interpreting it as decreased physical strength. Two patients (33.3%) had difficulty in differentiating the meaning of "sleepy" and "drowsy"; and did not seem to have interpreted the words "mood swings" (item 19). Two out of six patients did not include the meaning of "worried about never feeling better" (item 28) in their interpretation. The Chinese (HK) translation was revised based on the results of cognitive debriefing and the revised questionnaire was then field tested on 23 CHB patients before this study. The final Chinese (HK) CLDQ was formed and its back-translation is shown in the appendix.

### **Subjects**

One hundred and eighty four CHB patients were identified; 6 patients were excluded (3 had hepatitis B infection less than 6 mo, 2 had communication problems and 1 had co-infection with HCV) and 28 patients refused to participate in this study. One hundred and fifty Chinese adults consisting of 72 uncomplicated (normal liver function defined as liver enzymes persistently within the normal range and without any history of cirrhosis or HCC) and 78 complicated (cirrhosis or HCC) completed the study, giving a response rate of 84.3% (150/178). Table 1 shows their characteristics, overall and by disease severity groups. There were 8 patients in the complicated CHB group who had HCC without any cirrhosis and had normal liver function. There were no statistical differences in demographics between the uncomplicated and complicated CHB groups, except age and sex ( $P < 0.001$ ). Complicated CHB patients were older and there were more men than those in the uncomplicated group which was expected because CHB complications were more common in men than in women and the median age for the development of complications was 57.2 years<sup>[22,23]</sup>.

### **Score distribution**

Table 2 shows the distribution of the Chinese (HK) CLDQ and SF-36v2 scores. There was practically no

**Table 1** Socio-demographic and clinical characteristics of subjects *n* (%)

	Uncomplicated CHB ( <i>n</i> = 72)	Complicated CHB ( <i>n</i> = 78)	Overall ( <i>n</i> = 150)
Age, mean years ± SD <sup>1</sup>	50.2 ± 12.0	55.9 ± 9.5	53.2 ± 11.1
Sex <sup>1</sup>			
Male	42 (58.3)	65 (83.3)	107 (71.3)
Female	30 (41.7)	13 (16.7)	43 (28.7)
Education attainment			
No schooling	2 (2.8)	6 (7.7)	8 (5.3)
Primary	19 (26.4)	14 (17.9)	33 (22.0)
Secondary	35 (48.6)	46 (59.0)	81 (54.0)
Tertiary	16 (22.2)	12 (15.4)	28 (18.7)
Marital status			
Now married, living with spouse	59 (81.9)	67 (85.9)	126 (84.0)
Never married	6 (8.3)	5 (6.4)	11 (7.3)
Widowed	1 (1.4)	1 (1.3)	2 (1.3)
Divorced/separated	6 (8.3)	5 (6.4)	11 (7.3)
Occupation			
Managers, administrators & professional	19 (26.4)	17 (21.8)	36 (24.0)
Clerk, service and shop sales workers	16 (22.2)	19 (24.4)	35 (23.3)
Craft, machine operators & elementary	27 (37.5)	39 (50.0)	66 (44.0)
Others	10 (13.9)	3 (3.8)	13 (8.7)
Health status			
CHB	72 (100)		72 (48.0)
Cirrhosis		30 (38.5)	30 (20.0)
HCC		48 (61.5)	48 (32.0)
Child-Pugh Classification			
No cirrhosis/normal LF	72 (100)	8 (10.3)	80 (53.3)
Child A		47 (60.3)	47 (31.3)
Child B		8 (10.3)	8 (5.3)
Child C		15 (19.2)	15 (10.0)

CHB: Chronic hepatitis B; HCC: Hepatocellular carcinoma; LF: Liver function. Data are no. (%) of patients, unless otherwise indicated.

<sup>1</sup>Significant difference between uncomplicated and complicated CHB groups by independent sample *t* or Fisher's exact test, *P* < 0.05. Socio-demographic variables are recoded as binary categorical variables for performing Fisher's exact test: male vs female; no schooling vs any formal education; living with spouse vs other marital status; managers, administrators and professional vs other occupations.

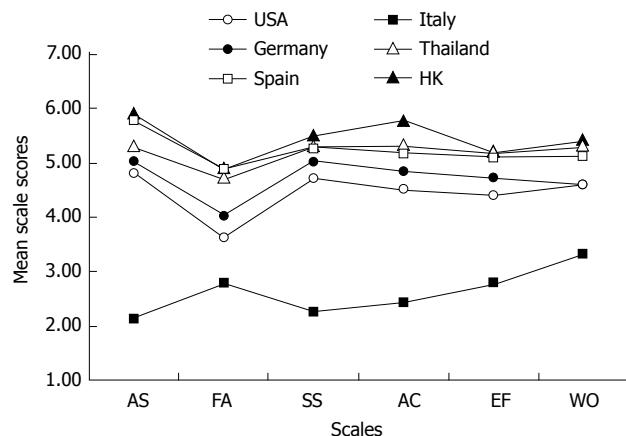
floor effect but there were significant ceiling effects in the Chinese (HK) CLDQ AS, AC and WO subscales, more so in the uncomplicated than the complicated group. Significant ceiling effects were also found in most SF-36v2 Health Survey scales. Sub-group analysis showed that the mean Chinese (HK) CLDQ scores were significantly lower in the complicated group than the uncomplicated group in all subscale and overall scores.

Figure 1 compares the distribution of the Chinese (HK) CLDQ scores with those from other countries. The distribution pattern of the Chinese (HK) CLDQ subscale scores was very similar to those of other countries<sup>[3,9,11,13]</sup>, except Italy, supporting cross-cultural conceptual equivalence.

### Scaling properties

Table 3 shows the mean item scores and standard deviation of the 29 CLDQ items grouped under their hypothesized subscales. All correlations between items and their hypothesized subscales score exceeded the standard of 0.4.

All but six items had a higher correlation with its hypothesized subscale than other subscales, i.e. 100% scaling success. Four items of the SS subscale and two items of the AC subscale correlated more highly with some other subscales than their own. Scaling success was the lowest in item 3 "bodily pain", which correlated more highly with four other subscales than with the SS subscale, with the highest found for EF, but the differences were not statistically significant. Items 6 "shortness of breath", 23 "dry mouth", 27 "itching", 7 "not able to eat as much as you



**Figure 1** The Chinese (HK) CLDQ scores by countries. AS: Abdominal Symptoms; FA: Fatigue; SS: Systemic Symptoms; AC: Activity; EF: Emotional Function; WO: Worry.

would like" and 9 "trouble in lifting or carrying heavy objects" correlated higher with one to three other subscales than its own, but the differences in the correlations were not statistically significant.

The overall scaling success rate on discriminant validity was 100% for four scales (AS, FA, EF and WO), but it was 73% for the AC subscale and 64% for the SS subscale.

### Factor analysis

Table 4 illustrates the rotated factor loadings between the

**Table 2 Distribution of the Chinese (HK) CLDQ and SF-36v2 Health Survey scores<sup>1</sup>**

	Uncomplicated CHB <sup>2</sup> (n = 72)				Complicated CHB <sup>2</sup> (n = 78)				Overall (n = 150)				
	Mean	(SD)	% floor <sup>3</sup>	% ceiling <sup>4</sup>	Mean	(SD)	% floor	% ceiling	Mean	(SD)	% floor	% ceiling	ES <sup>5</sup>
<b>C LDQ</b>													
AS	6.3	(1.0)	0.0	47.2	5.6	(1.5)	0.0	28.2	5.9	(1.3)	0.0	37.3	0.5 <sup>a</sup>
FA	5.2	(1.1)	0.0	6.9	4.6	(1.5)	0.0	5.1	4.9	(1.4)	0.0	6.0	0.4 <sup>a</sup>
SS	5.8	(1.0)	0.0	19.4	5.2	(1.2)	0.0	6.4	5.5	(1.2)	0.0	12.7	0.5 <sup>a</sup>
AC	6.2	(1.1)	0.0	48.6	5.4	(1.6)	0.0	28.2	5.8	(1.4)	0.0	38.0	0.6 <sup>a</sup>
EF	5.5	(1.0)	0.0	8.3	5.0	(1.4)	0.0	5.1	5.2	(1.3)	0.0	6.7	0.4 <sup>a</sup>
WO	5.9	(1.3)	1.4	37.5	5.0	(1.7)	0.0	17.9	5.4	(1.6)	0.7	27.3	0.6 <sup>a</sup>
Overall	5.8	(0.8)	0.0	0.0	5.1	(1.2)	0.0	1.3	5.4	(1.1)	0.0	0.7	0.6 <sup>a</sup>
<b>SF-36v2 Health survey (HK norm)</b>													
PF (90.6)	89.0	(14.7)	0.0	34.7	80.4 <sup>b</sup>	(15.4)	0.0	10.3	84.5	(15.6)	0.0	22.0	0.5 <sup>a</sup>
RP (90.2)	83.6 <sup>b</sup>	(21.0)	0.0	45.8	63.9 <sup>b</sup>	(29.4)	1.3	24.4	73.4	(27.4)	0.7	34.7	0.7 <sup>a</sup>
BP (82.6)	72.6 <sup>b</sup>	(23.0)	0.0	31.9	70.1 <sup>b</sup>	(28.6)	1.3	38.5	71.3	(26.0)	0.7	35.3	0.1
GH (53.2)	53.8	(21.7)	0.0	0.0	46.9 <sup>b</sup>	(25.6)	0.0	2.6	50.2	(24.0)	0.0	1.3	0.3
VT (60.2)	63.9	(19.8)	1.4	1.4	58.0	(26.5)	5.1	5.1	60.8	(23.6)	3.3	3.3	0.2
SF (92.4)	86.1 <sup>b</sup>	(19.3)	0.0	48.6	71.6 <sup>b</sup>	(29.0)	1.3	35.9	78.6	(25.8)	0.7	42.0	0.6 <sup>a</sup>
RE (88.5)	80.4 <sup>b</sup>	(19.8)	0.0	36.1	75.0 <sup>b</sup>	(26.9)	0.0	33.3	77.6	(23.8)	0.0	34.7	0.2
MH (72.0)	73.6	(18.4)	0.0	5.6	70.4	(21.9)	0.0	11.5	72.0	(20.3)	0.0	8.7	0.2
PCS (50)	46.4 <sup>b</sup>	(9.9)	0.0	0.0	39.5 <sup>b</sup>	(12.2)	0.0	0.0	42.8	(11.6)	0.0	0.0	0.6 <sup>a</sup>
MCS (50)	50.1	(10.4)	0.0	0.0	47.8	(13.9)	0.0	0.0	48.9	(12.3)	0.0	0.0	0.2

AS: Abdominal Symptoms; FA: Fatigue; SS: Systemic Symptoms; AC: Activity; EF: Emotional Function; WO: Worry; PF: Physical Functioning; RP: Role Physical; BP: Bodily Pain; GH: General Health; VT: Vitality; SF: Social Functioning; RE: Role Emotional; MH: Mental Health; PCS: Physical Component Summary; MCS: Mental Component Summary; ES: Effect Size. <sup>1</sup>The Chinese (HK) CLDQ score range 1-7 and SF-36v2 score range 0-100; higher scores indicate better health-related quality of life; <sup>2</sup>Uncomplicated CHB are patients who had no cirrhosis or hepatocellular carcinoma (HCC) and normal liver function; Complicated CHB refers to those with cirrhosis or HCC; <sup>3</sup>% floor: Percentage of CHB patients at the lowest possible score; <sup>4</sup>% ceiling: Percentage of CHB patients at the highest possible score; <sup>5</sup>Effect size was calculated as the difference between uncomplicated and complicated mean score, divided by the overall SD. <sup>a</sup>Significant difference between uncomplicated and complicated CHB patients by independent sample *t* (*P* < 0.05); <sup>b</sup>Significant difference between CHB groups and HK norm by independent sample *t* (*P* < 0.05).

29 items and 6 factors with eigenvalue > 1. The six factors explained 70.1% of total variance. The factor loadings of the items were not entirely consistent with the scaling hypothesis. Items of the FA and SS subscales, except bodily pain (item 3), decreased strength (item 8) and decreased energy (item 11), seemed to load on the same factor (factor 3). Two FA subscale items (8 “decreased energy” and 11 “decreased strength”) loaded more strongly on AC than its hypothesized factor. A new factor (factor 6) was found with the highest loading from two items assessing sleep (items 16 and 20). The items of EF, WO, AS and AC subscales loaded nicely on their hypothesized factors.

### Construct validity

Table 5 shows the correlations between the scores of the CLDQ and SF-36v2 Health Survey. As hypothesized, moderate to strong correlations were found between CLDQ FA and SF-36v2 VT scores; and between CLDQ SS and the SF-36v2 BP scores. The CLDQ AC score correlated significantly with all SF-36v2 Health Survey domain scores and the strongest was found with the SF-36v2 RP and SF scores. The CLDQ EF score correlated strongly not only with the SF-36v2 MH score but moderately with the SF-36v2 VT, RE, RP and GH scores.

### Sensitivity

As shown in Table 2, the CLDQ overall and subscale mean scores were all significantly higher in the uncomplicated than the complicated CHB group. The effect sizes of the group differences in the CLDQ scores all exceeded 0.4 (range 0.4-0.6). Only three of the eight SF-36v2 domain scores (PF, RP and SF) and the PCS

score detected a significant difference between the uncomplicated and the complicated groups. However, the greatest effect size difference between the two groups was found in the SF-36v2 RP score.

### Reliability

Across all subscales, the Cronbach's  $\alpha$  coefficients of the internal consistency reliability were higher than the recommended value of 0.7 (Table 5). ICC coefficients measuring the two-week test-retest reliability exceeded 0.7 in all but the AS (0.58) and AC (0.66) subscales. The reliability coefficients were comparable to those of the SF-36v2 Health Survey.

Table 5 also shows that the correlations (range 0.50-0.87) between the CLDQ subscales were smaller than the subscale internal reliability coefficients for all subscales, showing that each subscale measures a distinct concept. The overall CLDQ scores correlated strongly with all CLDQ subscale scores.

### DISCUSSION

The mean scores of the CLDQ found in our population were generally higher than those found in other countries. This might be the result of a sampling difference, in that over half of our subjects had uncomplicated CHB infection and most of the other studies included patients with more serious diseases and patients with HCV who tend to have more impairment in HRQOL than patients with CHB infection<sup>[24]</sup>. The other reason for a difference in the absolute HRQOL scores between different populations is a difference in the sociocultural norms. A comparison

**Table 3** Item descriptive statistics and item-subscale correlations of the Chinese (HK) CLDQ

Items	Mean	(SD)	Item-subscale correlations <sup>1</sup>						Success <sup>2</sup> (%)
			AS	FA	SS	AC	EF	WO	
<b>AS</b>									
1: Abdominal bloating	5.7	(1.6)	0.57 <sup>1</sup>	0.48	0.45	0.44	0.52	0.49	100
5: Abdominal pain	6.1	(1.4)	0.63 <sup>1</sup>	0.45	0.47	0.37	0.49	0.39	100
17: Abdominal discomfort	6.0	(1.5)	0.73 <sup>1</sup>	0.49	0.52	0.50	0.52	0.50	100
									(100)
<b>FA</b>									
2: Tiredness or fatigue	4.3	(1.6)	0.52	0.79 <sup>1</sup>	0.68	0.57	0.61	0.48	100
4: Feel sleepy during the day	4.4	(1.6)	0.35	0.70 <sup>1</sup>	0.55	0.45	0.49	0.36	100
8: Decreased strength	5.6	(1.7)	0.53	0.72 <sup>1</sup>	0.59	0.70	0.63	0.53	100
11: Decreased energy	5.0	(1.6)	0.40	0.77 <sup>1</sup>	0.59	0.61	0.66	0.47	100
13: Drowsiness	5.1	(1.7)	0.40	0.66 <sup>1</sup>	0.56	0.46	0.58	0.47	100
									(100)
<b>SS</b>									
3: Bodily pain	5.5	(1.7)	0.51 <sup>3</sup>	0.53 <sup>3</sup>	0.44 <sup>1</sup>	0.44 <sup>3</sup>	0.60 <sup>3</sup>	0.40	20
6: Shortness of breath	6.0	(1.5)	0.51	0.60 <sup>3</sup>	0.57 <sup>1</sup>	0.54	0.55	0.50	80
21: Muscle cramps	5.9	(1.4)	0.29	0.47	0.52 <sup>1</sup>	0.38	0.37	0.28	100
23: Dry mouth	4.7	(1.8)	0.37	0.47 <sup>3</sup>	0.47 <sup>1</sup>	0.41	0.51 <sup>3</sup>	0.54 <sup>3</sup>	40
27: Itching	5.4	(1.7)	0.30	0.49 <sup>3</sup>	0.49 <sup>1</sup>	0.41	0.42	0.41	80
									(64)
<b>AC</b>									
7: Not able to eat as much as you would like	5.6	(1.9)	0.42	0.59 <sup>3</sup>	0.46	0.56 <sup>1</sup>	0.48	0.47	80
9: Trouble in lifting or carrying heavy objects	5.8	(1.7)	0.40	0.51 <sup>3</sup>	0.55 <sup>3</sup>	0.42 <sup>1</sup>	0.52 <sup>3</sup>	0.40	40
14: Bothered by a limitation of the diet	6.0	(1.6)	0.37	0.47	0.44	0.65 <sup>1</sup>	0.50	0.50	100
									(73)
<b>EF</b>									
10: Anxiety	5.1	(1.8)	0.54	0.57	0.53	0.46	0.74 <sup>1</sup>	0.61	100
12: Unhappiness	5.2	(1.6)	0.43	0.58	0.52	0.44	0.72 <sup>1</sup>	0.52	100
15: Irritability	5.4	(1.5)	0.47	0.54	0.52	0.44	0.73 <sup>1</sup>	0.56	100
16: Difficulty in sleeping at night	5.1	(1.7)	0.45	0.46	0.46	0.45	0.59 <sup>1</sup>	0.48	100
19: Mood swings	5.5	(1.5)	0.46	0.60	0.57	0.50	0.79 <sup>1</sup>	0.54	100
20: Difficulty in falling asleep at night	4.5	(2.0)	0.36	0.49	0.50	0.45	0.52 <sup>1</sup>	0.41	100
24: Depression	5.5	(1.4)	0.42	0.56	0.52	0.41	0.76 <sup>1</sup>	0.60	100
26: Problems with concentration	5.4	(1.6)	0.44	0.69	0.61	0.61	0.69 <sup>1</sup>	0.61	100
									(100)
<b>WO</b>									
18: Worries about the impact of the liver disease	5.5	(1.8)	0.56	0.49	0.44	0.50	0.64	0.64 <sup>1</sup>	100
22: Worries that symptoms will develop into major problem	5.1	(1.8)	0.43	0.42	0.45	0.37	0.52	0.79 <sup>1</sup>	100
25: Worries that the condition is getting worse	5.2	(1.8)	0.44	0.49	0.54	0.49	0.59	0.85 <sup>1</sup>	100
28: Worries about never feeling any better	5.6	(1.7)	0.44	0.54	0.57	0.59	0.67	0.83 <sup>1</sup>	100
29: Availability of a liver for transplant	5.7	(2.0)	0.36	0.45	0.47	0.55	0.48	0.62 <sup>1</sup>	100
									(100)

<sup>1</sup>Spearman correlation between item and its hypothesized subscale corrected for overlap (relevant item removed from its subscale for correlation); <sup>2</sup>Level of scaling success, item-subscale correlation is higher for hypothesized subscale than competing subscale; <sup>3</sup>Item-subscale correlation is lower for hypothesized subscale than for competing subscale, but not statistically significant at the cutoff point of two standard errors (0.16).

with the population norms of generic HRQOL measures such as those of the SF-36 Health Survey will provide a more meaningful interpretation on the impact of CHB on HRQOL between different populations. Our study found that uncomplicated CHB patients had significant impairment in the SF-36v2 RP, BP, SF and RE domains, and complicated CHB patients had significantly lower SF-36v2 scores in six domains (PF, RP, BP, GH, SF and RE) than the norms of the HK population (Table 2)<sup>[25,26]</sup>. The findings suggested that CHB infection affected HRQOL only modestly unless complications develop. Surprisingly, there was no difference in the MH score between CHB patients and the HK population norm. It was unlikely that a potentially lethal chronic infection had no effect on mental health, the SF-36v2 Health Survey was probably not sensitive enough to detect the difference.

The high ceiling effects in the AS, AC and WO subscales in patients with uncomplicated CHB were

expected since they were usually asymptomatic. A pattern that was similar to that found in a Spanish population<sup>[9]</sup>. A high ceiling effect was also observed among patients with complicated CHB which was unexpected, this was probably because most of our subjects with complicated CHB were under anti-viral treatment that might have improved their HRQOL, or perhaps some patients had adjusted to their illnesses. On the whole, the CLDQ had a lower ceiling effect than the SF-36v2 Health Survey, suggesting that this disease-specific HRQOL measure would be more responsive than the generic measure in detecting improvements with treatment, which needs to be confirmed by prospective studies. The lack of floor effect indicates that the Chinese (HK) CLDQ would be able to capture any deterioration in patients' QOL as the disease progresses.

The item-subscale correlations and factor analysis results supported the scaling structure of the Chinese (HK)

**Table 4** Factor loadings of the Chinese (HK) CLDQ

Item	Factor 1 EF	Factor 2 WO	Factor 3 SS + FA	Factor 4 AS	Factor 5 AC	Factor 6 SL
Abdominal symptom (AS)						
1: Abdominal bloating	0.18	0.27	0.16	0.70	0.14	0.15
5: Abdominal pain	0.20	0.06	0.13	0.81	0.08	0.14
17: Abdominal discomfort	0.17	0.23	0.17	0.82	0.20	0.08
Fatigue (FA)						
2: Tiredness or fatigue	0.33	0.03	0.51	0.31	0.45	0.22
4: Feel sleepy during the day	0.28	-0.05	0.47	0.14	0.44	0.14
8: Decreased strength	0.37	0.14	0.37	0.24	0.60	0.24
11: Decreased energy	0.56	0.06	0.37	0.11	0.51	0.12
13: Drowsiness	0.49	0.09	0.47	0.15	0.36	-0.17
Systemic symptoms (SS)						
3: Bodily pain	0.49	0.00	0.18	0.39	0.14	0.22
6: Shortness of breath	0.22	0.22	0.59	0.39	0.18	0.03
21: Muscle cramps	0.09	0.08	0.76	0.17	0.10	0.14
23: Dry mouth	0.24	0.42	0.48	0.16	0.08	-0.02
27: Itching	0.10	0.27	0.65	-0.01	0.02	0.28
Activity (AC)						
7: Not able to eat as much as you would like	0.05	0.29	0.04	0.24	0.81	0.12
9: Trouble in lifting or carrying heavy objects	0.41	0.18	0.37	-0.05	0.35	0.17
14: Bothered by a limitation of the diet	0.11	0.43	0.09	0.10	0.67	0.12
Emotional function (EF)						
10: Anxiety	0.67	0.32	0.15	0.39	0.14	0.04
12: Unhappiness	0.77	0.25	0.15	0.20	0.13	0.01
15: Irritability	0.78	0.27	0.15	0.17	0.06	0.13
16: Difficulty in sleeping at night	0.27	0.17	0.17	0.18	0.14	0.79
19: Mood swings	0.78	0.24	0.10	0.19	0.20	0.21
20: Difficulty in falling asleep at night	0.16	0.14	0.25	0.22	0.25	0.71
24: Depression	0.8	0.31	0.15	0.08	0.03	0.22
26: Problems with concentration	0.52	0.28	0.29	0.18	0.37	0.27
Worry (WO)						
18: Worries about the impact of the liver disease	0.40	0.47	0.03	0.50	0.25	0.07
22: Worries that symptoms will develop into major problem	0.29	0.80	0.11	0.16	0.04	0.05
25: Worries that the condition is getting worse	0.29	0.84	0.13	0.17	0.17	0.09
28: Worries about never feeling any better	0.27	0.77	0.13	0.18	0.26	0.24
29: Availability of a liver for transplant	0.16	0.64	0.26	0.14	0.30	0.10

SL: Sleep.

**Table 5** Reliability and correlations of the Chinese (HK) CLDQ and SF-36v2 health survey scores

	AS	FA	SS	AC	EF	WO	Cronbach's $\alpha$	ICC <sup>1</sup>
<b>CLDQ</b>								
AS							0.84	0.58
FA	0.54						0.88	0.82
SS	0.54	0.72					0.74	0.86
AC	0.50	0.67	0.60				0.72	0.66
EF	0.58	0.72	0.68	0.61			0.90	0.86
WO	0.52	0.57	0.60	0.58	0.69		0.90	0.89
Overall	0.72	0.87	0.84	0.80	0.86	0.81	0.90	0.85
<b>SF-36v2 Health Survey</b>								
PF	0.47	0.56	0.65	0.54	0.47	0.46	0.81	0.93
RP	0.56	0.72	0.70	0.67	0.60	0.62	0.91	0.90
BP	0.44	0.44	0.62	0.42	0.48	0.44	0.89	0.77
GH	0.46	0.66	0.56	0.57	0.60	0.62	0.82	0.89
VT	0.51	0.79	0.63	0.58	0.67	0.54	0.86	0.85
SF	0.44	0.56	0.52	0.60	0.58	0.52	0.88	0.54
RE	0.53	0.51	0.52	0.46	0.66	0.50	0.89	0.74
MH	0.41	0.58	0.49	0.49	0.78	0.61	0.84	0.89

<sup>1</sup>All analyses were performed with the total sample of 150 patients, except for the Intra-class Correlation Coefficient (ICC), which was based on the results from 46 uncomplicated CHB patients.

CLDQ in general. However, the scaling success rates of items 3 (bodily pain), 23 (dry mouth) and 9 (trouble in

lifting or carrying heavy objects) seemed too low to be acceptable, raising the question whether they should be

**Table 6** Psychometric properties of the revised Chinese (HK) CLDQ subscales

Revised subscales	Mean	SD	% floor	% ceiling	Scaling success	Cronbach's $\alpha$	ICC
AS	5.9	1.3	0.0	37.3	100.0	0.84	0.58
SS + FA	5.2	1.1	0.0	4.7	95.0	0.84	0.88
AC	5.6	1.4	0.0	20.7	88.0	0.84	0.75
EF	5.3	1.3	0.0	12.0	93.3	0.92	0.87
WO	5.4	1.6	0.7	27.3	100.0	0.90	0.89
SL	4.8	1.7	1.3	17.3	100.0	0.78	0.79
Overall Scores	5.4	1.1	0.0	0.7	NA	0.89	0.85

grouped under other subscales than the originally hypothesized. It is interesting to note that bodily pain correlated the most with the EF subscale score and loaded the strongest on the EF factor (Table 4). It is a common observation that emotional state has a strong influence on pain perception and *vice versa*. Although the items on dry mouth or trouble in lifting or carrying heavy objects correlated more strongly with other subscales than their own, they should probably remain in the hypothesized subscale because the differences in the item-subscale correlations were not significant and the item-hypothesized subscale correlations were greater than 0.4. Furthermore, the factor loading results were not consistent with the results of the item-subscale correlations. The item "dry mouth" correlated most strongly with WO subscale score but the loading was the highest on the SS factor (0.48). The item "trouble in lifting or carrying heavy objects" correlated highest with the SS score but factor analysis showed that it loaded most strongly on the factor of EF.

The factor structure of the Chinese (HK) CLDQ version was almost identical to the original CLDQ in four subscales (EF, WO, AS and AC). The new factor of Sleep found in our Chinese population was also found in the Spanish, Italian and German population<sup>[9,10,27]</sup>. CHB patients may have sleep difficulties due to reasons other than emotional problems, such as pain and other symptoms. The items of the FA and SS subscales, except items 3, 8 and 11, loaded on one single factor since they all measure symptoms. Items 8 and 11 of the FA subscale loaded on the AC factor. Factor analysis with promax rotation was also performed to cross-validate the factor structure obtained by the varimax rotation, and it showed similar results with a new factor assessing sleep and items of the FA subscale loaded mostly on the AC factor instead of a separate factor.

An alternative scaling structure for the Chinese (HK) CLDQ based on the factor loading results could be formed. Items 16 (difficulty in sleeping) and 20 (difficulty in falling asleep at night) were grouped into a new Sleep subscale. Items 8 (decreased strength) and 11 (decreased energy) were grouped into the AC subscale. Items 2, 4 and 13 of the original FA subscale are grouped with items of the SS subscale to form the new SS subscale. Item 3 (bodily pain), although loaded most strongly and correlated the most with EF factor, remains in the SS subscale because this has better face validity. The psychometric properties of the revised Chinese (HK) CLDQ subscales with regrouping of the items are shown in Table 6. It can be seen that the new subscale structure greatly improves the scaling success rates of the SS and AC items, although it

reduces the success rate of the EF subscale slightly. The new scaling structure also reduced the ceiling effects of the SS and AC subscales. Further studies are needed to determine whether the revised subscale structure will translate into better sensitivity and responsiveness in clinical applications. Until such data are available, the original subscale structure of the CLDQ is recommended to allow better international comparability.

The expected correlations between the CLDQ and SF-36v2 Health Survey domains were observed confirming convergent construct validity. The correlation with the SF-36v2 RE domain was higher in the CLDQ EF than the AC subscale because conceptually the SF-36v2 RE measures the effect of emotional problems on daily activities.

The CLDQ subscales of AS and WO address domains that are not assessed by the generic measure (SF-36v2 Health Survey) and detected significant differences between the two groups of CHB patients. There were significant differences in the WO and EF subscales of the CLDQ between the CHB groups although this was not found in most of the mental-health related domains (RE, MH and MCS) of the SF-36v2 Health Survey, suggesting that the Chinese (HK) CLDQ was more sensitive than the generic measure in detecting the emotional impact of CHB. It is worth noting that although more domains in the CLDQ showed a significant difference between the complicated and uncomplicated CHB groups, the largest effect size difference was found in the SF-36v2 RP domain indicating that a disease-specific measure may not always be more sensitive than a generic measure. The two types of HRQOL measures should complement each other in the evaluation of the HRQOL of CHB patients.

Internal consistency and test-retest reliability were acceptable for all subscales. Test-retest reliability (ICC) of the AS subscale was relatively low (0.58) probably because these symptoms could fluctuate from day to day and pain intensity might vary noticeably in a relatively short period of time. Reliability of the CLDQ in our study was generally higher than those found in other studies (0.46-0.95)<sup>[9,12]</sup>. The SS subscale had very good test-retest reliability (ICC 0.86) in our population. The very low ICC (0.23) found in the US study was likely the result of an inappropriately long retest interval of six months<sup>[3]</sup>.

Our study administered the Chinese (HK) CLDQ using an interviewer since our populations had a relatively low literacy level. The performance of the instrument by self-completion will need to be confirmed by further studies. The responsiveness of the Chinese (HK) CLDQ in detecting changes with disease progression or anti-viral treatment will also need to be determined.

The Chinese (HK) CLDQ was validated in content and construct. It had satisfactory psychometric properties in terms of factor structure, scaling assumption, construct validity, reliability and sensitivity in Southern Chinese patients with CHB infection. It was more sensitive than the SF-36v2 Health Survey in detecting the impact of CHB on mental-health and symptom related HRQOL. The Chinese (HK) CLDQ should be applicable to all Cantonese-speaking Chinese in HK and other parts of Southern China. It is also likely to be applicable to the majority of Chinese populations in Australia, North America, and Europe who are mostly emigrants from HK. There was good equivalence in the score distribution pattern across several cultures indicating that it can be used as a cross-cultural HRQOL measure in multiethnic populations or global studies. Some modifications of the scaling structure of the CLDQ may improve its psychometric properties for CHB patients, which need to be explored by further clinical studies.

#### Appendix: Back-translation of Chinese (HK) CLDQ

##### Original Wording

This questionnaire is designed to find out how you have been feeling during the last 2 wk. You will be asked about you symptoms related to your liver disease, how you have been affected in doing activities, and how your mood has been. Please complete all of questions and select only one response for each question.

1 How much of the time during the last 2 wk have you been troubled by a feeling of abdominal bloating?

All of the time

Most of the time

A good bit of the time

Some of the time

A little of the time

Hardly any of the time

None of the time

2 How much of the time have you been tired or fatigued during the last 2 wk?

3 How much of the time during the last 2 wk have you experienced bodily pain?

4 How often during the last 2 wk have you felt sleepy during the day?

5 How much of the time during the last 2 wk have you experienced abdominal pain?

6 How much of the time during the last 2 wk has shortness of breath been a problem for you in your daily activities?

7 How much of the time during the last 2 wk have you not been able to eat as much as you would like?

8 How much of the time in the last 2 wk have you been bothered by having decreased strength?

9 How often during the last 2 wk have you had trouble lifting or carrying heavy objects?

10 How often during the last 2 wk have you felt anxious?

11 How often during the last 2 wk have you felt a decreased level of energy?

##### Backward Translation

The purpose of this questionnaire is to understand how you felt in the past 2 wk.

The questions are about the symptoms resulting from your liver illness and how these symptoms affect your participation in activities, and your emotions.

Please answer all questions. You can only choose one answer for each question.

1 In the past 2 wk, how much time you have been bothered by your bloating problem?

All the time

Most of the time

Quite Often

Sometimes

A Short Time

Hardly Any

Never

2 In the past 2 wk, how much time did you feel tired or exhausted?

3 In the past 2 wk, how much time did your body ache?

4 In the past 2 wk, how often did you feel sleepy during the daytime?

5 In the past 2 wk, how much time did you have abdominal pain?

6 In the past 2 wk, how much time were your daily activities affected by your shortness of breath?

7 In the past 2 wk, how much time were you unable to eat as much as you want?

8 In the past 2 wk, how much time have you been bothered by the decline in your physical energy?

9 In the past 2 wk, how often did you find it difficult when you were lifting or carrying heavy objects?

10 In the past 2 wk, how often did you feel anxious?

11 In the past 2 wk, how often did you find your energy level decreasing?

12 How much of the time during the last 2 wk have you felt unhappy? 12 In the past 2 wk, how much time did you feel unhappy?

13 How often during the last 2 wk have you felt drowsy? 13 In the past 2 wk, how often did you feel sleepy?

14 How much of the time during the last 2 wk have you been bothered by a limitation of your diet? 14 In the past 2 wk, how much time have you been bothered by your restricted diet?

15 How often during the last 2 wk have you been irritable? 15 In the past 2 wk, how often did you become irritable?

16 How much of the time during the last 2 wk have you had difficulty sleeping at night? 16 In the past 2 wk, how much time did you find it difficult to sleep at night?

17 How much of the time during the last 2 wk have you been troubled by a feeling of abdominal discomfort? 17 In the past 2 wk, how much time have you been bothered by your abdominal discomfort?

18 How much of the time during the last 2 wk have you been worried about the impact your liver disease has on your family? 18 In the past 2 wk, how much time did you worry that your liver illness will affect your family?

19 How much of the time during the last 2 wk have you had mood swings? 19 In the past 2 wk, how much time did your emotions fluctuate?

20 How much of the time during the last 2 wk have you been unable to fall asleep at night? 20 In the past 2 wk, how much time were you unable to sleep until sunrise?

21 How often during the last 2 wk have you had muscle cramps? 21 In the past 2 wk, how often did your muscle cramp?

22 How much of the time during the last 2 wk have you been worried that your symptoms will develop into major problems? 22 In the past 2 wk, how much time did you worry that your symptoms will become a serious problem?

23 How much of the time during the last 2 wk have you had a dry mouth? 23 In the past 2 wk, how much time did you have dry mouth?

24 How much of the time during the last 2 wk have you felt depressed? 24 In the past 2 wk, how much time did you feel depressed?

25 How much of the time during the last 2 wk have you been worried about your condition getting worse? 25 In the past 2 wk, how much time did you worry that your condition will deteriorate?

26 How much of the time during the last 2 wk have you had problems concentration? 26 In the past 2 wk, how much time did you find it hard to concentrate?

27 How much of the time have you been troubled by itching during the last 2 wk? 27 In the past 2 wk, how much time have you been bothered by itchiness?

28 How much of the time during the last 2 wk have you been worried about never feeling any better? 28 In the past 2 wk, how much time did you worry that your health condition will not improve?

29 How much of the time during the last 2 wk have you been concerned about the availability of a liver if you need a liver transplant? 29 In the past 2 wk, how much time have you worried that you could not get a liver if you have to undergo a liver transplant?

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#### COMMENTS

##### Background

Chronic hepatitis B (CHB) virus infection remains a global problem and a public health threat. CHB patients may suffer or die from liver-related complications, posing a threat to both mental and physical health, leading to impairment of quality of life.

##### Research frontiers

Health-related quality of life (HRQOL) outcomes should supplement traditional clinical outcomes in the evaluation of the impact and the effectiveness of treatment for patients with CHB infection.

### Innovations and breakthroughs

The Chronic Liver Disease Questionnaire (CLDQ) had been applied mainly to patients with hepatitis C virus infection in Western countries. This study was the first to show that a Chinese (Hong Kong) translation of the CLDQ was valid, reliable and sensitive for Southern Chinese patients with CHB infection. The CLDQ can be applied to millions of Southern Chinese CHB patients to evaluate their HRQOL. Some modifications might further improve its validity, reliability and sensitivity.

### Applications

The Chinese (Hong Kong) CLDQ can be used to evaluate the impact of CHB virus infection and assess the effectiveness of anti-viral drug treatments in Cantonese-speaking Southern Chinese. The CLDQ can be used as a cross-cultural HRQOL measure in international studies that include Southern Chinese.

### Terminology

CHB virus infection refers to those who are hepatitis B surface antigen-positive for more than six months. Validity is defined as the extent to which a test measures what it is intended to measure. Reliability refers to the consistency or stability of the measurement process across time, patients or observers.

### Peer review

The authors validated and tested the psychometric properties of a Southern Chinese translation of the CLDQ and determined that their questionnaire was valid, reliable, and sensitive for southern Chinese patients with hepatitis B virus infection. The study was well done and used appropriate methodology to validate and test the questionnaire.

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BRIEF ARTICLES

# Liver and spleen volume variations in patients with hepatic fibrosis

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morphological index of patients without liver cirrhosis. The SV is related to the severity of fibrosis, and the spleen of patients with advanced fibrosis is enlarged evidently. The LV/SV ratio and SLV/SV ratio are of a significant clinical value in the diagnosis of advanced liver fibrosis.

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**Key words:** Hepatic fibrosis; Volume measurement; Liver volume; Standard liver volume; Spleen volume; Liver volume/spleen volume ratio; Standard liver volume/spleen volume ratio

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Liu P, Li P, He W, Zhao LQ. Liver and spleen volume variations in patients with hepatic fibrosis. *World J Gastroenterol* 2009; 15(26): 3298-3302 Available from: URL: <http://www.wjgnet.com/1007-9327/15/3298.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.3298>

## Abstract

**AIM:** To study the liver and spleen volume variations in hepatic fibrosis patients at different histopathological stages.

**METHODS:** Multidetector computed tomography (MDCT) scan was performed in 85 hepatic fibrosis patients. Liver volume (LV) and spleen volume (SV) were measured. Fifteen healthy individuals served as a control group (S0). The patients were divided into stage 1 (S1) group ( $n = 34$ ), stage 2 (S2) group ( $n = 25$ ), stage 3 (S3) group ( $n = 16$ ), and stage 4 (S4) group ( $n = 10$ ) according to their histopathological stage of liver fibrosis.

**RESULTS:** The LV and standard LV (SLV) had a tendency to increase with the severity of fibrosis, but no statistical difference was observed in the 5 groups (LV:  $F = 0.245$ ,  $P = 0.912$ ; SLV:  $F = 1.902$ ,  $P = 0.116$ ). The SV was gradually increased with the severity of fibrosis, and a statistically significant difference in SV was observed among the 5 groups ( $P < 0.01$ ). The LV/SV ratio and SLV/SV ratio were gradually decreased with the aggravation of hepatic fibrosis, and statistically significant differences in both LV/SV and SLV/SV were found among the 5 groups ( $P < 0.01$ ).

**CONCLUSION:** The absence of obvious LV reduction in patients with chronic liver disease may be a

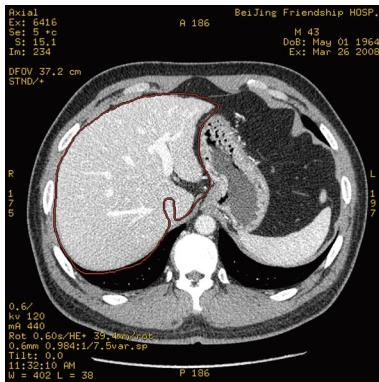
## INTRODUCTION

Hepatic fibrosis is an important histopathological stage of chronic liver disease, and can progress to hepatocirrhosis. Studies have shown that hepatic fibrosis can be reversed<sup>[1,2]</sup>. Therefore, hepatic fibrosis can be halted if it is early diagnosed and treated with intervention therapy. Liver biopsy is the golden diagnosis standard for hepatic fibrosis and cirrhosis, but it cannot be used widely and repeatedly due to its invasive nature. This study was to observe the liver volume (LV) and spleen volume (SV) variations in patients with hepatic fibrosis by 64-row multidetector computed tomography (MDCT).

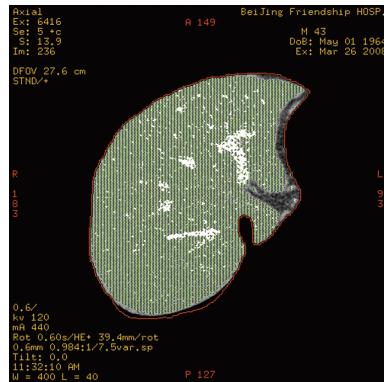
## MATERIALS AND METHODS

### Patients

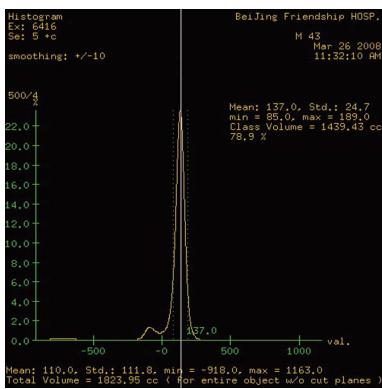
Eighty-five patients (66 males and 19 females, mean age 41 years) with liver fibrosis in our hospital between November 2007 and September 2008 were included in this study. All of them had chronic hepatitis B. The patients were divided into stage 1 (S1) group ( $n = 34$ ),



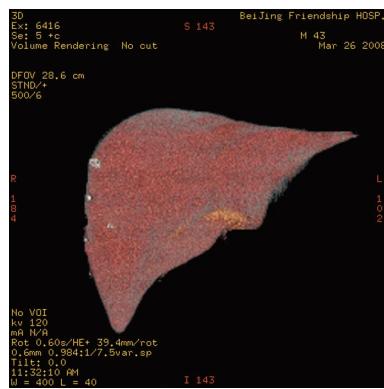
**Figure 1** Profile of the liver with an interval of several sections for the separation of liver from other organs and their tissues around.



**Figure 2** Regulation of threshold value for exclusion of gallbladder, main liver blood vessels and fat.



**Figure 3** Measurement of liver volume.



**Figure 4** Volume rendering image of liver after reconstruction.

stage 2 (S2) group ( $n = 25$ ), stage 3 (S3) group ( $n = 16$ ), and stage 4 (S4) group ( $n = 10$ ) according to their histopathological stage of fibrosis. The patients had no liver and spleen tumor. Fifteen patients (10 males and 5 females, mean age 35.5 years) with no history of liver and spleen disease served as a control group (S0). They underwent abdominal computed tomography (CT) scan for liver disease. Routine abdominal CT scanning showed no liver, bile and spleen disease. Liver test was normal and antigen test was negative for hepatitis B and C.

#### Diagnostic method

All patients with chronic hepatitis B were diagnosed according to the results of pathological examination of liver biopsy. The patients were divided into four groups according to the diagnostic criteria for virus hepatitis as previously described<sup>[3]</sup>.

#### Imaging parameters

Abdominal CT scan was performed with a 64-row MDCT scanner (GE LightSpeed VCT). Protocols included a non-enhancement scan and an enhancement scan of the upper abdomen from the dome of diaphragm to the entire liver and spleen (5 mm thickness, 80-120 mAs, and 120 kVp). All patients received an injection of 100 mL iopromide (300 mg I/mL; Ultravist, Schering) through a peripheral vein (generally an

antecubital vein) at a flow rate of 4.0 mL/s. The arterial phases were tracked with a scanning monitor and a time delay of 25 s after arterial phases was applied in all patients for the venous phases. Bolus-injection technique was used to administer contrast material with a power injector. All patients underwent CT scan at a supine position during a single breath.

#### Measurement of LV and SV

After scanning, 5 mm portal vein phase reconstruction images obtained from the raw data were divided (0.625 mm) and transferred to an interactive workstation (AW4.2 or AW4.3) to measure LV and SV. The profile of liver was outlined manually to exclude the inferior vena cava with an interval of several sections. Gallbladder, main liver blood vessels and fat were excluded by regulating the threshold value. The enclosed liver parenchymal area was then calculated automatically<sup>[4,5]</sup> and expressed as the LV (Figures 1-4).

Body weight (BW) and body height (BH) recorded at the time of CT examination were used to calculate the body surface area (BSA) following the equation:  $BSA (m^2) = [0.0071 \times BH (cm) + 0.0133 \times BW (kg) - 0.1971]$ . LV is significantly related with BW, BH, and BSA as described elsewhere<sup>[6,7]</sup>. Therefore, standard liver volume (SLV) was measured following the equation:  $SLV (cm^3/m^2) = LV (cm^3)/BSA (m^2)$ .

The measurement of SV and LV was similar. Since

**Table 1 Comparison of related volumes in patients with liver fibrosis and healthy controls (mean  $\pm$  SD)**

Group	Patients ( <i>n</i> )	Liver volume (cm <sup>3</sup> )	Standard liver volume (cm <sup>3</sup> )	Spleen volume (cm <sup>3</sup> )	Liver/spleen volume ratio	Standard liver/spleen volume ratio
Control	15	1133.39 $\pm$ 131.84	637.98 $\pm$ 65.93	190.94 $\pm$ 70.37 <sup>b,d</sup>	6.70 $\pm$ 2.56 <sup>b,d</sup>	3.81 $\pm$ 1.62 <sup>b,d,e</sup>
Stage 1	34	1117.33 $\pm$ 190.37	575.96 $\pm$ 73.82	213.20 $\pm$ 77.30 <sup>b,d</sup>	5.84 $\pm$ 2.05 <sup>b,d</sup>	3.05 $\pm$ 1.14 <sup>a,d</sup>
Stage 2	25	1158.30 $\pm$ 229.10	592.49 $\pm$ 65.31	253.53 $\pm$ 113.43 <sup>b,d</sup>	5.13 $\pm$ 1.55 <sup>d</sup>	2.69 $\pm$ 0.96 <sup>c</sup>
Stage 3	16	1168.23 $\pm$ 202.82	607.35 $\pm$ 69.81	358.67 $\pm$ 154.63	3.83 $\pm$ 1.77	2.00 $\pm$ 0.87
Stage 4	10	1126.40 $\pm$ 271.38	590.11 $\pm$ 114.70	479.65 $\pm$ 181.56	2.67 $\pm$ 1.12	1.39 $\pm$ 0.53
<i>F</i> value		0.245	1.902	12.383	9.702	9.784
<i>P</i> value		0.912	0.116	0.000	0.000	0.000

<sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 vs S3; <sup>c</sup>*P* < 0.05, <sup>d</sup>*P* < 0.01 vs S4; <sup>e</sup>*P* < 0.05 vs S2.

SV does not correlate with BW, BH and body mass index (BMI)<sup>[8-10]</sup>, it is not necessary to make the SV standard.

Then, the LV/SV ratio and SLV/SV ratio were calculated.

### Statistical analysis

Results were expressed as mean  $\pm$  SD. One way analysis of variance(ANOVA) was used for multiple mean comparisons. Bonferroni correction was used for comparison of two samples. If heterogeneity of variance was found, a log transformation was used to normalize the data distribution, and ANOVA was run with an equal variance. *P* < 0.05 was considered statistically significant. Statistical analysis was performed using SPSS (version 14.0, SPSS).

## RESULTS

The total LV and SLV were 1133.39  $\pm$  131.84 cm<sup>3</sup> and 637.98  $\pm$  65.93 cm<sup>3</sup> in the control group. The LV (*F* = 0.245) and SLV (*F* = 1.902) in patients with hepatic fibrosis tended to increase with the severity of fibrosis, but no statistically significant difference was observed among the 5 groups (Table 1).

A log transformation was used to normalize the data distribution and ANOVA was run with an equal variance due to the heterogeneity of variance in SV. The SV was larger in patients with liver fibrosis than in control group and increased gradually with the severity of fibrosis (Table 1). There was a significant difference among the 5 groups (*F* = 12.383, *P* = 0.000). Statistically significant differences were observed between S3, S4 and other groups(S0, S1 and S2, *P* < 0.01).

The LV/SV ratio and SLV/SV ratio decreased gradually with the severity of fibrosis (Table 1). The differences in LV/SV (*F* = 9.702, *P* = 0.000) and SLV/SV (*F* = 9.784, *P* = 0.000) were significant among the 5 groups. Statistically significant differences in LV/SV were observed between S3 and S0, S1, and between S4 and S0, S1, S2 (*P* < 0.01). There were statistically significant differences in SLV/SV between S3 and S0, S4 and S0, S1 (*P* < 0.01), and between S2 and S0, S3 and S1, S4 and S2 (*P* < 0.05).

## DISCUSSION

Measurement of LV and SV is a mature technique<sup>[4,5,10-12]</sup>.

The method, which combines profile outline and threshold value regulation, is convenient with a good veracity and repeatability<sup>[5]</sup>. Liver and spleen vary in size among different individuals<sup>[13,14]</sup>. LV is obviously related with BW, BH, and BSA in normal persons<sup>[15-17]</sup>. BSA is calculated according to BW and BH, and therefore can equalize the difference in BW and BH. It is more reasonable to choose SLV (per BSA volume) to analyze LV change in hepatic fibrosis patients at different histopathological stages. Because SV does not correlate with BW, BH, and BMI<sup>[8-10]</sup>, there is no need to make the spleen volume standard.

Reports are available on LV changes in patients with hepatic fibrosis<sup>[18,19]</sup>. Chen *et al*<sup>[18]</sup> reported that the left, right and caudate lobe volumes tend to increase with the aggravation of inflammatory activity and the severity of fibrosis, with significant changes in vertical diameter and volume index of the left lobe but no obvious change in the right and caudate lobe volume. Tarao *et al*<sup>[19]</sup> reported that the LV is significantly larger in grade 2 alcoholic liver fibrosis patients than in grade 1 alcoholic liver fibrosis patients and healthy controls, and the LV is larger in grade 3 alcoholic liver fibrosis patients than in grade 2 alcoholic liver fibrosis patients, suggesting that LV in patients with alcoholic liver fibrosis increases gradually with the severity of fibrosis. The results of this study show that the LV and SLV in patients with liver fibrosis tended to increase with the severity of fibrosis from S1 to S3, but decrease in S4, indicating that LV tends to increase gradually with the severity of chronic liver diseases or fibrosis. Since the number of hepatic cells accounts for 70%-80% of the liver parenchyma, and the reserved liver function is closely related to the total functional hepatic cells, LV measurement can partly show changes in hepatic cells and stages of liver fibrosis or cirrhosis, and thus can be used as an indicator of liver function. It has been shown that LV significantly correlates with Child-Pugh classification of cirrhosis<sup>[5,20,21]</sup>. In this study, LV and SLV changes were observed in patients with fibrosis (*P* > 0.05), which may be attributed to the limited number of cases. On the other hand, it may indicate that hepatic fibrosis has not yet led to obvious liver volume reduction and the decrease of hepatic cellular components.

It has been reported that changes in SV are related to the severity of fibrosis<sup>[19,22,23]</sup>. Tarao *et al*<sup>[19]</sup> showed that SV is significantly larger in grade 3 hepatic fibrosis

patients than in grade 1 and 2 hepatic fibrosis patients and healthy controls, indicating that SV in patients with hepatic fibrosis at an early stage is inconspicuously changed, but conspicuously at the advanced stage. Hoefs *et al*<sup>[22]</sup> reported that different percentages of SV above the upper normal limits can be noted in patients with histologically proven liver disease: no fibrosis (10%), or mild- moderate fibrosis (36.7%), early liver cirrhosis (52%), and advanced liver disease (75%), indicating that SV is closely related with the severity of liver fibrosis and cirrhosis. Ding *et al*<sup>[23]</sup> reported that SV is closely correlated with the histopathological stage of hepatic fibrosis and cirrhosis. In their study, the SV increased gradually in patients with hepatic fibrosis compared with normal subjects, and the differences between S3,S4 and the others were significant ( $P < 0.01$ ).

In our study, the SV was larger in patients with liver fibrosis and increased gradually with the severity of fibrosis, and statistically significant differences were seen between S3, S4 and other groups ( $P < 0.01$ ), suggesting that SV in patients with advanced fibrosis increases obviously compared with normal persons and patients with early fibrosis.

The underlying mechanism may be that extensive fibrosis with formation of fibrous septum occurs at the advanced stages of fibrosis, thus leading to portal congestion and hypertension, obstruction of spleen vein and splenomegaly.

It has been shown that the SV/LV ratio and SLV/SV ratio are significantly different between normal individuals and liver cirrhosis patients. The SV/LV ratio is considered a better index for the diagnosis of liver cirrhosis due to its minor coefficient of variation<sup>[11]</sup>. It was reported that the SV/LV ratio is of prognostic importance in patients with primary biliary cirrhosis<sup>[24]</sup>. The SV/LV ratio and SLV/SV ratio, calculated in this study, were  $6.70 \pm 2.56$  and  $3.81 \pm 1.62$ , respectively in the control group, and decreased gradually with the aggravation of fibrosis in the other groups. The differences in LV/SV between S3 and S0, S1, and between S4 and S0, S1, S2 were statistically significant ( $P < 0.01$ ). The differences in SLV/SV between S3 and S0, between S4 and S0, S1 and between S3 and S1, S4, S2 were statistically significant ( $P < 0.01$ ), indicating that the SV/LV ratio and SLV/SV ratio are smaller in patients with advanced hepatic fibrosis than in patients with early hepatic fibrosis and normal persons, which may contribute to the diagnosis of advanced fibrosis.

Liver biopsy is the golden standard for the differential diagnosis of liver fibrosis from cirrhosis. However, some CT image findings may be useful in pathologic staging of liver fibrosis and cirrhosis. Studies have shown that LV is smaller in cirrhosis patients than in normal individuals<sup>[5,11,20,21]</sup>. Since this study showed that there was no statistical difference in LV between the fibrosis and control groups, no obvious LV reduction in patients with chronic liver disease may be a morphological and predictive index for the absence of liver cirrhosis. However, its accuracy needs to be further evaluated.

The SV range is wide in normal cases<sup>[12]</sup>, and can be easily influenced by factors, such as venous congestion and disorders of the hematological system. Therefore, using SV as a dependable index for the severity of liver fibrosis needs to be further studied. However, SV can be used as a reference index based on the fact that changes in SV are related to the severity of liver fibrosis. The significance of changes in LV/SV ratio and SLV /SV ratio is similar to that of SV, because variations in LV/SV ratio and SLV/ SV ratio mainly depend on the increased SV.

When the LV and SV are measured, some other parameters, such as main portal and spleen vein diameter, and collateral circulation of portal vein, can be detected and evaluated, all of which are the indirect signs of liver disease and portal hypertension. However, changes in LV are a direct sign of the severity of chronic liver disease.

## COMMENTS

### Background

Hepatic fibrosis is an important histopathological stage of chronic liver disease, and can progress to liver cirrhosis. Its diagnosis and staging remain difficult.

### Research frontiers

Liver biopsy is considered the golden diagnosis standard for hepatic fibrosis and cirrhosis, but it is an invasive procedure. Changes in liver volume (LV) and spleen volume (SV) are the morphological index of chronic liver disease.

### Innovations and breakthroughs

Eighty-five cases of liver fibrosis underwent 64-row multidetector computed tomography (MDCT) scanning, and their LV and SV were measured. The results showed that the LV was not obviously reduced in liver fibrosis and cirrhosis patients, indicating that which may be a morphological and predictive index for the absence of liver cirrhosis.

### Applications

Measurement of LV and SV with a 64-row MDCT is a noninvasive method to display the morphological changes in liver fibrosis and can contribute to the clinical diagnosis and treatment of chronic liver disease.

### Peer review

The measurement of LV and SV with MDCT is a noninvasive method to estimate the severity of chronic liver disease, and is of potential usefulness in differentiating liver fibrosis from cirrhosis and in staging of liver fibrosis. Further study is needed to evaluate its accuracy.

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## Adult intussusception: A retrospective review of 41 cases

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Pathologically, 54.5% of the intussusceptions had a tumor, of which 27.3% were malignant. 9.1% comprised nontumorous polyps. Four intussusceptions had a gastrojejunostomy with intestinal intubation, and four intussusceptions had no organic lesion.

**CONCLUSION:** CT is the most effective and accurate diagnostic technique. Colonoscopy can detect most lead point lesions of non-enteric intussusceptions. Intestinal intubation should be avoided.

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**Key words:** Adult intussusception; Diagnosis; Management; Computed tomography scan; Intestinal intubation

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### Abstract

**AIM:** To optimize the preoperative diagnosis and surgical management of adult intussusception (AI).

**METHODS:** A retrospective review of the clinical features, diagnosis, management and pathology of 41 adult patients with postoperative diagnoses of intussusception was conducted.

**RESULTS:** Forty-one patients with 44 intussusceptions were operated on, 24.4% had acute symptoms, 24.4% had subacute symptoms, and 51.2% had chronic symptoms. 70.7% of the patients presented with intestinal obstruction. There were 20 enteric, 15 ileocolic, eight colocolonic and one sigmoidorectal intussusceptions. 65.9% of intussusceptions were diagnosed preoperatively using a computed tomography (CT) scan (90.5% accurate) and ultrasonography (60.0% accurate, rising to 91.7% for patients who had a palpable abdominal mass). Coloscopy located the occupying lesions of the lead point of ileocolic, colocolonic and sigmoidorectal intussusceptions. Four intussusceptions in three patients were simply reduced. Twenty-one patients underwent resection after primary reduction. There was no mortality and anastomosis leakage perioperatively. Except for one patient with multiple small bowel adenomas, which recurred 5 mo after surgery, no patients were recurrent within 6 mo.

### INTRODUCTION

Intussusception is defined as the telescoping of a segment of the gastrointestinal tract into an adjacent one. Intussusception is uncommon in adults compared with the pediatric population. It is estimated that only 5% of all intussusceptions occur in adults and approximately 5% of bowel obstructions in adults are the result of intussusception<sup>[1]</sup>. Adult intussusception (AI) often presents with nonspecific symptoms. Preoperative diagnosis remains difficult and the extent of resection, and whether the intussusception, should be reduced remains controversial<sup>[1]</sup>. The present study reviews our experience of AI, and discusses the optimal preoperative diagnosis and surgical management techniques.

### MATERIALS AND METHODS

The medical records of 41 adult patients (18 years of age and older) with a postoperative diagnosis of intussusception at the First Affiliated Hospital, China Medical University, from January 2001 to August 2008, were collected. The clinical features, diagnosis,

management and pathology of the 41 patients were reviewed.

An intussusception that involved only the jejunum or ileum was considered an enteric intussusception. An intussusception that involved the ileum and the colon was designated an ileocolic intussusception. An intussusception that involved only the colon was considered a colocolonic intussusception and one that involved the sigmoid colon and rectum was considered a sigmoidorectal intussusception<sup>[1]</sup>. A proximal segment of the bowel telescoped into the lumen of the adjacent distal segment was defined as antegrade intussusception. A distal segment of the bowel telescoped into the lumen of the adjacent proximal segment was defined as retrograde intussusception<sup>[2]</sup>.

Acute symptoms were defined as < 4 d, subacute symptoms were defined as 4–14 d, and chronic symptoms were defined as > 14 d<sup>[3]</sup>.

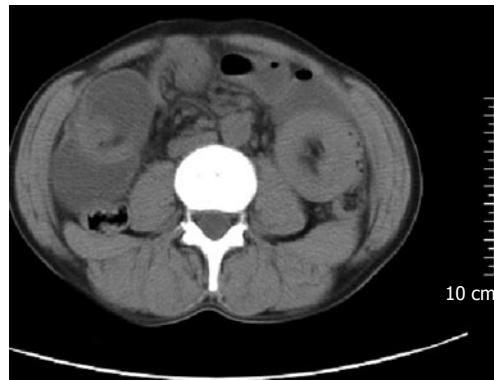
Intussusception was preoperatively diagnosed by abdominal ultrasonography with the target and doughnut signs on transverse view and the pseudokidney sign in the longitudinal view<sup>[1]</sup>. Intussusception was preoperatively diagnosed by multi-slice spiral computed tomography (CT) scans with the characteristic target or sausage sign, edematous bowel wall and mesentery in the lumen<sup>[4,5]</sup>.

## RESULTS

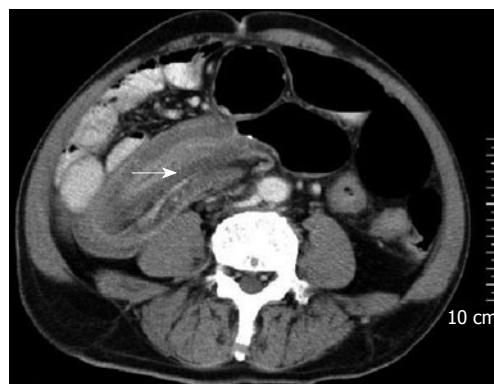
Of all the 41 patients, there were 18 males with an average age of 41.3 (15–71) and 23 females with an average age of 47.0 (18–87). The male:female ratio was 1:1.3. Three (7.3%) patients had two intussusceptions. In all, 44 intussusceptions were diagnosed, of which 20 were enteric intussusceptions (45.5%), 15 were ileocolic intussusceptions (34.1%), eight were colocolonic intussusceptions (18.2%) and one was a sigmoidorectal intussusception (2.3%). Forty-three intussusceptions were antegrade (97.7%) and only one enteric intussusception was retrograde (2.3%) (Table 1).

Of the 41 patients, 95.1% (39/41) had abdominal pain, 26.8% (11/41) had bloody stool, and 34.1% (14/41) had a palpable abdominal mass. This classic pediatric presentation triad was only seen in 9.8% (4/41). 70.7% (29/41) presented with intestinal obstructions of various extents. The duration of the symptoms varied from six hours to three years; 24.4% (10/41) with acute symptoms, 24.4% (10/41) with subacute symptoms, and 51.2% (21/41) with chronic symptoms.

Of the 41 patients, 65.9% (27/41) were preoperatively diagnosed with intussusception. Thirty patients had ultrasonography, of which 18 were diagnosed with intussusception (60.0% accuracy). However, the preoperative diagnostic accuracy of the patients who had palpable abdominal masses was 91.7% (11/12). Twenty-one patients had helical CT scans, of which 19 were diagnosed with intussusception (90.5% accuracy) (see Table 1, Figures 1–4). None of the patients who had experienced gastrojejunostomy and underwent an upper gastrointestinal water-soluble contrast study, were



**Figure 1** A 44-year-old man with two enteric intussusceptions due to multiple adenoma carcinomas. The intussusceptions appear as round target-shaped masses with a hypodense area of fat density close to its centre, the mesenteric fat. The beam is perpendicular to the axis of the intussusceptions.



**Figure 2** A 64-year-old man with an ileocolic intussusception due to a ileum B cell malignant lymphoma. A sausage-shaped mass with high density soft tissue above represents the edematous bowel wall of the intussuscipiens and the intussupceptum, with fat density below, representing mesenteric fat. The higher linear density within the mesenteric fat (arrow) is mesenteric blood vessels. This appearance is caused by the axis of the intussusception being parallel with the computed tomography (CT) beam.

diagnosed intussusception. One patient who had a small intestinal lipoma and underwent capsule endoscopy was diagnosed with regional mucosa puffiness. Eight patients underwent a colonoscopy. The etiologies were found in most of them by colonoscopy (Table 2).

Four intussusceptions in three patients, including two patients who had undergone gastrojejunostomy with an intestinal tube, and one patient with a retrograde idiopathic enteric intussusception, were simply reduced. One patient with a mobile cecum underwent appendectomy and cecum immobilization after primary reduction. Eighteen patients underwent segmental resection of the small bowel, 17 underwent a right hemicolectomy, one underwent a left hemicolectomy, and one patient with a sigmoidorectal intussusception underwent a segmental sigmoidectomy. One patient with multiple small and large intestinal adenomas underwent segmental resection of the small and large bowel. Of the 41 patients, 21 underwent resection after primary reduction (Table 1).

Of the 20 enteric intussusceptions, four of them (20%) underwent a simple reduction, nine (45%) had a

**Table 1** Preoperative diagnosis and treatment of 41 cases of adult intussusception (AI)

Age (yr)	Sex	US <sup>1</sup>	CT <sup>1</sup>	Histopathology	Type	Reduction <sup>2</sup>	Surgery
23	M	-	N	Small intestine hamartoma	Ileocolic	Y	Small intestine segmental resection
58	F	-	-	Intestinal inflammatory disease	Enteric	F	Small intestine segmental resection
41	M	N	-	Small intestine polyp	Ileocolic	F	Right hemicolectomy
46	F	N	Y	- (Mobile cecum)	Ileocolic	Y	Appendectomy, immobilization of the cecum
54	F	Y	-	Ascending colon adenocarcinoma	Colocolonic	N	Right hemicolectomy
34	F	Y	-	- Efferent Loop of Gastrojejunostomy with Tube	Enteric	Y	-
29	M	N	-	-	Enteric (retrograde)	Y	-
20	M	N	Y	GIST of small intestine	Ileocolic	Y	Right hemicolectomy
22	F	Y	-	Small intestine lipoma	Ileocolic	Y	Small intestine segmental resection
38	M	Y	-	Small intestine polyp	Enteric	F	Small intestine segmental resection
48	F	-	Y	Necrosis with bleeding	Enteric	F	Small intestine segmental resection
48	M	Y	Y	Suppurative appendicitis	Ileocolic	N	Right hemicolectomy
41	F	Y	-	GIST of small intestine	Ileocolic	Y	Small intestine segmental resection
38	M	Y	Y	Inflammation and ulcer of cecum	Ileocolic	Y	Right hemicolectomy
64	F	-	-	Small Intestine lipoma	Enteric	N	Small intestine segmental resection
49	F	Y	Y	- (After appendectomy)	Ileocolic	F	Right Hemicolectomy
50	M	N	-	Small intestine smooth muscle cell-derived borderline tumor	Enteric	Y	Small intestine segmental resection
18	F	N	-	Meckel diverticulum	Ileocolic	Y	Small intestine segmental resection
45	F	Y	Y	Small intestine malignant mesothelioma	Enteric	Y	Small intestine segmental resection
49	M	Y	Y	Small intestine polyp	Ileocolic	F	Right hemicolectomy
39	M	Y	Y	Cecum polyp	Colocolonic	Y	Right hemicolectomy
33	F	Y	-	Ileum adenoma with necrosis and bleeding	Ileocolic	N	Right hemicolectomy
65	M	-	-	Small intestine malignant Mesothelioma	Enteric	N	Small intestine segmental resection
38	M	N	Y	Colon Lipoma	Colocolonic	Y	Right hemicolectomy
25	F	-	-	Sigmoid Colon villous and tubular adenoma	Sigmoidorectal	Y	Partial resection of the sigmoid colon
19	M	Y	-	Mesenteric Lymphadenitis	Ileocolic	Y	Right hemicolectomy
23	F	N	N	Small Intestine Hamartoma	Enteric	Y	Small intestine segmental resection
51	F	-	Y	Colon Lipoma	Colocolonic	N	Right hemicolectomy
24	M	Y	-	Small and Large Intestine Multiple Adenomas	Enteric, Colocolonic	Y	Small intestine segmental resection and partial resection of the transverse colon
41	F	Y	-	Necrosis and Bleeding	Ileocolic	N	Right hemicolectomy
64	M	Y	Y	Ileum B Cell Malignant Lymphoma	Ileocolic	Y	Right hemicolectomy
58	F	N	Y	Ascending Colon Adenocarcinoma	Colocolonic	N	Right hemicolectomy
56	F	N	-	Intestinal Inflammatory Disease	Enteric	N	Right hemicolectomy
43	M	Y	-	- (Efferent Loop of Gastrojejunostomy with Tube)	Enteric, Enteric	Y	-
87	F	-	Y	Ascending Colon Adenocarcinoma	Colocolonic	N	Right hemicolectomy
44	M	-	Y	Small Intestine Multiple Adenomas Canceration	Enteric, Enteric	Y	Small intestine segmental resection
40	F	-	Y	Small Intestine Lipoma	Enteric	Y	Small intestine segmental resection
51	F	N	Y	GIST of Small Intestine	Enteric	Y	Small intestine segmental resection
70	F	Y	Y	- (Efferent Loop of Gastrojejunostomy with Tube)	Enteric	F	Small intestine segmental resection
68	F	-	Y	GIST of small Intestine	Enteric	Y	Small intestine segmental resection
71	M	N	-	Necrosis and Bleeding	Colocolonic	N	Left hemicolectomy

US: Ultrasonography; CT: Computed tomography; GIST: Gastrointestinal stromal tumor.<sup>1</sup>Y: Diagnosed with intussusception; N: Not diagnosed with intussusception; -: Did not undergo this diagnostic modality.<sup>2</sup>Y: Reduction succeeded; F: Reduction failed; N: Reduction not attempted.

segmental resection with primary reduction, four (20%) failed in reduction, and three (15%) had segmental resection without reduction.

Of the 15 ileocolic intussusceptions, nine (60%) were reduced successfully. Due to the reduction, five patients had limited resection with preservation of the antireflux ileocecal valve. Reduction failed in three patients (20%). Three of them (20%) had a right hemicolectomy without reduction.

Of the eight colocolic intussusceptions, three of them were reduced successfully before resection.

The other five had resection without reduction. The sigmoidorectal intussusception was reduced and had a segmental sigmoidectomy.

There was no perioperative mortality and anastomosis leakage. Except for one patient with multiple small bowel adenomas recurrent 5 mo after surgery, none of them were recurrent within 6 months postoperatively.

Pathological examinations of the 44 intussusceptions showed that a tumor occupied 54.5% (24/44), with 27.3% (12/44) malignant, 25.0% (11/44) benign, and

**Table 2** Coloscopy results for intussusceptions

Intussusception type	Etiology	Coloscopy diagnosis	
		Diagnosis of intussusception	Etiological diagnosis
Enteric	Intestinal inflammatory disease	No	Intestinal inflammatory disease
Ileocolic	- (After appendectomy)	Yes	-
Ileocolic	Ileum B cell malignant lymphoma	Yes	Occupying lesion
Colocolonic	Colon lipoma	No	Occupying lesion
Colocolonic	Colon lipoma	Yes	Occupying lesion
Colocolonic	Ascending colon adenocarcinoma	No	Adenocarcinoma
Colocolonic	Ascending colon adenocarcinoma	No	Adenocarcinoma
Sigmoidorectal	Sigmoid colon villous and tubular adenoma	Yes	Villous and tubular adenoma

**Table 3** Etiologies of the 44 AIs

		Enteric	Ileocolic	Colocolonic	Sigmoidorectal	Percentage
Tumor	Malignant	6	3	3	0	27.3 (12/44)
	Borderline	1	0	0	0	2.3 (1/44)
	Benign	4	3	3	1	25.0 (11/44)
Nontumorous polyp		1	2	1	0	9.1 (4/44)
Intestinal inflammatory disease		2	3	0	0	11.4 (5/44)
Anatomy abnormality		0	2	0	0	4.5 (2/44)
Iatrogenic		4	1	0	0	11.4 (5/44)
Idiopathic		2	1	1	0	9.1 (4/44)
Percentage		45.5 (20/44)	34.1 (15/44)	18.2 (8/44)	2.3 (1/44)	100.0 (44/44)



**Figure 3** Efferent loop intussusception with a tube. A 70-year-old woman underwent Billroth II gastrectomy and efferent loop intubation for enteral nutrition. One month postoperatively, CT at the level of the lower abdomen shows a round, target-shaped mass in the left abdomen. The mass consists of a hyperdense tube (arrow), a "half-moon" shaped hypodense area medial to it, the intussuscepted mesenteric fat and a soft tissue rim representing the opposing walls of the intussuscipiens and the intussusceptum.

2.3% (1/44) borderline. 9.1% (4/44) were nontumorous polyps. 4.5% (2/44) were due to intestinal inflammatory disease. Meckel's diverticulum and mobile cecum accounted for 4.5% (2/44). Apart from three cases (four intussusceptions), who had already undergone a gastrojejunostomy, no organic lesion was found in four intussusceptions by pathology or exploration, one of which one was the only retrograde intussusception (see Tables 1 and 3).

## DISCUSSION

Intussusception is the leading cause of intestinal obstruction in children and ranks second only to



**Figure 4** An 87-year-old woman with a colocolonic intussusception due to ascending colon carcinoma.

appendicitis as the most common cause of acute abdominal emergency in children. AI is distinct from pediatric intussusception in that it is rare, accounting for only 5% of all cases of intestinal obstructions, and about 90% of it is secondary. The exact mechanism is still unknown. However, it is believed that any lesion in the bowel wall or irritant within the lumen that alters normal peristaltic activity is able to initiate an invagination. Ingested food and subsequent peristaltic activity of the bowel produces an area of constriction above the stimulus and relaxation below, thus telescoping the lead point through the distal bowel lumen<sup>[1,3,4]</sup>.

Similar to the results of Zubadi *et al*<sup>[1]</sup>, enteric type intussusception is the most common type in our series. However, in the report of Goh *et al*<sup>[4]</sup> of 60 cases of AI, ileocolic (25%) and ileocecal-colic (13.3%) types were the most common. Their enteric type occupied 26.7%. Similar to our results, their colocolic and sigmoidorectal

types were the least common types.

Most patients present with subacute (24.4%) or chronic (51.2%) symptoms; therefore the characteristic pediatric presentation triad of abdominal pain, palpable abdominal mass and bloody stool was only seen in 9.8% of cases. It is one of the reasons why preoperative diagnosis is difficult.

Ultrasound is apt to be masked by gas-filled loops of bowel, and most AIs present with intestinal obstruction<sup>[6]</sup>. The preoperative diagnosis accuracy (60.0%) of ultrasonography is not satisfying. However, the preoperative diagnosis accuracy of the 12 patients who had palpable abdominal mass was 91.7%, indicating that in cases of palpable abdominal mass, the diagnostic accuracy of ultrasonography would increase significantly.

Recently, with the signs of target or sausage, mesenteric fat and vessels, abdominal CT scan has been reported to be the most useful imaging technique, with a diagnostic accuracy is 58%-100%<sup>[3-5,7,8]</sup>. Recent studies have demonstrated the superiority of CT in revealing the site, level, and cause of intestinal obstructions and in demonstrating threatening signs of bowel nonviability<sup>[9,10]</sup>. As was shown in our study, the majority of AIs presented with partial or complete intestinal obstruction. Moreover, 90.5% (20/22) of AIs were diagnosed by CT in our series. One case of AI that was not diagnosed by CT, however, was correctly diagnosed as having an intestinal occupying lesion. In contrast to ultrasound, CT is not affected by the presence of gas in the bowel and will clearly demonstrate the intussusception, whether in the small bowel or in the colon. Additional valuable information, such as metastasis or lymphadenopathy, is readily obtained by CT and may point to an underlying pathology<sup>[3]</sup>. Therefore, we suggest that all patients presenting with an intestinal obstruction should have an abdominal CT scan as a regular diagnostic test.

Wang *et al*<sup>[3]</sup> considered that most lesions lead distally, can easily be seen by coloscopy, and intraoperative coloscopy might help to distinguish benign from malignant lesions before reduction of intussusceptions. Using the results of a coloscopy, limited surgical management by appendectomy, polypectomy or diverticulectomy might be performed in specific situations, resulting in an uncompromised bowel after reduction<sup>[3]</sup>. In our study, all lesions of the lead points of ileocolic, colocolic or sigmoidorectal intussusceptions were found in the seven patients who had a colonoscopy. Moreover, all the adenoma and adenocarcinomas were diagnosed pathologically through colonoscopy. However, some lesions, such as lymphoma and lipoma, which were not in the mucosa, were not diagnosed pathologically. Therefore, we think that the pathological diagnosis of most of the lead points of ileocolic, colocolic and sigmoidorectal intussusceptions, which locate in the mucosa, could be made by coloscopy. Perhaps some lesions, such as appendicitis and polyps, could be diagnosed by coloscopy to avoid undue surgery.

It is reported that 8%-20% of AIs are idiopathic and are more likely to occur in the small intestine<sup>[6]</sup>. In

our series, there were four [9.1% (4/44)] patients whose etiologies were not found by surgical exploration and/or pathology. One of them occurred in the colon (see Tables 1 and 3). Three of them had intestinal necrosis with bleeding. Segments of intestine had to be resected. The only retrograde intussusception found in our patients was cured by simple reduction.

Most AIs have underlying pathological lesions; therefore, most authors agree that laparotomy is mandatory. However, whether or not the intussusception should be reduced before resection remains controversial. The theoretical objections to reductions are intraluminal seeding and venous dissemination of malignant cells, possible perforation during manipulation and increased risk of anastomotic complications in the face of edematous and inflamed bowel<sup>[11]</sup>.

Although only 30% (6/20) of the etiologies of our enteric intussusceptions were malignant, borderline leiomyoma is potentially malignant. Therefore, reduction before resection would be more prudent. We suggest that if the underlying etiology and/or the lead point is suspected to be malignant, or if resected area required without reduction is not massive, an en bloc resection of the intussusception should be considered.

There were only three cases (37.5%) of colocolic intussusceptions caused by a malignant tumor—adenocarcinoma in our series, but most authors report malignant pathology presents in 69%-100%<sup>[3,4]</sup>. Only one case of ileocolic intussusception had a malignant organic lesion (terminal ileum B cell malignant lymphoma) in our series. Wang *et al*<sup>[3]</sup> reported 5/12 patients had malignant lesions in this type of intussusception. They think intraoperative colonoscopy might help to distinguish benign from malignant lesions before reduction. This technique can identify benign lesions of the ileum and be used to perform limited resection with preservation of the antireflux ileocecal valve. Moreover, appendectomy, polypectomy or diverticulectomy might be performed in specific situations to produce an uncompromised bowel after reduction, when definitively diagnosed by coloscopy<sup>[3]</sup>. Our colonoscopies found all (7/7) of the lead point lesions and diagnosed all of the adenoma and adenocarcinomas of non-enteric intussusceptions. In our study, the lesions of appendicitis, benign tumors and polyps might have been diagnosed by coloscopy; organic lesion might have been excluded on the patient who had undergone appendectomy before exploration for intussusception. If coloscopy had been undertaken, unnecessary surgery could have been avoided. Therefore, we consider that in ileocolic, colocolic and sigmoidorectal intussusceptions, coloscopy is necessary, either preoperatively or intraoperatively.

The sigmoidorectal intussusception caused by villous and tubular adenoma of the sigmoid colon in our study was successfully reduced before partial resection of the sigmoid colon and the patient was spared a Miles operation. Zubaidi *et al*<sup>[11]</sup> considered that even if an intussusception of this type is secondary to a carcinoma, to avoid an abdominoperineal resection and a permanent colostomy, reduction should be performed first.

There were three patients in our study who underwent gastrojejunostomy and nasojejunal intubation for enteral nutrition and had efferent loop intussusceptions. All of them were misdiagnosed as gastropexia initially. The tube had been intubated together with a nasogastric tube into the stomach. During the operation, before completion of the gastrojejunostomy, a candy ball enveloped with a perturbed surgery glove finger end, which was tied on the tip of the tube, was put into the efferent loop, then the candy ball was drawn from the outside of the efferent loop more than 40 cm distally to the anastomosis. Gayer et al<sup>5</sup> and Erkan et al<sup>6</sup> also consider intestinal intubation in the etiology of intussusception. However, they did not illuminate the mechanism of it. It is well known that the mucosal and seromuscular layers of the intestine connect loosely with each other. We think that when the candy ball on the tip of the tube is drawn distally, because of friction, the proximal mucosa will be pushed, folded and prolapsed distally. If the muscularis of mucosa is injured, then the distally pushed mucosa will not be able to recover by itself. As a result, the mucosa could prolapse distally and protrude into the intestinal lumen like a ring shaped tumor, and then an intussusception could occur.

In conclusion, AI is an infrequent problem. Most AIs present with subacute and chronic symptoms have intestinal obstructions to various extents. CT is the most effective and accurate diagnostic technique. In the case of a palpable abdominal mass, ultrasonography is also helpful for diagnosis. Enteric intussusception should be reduced when the underlying etiology is suspected to be benign or the resection is massive without reduction. Most lead point lesions of the ileocolic, colocolic or sigmoidorectal intussusceptions can be found by coloscopy. For these types of intussusceptions, coloscopy might provide information allowing the avoidance of unnecessary surgery. Intestinal intubation is the main cause of iatrogenic intussusception and should be avoided.

## COMMENTS

### Background

Intussusception is uncommon in adults. It is estimated that only 5% of all intussusceptions occur in adults and approximately 5% of bowel obstructions in adults are the result of intussusception. Adult intussusception (AI) often presents with nonspecific symptoms. Preoperative diagnosis remains difficult. The extent of resection and whether the intussusception should be reduced remains controversial.

### Research frontiers

Recent researches are focused on accurate preoperative diagnosis and proper treatment of it.

### Innovations and breakthroughs

According to their data, the authors found computed tomography (CT) is the most effective and accurate diagnostic technique. In case of a palpable abdominal mass, ultrasonography is also helpful for diagnosis. Enteric intussusception should be reduced when the underlying etiology is suspected to be benign or the resection is massive without reduction. Most lead point lesions of the ileocolic, colocolic or sigmoidorectal intussusceptions can be found

by coloscopy. For these types of intussusception, coloscopy might provide information that allows avoidance of unnecessary surgery. Intestinal intubation is the main cause of iatrogenic intussusception and should be avoided. They also propose a hypothesis for the occurrence of this kind of intussusception.

### Applications

The authors suggest that all patients presenting with an intestinal obstruction or the patients who are suspected intussusception, have an abdominal CT scan as a regular diagnostic test. In cases with a palpable abdominal mass, ultrasonography should be applied. If non-enteric intussusception is suspected, coloscopy might provide information allowing the avoidance of unnecessary surgery. Enteric intussusception should be reduced when the underlying etiology is suspected to be benign or the resection is massive without reduction. Intestinal intubation should be omitted unless very necessary or carried out very gently or with lubrication.

### Terminology

Intussusception: defined as the telescoping of a segment of the gastrointestinal tract into an adjacent one; enteric intussusception: an intussusception involved only jejunum or ileum is considered an enteric intussusception; ileocolic intussusception: an intussusception that involves the ileum and the colon is designated an ileocolic intussusception; colocolonic intussusception: an intussusception involving only the colon is considered a colocolonic intussusception; sigmoidorectal intussusception: involving the sigmoid colon and rectum; non-enteric intussusception: including ileocolic, colocolonic and sigmoidorectal intussusceptions. Acute symptoms: defined as < 4 d; Subacute symptoms: defined as 4-14 d. Chronic symptoms: defined as > 14 d. Antegrade intussusception: the proximal segment of the bowel telescoped into the lumen of the adjacent distal segment is defined as antegrade intussusception. Retrograde intussusception: the distal segment of the bowel telescoped into the lumen of the adjacent proximal segment is defined as retrograde intussusception.

### Peer review

The authors reviewed 41 cases of adult intussusception. The article is well written and worthy of publication.

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# Unexplainable development of a hydatid cyst

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## Abstract

Echinococcosis is a cyclozoanosis characterized by cystic lesions usually situated inside or outside the liver. We discuss the case of a 77-year-old woman with a recurrent hydatidosis with a cyst arising from the liver, growing through the lateral right abdomen wall, and reaching the subcutaneous tissue of the lumbar region. In the literature, rare subcutaneous or muscular localizations of hydatid cysts are described, however, there is no mention of a cyst growing over the abdominal wall muscles, shaped like an hourglass, partially in the liver and partially in the subcutaneous tissue, as in our case. We have not found any pathogenetic explanation for this growth pattern which is not typical of the biological behaviour of a hydatid cyst.

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**Key words:** Echinococcosis; Growth; Hepatic; Hydatid cyst; Pathogenesis

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## INTRODUCTION

Hydatid disease is a cyclozoanosis caused by *Echinococcus granulosus* or *Echinococcus multilocularis*, and is characterized by cystic lesions usually situated in the liver. These lesions can be asymptomatic for a long time, or cause an ache, however, there are no specific local or general symptoms and signs are related to size, location or complications of the cyst. The case of recurrent hydatidosis in a 77-year-old woman is discussed where the cyst, probably arising from the liver, grew through the lateral right abdomen wall and reached the subcutaneous tissue of the lumbar region.

## CASE REPORT

A 77-year-old woman was evaluated due to of the growth of an aching mass in the right lumbar region. Three years previously surgery was performed for hydatidosis of the mesentery. Objective examination revealed a painful swelling mass in the right lumbar region about 7-8 cm in diameter and mobile with respect to the upper and lower layers, which had distinct margins and a smooth surface (Figure 1). On palpating the abdomen the patient had pain in the right upper abdominal quadrant. CT of the abdomen showed a voluminous low-density mass (74 cm × 58 mm) in the low-external side of the VI liver segment. The mass was pedunculated, growing over the right posterior abdominal wall muscles and reaching the subcutaneous tissues (Figure 2).

Surgical treatment confirmed that the voluminous cyst was connected to the VI liver segment by a peduncle. Growing sideways and then behind, this mass reached the muscular layer arriving at the subcutaneous tissue of the right lumbar region. The mass was removed from the perihepatic side of the peduncle, 2-3 cm inside the liver, and from the abdominal external oblique muscle and the latissimus dorsi up to the subcutaneous tissue of the lumbar region (Figure 3). The post-operative period was problem-free, and the patient was discharged after 1 wk on anti-helminthic therapy. Histology confirmed the suspicion of hydatid cyst.

## DISCUSSION

Hydatid disease is caused by *Echinococcus granulosus*, an endemic infection in several mediterranean countries, or by *Echinococcus multilocularis*. Hydatid cysts can



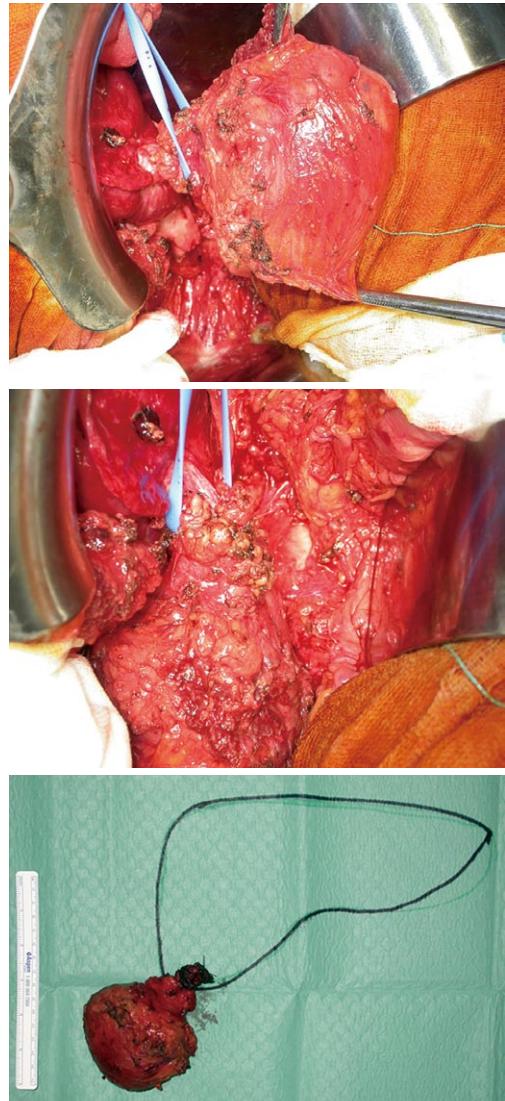
**Figure 1** Mass in the right lumbar region.



**Figure 2** CT. The mass and the peduncle which connected it to the liver, as shown by the scheme with the surgical specimen.

be observed in all parts of the body, but are more frequent in the liver (50%-70%), lung (20%), and spleen (5%-8%)<sup>[1]</sup>, while rare localizations are the kidneys, adrenal glands<sup>[2]</sup>, spleen, soft tissues and brain<sup>[3]</sup>.

Echinococcosis may be asymptomatic, detected only when imaging scans of the liver are carried out for different reasons, but it may cause pain or lead to complications. Cysts can rupture into the peritoneum, resulting in anaphylaxis or peritonitis<sup>[4]</sup>, or into the biliary tract, causing cholangitis or cholestasis, and into the bronchi, causing bronchial fistula, or the cysts may become infected and form liver abscesses. Compression of the bile duct, portal vein, and hepatic veins can cause cholestasis, portal hypertension, and Budd-Chiari syndrome, respectively<sup>[5]</sup>.



**Figure 3** Pedunculated mass arising from the sixth liver segment and growing over the posterior abdominal wall muscles and reaching the subcutaneous tissue.

The diagnosis of hydatid cysts relies on epidemiologic data (geographical location and profession) clinical manifestations, serologic tests and radiologic imaging.

The treatment of choice is surgery. Chemotherapy (albendazole and mebendazole) is currently indicated in patients who cannot undergo major operations or for the prevention of secondary echinococcosis after surgery<sup>[6]</sup>, but is contraindicated for large, superficially situated, or infected cysts, which are prone to rupture<sup>[7,8]</sup>.

Our case shows some particular features that deserve closer examination. Usually the hydatid cyst grows in the liver in every direction, both inside the hepatic parenchyma and outside towards the peritoneal cavity. In our case, the cyst was connected to the liver by a peduncle 3-4 cm inside the liver. The surgeon who operated on our patient three years previously informed us of two hydatid cysts (diameter 9 cm and 12 cm) located in the mesocolon, which were connected by a peduncle, making it easy to remove them without spreading the disease. The hypothesis that the cyst we treated could have been a secondary echinococcosis due to spillage

of cystic fluid and seeding during the first operation<sup>[5]</sup> has been denied. In the literature, rare subcutaneous or muscular localizations of hydatid cysts have been described<sup>[9]</sup>, however, a cyst growing over the abdominal wall muscles, shaped like an hourglass, partially in the liver and partially in the subcutaneous tissue, as in our case has never been described. This growth pattern is not typical of the biological behaviour of a “benign tumor” such as a hydatid cyst, and seems to be more similar to the behaviour of an infiltrating malignant neoplasm. The only plausible explanation is that it could be related to seeding during the first surgery through drainage, however, the only drainage after the first surgery was located in the right lower abdominal quadrant, far away from the lumbar localization of the cyst we treated.

In the literature, Parmar *et al*<sup>[10]</sup> reported a case showing some similarities to our patient: a hepatic hydatid cyst which had ruptured into the subcutaneous tissue of the anterior abdominal wall. While the superficial and subcutaneous localization of the mass was similar, its connection with the right lobe of the liver was different. In Parmar's case the right lobe of the liver was almost entirely occupied by the cyst, in our case the cyst was connected only by a peduncle. Parmar did not suggest any pathogenetic explanation, but just describes the kind of rupture that can involve a hydatid cyst.

In conclusion, we have not found any evidence in the literature for a pathogenetic explanation for the growth of the hydatid cyst over the abdominal wall muscles, shaped like an hourglass, partially in the liver and partially in the subcutaneous tissue. A similar growth pattern may occur in cysts found in the liver cupola where they can damage the diaphragm through inflammatory processes. The cysts then grow towards the pleural opening to the bronchial tubes, resulting in an uncommon and severe complication,

a hydatid bronchobiliary fistula. In this situation the pathogenetic mechanism is clearly caused by the infective process resulting in adhesions in the diaphragm. In our case no infective process was present, the cyst was connected to the liver by a peduncle which was easily removed from the transverse colon and from the muscles.

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CASE REPORT

## Excision of a large abdominal wall lipoma improved bowel passage in a Proteus syndrome patient

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### INTRODUCTION

Proteus syndrome is an extremely rare disorder characterized by postnatal overgrowth of multiple tissues derived from different cell lineage<sup>[1-3]</sup>. The patient with Proteus syndrome described in this paper experienced constipation, nausea, vomiting, and abdominal pain due to the encasement and pressure of the intestinal tract by a large fatty mass which had developed in the left abdominal wall. The excision of this mass improved the symptoms.

### CASE REPORT

A 4-year-old female underwent a resection of a soft mass in her left inguinal area. This mass was pathologically diagnosed to be a lipoma. She was sent to the Department of Orthopedic Surgery because the lesion recurred and spread to the surrounding area. The recurrent tumors in her left inguinal area, left thigh and knee increased in size, and additional excisions were performed when the patient was 8- and 11-years-old. The pathological diagnosis was an infiltrating lipoma. She was diagnosed to have Proteus syndrome based on the diagnostic criteria reported<sup>[2-4]</sup>. The neoplasm increased in size gradually, and produced hemihypertrophy of the left lower extremity and trunk, and spread to the left abdominal wall and the retroperitoneum. Since the increasing abdominal mass encased and pressed the digestive organs, she gradually developed constipation. Bowel movements occurred about once a week at the time of presentation. A bowel obstruction caused symptoms including constipation, nausea, vomiting and abdominal pain. She had two healthy sisters and the rest of her family were also healthy. She had iron-deficient anemia with hypermenorrhea and she had a

### Abstract

Proteus syndrome is an extremely rare congenital disorder that produces multifocal overgrowth of tissue. This report presents a surgical case of a large lipoma in the abdominal wall of a patient with Proteus syndrome. She was diagnosed with Proteus syndrome based on certain diagnostic criteria. The neoplasm increased in size gradually, producing hemihypertrophy of her left lower extremity and trunk, and spread to her retroperitoneum and her left abdominal wall. She experienced gradually progressive constipation, nausea, vomiting, and abdominal pain. Computed tomography (CT) of the abdomen demonstrated a large mass in the subcutaneous adipose tissue of the left lower abdominal wall which measured 12 cm × 8 cm × 6 cm in diameter and encased the left colon. This mass in the abdominal wall was excised. The weight of the excised mass was 1550 g. The histopathological diagnosis of this mass was lipoma. After surgery, the encasement of the left colon was improved, and the patient was able to move her bowels twice per day. The excision of the large lipoma in the abdominal wall contributed to the improved bowel passage in this patient with Proteus syndrome.

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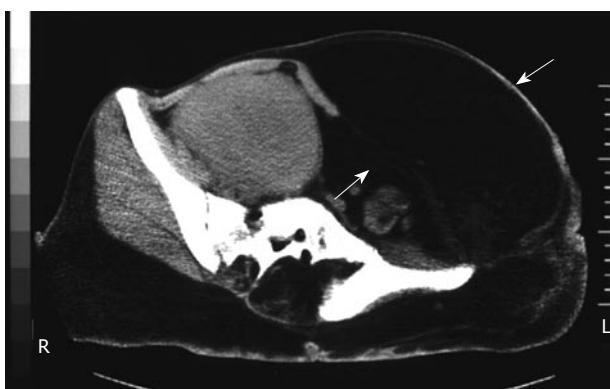
**Key words:** Proteus syndrome; Lipoma; Bowel obstruction; Abdominal wall; Bowel passage



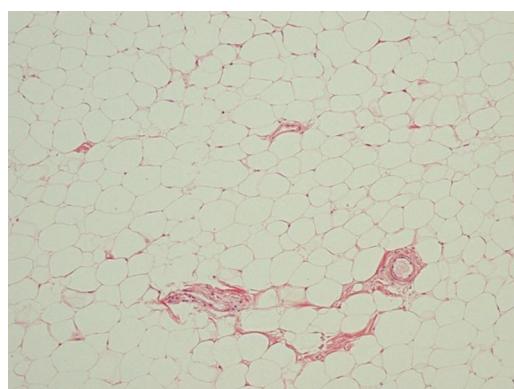
**Figure 1 Clinical findings of the abdomen.** There was a child-head sized mass at the left lower abdomen (arrows).



**Figure 2 A preoperative plain abdominal X-ray examination indicated that colon gas in the left colon (arrows) was shifted to the upper right side.**



**Figure 3 Preoperative computed tomography (CT) of the abdomen.** A large mass in the subcutaneous adipose tissue in the left lower abdominal wall was identified (arrows) and this encased the peritoneal organs to the right side.



**Figure 4 Histopathological findings of the excised mass.** Lobular arrangement of mature adipocytes diffusely infiltrating into the skeletal muscle was observed.

transfusion history. On physical examination, she had a temperature of 36.4°C, and blood pressure of 120/70 mmHg. A large mass with unclear margins was found in the left lower quadrant of her abdominal wall (Figure 1). She showed hemihypertrophy and deformity of her left lower extremity. There were large and bizarre skin tumors on her left lower extremity.

Laboratory data showed a white blood cell count of 4800 cells/mm<sup>3</sup>, hemoglobin 7.7 g/dL, hematocrit 25%, platelets 321 000 cells/mm<sup>3</sup>, normal electrolytes and blood urea nitrogen, and the liver function was normal.

A plain abdominal X-ray examination indicated scoliosis and deformity of the pelvic bone. Colon gas in the left colon shifted to the right upper side (Figure 2). Computed tomography (CT) of the abdomen demonstrated a large mass in the subcutaneous adipose tissue at the left lower abdominal wall which was 12 cm x 8 cm x 6 cm in diameter and encased the peritoneal organs to the right side (Figure 3). An upper gastrointestinal series revealed a mass encasing the stomach toward the upper side and the small intestine toward the right upper side (data not shown).

The mass in the abdominal wall was excised in 1997. The weight of the excised mass was 1550 g. A histopathological examination revealed a lobular arrangement of mature adipocytes (Figure 4), and these cells diffusely invaded into the skeletal muscle.

These findings indicated the diagnosis of a non-malignant lipoma.

The postoperative course was uneventful, the encasement of the left colon was improved, and she left the hospital on the 15th postoperative day. At present, she continues to receive medical treatment on an outpatient basis. Postoperatively, bowel movement occurred twice a day. A postoperative plain X-ray examination indicated that the shift of the left colon gas was improved (Figure 5). Postoperative CT also revealed reduced encasement of the peritoneal organs (Figure 6).

## DISCUSSION

Wiedermann *et al*<sup>[2]</sup> proposed diagnostic criteria for Proteus syndrome: (1) gigantism of the hands and/or feet, (2) pigmented nevus, (3) hemihypertrophy, (4) subcutaneous tumors, (5) skull anomalies, (6) accelerated growth and (7) visceral abnormalities. Recently, the main diagnostic criteria for Proteus syndrome are that it is sporadic, mosaic and has a progressive course<sup>[3]</sup>. The current case presented with lipomatosis, severe hemihypertrophy of the left trunk and left lower extremity, scoliosis, endometriosis and huge bizarre-shaped body tumors. Consequently, this patient was diagnosed to have Proteus syndrome based on the above-mentioned diagnostic criteria<sup>[5]</sup> and was the 6th



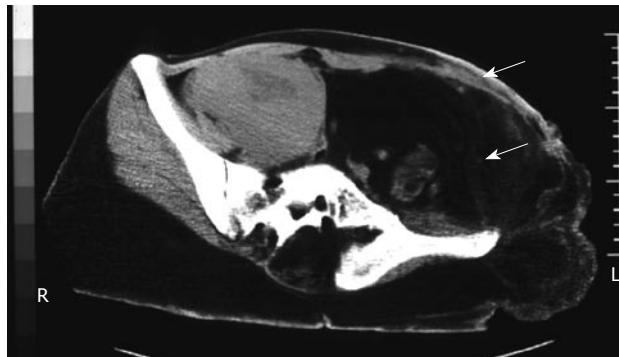
**Figure 5** A postoperative plain abdominal X-ray examination indicated that the shift of the left colon gas (arrows) was improved as compared to the preoperative state.

Japanese case described in the English literature<sup>[5-9]</sup>.

This syndrome is caused by the germline loss of functional mutations in PTEN<sup>[10]</sup> and is an overgrowth syndrome<sup>[11]</sup>. PTEN acts as a negative regulator of PI3-kinase signaling by catalyzing the dephosphorylation of PIP3<sup>[12,13]</sup>. This case was diagnosed based on the previous criteria for Proteus syndrome<sup>[2-4]</sup>, and the germline mutation in PTEN was not examined.

Lipomatosis of the colon complicating Proteus syndrome was reported previously<sup>[14]</sup>. A 12 year-old boy with Proteus syndrome presented with gross abdominal distension and severe intractable constipation. Magnetic resonance imaging showed adipose tissue widely spread throughout the abdomen and pelvis. A laparotomy revealed a huge infiltrating lipomatosis encasing the left colon, including the rectum. On the other hand, the symptoms of bowel obstruction in the current case may have been caused by the encasement and pressure of the intestinal tract by a child-head sized lipoma that developed in the left abdominal wall. This lipoma was excised from the abdominal wall and removal of this huge lipoma produced the improvement in the patient's symptoms.

Proteus syndrome is a complex disorder associated with varied, disproportionate, asymmetric overgrowth of many body parts and unregulated adipose tissue. The overgrowth seen in Proteus syndrome is progressive and difficult to manage. Patients with Proteus syndrome require repeated treatment for the progressive overgrowth of tissue over a long period. Aggressive treatment may cause severe functional and cosmetic consequences, so surgical intervention is often delayed until it is absolutely necessary<sup>[15]</sup>. In the current case, other sites of the lipomatosis, such as the left extremity and back, were excised when it affected the patient's quality of life. This report presented an extremely rare case of bowel obstruction due to a large lipoma in the abdominal wall. The excision of this large mass improved the bowel passage in a patient with Proteus syndrome.



**Figure 6** Postoperative CT of the abdomen. The encasement of the peritoneal organs was improved as compared to the preoperative state (arrows).

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CASE REPORT

# Granular cell tumor of the cecum with extensive hyalinization and calcification: A case report

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## INTRODUCTION

### Abstract

A granular cell tumor (GCT) is a benign neoplasm of unclear histogenesis that is generally believed to be of nerve sheath origin. GCT is not common and most often affects the tongue, skin and soft tissue, although it may occur anywhere in the body. We experienced a case of GCT that arose in the cecum of a 55-year-old man. The GCT was removed by laparoscopic resection. In addition to the tumor, endoscopic examination revealed the presence of a 5-mm-polyp in the descending colon and multiple tiny polyps in the sigmoid colon and rectum. Histological examination demonstrated a cecal tumor 1.5 cm × 1.0 cm × 0.7 cm with a hard consistency; in cut sections, mixed cells with yellowish and whitish portions were seen. The tumor was located between the mucosa and subserosa, and was composed of plump histiocyte-like tumor cells with abundant granular eosinophilic cytoplasm, which were immunoreactive for S-100 protein, vimentin, neuron-specific enolase, inhibin- $\alpha$  and calretinin. The tumor showed extensive hyalinization and focal dystrophic calcification. Immunohistochemical profiles did not confirm any particular cell type for the histogenetic origin of the GCT, including a nerve sheath origin. Extensive hyalinization and calcification showing involution of tumor cells suggest benign clinical behavior of GCT.

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**Key words:** Granular cell tumor; Cecum; Histogenesis; Calcification

## CASE REPORT

A 56-year-old man was admitted to the Gastroenterology Department of Chosun University Hospital with a 2-mo history of abdominal pain and diarrhea. Colonoscopy



**Figure 1** Endoscopic examination revealed an approximately 2-cm-sized protruding mass in the cecum.

revealed a protruding mass approximately 2 cm in diameter, with central umbilication in the cecum (Figure 1), as well as a 5-mm polyp and tiny sized multiple polyps in the descending colon and rectum, respectively.

A polyp in the descending colon was removed by snare polypectomy, and the polyp was diagnosed histologically as tubular adenoma. The cecal mass was resected by laparoscopic myotomy. Grossly, the mass was 1.5 cm × 1.0 cm × 0.7 cm, yellowish-white in color, and hardly palpable. Microscopically, the well-circumscribed mass was located mainly between the submucosa and subserosa, with partial involvement of the mucosa (Figure 2A). The mass was composed of round to polygonal cells with abundant granular eosinophilic cytoplasm. Characteristically, much of the tumor showed extensive hyalinization and focal dystrophic calcification (Figure 2B and C). There was no tumor necrosis, spindling and vesicular nuclei with large nucleoli, increased mitotic activity, a high nuclear to cytoplasmic ratio, and pleomorphism. The tumor was seen as a small, well-circumscribed mass, but with no infiltrative growth pattern; findings that favor a benign rather than a malignant lesion. Immunohistochemical analysis demonstrated that the tumor cells were reactive for S-100 protein (Figure 2D), neuron-specific enolase (NSE), vimentin, calretinin and inhibin (Figure 3). The tumor cells were non-reactive for smooth muscle actin (SMA), c-kit (CD117) and CD34. Based on the morphological and immunohistochemical findings, the resected tumor was diagnosed as a benign GCT that occurred in the cecum.

## DISCUSSION

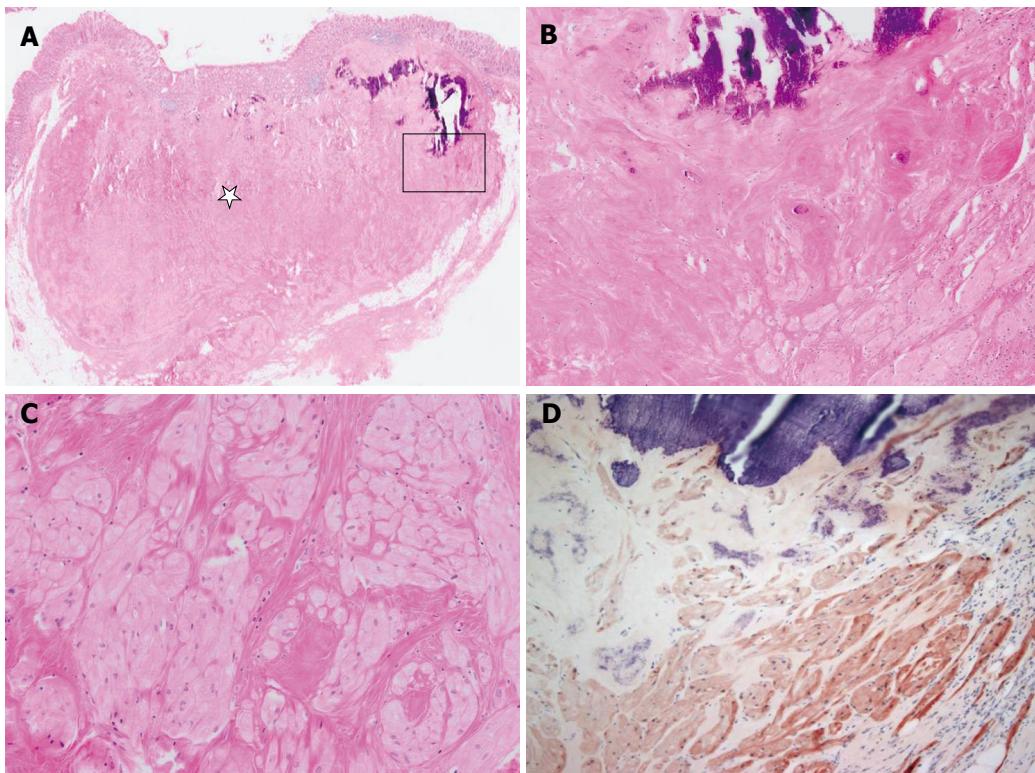
GCT is diagnosed only rarely, based on macroscopic and endoscopic examinations, as a result of its small size and shape that resemble a diminutive polyp<sup>[12]</sup>. Recently, endoscopic ultrasonography (EUS) has been used more frequently to determine the depth of tumor invasion in the gastrointestinal wall, and, it is useful for evaluating gastrointestinal tract submucosal tumors<sup>[13]</sup>. However, EUS cannot sufficiently distinguish a benign submucosal tumor from other tumors such as malignant neoplasia<sup>[14]</sup>.

The final diagnosis of GCT is dependent on the pathological findings. The histological markers for GCT are plump histiocyte-like, bland-looking neoplastic cells

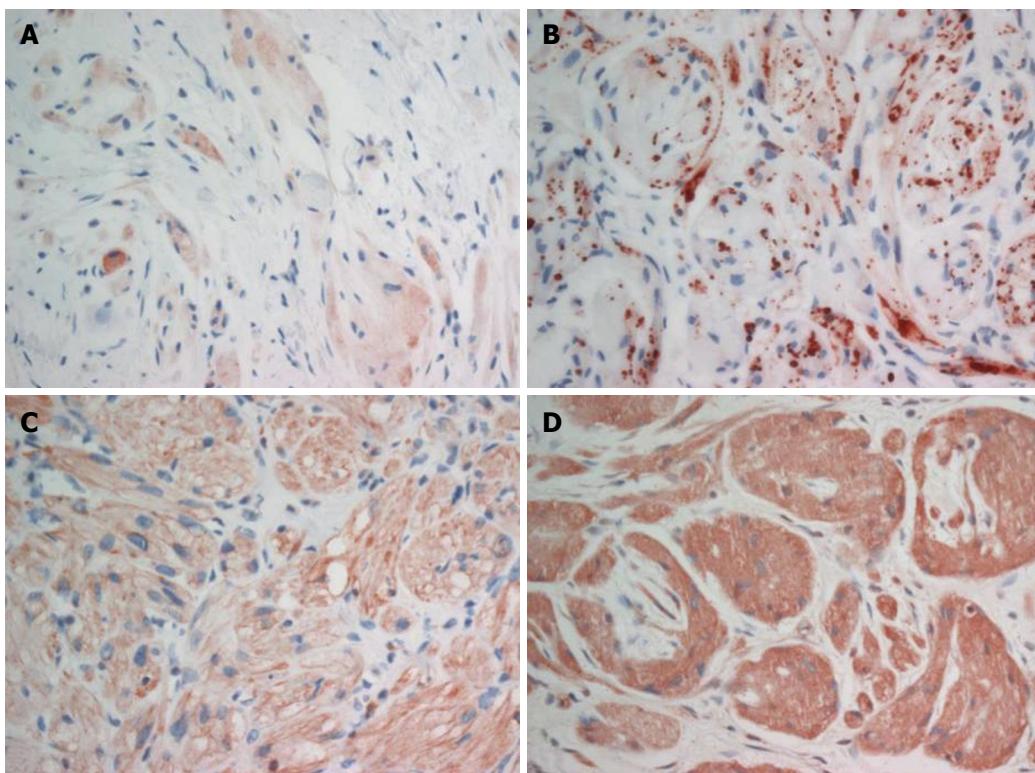
with abundant granular eosinophilic cytoplasm, which contains acidophilic, periodic acid-Schiff-positive, diastase-resistant granules. The cells contain small, uniform nuclei where mitotic figures are absent and neural markers, including S-100 protein or NSE, are expressed uniformly<sup>[15,16]</sup>.

The histogenesis of GCT has remained enigmatic in spite of a vast number of immunohistochemical and ultrastructural studies<sup>[7]</sup>. Neural origin or differentiation, in particular of the Schwann cell type, is currently in favor. However, recent findings have cast doubt on the neural origin of these tumors<sup>[7]</sup>. Vered *et al*<sup>[7]</sup> have suggested that immunoreactivity of the granular cells to a broad panel of antibodies including S-100 protein, CD68, vimentin, calretinin, NKI/C3, protein gene product 9.5, nerve growth factor receptor and inhibin- $\alpha$  that characterize different tissue do not confirm any particular cell type for the histogenesis of GCT. In the present case, tumor cells were reactive for S-100 protein, NSE, calretinin, vimentin, and inhibin- $\alpha$ , which agrees with a previous study<sup>[7]</sup>.

In most cases of colonic GCT, the tumor was less than 2 cm and well separated from the muscularis propria<sup>[17]</sup>. Since this tumor is considered as benign, and no patients with recurrence or metastasis have been documented, it is usually accepted that endoscopic tumor excision may be the best treatment for GCT in the gastrointestinal tract<sup>[14]</sup>. However, Nakachi *et al*<sup>[14]</sup> have suggested that GCT in the gastrointestinal tract is usually small and asymptomatic, and the tumor tends to be found incidentally during endoscopy performed for other reasons. Observation of these GCTs with the use of endoscopy and EUS is indicated unless the patient is symptomatic, or the tumor is larger than 2 cm or demonstrates atypical EUS or histological features. In the present case, there was extensive hyalinization and focal dystrophic calcification of tumor cells, which indicated a long-standing tumor with no atypical changes, which supports the above description. Vered *et al*<sup>[7]</sup> have advocated that GCT can be regarded as a lesion that reflects local metabolic or reactive changes rather than a true neoplasm. In the large series of GCTs evaluated by Vered *et al*<sup>[7]</sup>, lesions displayed three main architectural patterns, including small and well-circumscribed nodules, larger and poorly circumscribed lesions, and an impressive infiltrative pattern with remote satellite nodules. The pattern of small and well-circumscribed nodules may represent benign mesenchymal tumors that have undergone extensive or complete granular cell change, and the remaining patterns may be compatible with a diffuse process of metabolically induced cytoplasmic granular changes in mesenchymal cells. Most lesions with an infiltrative pattern and positive margin almost never recur, whereas for a definite tumor, recurrent lesions are expected. It has been suggested that the lesions may be metabolic or reactive in nature and not neoplastic. The present case displayed extensive hyalinization and calcification in between granular cells, which suggestss a long duration; findings that favor GCT with reactive changes, or a true neoplasm.



**Figure 2** Microscopically and immunohistochemically, the appearance of the tumor was compatible with that of granular cell tumor. A: The tumor was located mainly between the submucosa and the subserosa. Much of the tumor showed extensive hyalinization (star) and dystrophic calcification. B and C: A high magnification view (square of A) showed dystrophic calcification, hyalinization and some viable tumor cell nests in the peripheral portion of the tumor. The tumor cells were composed of round to polygonal cells with abundant granular eosinophilic cytoplasm. D: Immunohistochemically, tumor cells were reactive for S-100 protein.



**Figure 3** Tumor cells of the granular cell tumor were immunoreactive for inhibin- $\alpha$  (A), calretinin (B), vimentin (C) and NSE (D).

Several GCTs with adjacent benign or malignant tumors have been reported previously. In 2006, Eriksen *et al*<sup>[18]</sup> reported the first case of a synchronous adenoma

and GCT, and Sarsik *et al*<sup>[19]</sup> also observed a tubular adenoma in the vicinity of a GCT. Caltabiano *et al*<sup>[20]</sup> reported a GCT covered by squamous cell carcinoma

of the tongue. The present case also had adjacent multiple polyps, including tubular adenoma. Based on these findings, it is suggested that GCT shows reactive granular cell changes in the process of spontaneous regression of a preceding tumor. The coincidence of the adjacent tumor can be regarded as favoring a non-neoplastic or reactive process. However, to date, there is no evidence of any association or disposing factors between GCT in the colon and colonic adenoma or other malignancy<sup>[18]</sup>.

In summary, we experienced a 1.5 cm × 1.0 cm × 0.7 cm cecal GCT that showed extensive hyalinization and focal dystrophic calcification with synchronous tubular adenoma in the descending colon. Immunohistochemical profiles did not confirm any particular cell type for the histogenesis of GCT. Endoscopists and pathologists should consider the possibility of this tumor in the gastrointestinal tract. When a patient is asymptomatic, and unless the tumor is larger than 2 cm or shows atypical features, observation of this tumor with the use of endoscopy and EUS is indicated.

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CASE REPORT

## Large cavernous hemangioma in the cecum treated by laparoscopic ileocecal resection

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### Abstract

A cavernous hemangioma of the cecum is a rare vascular malformation but is clinically important because of the possibility of massive bleeding. We report a case of a large cavernous hemangioma with pericolic infiltration in the cecum which was removed successfully using minimally invasive surgery.

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**Key words:** Cavernous hemangioma; Cecum; Laparoscopic surgery

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### INTRODUCTION

Hemangiomas of the large intestine are relatively

uncommon benign tumors which can occur anywhere in the gastrointestinal tract<sup>[1]</sup>. These are clinically important vascular malformations because of the possibility of massive bleeding. Although a variety of histological and clinical types of hemangioma exist, the cavernous and capillary subtypes are most commonly encountered. Recent advances in colonoscopic techniques have led to successful endoscopic resection in selected cases<sup>[2-4]</sup>, but most large lesions have been treated surgically<sup>[5,6]</sup>. We report the case of a large cavernous hemangioma in the cecum with pericolic infiltration which was removed successfully using minimally invasive surgery.

### CASE REPORT

A 37-year-old woman was referred to her local hospital because of intermittent rectal bleeding. Colonoscopy revealed a blue polypoid lesion with a nodular surface in the cecum (Figure 1). A hemangioma was suspected. She was transferred to our hospital for further evaluation and surgical treatment.

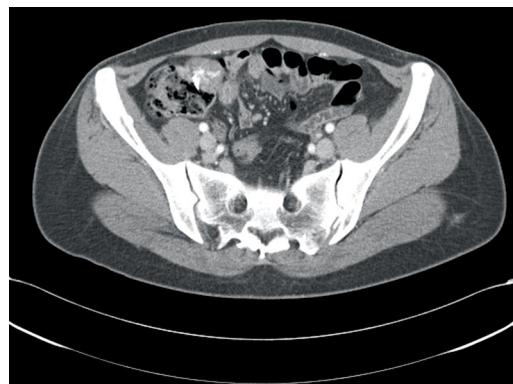
The physical examination revealed no specific signs. Abdominopelvic computed tomography (CT) showed a lesion in the cecum protruding extraluminally with heterogeneous enhancement (Figure 2). The operative findings suggested a tumor involving the cecum and extending into the pericolic fat. Laparoscopic-assisted ileocecal resection and side-to-end ileo-ascending colon anastomosis were carried out. Macroscopically, the surgical specimen was a 5.8 cm × 4.2 cm cavernous hemangioma involving the entire wall of the colon and extending into the pericolic fat (Figure 3). Microscopically, the tumor consisted of large, dilated, blood-filled vessels lined by flattened endothelium. The vascular walls were thickened focally by adventitial fibrosis (Figure 4). Postoperatively, the patient has recovered well with no recurrence of symptoms.

### DISCUSSION

Hemangiomas are rare lesions of the colon, but vascular malformations of the gastrointestinal tract have been reported since 1839<sup>[7]</sup>. According to the literature, these lesions originate from embryonic sequestrations of mesodermal tissue<sup>[8]</sup>. Histologically, hemangiomas are distinct from telangiectasias and angiomyolipomas, and most colonic hemangiomas are the capillary or



**Figure 1** Colonoscopy of the cecum showing a bluish nodular lesion.



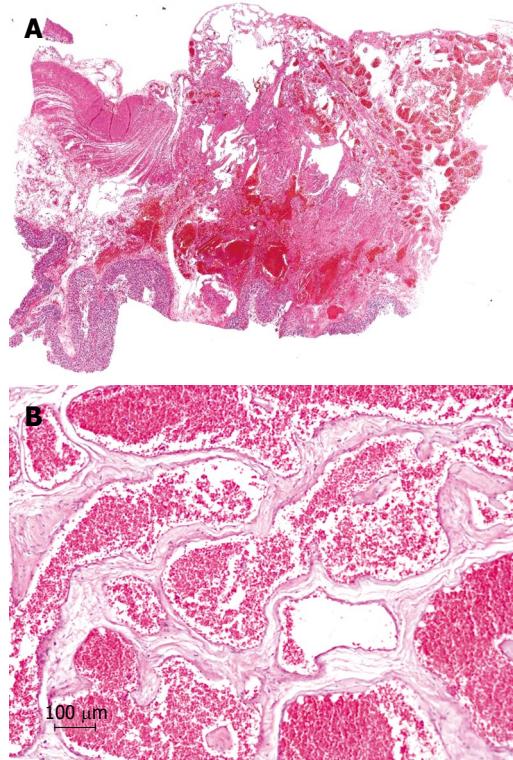
**Figure 2** Abdominopelvic computed tomography showing a lesion in the cecum protruding extraluminally with heterogeneous enhancement.



**Figure 3** Macroscopically, the surgical specimen was a cavernous hemangioma.

cavernous subtype. Capillary hemangiomas consist of a proliferation of small capillaries composed of thin-walled spaces lined by endothelial cells, while cavernous hemangiomas consist of large spaces lined by single or multiple layers of endothelial cells<sup>[9]</sup>. The capillary subtype is usually solitary and causes no symptoms, while the cavernous subtype presents with bleeding (60%-90%), anemia (43%), obstruction (17%), and, rarely, with platelet sequestration, although approximately 10% of patients remain asymptomatic<sup>[10]</sup>. The endoscopic findings of hemangiomas of the colon vary. Grossly, hemangiomas appear as soft, compressible bluish or deep red submucosal lesions, with dilated, engorged veins in the rectal wall<sup>[5]</sup>. However, they can be difficult to diagnose in some cases, especially if the hemangioma has an unusual color or is covered. A histological diagnosis before treatment may be difficult because of the risk of uncontrollable bleeding following a biopsy<sup>[1,4]</sup>. Hence, these lesions should not be biopsied. Abdominal CT can provide useful information about the size of the lesion and its extension to adjacent organs. Visceral angiography may also be useful in excluding coexisting hemangiomas at other sites in the gastrointestinal tract<sup>[5]</sup>.

Complete surgical resection is the best treatment for large or diffuse lesions<sup>[5]</sup>, although endoscopic resection has sometimes been recommended for hemangiomas that are pedunculated, polypoid, small, and limited



**Figure 4** HE staining of the hemangioma. A: Histological section showing large, dilated, blood-filled vessels lined by flattened endothelium (HE,  $\times 1$ ); B: The vascular walls were thickened focally by adventitial fibrosis (HE,  $\times 100$ ).

to the submucosal layer according to endoscopic ultrasonography<sup>[4]</sup>. Polypoid hemangiomas sometimes involve the entire wall of the colon, extending through the mesocolon and mesentery, as in our case<sup>[5]</sup>. In summary, we performed laparoscopic resection of a large cavernous hemangioma in the cecum. Although these lesions are rare, a better understanding of these lesions should help to obtain a definite diagnosis and devise an appropriate treatment plan.

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LETTERS TO THE EDITOR

## Different modalities of arterial reconstruction in hepatic retransplantation using right partial graft

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### TO THE EDITOR

Hepatic retransplantation (HR) is a major challenge due to inferior results when compared to primary transplantation, ethical questions related to the use of a limited resource, major economic costs, and more demanding surgical techniques<sup>[1,2]</sup>. Arterial revascularization of a graft can be challenging, especially if hepatic artery thrombosis or vascular abnormalities are present<sup>[3,4]</sup>.

The chronic shortage of cadaveric livers has led surgeons to alternative approaches, namely split-liver<sup>[5]</sup> and living-related transplantation<sup>[6]</sup>, which are now routinely performed, but almost exclusively for primary liver transplantation.

According to UNOS data, of the HRs performed between 1996 and 2007, only 8.7% were done using right or extended right grafts from deceased donors, and 14.3% using right grafts from live donors<sup>[7]</sup>.

In our institute, as well as in most European centers, both the right lobe from a living donor and the right extended graft from the deceased donor are procured with only the right branch of the hepatic artery for arterial supply. When a right partial graft is transplanted, the need to use the right branch of the hepatic artery instead of the entire hepatic artery, or the celiac trunk, or the aortic patch, can discourage surgeons from considering the possibility of an interposition graft.

Here we report our experience with 5 HRs in which right partial grafts resulting from conventional *in situ* extended right graft split livers (ERSL), and one right lobe resulting from an adult-to-adult living-related transplant (LRLT) were used.

For arterial reconstruction, we used, in the first case, an end-to-end anastomosis between the right branch of the ERSL and the recipient proper hepatic

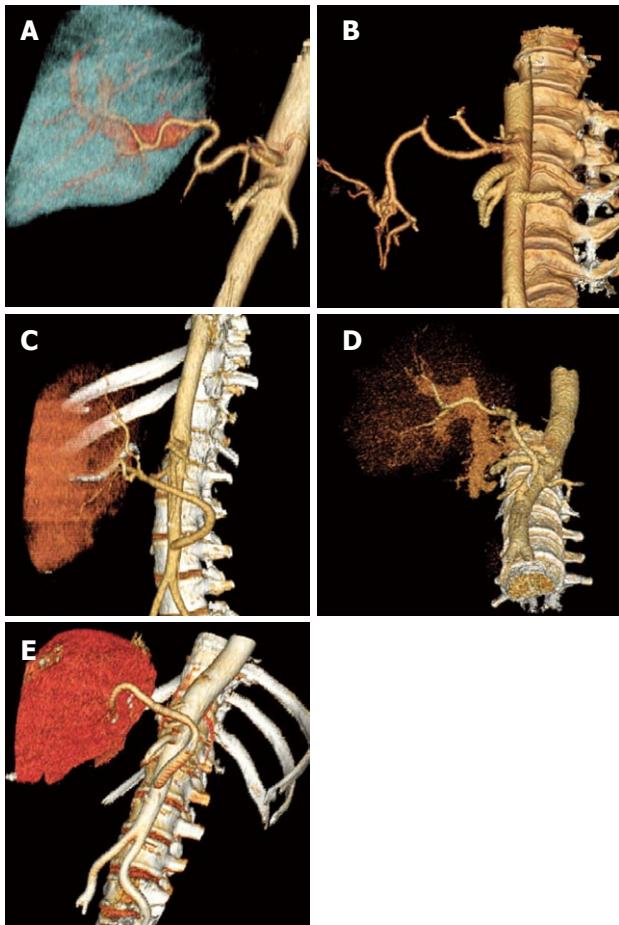
### Abstract

Though split-liver and living-related transplantation are routinely performed, they are done almost exclusively for primary liver transplantation because of potential surgical difficulties. These difficulties are generally related to arterial revascularization, particularly if there is hepatic artery thrombosis. According to UNOS data, of the hepatic retransplantations performed between 1996 and 2007, only 8.7% were done using right or extended right grafts from deceased donors, and 14.3% using right grafts from live donors. Here we report our experience with 5 hepatic retransplantations in which right partial grafts resulting from conventional *in situ* splits, and one right lobe resulting from an adult-to-adult living-related transplant, were successfully used with different modalities of graft arterialization.

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**Key words:** Hepatic artery; Liver transplantation; Split liver; Living related transplantation; Hepatic retransplantation

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**Figure 1 Arterial Reconstruction.** A: End-to-end anastomosis between the right branch of the extended right graft split livers (ERSL) and the recipient proper hepatic artery in a patient who needed retransplantation after living-related transplant (LRLT); B: End-to-end anastomosis between the right branch of the ERSL and recipient splenic artery in a patient transplanted because of hepatic artery thrombosis after LRLT; C: Cadaveric donor iliac interposition graft between the infra renal aorta and the right branch of the LRLT in a patient who had hepatic artery thrombosis after the standard primary transplant; D: Arterial revascularization using a cadaveric donor iliac interposition graft between the infra renal aorta and the right branch of the ERSL; E: Cadaveric donor iliac interposition graft between the infra renal aorta and the right branch of the ERSL.

artery in a patient who needed retransplantation after LRLT (Figure 1A). In the second case, we performed an end-to-end anastomosis between the right branch of the ERSL and the recipient splenic artery in a patient transplanted because of hepatic artery thrombosis after LRLT (Figure 1B). In the third case, a living-related transplant involving a patient who had hepatic artery thrombosis after the standard primary transplant, the arterial reconstruction was obtained using a cadaveric donor iliac interposition graft between the infra renal aorta and the right branch of the right lobe (Figure 1C). In the fourth case, an ERSL was used to re-transplant a patient having undergone a standard primary liver transplant. In this case, arterial revascularization was obtained using a cadaveric donor iliac interposition graft between the infra renal aorta and the right branch of

the ERSL (Figure 1D). In the last case, after the failure of an LRLT, an ERSL was used, with the need again for a cadaveric donor iliac interposition graft between the infra renal aorta and the right branch of the ERSL (Figure 1E). All the patients here reported received a mean follow-up of 28 mo (3-42) and are alive and free of biliary complications, or required relaparotomy due to graft loss.

The HR rate in our center is 8.6% out of a total of 580 liver transplants performed since 1999. Recipients are considered candidates for HR if they fit our listing criteria for primary transplantation. During HR, from a technical stand point, we always perform the standard technique with the use of veno-venous bypass. Portal vein and hepatic artery reconstruction are realized according to the vascular anatomy of each single patient. Vessel mismatch is not problematic when a wider iliac graft, which does not require spatulating or modifying in any way the larger artery, is used.

In regions like ours, with a rate of 9.3 cadaveric donor pmp, the chance of using right partial graft gave us the possibility to re-transplant patients that otherwise could not be cured. Although the surgical technique of arterial reconstruction here described is not a novelty, it should be emphasized that right partial grafting for HR achieves good results and allows optimization of a limited resource.

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## Meetings

### Events Calendar 2009

January 12-15, 2009  
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January 21-24, 2009  
Westin San Diego Hotel, San Diego, CA  
Advances in Prostate Cancer Research

February 3-6, 2009  
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)  
Second AACR Conference  
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009  
Hyatt Regency Boston, Boston, MA  
Translation of the Cancer Genome

February 8-11, 2009  
Westin New Orleans Canal Place, New Orleans, LA  
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009  
Hong Kong Convention and Exhibition Centre, Hong Kong, China  
19th Conference of the APASL  
<http://www.apasl2009hongkong.org/en/home.aspx>

February 27-28, 2009  
Orlando, Florida  
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

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EASL/ AASLD Monothematic: Nuclear Receptors and Liver Disease  
[www.easl.ch/vienna2009](http://www.easl.ch/vienna2009)

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Phoenix, Arizona  
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009  
Marriott Wardman Park Hotel  
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2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009  
Colorado Convention Center, Denver, CO  
AACR 100th Annual Meeting 2009

April 22-26, 2009  
Copenhagen, Denmark  
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)  
<http://www.easl.ch/>

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Denver, Colorado, USA  
Digestive Disease Week 2009

May 29-June 2, 2009  
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Methods in Clinical Cancer Research (Europe)

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World Conference on Interventional Oncology  
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Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

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Molecular Biology in Clinical Oncology

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Methods in Clinical Cancer Research

August 14-16, 2009  
Bell Harbor Conference Center, Seattle, Washington, United States  
Practical Solutions for Successful Management  
<http://www.asge.org/index.aspx?id=5040>

September 23-26, 2009  
Beijing International Convention Center (BICC), Beijing, China  
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September 27-30, 2009  
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For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.



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- 1 Jung EM, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

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- 2 Lin GZ, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

*In press*

- 3 Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

*Organization as author*

- 4 Diabetes Prevention Program Research Group. Hypertension,

insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

*Both personal authors and an organization as author*

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

*No author given*

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

*Volume with supplement*

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

*Issue with no volume*

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900 DOI:10.1097/00003086-20020800-00026]

*No volume or issue*

- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

## Books

*Personal author(s)*

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

*Chapter in a book (list all authors)*

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

*Author(s) and editor(s)*

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

*Conference proceedings*

- 13 **Hardden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

*Conference paper*

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming-EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

**Electronic journal** (list all authors)

- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

**Patent** (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc, assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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[1]Passed away on October 20, 2007

[2]Passed away on June 11, 2007

[3]Passed away on June 14, 2008



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# Bile acids as endogenous etiologic agents in gastrointestinal cancer

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## Abstract

Bile acids are implicated as etiologic agents in cancer of the gastrointestinal (GI) tract, including cancer of the esophagus, stomach, small intestine, liver, biliary tract, pancreas and colon/rectum. Deleterious effects of bile acid exposure, likely related to carcinogenesis, include: induction of reactive oxygen and reactive nitrogen species; induction of DNA damage; stimulation of mutation; induction of apoptosis in the short term, and selection for apoptosis resistance in the long term. These deleterious effects have, so far, been reported most consistently in relation to esophageal and colorectal cancer, but also to some extent in relation to cancer of other organs. In addition, evidence is reviewed for an association of increased bile acid exposure with cancer risk in human populations, in specific human genetic conditions, and in animal experiments. A model for the role of bile acids in GI carcinogenesis is presented from a Darwinian perspective that offers an

explanation for how the observed effects of bile acids on cells contribute to cancer development.

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**Key words:** Bile acids; Cancer; Adenocarcinoma; Esophagus; Stomach; Small intestine; Pancreas; Colon; Apoptosis; DNA damage

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## INTRODUCTION

Although it was proposed that bile acids are carcinogens as early as 1939 and 1940, there was little evidence at that early time that bile acids act as carcinogens in the gastrointestinal (GI) tract (reviewed in<sup>[1]</sup>). Since then, however, evidence has accumulated that exposure of cells of the GI tract to repeated high physiologic levels of bile acids is an important risk factor for GI cancer. Here we review the substantial evidence, much of it obtained in the last few years, for a role of bile acids in cancers of the esophagus, stomach, small intestine, liver, biliary tract, pancreas and colon/rectum. High exposure to bile acids may occur in a number of settings, but, most importantly, is prevalent among individuals who have a high dietary fat intake<sup>[2]</sup>. A rapid effect on cells of high bile acid exposure is the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Increased production of ROS/RNS, can lead to increased DNA damage and then increased mutation. The production of ROS/RNS following bile acid exposure likely occurs through multiple pathways involving disruptions of the cell membrane and mitochondria<sup>[1]</sup>. For each organ of the GI tract, we

review evidence, where available, on deleterious effects of bile acids, including the induction of ROS/RNS, induction of DNA damage, mutation and apoptosis, and the development of reduced apoptosis capability upon chronic exposure. Reduced ability to undergo apoptosis is important because apoptosis is a beneficial process that rids the body of cells with unrepaired DNA damage that can cause mutation. Reduced apoptosis capability has been linked to increased mutagenesis<sup>[3-5]</sup>. We also review epidemiologic evidence and results of animal experiments indicating that long-term exposure to elevated levels of bile acids increases GI cancer risk.

The annual world-wide number of deaths due to cancer is about 7.6 million, and among these about 2.8 million (36%) are due to cancers of the GI tract<sup>[6]</sup>. A recent prospective study was carried out on red and processed meat in relation to cancer incidence in a cohort of approximately half a million men and women<sup>[7]</sup>. Individuals in the highest quintile of red meat intake, compared with those in the lowest, had a statistically significant elevated risk of esophageal, colorectal and liver cancer. Also, for processed meat, the risk of colorectal cancer was elevated. Both types of meat are sources of saturated fat and iron, which have independently been associated with carcinogenesis. In addition, processed meats contain nitrates and nitrites, precursors of N-nitroso mutagenic compounds.

## ESOPHAGUS

The estimated yearly number of deaths world-wide from esophageal cancer is 300 034 for men and 142 228 for women<sup>[6]</sup>, making it the sixth leading cause of cancer deaths among men and women combined. There are two principal histologic types of esophageal cancer, adenocarcinoma and squamous cell carcinoma. In the United States, the incidence of adenocarcinoma has increased four-fold between 1973 and 2002, whereas squamous cell carcinoma has declined 30% over the same period, making adenocarcinoma the predominant form of esophageal cancer<sup>[8]</sup>. Barrett's metaplasia of the esophagus is an important predisposing condition for the development of esophageal adenocarcinoma<sup>[9]</sup>. Barrett's esophagus (BE) is a metaplastic lesion of the distal esophagus, characterized by the replacement of the normal squamous epithelium by columnar intestinal epithelium containing goblet cells. BE is associated with increased duodeno-gastro-esophageal reflux<sup>[10,11]</sup>, which causes increased exposure of the esophagus to bile acids from the duodenum and acidity (gastric acidity) from the stomach. Individuals with esophageal adenocarcinoma experience even greater exposure to bile than persons with uncomplicated BE<sup>[12]</sup>. Expression of bile acid transporter proteins is increased in BE tissues, suggesting that the development of BE metaplasia may be an adaptation to protect cells from bile acids<sup>[13]</sup>. Thus progression to BE and to adenocarcinoma may be strongly influenced by bile acid exposure. As discussed next, evidence indicates that short-term exposure of esophageal cells to bile acids induces oxidative stress, DNA damage, mutation and apoptosis; and among surviving cells selects over the long-run for resistance to apoptosis and ultimately cancer.

Five studies have shown that bile acids cause increased production of ROS in esophageal cells, including those from BE metaplasia. A cocktail of five bile acids designed to mimic the bile acids present in gastroesophageal reflux was used to test whether reflux induces ROS<sup>[14]</sup>. The five bile acids were glycocholic acid (GCA), taurocholic acid (TCA), glycdeoxycholic acid (GDCA), glycochenodeoxycholic acid (GCDC) and deoxycholic acid (DCA). This cocktail induced ROS in biopsies from human BE metaplastic tissue. The bile acid cocktail also induced ROS in cultured SV40-transformed squamous esophageal epithelial cells (HET1-A). DCA induced ROS in cultured human esophageal adenocarcinoma cells (OE33) and squamous cell carcinoma cells (KYSE-30)<sup>[15]</sup>. GCDC in acidic media induced ROS in cultured esophageal squamous cell lines derived from patients with gastroesophageal reflux disease (GERD) with BE, or without BE<sup>[16]</sup>. When mice were fed a zinc deficient diet containing a DCA supplement, ROS production was increased and BE-like lesions developed<sup>[17]</sup>.

Six studies showed that bile acids induce DNA damage in esophageal cells (Table 1), and five of these reported evidence for oxidative DNA damage.

The findings that bile acids induce DNA damage suggest that bile acids may also increase the frequency of mutation, since replication of a damaged DNA template strand often results in a replication error and thus a mutation.

Esophagoduodenostomies were performed on Big Blue F1 *lacI* transgenic rats to surgically increase duodeno-gastro-esophageal reflux<sup>[21]</sup>. The frequency of *lacI* mutant cells proved to be significantly higher in the esophageal mucosa of the surgically altered rats than in the unaltered control rats, indicating that components of refluxate, such as bile acids, increase mutation. Forty-six percent of the mutant cells were altered at CpG dinucleotide sites, and the majority of these mutations (61%) were C to T or G to A transitions. This pattern of mutation is similar to that in human esophageal adenocarcinoma, suggesting that reflux is not only mutagenic, but also carcinogenic. Consistent with these findings, it was found that DCA treatment of cultured esophageal cells cause an increase in the frequency of GC to AT mutations in the *p53* gene<sup>[15]</sup>. In addition, increased duodeno-gastro-esophageal reflux was observed to increase mutagenesis using a surgical model in Big Blue mice (rather than rats)<sup>[22]</sup>.

Bile acids induce apoptosis in esophageal cells, perhaps through the mediation of damaging ROS. DCA induced apoptosis in esophageal biopsies from normal human squamous epithelium<sup>[23]</sup>. Also, five different bile acids [GCDC, GDCA, TCA, taurochenodeoxycholic acid (TCDC) and taurodeoxycholic acid (TDCA)] individually, and also in a mixture, induced apoptosis of cultured human normal esophageal mucosal epithelial cells<sup>[24]</sup>.

Although a short-term effect of high bile acid exposure is induction of apoptosis, a longer-term effect of repeated high exposure to apoptosis-inducing agents, such as bile acids, appears to be selection for apoptosis resistant cells. When tissue samples from patients with normal esophagus, esophagitis, BE lesions and

**Table 1** Bile acids induce DNA damage in cells of the esophagus

Cells/tissues	Bile acids that induce DNA damage	Assay for damage	Ref.
Cultured SV40-transformed, squamous esophageal epithelial cells (HET1-A) and Barrett's associated adenocarcinoma cells (FLO-1)	DCA; also cocktail containing GCA, TCA, TCDCA	Comet assay <sup>1</sup> for strand breaks	[18]
Cultured SV40-transformed, squamous esophageal epithelial cells (HET1-A)	DCA	Comet assay for strand breaks; evidence for oxidative mechanism involving nitric oxide	[19]
Cultured human adenocarcinoma cells (OE33)	DCA	Micronuclei assay; induction of micronuclei by DCA, reduced by antioxidants	[15,20]
Biopsies from human Barrett's esophageal metaplastic tissue	Cocktail containing DCA, GCA, TCA, GDCA, GCDCA	8-OHdG, an oxidized form of the DNA base guanine; assayed by IHC	[14]
Mouse model of esophagitis and Barrett's esophagus	DCA (as dietary supplement; also zinc deficiency)	8-OHdG assayed by IHC	[17]

<sup>1</sup>Comet assay, also known as the single cell gel electrophoresis assay; 8-OHdG: 8-hydroxydeoxyguanosine; IHC: Immunohistochemical assay.

adenocarcinomas were studied for apoptosis capability, it was found that apoptosis is inhibited early in the dysplasia-carcinoma sequence of BE by over-expression of the anti-apoptotic protein, Bcl-2<sup>[25]</sup>, presumably as a result of chronic gastroesophageal reflux containing bile acids. BE cells have high levels of the anti-apoptotic proteins IL-6, Bcl-xL and Mcl-1<sup>[26]</sup>. Studies of tissues obtained from patient biopsies, indicated that BE cells are resistant to apoptosis induction by DCA compared to esophageal squamous epithelium and normal colon epithelium<sup>[23]</sup>. Reduced apoptosis competence may arise by mutation in genes encoding proteins necessary for apoptosis. Since cells resistant to apoptosis have a growth advantage in the presence of agents that ordinarily induce apoptosis, such as bile acids, these cells will tend to proliferate to form a field of apoptosis resistant cells<sup>[27]</sup>. Within such a defective field, repeated encounters with bile acids in reflux would cause further DNA damage. Such DNA damage, leading to further mutation, may give rise to malignancy.

Considerable evidence indicates an association of bile acid exposure with esophageal cancer. In rats, reflux of duodenal or gastro-duodenal contents, that include bile acids, induced esophageal carcinoma in the absence of exogenous carcinogen<sup>[28]</sup>. Rat surgical models with increased duodenal reflux into the esophagus, but without added carcinogen, caused esophagitis, BE-like lesions and adenocarcinomas<sup>[29-32]</sup>. Persons with BE were found to have increased duodenoesophageal reflux and increased exposure to bile acids in their refluxate, suggesting that the BE premalignant lesion is linked to bile acid exposure<sup>[10,11]</sup>. In a rat duodenal-contents reflux model, a high animal-fat intake changed the bile acid composition of bile juice and increased the development of BE and esophageal adenocarcinoma<sup>[33]</sup>.

In summary, evidence indicates that, in esophageal cells and tissues, bile acids have the short-term effect of inducing oxidative stress, oxidative DNA damage, mutation and apoptosis. Over a longer period, bile acids are implicated in the development of apoptosis resistance and eventually the development of adenocarcinoma.

## STOMACH

The estimated yearly number of deaths world-wide from gastric cancer is 511 549 for men and 288 681 for women<sup>[7]</sup>,

making it the second leading cause of cancer deaths among men and women combined. Infection by the bacterium *Helicobacter pylori* is the major etiologic risk factor in gastric carcinogenesis. However, gastroesophageal reflux appears to have an important role in the development of gastric cardia adenocarcinoma<sup>[34,35]</sup> which may have an etiology similar to that of esophageal adenocarcinoma<sup>[34]</sup>.

Exposure of cultured gastric carcinoma cells (St23123) to TCDCA increased production of ROS<sup>[36]</sup>. DCA induced apoptosis in cultured human gastric epithelial cells<sup>[37]</sup>. In rats, TCA increased stomach tumorigenesis induced by the carcinogen N-methyl-N'-nitro-N-nitrosoguanidine<sup>[38]</sup>. Carcinoma in the gastric stump (generated in rats by surgical gastrectomy) was increased by dietary fat intake and increased bile acid output<sup>[39]</sup>. Gastric adenocarcinomas were found to develop in a rat surgical model of duodenal reflux<sup>[40]</sup>. Gastroesophageal reflux in humans is implicated in adenocarcinoma of the gastric cardia<sup>[34,35,41]</sup>. Thus, elevated bile acid exposure is associated with increased ROS, induction of apoptosis and increased development of cancer of the gastric cardia.

## SMALL INTESTINE

Small intestinal cancer is relatively infrequent compared to other cancers of the GI tract. In the United States, only 0.2% of all cancer deaths are due to cancer of the small intestine. Elevated risk of carcinoid tumor of the small intestine is associated with saturated fat intake<sup>[42]</sup>, consistent with an etiologic role of bile acids. Fifty-three percent of adenocarcinomas of the small intestine arise in the duodenum, although the length of the duodenum is only 4% of the entire length of the small intestine. In addition, 57% of these duodenal cancers arise in the 6-7 cm segment that includes the outlet (Ampulla of Vater) of the common bile duct where bile (including bile acids) and pancreatic secretions empty into the small intestine<sup>[43]</sup>. Most adenomas and carcinomas of the small intestine and extrahepatic bile ducts arise in the region of the Papilla of Vater (which includes the Ampulla of Vater)<sup>[44]</sup>. Patients who have undergone a cholecystectomy are at increased risk of cancer of the small intestine, a risk that declines with increasing distance from the common bile duct<sup>[45]</sup>. These findings indicate that exposure to high levels of bile might be the

**Table 2** Bile acids induce apoptosis in liver cells

Cells/tissues	Bile acid(s) that induced apoptosis	Ref.
Isolated rat hepatocytes	GDCA GCDCA	[64,65] [55,66]
Isolated rat and mouse hepatocytes	GCDCA, GCA	[57]
Liver tissue sections from rats fed DCA, and cultured human hepatocellular carcinoma cells (HuH-7)	GCDCA DCA	[67] [68]
Cultured rat hepatocytes (McNtcp.24 cells)	DCA	[58]
Cultured human hepatocellular carcinoma cells (HuH-7)	GCDC	[69,70]
Rat hepatocytes and human hepatoma carcinoma cells (HuH-7)	GCDCA	[71]
	Taurolithocholate-3-sulfate	[59]

underlying cause of carcinomas of the small intestine.

Individuals with familial adenomatous polyposis (FAP) have an increased risk of developing adenomas and cancer of the small and large intestine. In the small intestine, these lesions arise mostly near the outlet of the common bile duct, where their distribution parallels bile acid exposure<sup>[46,47]</sup>. In a mouse model of FAP (*Apc*<sup>min/+</sup>), higher dietary fat intake was associated with an increase in small intestinal tumors<sup>[48]</sup>. Administration of CDCA in this FAP mouse model increased duodenal tumors, suggesting that unconjugated bile acids contribute to periampullary tumor formation in the setting of an *Apc*<sup>min/+</sup> genotype<sup>[49]</sup>.

The farnesoid X receptor (FXR) is a member of the nuclear receptor superfamily, and bile acids are endogenous ligands of FXR. FXR is necessary for maintaining bile acid homeostasis, and activation of FXR induces the expression of ileal bile acid binding protein (IBAB) and ileal bile acid transporters. In *Apc*<sup>min/+</sup> mice, FXR deficiency led to an increase in the size of small intestine adenocarcinomas<sup>[50]</sup>. Taken together, these results indicate that bile acids play a central role in cancer of the small intestine.

## LIVER

The estimated yearly number of deaths world-wide from liver cancer is 474 215 for men and 205 656 for women<sup>[6]</sup>, making it the third leading cause of cancer deaths among men and women combined. The majority of liver cancers world-wide arise as a result of chronic infection by hepatitis B or C virus, or from exposure to aflatoxin B1, a carcinogenic food contaminant. Excessive alcohol consumption is another risk factor. However, the risk of hepatocellular carcinoma is elevated in individuals with late stage primary biliary cirrhosis, a possible autoimmune disease<sup>[51]</sup>. Liver cancer can also arise in children with a defect in the bile acid export pump<sup>[52]</sup>. Thus bile acids are implicated in at least some cases of liver cancer.

Several studies have shown that bile acids induce ROS in cells of the liver. TCDCA induced ROS in isolated rat hepatocytes<sup>[53,54]</sup>. ROS were also induced in rat hepatocytes by GCDCA<sup>[55-57]</sup> and by DCA<sup>[58]</sup>. Taurolithocholate-3-sulfate induced ROS both in rat hepatocytes and a human hepatoma cell line (HuH7)<sup>[59]</sup>.

Treatment of human hepatoma cells (HepG2) with DCA activated the *gadd153 promoter*<sup>[60]</sup>. This promoter is activated by DNA damage, suggesting that DCA induces

DNA damage in hepatoma cells.

DCA is a promoter of preneoplastic lesions (hyperplastic nodules) in hepatocellular carcinogenesis<sup>[61,62]</sup>. Evidence has also been presented that DCA, given as a dietary supplement in rats, possess initiating activity for hepatocarcinogenesis<sup>[63]</sup>. At least 12 studies have shown that bile acids induce apoptosis in liver cells. These are listed in Table 2. Apoptosis induced in liver cells by hydrophobic bile acids is likely caused by oxidative stress<sup>[59]</sup>.

Four studies indicated that bile acid-induced apoptosis in liver cells is mediated by ROS. A lazaroid antioxidant (U83836E) inhibited induction of apoptosis in isolated rat hepatocytes<sup>[55]</sup>. The antioxidants  $\alpha$ -tocopherol, ebselen or idebenone (a coenzyme Q analogue) inhibited apoptosis of isolated rat hepatocytes by GCDCA and GCA<sup>[57]</sup>. Also in isolated rat hepatocytes, the antioxidants  $\beta$ -carotene and  $\alpha$ -tocopherol inhibited GCDCA induced apoptosis<sup>[67]</sup>. LCA and CDCA activated the antioxidant responsive element Nrf2 in human hepatoma-derived cells (HepG2), mouse hepatoma-derived cells (Hepa1c1c7) and primary human hepatocytes<sup>[72]</sup>. Nrf2 activation inhibits apoptosis, and the target genes of activated Nrf2 include the genes that encode the rate-limiting enzyme in glutathione biosynthesis and thioredoxin reductase 1. The general finding that induction of apoptosis in liver cells by bile acids can be reduced by anti-oxidants implies that this induction is mediated by ROS.

The bile salt export pump conveys bile acids from the hepatocyte cytoplasm into bile canaliculi. Mutations in the *ABCB11* gene cause a deficiency in the bile salt export pump, leading to intrahepatic accumulation of toxic bile salts. Children with such mutations have an increased incidence of hepatocellular carcinoma<sup>[52,73]</sup>. Mice lacking the farnesoid X receptor, which controls the synthesis and export of bile acids, have increased hepatic bile acids. These mice have a high incidence of liver tumors<sup>[74,75]</sup>. Such findings led to the suggestion that in cholestatic liver disease, chronic exposure to bile acids may play an important role in hepatocellular carcinogenesis<sup>[51]</sup>.

## BILIARY TRACT

Cholangiocarcinoma (CC) is an adenocarcinoma that arises from the bile duct epithelium. The CCs that occur within the liver are referred to as intrahepatic CCs. Those that occur at the confluence of the left and right hepatic duct are termed hilar CCs. The CCs that arise between the hepatic hilum and the duodenal papilla (or

Ampulla of Vater) are called extra hepatic CCs<sup>[76]</sup>.

The gallbladder and bile duct are exposed to high concentrations of bile acids. The bile acids excreted from the liver into the gall bladder are at a concentration of approximately 100 mmol/L<sup>[77]</sup>. The lifetime risk for developing cholangiocarcinoma in patients with primary sclerosing cholangitis is estimated at 7%-13%<sup>[78]</sup>, and it was suggested that chronic exposure to bile acids may play an important role in cholangiocellular carcinogenesis<sup>[51]</sup>. Two children with progressive familial intrahepatic cholestasis and cholangiocarcinoma were found to have an absence of bile salt export pump expression and mutations in the *ABCB11* gene<sup>[79]</sup>. Loss of a functional bile salt export pump may cause cholangiocarcinoma through intracellular accumulation of bile acids. Incubation of immortalized mouse cholangiocytes with GCDC resulted in the generation of ROS and an increase the percentage of cells with oxidative DNA damage (8-OHdG), suggesting that the a long-term effect of excessive exposure of the biliary tract to GCDC may be carcinogenesis<sup>[80]</sup>.

## PANCREAS

The estimated yearly number of deaths world-wide from pancreatic cancer is 137 206 for men and 122 185 for women<sup>[6]</sup>, making it the eighth leading cause of cancer deaths among men and women combined. Most adenocarcinomas of the pancreas occur in the head of the gland, which is in close proximity to bile<sup>[81]</sup>. In a hamster surgical model, bile reflux into the pancreatic duct was shown to induce development of intraductal papillary carcinomas of the pancreas<sup>[82]</sup>, suggesting that bile acid may be an etiologic agent in pancreatic cancer. Consistent with this idea, epidemiological studies found a positive correlation between ingestion of a western style high fat diet and the incidence of pancreatic cancer<sup>[83-85]</sup>. Treatment of human pancreatic cancer cell lines with bile acids (CDCA, DCA or TCDCA) induced cyclooxygenase-2 (COX-2) expression<sup>[81]</sup>. Since COX-2 is overexpressed in human pancreatic adenocarcinomas, these results also suggest a possible role for bile acids in pancreatic carcinogenesis.

## COLON AND RECTUM

The estimated yearly number of deaths world-wide from cancer of the colon and rectum is 318 798 for men and 284 169 for women<sup>[6]</sup>, making it the fourth leading cause of cancer deaths among men and women combined. Although both inherited mutations, environmental factors (e.g. smoking) and dietary factors are involved in colorectal cancer development, sporadic colorectal cancer appears to be caused predominantly by dietary factors.

The association of risk of colorectal cancer and consumption of red meat and processed meat was assessed in a meta-analysis of 15 prospective studies on red meat and 14 studies on processed meat<sup>[86]</sup>. The results showed consistent associations between high consumption of red and of processed meat and risk of colorectal cancer. In another recent study, a dose-dependent positive

**Table 3** Bile acids induce ROS/RNS in colon cells

Cells/tissues	Bile acid(s) that induced ROS/RNS	Ref.
Human colon surgical resections	DCA (RNS)	[110]
Cultured human adenocarcinoma cells (CACO-2)	DCA, LCA (ROS)	[111]
Cultured human adenocarcinoma cells (HT-29)	DCA, LCA (ROS) DCA (ROS) DCA (RNS)	[112] [36,113] [114]
Cultured human adenocarcinoma cells (HCT116)	DCA (ROS) DCA (RNS)	[109,115,116] [117]
Rat colonic mucosa	DCA (ROS)	[118]
Mouse colonic mucosa	DCA (ROS, RNS)	[119]

association between saturated fat intake and localized colorectal cancer was found in women, but not in men<sup>[87]</sup>. In earlier work, a positive association between dietary fat consumption and cancer incidence was reported<sup>[88-93]</sup>. Dietary total fat intake and saturated fat intake, but not polyunsaturated fat intake, are positively associated with colon cancer incidence<sup>[94]</sup>. In cancer prone *Apc*<sup>min/+</sup> mice, a high fat diet results in a significant increase in tumors<sup>[48]</sup>. A Western-style diet, containing elevated lipids and decreased calcium and vitamin D, induced colonic tumors in normal CB7Bl/6 mice<sup>[95-97]</sup>. Taken together, these studies implicate dietary fat (primarily from red and processed meat) in the etiology of human colorectal cancer.

Dietary intake of high-fat and high-beef foods results in a significantly higher excretion of fecal secondary bile acids, mainly DCA and LCA<sup>[98]</sup>. Presumably the increase in DCA and LCA reflects increased production of bile acids in order to emulsify the increased level of dietary fat. Epidemiologic studies have also found that fecal bile acid concentrations are increased in populations with a high incidence of colorectal cancer<sup>[99-106]</sup>. The most significant bile acids with respect to human colorectal cancer appear to be the secondary bile acids, DCA and LCA<sup>[99]</sup>.

Although repeated exposure of the colorectal epithelium to high physiological concentrations of bile acids appears to be the major etiologic factor in colorectal carcinogenesis, other factors may also be significant. Intake of dietary heme iron is associated with increased risk of colorectal cancer<sup>[107]</sup>, suggesting that iron catalyzed formation of ROS may play a role. The risk of colorectal cancer is also increased by smoking<sup>[108]</sup>. Bile acids and nicotine from smoking can interact synergistically in colon cells to increase oxidative stress and DNA damage<sup>[109]</sup>.

Twelve studies have reported that bile acids induce production of ROS or RNS in colon cells (Table 3).

Fourteen studies showed that bile acids induce DNA damage in colon cells (Table 4), of which a component is likely oxidative DNA damage. Defective repair of oxidative DNA damage is linked to increased risk of colon cancer. The base excision repair pathway deals with oxidative damages in DNA caused by ROS. 8-OHdG is a major oxidative damage in DNA that can mispair with adenine causing G:C to T:A transversion mutations, unless the mispair is corrected. MUTYH is a mammalian DNA glycosylase that initiates base excision repair by excising adenine opposite 8-OHdG. Genetic

**Table 4** Bile acids induce DNA damage in colon cells

Cells/tissues	Bile acid(s)	Assay for DNA damage	Ref.
Isolated mouse colon crypt cells	LCA	Nucleoid sedimentation for strand breaks	[122]
Isolated human and rat colon cells LCA	LCA	Comet assay for strand breaks	[123]
Isolated rat colon cells	DCA	Immunostaining for poly (ADP-ribose) an indicator of DNA damage	[124]
Freshly isolated normal human colonocytes	DCA, CDCA	Comet assay for strand breaks	[125]
Cultured human adenocarcinoma cells (HT-29)	DCA, CDCA	Comet assay for strand breaks and modified comet assay for oxidative DNA damage	
Cultured human adenocarcinoma cells (HT-29)	DCA, LCA	Comet assay for strand breaks	[112,126]
Cultured human adenocarcinoma cells (CACO-2)	DCA, LCA	Comet assay for oxidative DNA damage	[111]
Cultured human colon adenocarcinoma cells (HCT-116 & HCT-15)	DCA	Comet assay for strand breaks	[127]
Cultured human colon adenocarcinoma cells (HCT-116 & HT-29)	DCA	Comet assay for strand breaks	[128]
Cultured human colon adenocarcinoma cells (HCT-116)	DCA	Induction of the DNA repair protein BRCA-1 Induction of DNA damage inducible gene GADD153 Comet assay	[129] [130] [116]
Cultured human colon adenocarcinoma cells (HCT-116 and HCT-15)	DCA	Induction of DNA damage inducible genes GADD34, GADD45, GADD153	[131]
Colon samples from mouse dietary colitis model	DCA	Oxidative DNA damage: 8-OHdG assayed by immunohistochemistry	[119]

**Table 5** Bile acids induce apoptosis in colon cells

Cells/tissues	Bile acid(s) that induced apoptosis	Ref.
Biopsies from normal human colonic mucosa	DCA	[135-139]
Colon adenoma cell lines (AA/C1 and RG/C2), and carcinoma cell line (PC/JW/F1)	DCA	[140]
Cultured human adenocarcinoma cells (HT-29 and CaCo-2)	DCA	[141,142]
Cultured human adenocarcinoma cells (HCT-116)	DCA, CDCA DCA	[130,143-146] [116,147-149]
Cultured human adenocarcinoma cells (HT-29)	DCA	[114]
Cultured human adenocarcinoma cells (HT-29 and HCT-116)	DCA DCA DCA	[150] [128]
Cultured human adenocarcinoma cells (HT-29) and human fetal colonic mucosal cells (FHC)	DCA, LCA, CDCA	[151]
Cultured human adenocarcinoma cells (HT-29, SW480, SW620)	DCA, LCA, CA, CDCA	[152]
Cultured human adenocarcinoma cells [HCT-116 (p53 <sup>+</sup> ) and HCT-15 (p53 <sup>-</sup> )]	DCA, CDCA	[153,154]
Cultured human adenocarcinoma cells (HCT-116SA apoptosis-sensitive and HCT-116RB, HCT-116RC and HCT-116RD apoptosis resistant)	DCA	[127]
Human colonic mucosal samples from surgical resections	DCA	[155]

defects in MUTYH cause multiple polyps<sup>[120]</sup> and greatly increased risk of colorectal cancer<sup>[121]</sup> in humans.

The numerous studies showing that bile acids induce DNA damage in colon cells suggest that bile acids may also induce mutation and genomic instability. In a model system for inducing tumors in the rat using the carcinogen azoxymethane, DCA not only increased the incidence of colon tumors, but also increased the incidence of tumors with *K-ras* point mutations<sup>[132]</sup>, suggesting that DCA may induce *K-ras* mutations. Hydrophobic bile acids cause aneuploidy and micronuclei formation, indicators of genomic instability, in a variety of cell types including colon epithelial cells<sup>[133]</sup>. Persistent exposure of cultured colon epithelial cells to DCA results in alterations in expression of chromosomal maintenance/mitosis-related genes that might give rise to the observed genomic instability<sup>[133]</sup>.

The 27 studies listed in Table 5 indicate that hydrophobic bile acids induce apoptosis in colon cells. Exposure of colon epithelial cells to DCA causes induction of growth arrest and DNA damage-inducible genes *GADD34*, *GADD45* and *GADD153*, probably in

response to the DNA damage caused by DCA<sup>[131]</sup>. DCA induced expression of *GADD153* is essential for DCA induction of apoptosis<sup>[130]</sup>. These findings suggest that induction of DNA damage by DCA results in apoptosis. Induction of apoptosis by DCA may protect against the survival of cells with damaged template DNA that upon replication might undergo mutation leading to cancer<sup>[134]</sup>.

Repeated long-term exposure of colonic epithelial cells to high physiologic concentrations of bile acids appears to select for cells that are resistant to induction of apoptosis by bile acids. Such apoptosis-resistant cells might arise and clonally expand through the processes of mutation (or epimutation) and natural selection. Several studies of colon cancer patients have shown that epithelial cells in areas of the colonic mucosa that do not contain the cancer itself have increased resistance to induction of apoptosis by DCA<sup>[115,135,137-139]</sup>. The expression of anti-apoptotic protein Bcl-xL is elevated in the colorectal mucosa adjacent to colorectal adenocarcinomas<sup>[157]</sup>. These findings suggest that tumors may often arise in a field of apoptosis-resistant epithelial cells. A variant of ileal bile acid binding protein (IBABP), termed IBABP-L,

is upregulated in colorectal cancer and is necessary for survival of HCT116 colon cancer cells in the presence of physiologic levels of hydrophobic bile acid<sup>[158]</sup>. This finding suggests that IBABP-L is a key factor in the development of resistance to bile acids in colon cancer cells. Furthermore, repeated long-term exposure of HCT-116 human colonic epithelial cells in culture to sublethal concentrations of DCA selects for cells that have further increased resistance to DCA-induced apoptosis<sup>[159]</sup>. These observations suggest a link between development of resistance to bile acid-induced apoptosis and colon cancer.

In summary, evidence indicates that, in colonic epithelial cells and tissues, bile acids have the short-term effect of inducing oxidative stress that causes DNA damage leading to mutation and apoptosis. Over a longer period, repeated exposure to high levels of bile acid may select for the development of apoptosis resistant fields of cells and eventually to the development of adenocarcinoma.

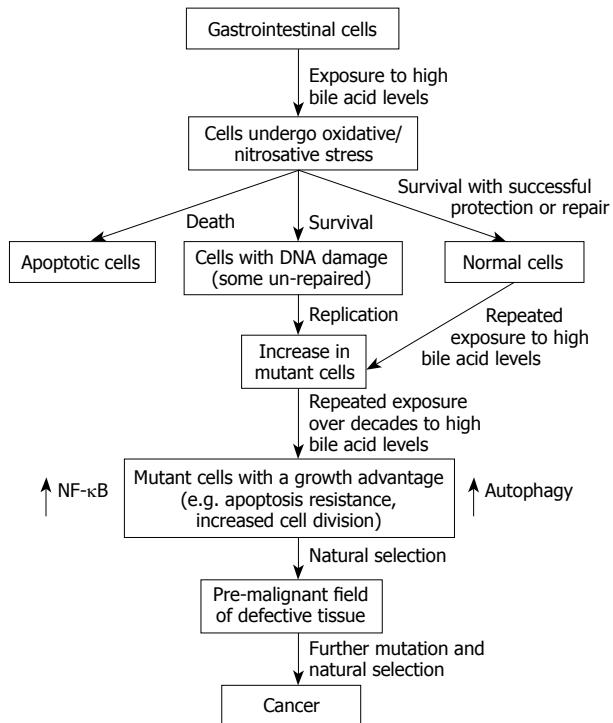
### DNA DAMAGE COUPLED WITH RESISTANCE TO CELL DEATH DRIVES TUMORIGENESIS

We have emphasized, above, the role of bile acids in inducing ROS/RNS and DNA damage in cells of the GI tract. These stresses, if excessive, can overwhelm cellular defenses resulting in cell death<sup>[139,160-163]</sup>. However, we have also shown that bile acids can activate two major cell survival pathways, NF-κB<sup>[115,124]</sup> and autophagy<sup>[164]</sup> (Figure 1). Both of the pathways are known to be activated by ROS<sup>[165,166]</sup>. Results from our laboratory indicate that the activation of both pathways by DCA can be attenuated by the use of antioxidants<sup>[113,115,124,164]</sup>. We have also shown that the NF-κB and autophagy pathways contribute to the stable apoptosis resistance that characterizes cell lines persistently exposed to DCA<sup>[159,164]</sup>. The sensitization to DOC-induced cell death after interfering with these pathways was documented using antisense oligonucleotides against the p65 subunit of NF-κB<sup>[159]</sup> and pharmacologically through the use of 3-methyladenine<sup>[164]</sup>, an inhibitor of autophagy.

The induction of persistent DNA damage in apoptosis-resistant cells is a dangerous situation that can lead to further mutation and ultimately cancer (Figure 1). An increase in Bcl-2 (an anti-apoptotic protein), for example, may also downregulate Ku DNA binding activity, thereby further amplifying genomic instability through interference with the non-homologous end-joining pathway of DNA repair<sup>[167]</sup>. The cross-talk between anti-apoptotic proteins and DNA repair proteins is a current area of investigation.

### NUCLEAR BILE ACID RECEPTORS FXR, VDR AND PXR/SXR

Recently, it has become apparent that nuclear bile acid receptors FXR, VDR and PXR/SXR play an



**Figure 1** The role of bile acids in the sequence of events leading to gastrointestinal cancer.

important role in protecting against carcinogenic effects of bile acids. FXR, a member of the nuclear receptor superfamily, responds to bile acids as physiological ligands<sup>[168-170]</sup>. FXR has a key role in activating pathways that maintain bile acid homeostasis<sup>[50]</sup>. FXR protects against intestinal tumorigenesis, possibly by a mechanism involving induction of apoptosis<sup>[50,171]</sup>.

The vitamin D receptor (VDR) functions as a receptor for the secondary bile acid lithocholic acid, and has a key role in activating a pathway that detoxifies lithocholic acid<sup>[172]</sup>. Similarly, the human xenobiotic receptor SXR (steroid xenobiotic receptor) and its rodent homolog PXR (pregnane X receptor) are bile acid receptors that, when activated, induce a response that detoxifies bile acids<sup>[173,174]</sup>. PXR promotes bile acid detoxification by activating bile acid metabolizing enzymes and transporters. In both human colon cancer cells and normal mouse colon epithelium PXR/SXR protects against bile acid induced apoptosis<sup>[149]</sup>.

### CONCLUSION

In Figure 1, we suggest a possible general pathway for bile acid induced carcinogenesis based on evidence reviewed above. An immediate effect on cells of the GI tract to exposure to a high physiologic level of bile acids is the induction of ROS/RNS. This can lead to DNA damage and apoptosis in some cells. Among surviving cells, some may remain normal by successfully employing protective and repair mechanisms. Other surviving cells, however, may retain unrepaired DNA damage. When such cells undergo DNA replication using a damaged strand as template, mutations will likely arise. Over years of frequently repeated exposure to high

levels of bile acids many mutations will occur, and some of these mutations may provide a growth advantage to the cell in which they occur. The growth advantage may involve apoptosis resistance, and increased and/or aberrant proliferation. Such cells will tend to expand clonally at the expense of neighboring cells to form a field of defective cells. Further repeated exposure to high levels of bile acids will lead to additional mutations. Should some of these mutations arise within a defective field and also provide additional growth advantages, a secondary field will spread within the first field by natural selection. Repetition of this "mutation-and-selection" process over many years, perhaps decades, will lead to a pre-malignant field and eventually to cancer.

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## Focus on acute diarrhoeal disease

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### Abstract

Diarrhoea is an alteration of normal bowel movement characterized by an increase in the water content, volume, or frequency of stools. Diarrhoea needs to be classified according to the trends over time (acute or chronic) and to the characteristics of the stools (watery, fatty, inflammatory). Secretory diarrhoeas, mostly acute and of viral aetiology in more than 70% of cases, are by far the most important subtype of diarrhoeas in terms of frequency, incidence and mortality (over 2.5 million deaths/year in developing countries). Natural and synthetic opiates such as morphine, codeine, and loperamide which react with endogenous opiates (enkephalins, beta-endorphins, dynorphins) mainly act on intestinal motility and slow down transit. An antidiarrhoeal drug developed in recent years, racecadotril, acts as an enkephalinase inhibitor. Clinical studies have shown that it is just as effective as loperamide in resolving acute diarrhoea but with greater reduction in pain and abdominal distension. Some studies have explored the prevalence of diarrhoea in old age. An epidemiological study carried out in Italy by 133 General Practitioners on 5515 elderly outpatients reported a prevalence of diarrhoea, defined according to the Rome criteria, of 9.1%. Infectious diseases (19%) and drug use (16%) were the most common causes of diarrhoea in old age. Regardless of the cause, the treatment of elderly patients with diarrhoea must include rehydration and nutritional support. Every year, more than 50 million tourists travel from industrialized countries to places where hygiene levels are poor. At least 75% of those travelling for short periods mention health problems, and in particular traveller's diarrhoea.

### INTRODUCTION

A decrease in consistency (i.e. soft or liquid) and an increase in frequency of bowel movements to > 3 stools per day has often been used as a definition of diarrhoea for epidemiological investigations. Diarrhoea is an alteration of normal bowel movement characterized by an increase in the water content, volume, or frequency of stools. Intestinal water balance results from a complex regulation involving inflammatory mediators (prostaglandins, leukotrienes, bradykinin, nitric oxide), hormones, neuropeptides, integrity of the intestinal wall, efficiency of the circulatory system and of the enteric nervous system<sup>[1]</sup>.

From a clinical point of view, diarrhoea needs to be classified taking into account certain characteristics such as trends over time (acute or chronic, using a limit of 4 wk to separate the two conditions) and the characteristics of the faeces (watery, fatty, inflammatory, etc)<sup>[2]</sup>. Using all these characteristics, a doctor can better understand the diarrhoea symptom and decide upon diagnosis and therapy more rationally. The duration of diarrhoea is important, because acute forms are usually due to some infectious agent, intoxication, or food allergy. However, acute diarrhoea may be a symptom of the onset of chronic organic or functional disease. Also important is a chemical/physical examination of the stools. Watery diarrhoea is a symptom of some defect in the re-absorption of water due to an imbalance between the secretion and absorption of electrolytes (secretory diarrhoea) or to the ingestion of substances which the intestine has failed to absorb (osmotic diarrhoea).

Excessively fatty diarrhoea may be due to low intestinal absorption of lipids, which may be due in turn to poor digestion thereof, and inflammatory diarrhoea, if there is mucus and pus. The distinction between secretory and osmotic diarrhoea may be made clinically by trying to eliminate the various causes of osmotic diarrhoea, which are relatively few. The latter is due to the ingestion of salts (magnesium sulphate or phosphate) or polysaccharides (mannitol, sorbitol) which are not readily absorbed, or to some enzyme defect in the intestinal mucosa (e.g. a lack of lactase). Osmotic diarrhoea stops when the patient fasts, or when substances which cannot be readily absorbed are no longer ingested; secretory diarrhoea, however, continues even when the patient has stopped eating. Secretory diarrhoea may be caused by several factors, either endogenous or exogenous, which determine an imbalance between the absorption and secretion of electrolytes. Among the causes of secretory diarrhoea there are also intestinal motility abnormalities, both primitive and secondary to systemic neuro-endocrine or metabolic diseases. A significant proportion - usually about one third - of patients with irritable bowel syndrome (IBS) have diarrhoea as their main symptom. The clinical characteristics of this type of diarrhoea, called "functional," are its periodicity, its occurrence solely during the day, after meals, and faecal urgency or incontinence. The pathogenic mechanisms most often seen in these patients are stress (*via* central or peripheral mediators, the most important of which is serotonin), food factors (allergies) and hormones (oestrogen, prostaglandin)<sup>[3]</sup>.

## ETIOLOGICAL FACTORS OF THE ACUTE DIARRHOEA IN ADULT AGE

Secretory diarrhoeas, mostly acute and due to infections (bacteria, viruses, parasites), are by far the most important subtype of diarrhoeas in terms of frequency, incidence and mortality (over 2.5 million deaths/year). In developing countries, they represent the primary cause of child mortality, whereas in developed and developing countries alike secretory diarrhoeas are an important cause of hospitalisation and health expenditure. It is estimated that about 200-300 million new cases occur yearly in the USA with 900 000 hospital admissions and an overall expenditure of about 23 million dollars<sup>[4]</sup>. Despite these numbers, the true prevalence of infectious diarrhoea is probably underestimated since the pathogen may not be searched for in stool samples or the patient may not seek medical or hospital attention<sup>[5]</sup>. A Canadian study showed that only 22% of patients with diarrhoea seek medical attention and only 5% of these has a stool examination<sup>[5]</sup>. Infectious diarrhoeas are of viral aetiology in more than 70% of cases. Rotavirus is the major cause of infantile gastroenteritis and each year causes 600 000-800 000 deaths worldwide<sup>[6]</sup>. The virus infects the mature enterocytes of the villus tip of the small intestine and induces watery diarrhoea. Rotavirus impairs activities of intestinal disaccharidases and Na<sup>+</sup>-solute transport and inhibits water reabsorption through

the production of NSP4 enterotoxin. An additional secretion component is due to activation of the enteric nervous system, producing an increased chloride secretory response. The other viral etiologic agent is Norovirus which exerts a direct action on the activity of enzymes of the brush border<sup>[7]</sup>.

Bacterial aetiology occurs in 1.5%-5.6% of cases. The most frequently identified bacteria are *Campylobacter* (2.3%), *Salmonella* (1.8%), *Shigella* (1.1%) or *Escherichia Coli* (0.4%). Symptoms such as fever and bloody diarrhoea are strongly suggestive of the presence of an invasive bacterium (*Shigella spp*, *Salmonella spp*, *Camp. jejuni*, *Clostridium difficile*). These orally ingested micro-organisms overtake immune defences and adhere to the intestinal wall. Subsequently they alter the metabolism of the cell by penetrating the cell, either directly or through the production of toxins. Both toxins and bacteria cause cell death and can invade bloody circulation with systemic symptoms such as fever, chills, hypotension, nausea and vomiting. Depending on the pathogenetic mechanism, infectious bacterial diarrhoeas can be divided in cytotoxic (pathogens stimulate secretory function by activating intracellular enzymes without damaging the epithelial layer e.g. *Vibrio cholerae*, some strains of *E. coli*, *Bacillus cereus*) and cytotoxic (pathogens damage directly epithelial cell e.g. *Shigella*, *C. perfringens*, *C. difficile*, *Staphylococcus aureus*, *Salmonella* and *Campylobacter*)<sup>[8]</sup>.

A striking example of infectious diarrhoea of bacterial origin is that caused by *V. cholerae*, a Gram-negative bacterium causing a severe acute watery diarrhoea burdened with a 25%-50% mortality rate<sup>[9]</sup>. Every year, more than 100 000 cholera cases and 2000-3000 deaths are officially reported to WHO. Cholera toxin activates adenylate cyclase and elevated intracellular cAMP levels provoke loss of water and electrolytes which is manifested as the typical diarrhoea. The massive outpouring of electrolyte-rich isotonic fluid into the bowel can lead to volume depletion and shock, followed by renal and cardiac failure. Rehydration therapy, either intravenous or oral, considerably decreases the number of deaths from 25%-50% to less than 1%<sup>[9]</sup>.

*E. coli*, a normal saprophyte of the gastrointestinal tract, is another important cause of acute infectious diarrhoea<sup>[10]</sup>. According to pathogenetic mechanisms, as well as clinical syndromes, 5 strains can be distinguished (enterotoxigenic *E. coli*, enteropathogenic *E. coli*, enterohaemorrhagic *E. coli*, enteroinvasive *E. coli*, enteroaggregative *E. coli*). Transmission is *via* the orofaecal route or *via* direct person-to-person contact.

Shigellosis is a major cause of diarrhoea-related morbidity and mortality, especially in developing countries, with an estimated annual incidence of 165 million cases and 1 million deaths<sup>[11]</sup>. Transmission usually occurs *via* contaminated food and water or through person-to-person contact. Shigella bacteria multiply within colonic epithelial cells causing inflammation, mucosal ulceration, and bleeding. The symptoms of shigellosis include diarrhoea and/or dysentery with frequent mucoid bloody stools, abdominal cramps and tenesmus. The severity of the clinical picture is directly related to the infecting strain; *Sh. sonnei* causes mild diarrhoea, whereas *Sh. dysenteriae* and

*Sh. flexneri* usually cause mucoid bloody diarrhoea<sup>[12]</sup>.

*C. difficile* is an important nosocomial pathogen and the most frequently diagnosed cause of infectious hospital-acquired diarrhoea<sup>[13]</sup>. The causative organism is acquired by the oral route from an environmental source or by contact with an infected person or a health care worker who serves as a vector. Disruption of the bowel microflora, generally by antibiotics (clindamycin, cephalosporine and chinolones) creates an environment that allows *C. difficile* to proliferate. Toxigenic strains usually produce toxin A and toxin B, which cause intense inflammation of the colonic mucosa with fluid and electrolyte secretion<sup>[14]</sup>. The syndrome that results includes severe watery diarrhoea, fever, abdominal pain, and leukocytosis, sometimes complicated by toxic megacolon.

Salmonella species are Gram-negative aerobic/anaerobic bacilli that cause substantial morbidity, mortality and burden of disease globally. Salmonella can colonize both the small bowel and colon causing different clinical pictures. Typhoid fever (*S. typhi* and *S. paratyphi*) and enteritis (*S. enteritidis* and *S. typhimurium*) are the most common disease syndromes. Typhoid fever is particularly frequent in under-developed countries. In year 2000, a total of 21.6 million cases occurred with more than 216000 deaths<sup>[15]</sup>. Intestinal parasitic infections account for 20%-25% of infectious diarrhoeas, which are mostly chronic and endemic in developing countries<sup>[6]</sup>. *Giardia lamblia* is the most frequent cause of parasitic diarrhoea in immunocompetent patients. Giardiasis is common in developing countries but also in industrialized countries e.g. endemic areas in Russia<sup>[16]</sup>. *G. lamblia*, the cause of human giardiasis, is among the most common intestinal protozoa worldwide. Human infection may range from asymptomatic shedding of giardial cysts (60% of cases) to symptomatic giardiasis, causing abdominal cramps, nausea, acute or chronic diarrhoea, with malabsorption and failure of children to thrive. Factors affecting different clinical manifestations include virulence of the strain, amount of parasites ingested, and host immune response. Quite interestingly, giardiasis does not induce leukocytosis or eosinophilia.

Amoebiasis is the second leading cause of death from parasitic disease worldwide. The causative protozoan parasite, *Entamoeba histolytica*, is a potent pathogen infecting about 50 million people and resulting in 40 000 deaths per year<sup>[17]</sup>. The infection prevails in developing countries, particularly India, Africa, Mexico and South America. People at risk of infection include immigrants, travellers returning from countries of high endemicity, and men who have sex with men. Clinical manifestations range from asymptomatic carriage to invasive disease (bloody diarrhoea), to extraintestinal disease with liver abscess.

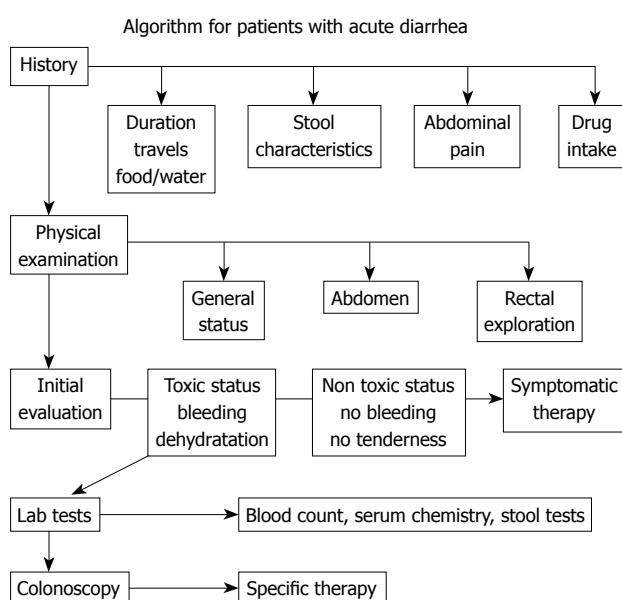
Acute infectious diarrhoea is among the most common illnesses worldwide, particularly in developing countries, and its associated morbidity and mortality are greatest among those at the extremes of age.

The presence of leukocytes in the stools is extremely important for the differential diagnosis of infectious diarrhoea. Faecal leukocytes are present in patients with diarrhoea caused by *Shigella*, *Campylobacter*, enteroinvasive *E. coli*, and absent in cases of infection by *V. cholerae*,

enterotoxigenic *E. coli*, *Rotavirus*, *Norovirus*, *G. lamblia*, *Entamoeba histolytica*, *Staph. aureus*, *Clostridium*.

## RATIONAL APPROACH TO DIAGNOSIS AND TREATMENT

Clinical classification of diarrhoea and an understanding of the main pathogenic mechanisms that bring it about are vital for a diagnostic and therapeutic approach. The initial diagnostic strategy (Figure 1) in patients with acute diarrhoea is mainly based upon a proper collection of medical history and an evaluation of the patient's clinical condition. This first diagnostic step often allows a good empirical therapy to be prescribed, whereas in patients with a poor general condition, or in those that do not respond to treatment, laboratory tests and, if required, an endoscopic examination of the intestine, must be undertaken. In both adults and children, rehydration is essential in managing patients with acute diarrhoea. Millions of lives have been saved thanks to the introduction of oral re-hydration therapy but this therapeutic approach, although essential in correcting dehydration, does not resolve the process that is at the base of diarrhoea and does not change the volume of faeces and evacuation frequency. In patients where the diagnosis leads to ascertainment of the cause of diarrhoea, therapy is usually aimed at removing the identified cause or aetiological agent. In many patients, therapy must begin before all the diagnostic tests have been exhausted (empirical therapy), since acute diarrhoea can greatly affect the quality of a patient's life and may compromise the health of children and elders. Anti-diarrhoeal therapy, known as symptomatic therapy, may be adopted alongside an aetiological therapy to improve the patient's clinical condition. The rational basis for an empirical or symptomatic therapy is determined by the main pathogenetic mechanism, that is the inability of the intestine to re-absorb water. Table 1 lists drugs for treatment of diarrhoea. The most widely used drugs in treating the symptoms of diarrhoea are those containing substances which react with endogenous opiates. These are peptides present not only in the central nervous system but also in the EC cells and the nervous plexus of the intestinal wall. There are three main types of endogenous peptide opiates: (1) enkephalins, which are found in the epithelial cells of the intestine; when they bind with delta receptors, they reduce cAMP levels and thus reduce the secretion of water and electrolytes; (2)  $\beta$ -endorphins, which bind with the mu receptor and mostly act by blocking gastrointestinal motility; (3) dynorphins, which bind with kappa receptors and lower nociceptive sensitivity. Natural and synthetic opiates such as morphine, codeine, and loperamide, are ineffective as antidiarrhoeal drugs; however, they mainly act on intestinal motility and slow down transit. This is why they have some side-effects, such as secondary constipation and abdominal bloating, which, together with some effects on CNS, contraindicate their use in children and in the elderly. An understanding of the molecular and cellular mechanisms causing intestinal

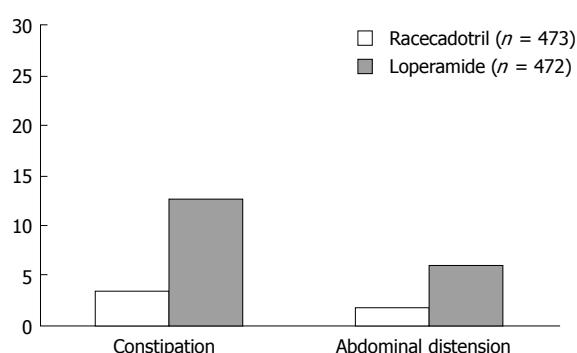


**Figure 1** Algorithm for patients with acute diarrhoea (modified from Schiller LR. Diarrhea. *Med Clin North Am* 2000; 84: 1259).

**Table 1** Symptomatic therapy of diarrhea

Opiates	Drug	Dose
μ-receptor agonists	Diphenoxylate	2.5–5 mg qid
	Loperamide	2–4 mg qid
	Codeine	15–60 mg qid
	Morphine	2–20 mg qid
Enkephalinase inhibitor δ-receptor	Racecadotril	1.5 mg/kg tid
Adrenergic agonists	Clonidine	0.1–0.3 mg tid
Somatostatin analogue	Octreotide	30–250 μg tid
Bile acids binding substances	Cholestyramine	4–16 g/die
Fibers	Psyllium	10–20 g/die

secretion has brought about developments in substances which can act selectively as anti-secretory drugs. Pharmacological research has recently been mainly oriented on enkephalins, which are endogenous opiates and are fairly widespread in the enteric nervous system. Enkephalinergic nerves reach the basolateral membrane of enterocytes where, *via* the delta receptors, they inhibit the action of adenylyl cyclase thus blocking the secretion of water and chloride. Enkephalins are rapidly broken down by a specific enzyme, enkephalinase, which determines the biological half-life of these peptides. In this context, racecadotril is an antidiarrhoeal drug developed in recent years. Racecadotril acts as an enkephalinase inhibitor thus promoting the anti-secretory action of enkephalins at the gastrointestinal level. After ingestion racecadotril is rapidly absorbed and becomes an active metabolite (Tiorfan). The anti-diarrhoeal action of this substance has been studied many times in pre-clinical and clinical trials, and it has been shown that it is purely anti-secretory. In fact, this drug has no effect on intestinal motility. Clinical studies<sup>[18–21]</sup> have shown that it is just as effective as loperamide in resolving acute diarrhoea but with greater reduction in pain and abdominal bloating and less secondary constipation (Table 2, Figure 2). Racecadotril



**Figure 2** Treatment-related adverse events associated with antidiarrhoeal therapy (from Prado et al<sup>[20]</sup>).

**Table 2** Comparison of Racecadotril (100 mg *tid*) and Loperamide (1·6 mg/die) in the treatment of acute diarrhea in adults

Author, yr	Patients (n)	Study design	Time to resolution (h)	P
Freixinos J, 1996	574	d.b. randomized	28.9 vs 26.8	NS
Vetel JM, 1999	147	d.b. randomized	14.9 vs 13.7	NS
Prado D, 2002	945	s.b. randomized	55 vs 55	NS
Wang HH, 2005	62	s.b. randomized	19 vs 13	NS

NS: Not significant.

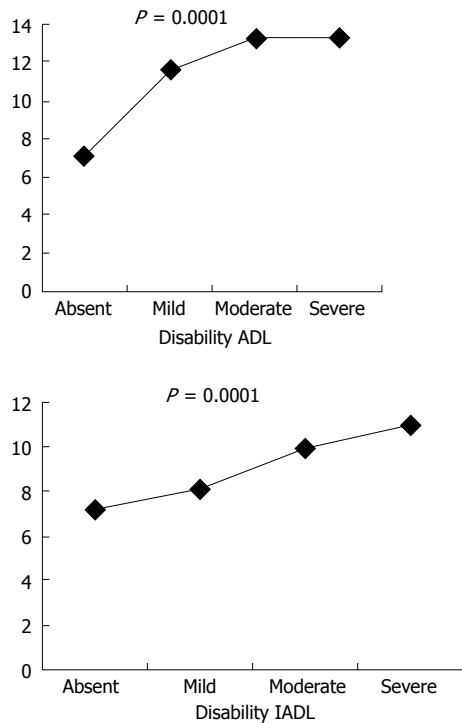
therefore represents a safe and rational therapy for acute diarrhoea, and is easy to use either alone or together with an aetiological therapy. The particular features of this drug make it very effective and suitable for use in children as well as in geriatric age groups.

Other drug categories include: (1) somatostatin analogues (octreotide), which are used in carcinoid syndrome or in other endocrinous diarrhoeas; (2) adrenergic agonists, such as clonidine, which affect intestinal motility and transport; (3) chelating agents, such as colestyramine, which are used with diarrhoea caused by bile acids, such as in post-cholecystectomy or maldigestion syndromes; (4) dietary food supplements, such as psyllium, which increase stool consistency and are useful for patients with incontinence.

## ACUTE DIARRHOEA IN THE ELDERLY

### Epidemiology

Some studies have explored the prevalence of diarrhoea in old age but the data does not give homogeneous results. A previous study performed in 328 non-institutionalized elderly subjects from U.S.<sup>[22]</sup> reported a prevalence of diarrhoea of 14.2%. More recently, a cross-sectional survey carried out in Australia, Canada, Ireland and the U.S. reported a prevalence of diarrhoea of 3.9% in elderly subjects aged 65 years or more<sup>[23]</sup>. Differences in the definitions of diarrhoea and the methodology of study recruitment may explain the discrepancies in the results. Indeed, in the first study, collection of data was made through a mailed self-administered questionnaire and patients with diarrhoea included in the survey were determined according to the Rome criteria for functional



**Figure 3** Prevalence of diarrhoea and disability according to ADL and IADL. 423 patients with diarrhea (GSRS score  $\geq 2$ ), M = 178, F = 245, mean age =  $75.0 \pm 6.3$  years, range = 65-100 years.

diarrhoea, i.e. both “the subjects with a stool frequency of more than three stools per day” and “the subjects who passed loose or watery stool and/or with urgent need for defecation”. In the second study, diarrhoea was defined according to the World Health Organization definition as three loose stools or bowel movements in any 24 h period during the 4 wk before the interview. In this study, moreover, data were collected by telephone call, thus excluding persons who did not have access to a fixed line telephone in their home and therefore probably the older and more disabled elderly people.

Very recently an epidemiological study carried out in Italy by 133 General Practitioners on 5515 elderly outpatients, reported a prevalence of diarrhoea, defined according to the Rome criteria, of 9.1%; the prevalence of diarrhoea significantly increased with an increase of both age and the grade of disability as evaluated by the ADL and the IADL (Figure 3). The study, moreover, demonstrated that, in the past 6 mo, elderly patients with diarrhoea underwent a significantly higher number of gastroenterological visits and instrumental examinations of the gastrointestinal tract such as barium X-ray, colonoscopies, abdominal ultrasound and CT than elderly subjects without diarrhoea. This finding is indirectly in agreement with previous studies supporting the concept that diarrhoea in old age may significantly impair the quality of life and functional status of affected individuals<sup>[24]</sup> and it may be a cause of morbidity and complications leading to a severe burden in hospitalized elderly patients<sup>[25]</sup>.

### Etiology

A previous study carried out in the U.S. in hospitalized patients aged 70 years and over reported that infectious

diseases (19%) and drug use (16%) were the most common causes of diarrhoea in old age. Gastrointestinal disorders, such as ischaemic colitis, malabsorption, diverticular disease, IBS and tumors of the colon and/or the small intestine accounted for about 15% of cases. Over 20% of diarrhoea observed in this population, however, was associated with the presence of constipation, and diarrhoea was a clinical expression of faecal incontinence in the presence of coprostasis<sup>[26]</sup>.

**Infectious diarrhoea:** A study from the U.S. reported that *Salmonella* (16.1 cases/100 000 persons), *Campylobacter* (13.4 cases/100 000 persons), *Shigella* (10.3 cases/100 000 persons) and *E. coli* O157:H7 (1.7 cases/100 000 persons) were the pathogens most frequently associated with diarrhoea. *Vibrio*, *Yersinia*, *Listeria* and *Cyclospora* were found in less than one case per 100 000 subjects<sup>[8]</sup>. A positive stool culture was found in 1.5%-5.6% of cases when performed three days before the beginning of symptoms. After 72 h from the beginning of diarrhoea, the diagnostic accuracy of the stool culture was less than 0.8%. Thus the microbiologic evaluation of hospitalized patients or those with recent exposure to antibiotics in whom diarrhoea develops should focus on the diagnosis of toxigenic *C. difficile*, the most common cause of nosocomial diarrhoea. Testing for other pathogens in patients who have been hospitalized for more than 72 h is discouraged, except in elderly patients 65 years of age or older with comorbidity, patients with Human Immunodeficiency Virus infection, patients with neutropenia and patients with suspected systemic spread of enteric infection<sup>[27]</sup>.

In elderly patients who have taken antibiotics the most frequent cause of infectious diarrhoea is *C. difficile*. Hospitalization and institutionalization are independent risk factors for *C. difficile* infection; indeed, in these settings the infection may be observed in 30% of subjects, even if asymptomatic in over 2/3 of subjects, with a high rate (10%-20%) of relapses. In elderly subjects *C. difficile* infection may have a nonspecific clinical presentation, including hyperpyrexia, abdominal pain or leukocytosis, and is sometimes complicated by toxic megacolon and sepsis.

**Drug-related diarrhoea:** Diarrhoea is a relatively frequent adverse event, accounting for about 7% of all drug adverse effects<sup>[28]</sup>. More than 700 drugs have been implicated in causing diarrhoea. Several mechanisms have been reported to be involved in inducing drug-related diarrhea, such as altered gastrointestinal defences, mucosal damage of the small and large intestine and/or disruption of normal pathophysiological processes of fluid and electrolyte absorption and secretion; sometimes more than one mechanism may be involved<sup>[29]</sup>.

A recent epidemiological survey carried out in Italy demonstrated that elderly patients with diarrhoea were taking a significantly higher number of drugs than patients without diarrhoea. Moreover, a significant increase in the prevalence of diarrhoea occurred in patients who were treated with a progressively higher number of drugs, reaching a prevalence of 11.0% in patients who were taking 3-5 drugs and a prevalence of 11.7% in patients who were taking 6 drugs or more.

In this elderly population of outpatients, the drugs significantly associated with the presence of diarrhoea were antibiotics, proton pump inhibitors, allopurinol, psycholeptics, selective serotonin reuptake inhibitors and the antihypertensive angiotensin II receptor blockers.

### **Approaches to therapy**

**Rehydration and nutrition:** Regardless of the cause, the treatment of elderly patients with diarrhoea must include rehydration and nutritional support. Patients should be encouraged to drink fluids and take salts both in liquids and in crackers. If necessary i.v. electrolyte solutions may be used.

**Antimicrobials:** Since in over 90% of cases of diarrhoea the pathogen may not be identified, the clinical benefit of an empiric antibiotic treatment should be evaluated taking into account the risk of adverse event reactions and the risk of harmful eradication of normal flora. In elderly patients with community-acquired diarrhoea with fever, dysentery and severe clinical conditions, and in whom diarrhoea is not thought to be attributable to fluoroquinolone-resistant bacteria, empirical treatment with an agent such as a fluoroquinolone is reasonable. Alternatively, treatment in severely ill elderly patients may include macrolides such as erythromycin or azithromycin<sup>[8]</sup>. The treatment of *C. difficile*-associated diarrhoea usually includes cessation of the initiating antibiotic. The choice to immediately re-treat the patient with another antibiotic is poor, supported by currently available evidence. Oral metronidazole is effective; vancomycin has been tested but it is more prone to serious adverse drug reactions. Recent evidence suggest that teicoplanin is better than vancomycin for bacteriological cure and that it has borderline greater efficacy in reducing symptomatology<sup>[30]</sup>.

**Symptomatic therapy:** Over 300 over-the-counter products are currently used for their antidiarrhoeal properties; of these, only loperamide, bismuth subsalicylate and kaolin have been tested in controlled studies. Curiously, none of these studies have been carried out in elderly patients. Recently, a multicenter study carried out on 945 outpatients, reported that racecadotril, a potent inhibitor of enkephalinase which exerts an anti-hypersecretory effect without increasing intestinal transit time, was as effective as loperamide in reducing diarrhoea with a significantly lower prevalence of side effects such as constipation, anorexia and abdominal pain than loperamide<sup>[20]</sup>.

Because of its characteristics, racecadotril can be an effective pharmacologic option in the treatment of diarrhoea in elderly patients; however, further studies are needed to extensively evaluate the role of racecadotril in the treatment of diarrhoea in elderly patients.

## **TRAVELLER'S DIARRHOEA**

Every year, more than fifty million tourists travel from industrialized countries to places where hygiene levels are poor. At least 75% of those travelling for short

periods mention health problems - mostly mild - but occasionally they require help from a doctor (7%) or even admission to a hospital (1%).

Traveller's diarrhoea (TD) is the most frequent of these problems; it occurs during or immediately after a trip to a less hygienic country, lasts for 3-4 d, and is characterised by the elimination of watery or soft faeces.

TD is usually brought about by eating contaminated water or foodstuffs, and affects 20%-50% of those travelling to countries with poor hygiene standards. In certain geographical areas, up to 60% of tourists can be affected if they stay for longer than 2 wk.

Based on hygiene conditions, there are 3 risk levels in the world: high risk [most of Asia, the Middle East, Turkey, Africa - except South Africa - Central America, and part of Latin America (20%-56%)], average risk (Eastern Europe, South Africa, some countries in Latin America, and some Caribbean islands) and low risk (Western Europe, the U.S., Canada, Australia, and Japan (4%-8%) (Figure 4).

The risk of contracting TD depends very closely upon the hygiene conditions in the country, and is very high for tourists from low-risk countries travelling to other countries (Table 3).

### **Aetiology**

TD aetiology is varied, and includes viruses, bacteria, protozoans, and mycetes. The main aetiological agents of secretory diarrhoea are *E. coli* (enteropathogenic, enteroadhesive, enterotoxic), *V. cholerae*, *C. difficile*, *Rotavirus* or *Norwalk astrovirus*, and for dysentery-related diarrhoea they are *E. coli* (enteroinvasive, enterohaemorrhagic), *Shigella spp*, *Camp. jejuni*, *S. typhi*, *S. paratyphi*, other salmonellas, *Aeromonas spp*, *Yersinia enterocolitica*, non-cholera bacilli, *Entamoeba histolytica*, *G. lamblia*, *Cryptosporidium*, *Cyclospora*.

### **Prevention**

Risk depends greatly upon the foodstuffs consumed (Table 4). The place in which food is prepared is also essential: in fact, the risk increases from private homes to restaurants, to food purchased at the road side.

When assessing risk, due account must be kept of certain variables which affect TD. Among the various predisposing factors are the type of trip, so an adventure holiday is more risky than one in a five-star hotel. Young people who do not eat at the table, especially children, seem to be at much greater risk than adults. There may also be certain genetic factors which might explain why some people are more easily affected by TD than others. Some conditions such as hypochlorhydria and achlorhydria should not be neglected: gastric acidity is an important barrier against pathogens. People with genetic or food-based hypochlorhydria, or who have had stomach surgery and need to take proton pump inhibitors, have a higher risk of TD<sup>[31]</sup>.

Indeed, it is interesting to notice that a trip to a high-risk area less than 6 mo after a previous trip there has a much lower risk of TD. This may be due to some sort of immunity to gastroenteral pathogens developed since the previous visit.

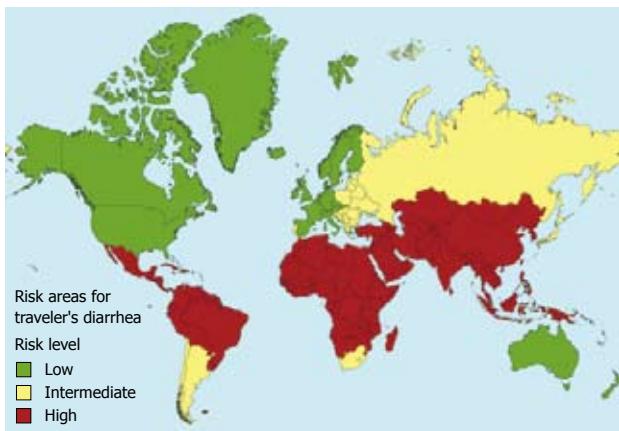


Figure 4 TD risk areas (Health Information for International travel, CDC 2005-2006).

Table 3 TD risk according to country and destination

Country of origin	Country of destination (%)		
	Low	Average	High
Low	2-4	10-20	20-40
Average	2-4	Indeterminate	90 <sup>1</sup>
High	2-4	ND	8-18

<sup>1</sup>Selected groups (e.g. Nile Cruises); ND: Not available.

Table 4 Food risk scale

Low risk (increasing)	High risk (decreasing)
Coffee, tea (served hot)	Puddings (> with unbaked creams)
Foods served at > 60°C	Tap water, ice
Fruit peeled by consumer	Cooked shellfish
Freshly-squeezed fruit	Cheese
Soft drinks in general	Cold collation
Bread	Spicy sauces
Bottled mineral water	Salads and raw vegetables
Butter	Milk

TD prevention is based on: suitable treatment of food, vaccination, and good personal hygiene, especially washing one's hands properly.

**Treatment of food:** To reduce the risk of TD as much as possible, one must take care when choosing food and drink, and avoid as far as possible any which do not appear to have been prepared properly.

These choices are extremely simple, but are very often ignored. Indeed, only a tiny fraction of all travellers take any notice of them. An inquiry carried out at big European airports<sup>[32]</sup> found that only 5.5% of travellers were willing to do anything about food safety.

**Vaccinations:** Anti-typhus, anti-hepatitis A, and anti-cholera vaccines can be effective against certain levels of TD. (1) Anti-typhus. There are two types of vaccines: tablets and injections. The first requires three tablets to be taken every other day. It is effective in 75% of cases, and provides protection for 2-3 years. The second type of

vaccine requires a single injection, and is 77% effective for three years. The oral vaccine must not be taken if one is taking malaria tablets, because it reduces the effectiveness of both. The injection, however, can be given along with other vaccinations (including malaria). In the oral and injected forms, efficacy is 55%-80% for three years (Levine, 2001); (2) Anti-hepatitis A. A subcutaneous injection can be given, and it becomes very effective two weeks thereafter; it will continue to provide protection for up to 20 years. The vaccination requires two injections: the second is given 6-12 mo after the first; (3) Anti-cholera. Two types of oral vaccine are given against *V. cholerae*: live but weakened, genetically a sub-unit A (CVD 103 HgR), and inactive cells deactivated using a sub-unit B mix (WC/rBS). The latter, designed to protect travellers against cholera, has been proven effective against TD caused by enterotoxic *E. coli*, the most frequent cause of this type of disease. Protection against TD can reach 80%<sup>[33,34]</sup>.

### Therapy

Rest must be taken for 2-3 d, and only water drunk. If symptoms persist, intestinal disinfectants can be given. To reduce the risk of dehydration and the duration of diarrhoea, antidiarrhoeal drugs can be used. Loperamide is effective but has side effects such as constipation and meteorism and/or abdominal swelling, which may cause intestinal occlusion, especially in elderly patients<sup>[35,36]</sup>. Racecadotril, recently made available in Italy, is equally effective as loperamide and does not cause any of these problems.

The General Medical Council in Italy has designed a solution to be taken by mouth, made up as follows: sodium chloride (3.5 g/L), potassium chloride (1.5 g/L), glucose (20.0 g/L), and sodium citrate (2.9 g/L). A solution using 2.5 g/L of sodium bicarbonate has a shorter half-life but is physiologically equivalent and can be found in most countries. Alternatively, the following solution can be used: 6 tsp. of sugar, 1 tsp. of kitchen salt, 2 pts. of drinking water<sup>[37,38]</sup>.

Antibiotics should only be given in serious cases, and where a specific bacterial aetiology is suspected.

### CONCLUSION

In summary, diarrhoea is an alteration of normal bowel movement characterized by an increase in the water content, volume, or frequency of stools. Secretory diarrhoeas, mostly acute and due to infections (bacteria, viruses, parasites), are by far the most important subtype of diarrhoeas in terms of frequency, incidence and mortality. Clinical classification of diarrhoea and an understanding of its main pathogenic mechanisms are fundamental for a diagnostic and therapeutic approach. A symptomatic anti-diarrhoeal therapy may be adopted alongside an aetiological therapy to improve the patient's clinical conditions and among the anti-diarrhoeal drugs racecadotril represents a safe and rational therapy. Acute diarrhoea is frequently reported in old age with a prevalence of 3.9%-14.2%. The treatment of elderly patients with diarrhoea, regardless of the cause, must include rehydration and nutritional support. TD may

represent a relevant problem since every year more than fifty million tourists travel from industrialized countries to places where hygiene levels are poor and at least 75% of those travelling for short periods mention health problems.

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# Vitamin D for the prevention and treatment of pancreatic cancer

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$1\alpha,25(\text{OH})_2\text{D}_3$  and its analogs are potentially attractive novel therapies for pancreatic cancer.

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## Abstract

Pancreatic cancer is ranked fifth among cancer-related deaths worldwide with a 5-year survival rate of less than 5%. Currently, surgery is the only effective therapy. However, most patients are diagnosed in the late stage and are not suitable for receiving curative surgery. Moreover, pancreatic cancer doesn't respond well to traditional chemotherapy and radiotherapy, leaving little effective treatment for advanced pancreatic cancer cases.  $1\alpha,25$ -dihydroxyvitamin  $\text{D}_3$  [ $1\alpha,25(\text{OH})_2\text{D}_3$ ], the biologically active form of vitamin  $\text{D}_3$ , was originally identified during studies of calcium and bone metabolism, though it is now recognized that it exerts biological effects in almost every tissue in the body. Abundant evidence has shown that  $1\alpha,25(\text{OH})_2\text{D}_3$  has antiproliferative, apoptotic, pro-differentiation and antiangiogenesis effects in many types of cancer cells *in vivo* and *in vitro*, including breast, prostate, and colon. Similarly, the antitumor growth effect of  $1\alpha,25(\text{OH})_2\text{D}_3$  on pancreatic cells has been demonstrated. The clinical use of  $1\alpha,25(\text{OH})_2\text{D}_3$  is impeded by the lethal side effects of hypercalcemia and hypercalciuria. Therefore,  $1\alpha,25(\text{OH})_2\text{D}_3$  analogs, which are either equipotent or more potent than  $1\alpha,25(\text{OH})_2\text{D}_3$  in inhibiting tumor cell growth but with fewer hypercalcemic and hypercalciuric side effects, have been developed for the treatment of different cancers. Recently, a pre-clinical study demonstrated that a less calcemic analog of  $1\alpha,25(\text{OH})_2\text{D}_3$ , 19-nor- $1\alpha,25(\text{OH})_2\text{D}_2$  (Paricalcitol), is effective in inhibiting tumor growth *in vitro* and *in vivo*, *via* upregulation of p21 and p27 tumor suppressor genes. Studies on the anti-tumor effects of a more potent analog of Paricalcitol are underway.

## INTRODUCTION

Pancreatic adenocarcinoma is one of the most lethal human malignancies. It ranks as the fourth most common cancer-related mortality in the Western world and fifth most common worldwide<sup>[1]</sup>. There were 37 680 new cases of pancreatic cancer and 34 290 deaths due to this disease in 2008 in the United States alone<sup>[2]</sup>. The overall 5-year survival rate of pancreatic cancer is estimated to be around 1%-4%, which is attributed to its aggressive growth behavior, such as early local spread and metastasis, and resistance to radiation and most systemic chemotherapies<sup>[1]</sup>. The poor prognosis is also due to the lack of effective early diagnosis. Consequently, most patients have been diagnosed with late stage disease. Currently, surgery remains the cornerstone of treatment. After resection, the 5-year survival rate is 10%-29%<sup>[3-5]</sup>, but only 10%-15% of pancreatic cancer patients are suitable candidates for resection. For the non-operable pancreatic cancer cases, the most frequently used treatment methods include radiation and chemotherapy with 5-fluorouracil, cisplatin or gemcitabine<sup>[6-8]</sup>, or a combination of these modalities<sup>[9-11]</sup>. Growing evidence supports neoadjuvant therapy to downstage some pancreatic cancer patients from borderline resectable to resectable disease<sup>[12,13]</sup>,

but its impact on long-term survival still needs further examination<sup>[14]</sup>. With this bleak background, there is an urgent need to develop novel treatments for pancreatic cancer.

## SOURCES AND METABOLISM OF VITAMIN D

The production of vitamin D<sub>3</sub> depends on solar ultraviolet B radiation (wavelength between 290–315 nm), which converts 7-dehydrocholesterol stored in the skin to previtamin D<sub>3</sub>. Previtamin D<sub>3</sub> is then thermoisomerized to vitamin D<sub>3</sub>, which then enters the bloodstream and is bound to the vitamin D binding protein. Very little food naturally contains vitamin D. The cutaneous synthesis of vitamin D<sub>3</sub> is normally responsible for over 90% of our vitamin D requirement<sup>[15]</sup>. Vitamin D (including both vitamin D<sub>2</sub> and vitamin D<sub>3</sub>) obtained from sunlight or dietary sources is then converted to 25-hydroxyvitamin D [25(OH)D], catalyzed by vitamin D-25-hydroxylase in the liver. 25(OH)D is the major circulating form of vitamin D and is widely accepted as an index of vitamin D status in humans. However, it is biologically inert until it is hydroxylated in the kidney to form 1 $\alpha$ ,25-dihydroxyvitamin D [1 $\alpha$ ,25(OH)<sub>2</sub>D]. 1 $\alpha$ ,25(OH)<sub>2</sub>D is a lipid-soluble hormone that interacts with the vitamin D receptor (VDR) to exert a variety of functions, including genomic and non-genomic actions.

## VITAMIN D AND CANCER

1 $\alpha$ ,25(OH)<sub>2</sub>D, the biologically active form of vitamin D, was originally discovered because of its effects on calcium and bone metabolism. It is now recognized that the hormone has activities in almost every tissue in the body. 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> implements this effect through binding to the nuclear VDR and then binding to a specific DNA sequence, the vitamin D response elements (VDREs)<sup>[16]</sup>. *Via* this genomic pathway, 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> can modulate gene expression in a tissue-specific manner, mainly leading to inhibition of cellular proliferation, induction of differentiation and apoptosis, which in turn, protect cells from malignant transformation and repress cancer cell growth.

However, the use of vitamin D to treat cancers has been impeded by lethal hypercalcemia induced by systemic administration of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>. To overcome this drawback, analogs of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, exhibiting more potent growth inhibition but less calcemic effects, have been developed as anticancer drugs<sup>[17]</sup>, and some of them have shown great results in pre-clinical studies. For example, Akhter *et al*<sup>[18]</sup> demonstrated that a 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> analog, EB 1089, exhibited an antiproliferative effect on colon cancer in a xenograft animal model. Abe-Hashimoto *et al*<sup>[19]</sup> showed that another 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> analog, OCT, displayed an antitumor effect on a xenograft model using MCF-7, a

breast cancer cell line, in combination with tamoxifen. Furthermore, Polek *et al*<sup>[20]</sup> showed that another analog, LG190119, possessed antiproliferative activity when tested in an LNCaP prostate cancer cell xenograft model.

There are some limited clinical trials that show 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> and its analogs have antitumor effects in humans when used alone or in combination with other chemotherapy drugs. For example, Beer *et al*<sup>[21]</sup> conducted a phase II clinical trial in which they showed the combination of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> and docetaxel could induce > 50% decline in PSA, a prostate cancer tumor marker, and improve survival of prostate cancer patients. However, most of the clinical trials only confirmed the less- or non-calcemic effect of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> or its analog when used alone or in combination with other chemotherapy drugs without prolonging the survival of cancer patients. There are still many ongoing clinical trials attempting to demonstrate the antitumor effect of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> and its analogs in humans. More effort is required in order to fully appreciate the clinical utilities of vitamin D-based therapies in treating human diseases, including cancers.

## MECHANISMS OF VITAMIN D ACTIONS FOR CANCER TREATMENT

Vitamin D reduces the risk of cancer through its biologically active metabolite, 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, which regulates cellular proliferation and differentiation, inhibits angiogenesis, and induces apoptosis.

Stumpf *et al*<sup>[22]</sup>, in 1979, reported that the VDR existed not only in the intestine, bone and kidney, but in almost all tissues in the body. Suda *et al*<sup>[23]</sup> (1982) first noted that 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> caused a marked inhibition of cell growth on VDR-positive M-1 leukemic cells. In the same year, Tanaka *et al*<sup>[24]</sup> reported that 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> showed the same effect on HL-60 leukemic cells, which had the VDR. Since then, many VDR-containing cancer cell lines, including prostate, colon, breast, lung, and melanoma, have shown growth inhibition when exposed to 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub><sup>[15,25-27]</sup>.

The antiproliferative effects of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> are mainly due to alterations in several key regulators of the cell cycle, culminating in dephosphorylation of retinoblastoma protein and arrest of cells in G<sub>0</sub>/G<sub>1</sub><sup>[28]</sup>. Progression of the cell cycle is regulated by cyclins and their associated cyclin dependant kinases and cyclin dependant kinase inhibitors (CKIs). The CKI genes, such as p21 and/or p27, have VDRE within their promoter regions, and are genomic targets of the 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>/VDR complex in many cell types, which in turn, induces G<sub>1</sub> cell-cycle arrest and withdrawal from the cell cycle<sup>[29-31]</sup>. However, some genes are transcriptionally affected by 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> but lack VDREs in their promoter regions, which suggests that 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> induces indirect effects on cell-cycle regulation through another signaling pathway.

For example,  $1\alpha,25(\text{OH})_2\text{D}_3$  could downregulate the expression of estrogen, epidermal growth factor, insulin-like growth factor 1, and keratinocyte growth factor and upregulate inhibitory growth factors, such as transformation growth factor- $\beta$ <sup>[32-35]</sup>.

$1\alpha,25(\text{OH})_2\text{D}_3$  can also induce differentiation to control tumor cell proliferation through the pathways of phosphatidylinositol 3-kinase, which is VDR dependent<sup>[36]</sup>, and by suppression of interleukin 12 protein secretion<sup>[37]</sup>, which is VDR independent.

Induction of apoptosis is another important function of  $1\alpha,25(\text{OH})_2\text{D}_3$ , which represses the expression of the antiapoptotic protein, BCL<sub>2</sub>, and prosurvival protein, BCL-X<sub>L</sub>.  $1\alpha,25(\text{OH})_2\text{D}_3$  also enhances the expression of proapoptotic proteins such as BAX and BAD<sup>[38]</sup>. In addition to the BCL<sub>2</sub> family,  $1\alpha,25(\text{OH})_2\text{D}_3$  can activate the caspase effector molecules directly to induce apoptosis<sup>[38]</sup>. Furthermore, it has been shown that  $1\alpha,25(\text{OH})_2\text{D}_3$ , in combination with radiation or chemotherapeutic agents, causes an additive effect on cancer cell death<sup>[39-42]</sup>.

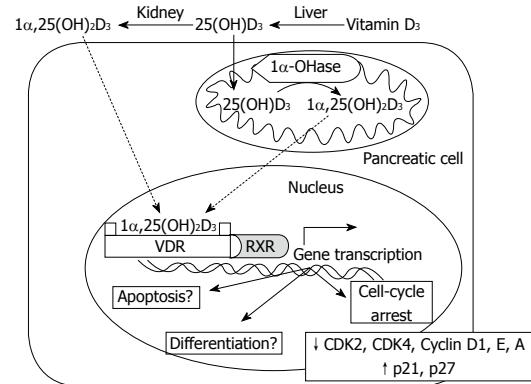
Inhibition of angiogenesis is also an important anticancer mechanism of  $1\alpha,25(\text{OH})_2\text{D}_3$ .  $1\alpha,25(\text{OH})_2\text{D}_3$  can repress endothelial cell growth *in vitro* and reduce angiogenesis *in vivo*<sup>[43-45]</sup>. The anti-angiogenic effects of  $1\alpha,25(\text{OH})_2\text{D}_3$  might subsequently lead to the inhibition of metastasis, as demonstrated in murine prostate and lung models treated with  $1\alpha,25(\text{OH})_2\text{D}_3$ <sup>[46,47]</sup>.

The precise anticancer mechanisms of  $1\alpha,25(\text{OH})_2\text{D}_3$ , including VDR-dependent and VDR-independent pathways are not fully understood. For the further use of  $1\alpha,25(\text{OH})_2\text{D}_3$  and its analogs in the treatment of cancers, there should be more studies addressing this issue to pave the way for the development of more potent  $1\alpha,25(\text{OH})_2\text{D}_3$  analogs.

## ASSOCIATION OF VITAMIN D AND PANCREATIC CANCER-EPIDEMIOLOGICAL EVIDENCE

Epidemiologic studies have shown that vitamin D status, influenced by living at high or low latitude, solar UV exposure and dietary intake of vitamin D, was inversely associated with the incidence of some cancers such as prostate, colon and breast<sup>[48-50]</sup>. It was also reported that the risk of developing prostate, breast and colon cancer was decreased by 30%-50% if the serum concentration of  $25(\text{OH})\text{D}$  exceeded 50 nmol/L<sup>[51,52]</sup>. Tangpricha *et al*<sup>[53]</sup> demonstrated that vitamin D deficiency enhanced the growth of MC-26 colon cancer xenografts in BALB/c mice, which supported the hypothesis that vitamin D sufficiency could reduce the proliferation of tumor cells *in vivo*.

To date, there have been only two epidemiological studies discussing the vitamin D status and the incidence of pancreatic cancer. One was conducted by Skinner *et al*<sup>[54]</sup> on U.S. nurses and health professionals, which demonstrated that higher dietary intake of vitamin D could be associated with lower incidence of pancreatic



**Figure 1 Mechanisms of Vitamin D<sub>3</sub> action on pancreas cells.** Both  $1,25(\text{OH})_2\text{D}_3$  and  $25(\text{OH})\text{D}_3$  can enter pancreas cells, where  $25(\text{OH})\text{D}_3$  can be converted to  $1,25(\text{OH})_2\text{D}_3$  by  $1\alpha$ -OHase.  $1,25(\text{OH})_2\text{D}_3$  can bind the VDR and further combine with the retinoid X receptor (RXR) to form a heterodimer, VDR-RXR. The VDR-RXR heterodimer binds to specific vitamin D response elements located in the promoter region of vitamin D-responsive genes, which in turn induces gene transcription. So far, in terms of pancreas cells, inhibition of CDK2, CDK4, Cyclin D1, Cyclin E, Cyclin A and upregulation of p21 and p27 have been demonstrated, leading to a block in the cell cycle at G<sub>0</sub>/G<sub>1</sub>. Regarding induction of differentiation, apoptosis and other anticancer mechanisms, further studies are required.

cancer. The other report, however, conducted in the observational cohort of the Finnish Alpha-Tocopheral Beta-Carotene (ATBC) trial, showed a discrepancy in results compared to the report by Skinner *et al*<sup>[54]</sup>. The ATBC data suggested that a higher serum concentration of  $25(\text{OH})\text{D}$  in male smokers was associated with a higher incidence of pancreatic cancer<sup>[55]</sup>. The reason behind these contradictory results was not clear, but they employed different measures of dietary intake of vitamin D *versus*  $25(\text{OH})\text{D}$ , and the participants in the ATBC trials were all male smokers, which might have contributed to the different conclusions in the two studies. To clarify this, more studies addressing the relationship between vitamin D status and incidence of pancreatic cancer are required.

## VITAMIN D AND PANCREATIC CANCER-BIOCHEMICAL EVIDENCE

While the precise anticancer mechanisms, including VDR-dependent and VDR-independent actions, induced by  $1\alpha,25(\text{OH})_2\text{D}_3$  still require further investigation, it is known that in pancreatic cancer cells,  $1\alpha,25(\text{OH})_2\text{D}_3$  induces the expression of p21 and p27 and inhibits the production of cyclins (D<sub>1</sub>, E and A) and cyclin dependent kinases 2 and 4, which in turn, elicit cell cycle arrest in G<sub>0</sub>/G<sub>1</sub> *in vitro*<sup>[56]</sup>. Similar effects have been observed with 19-nor- $1\alpha,25(\text{OH})_2\text{D}_2$  (Paricalcitol) in human pancreatic cancer cells *in vitro* and *in vivo* by upregulating the expressions of p21 and p27<sup>[57]</sup> (Figure 1).

Currently, very little is known about the mechanisms of vitamin D actions on pancreatic cancer cells. More emphasis in this area is needed, because very few efficient therapeutic options are available to benefit pancreatic cancer survival.

## POTENTIAL OF VITAMIN D ANALOGS FOR PANCREATIC CANCER TREATMENT

Since the VDR exists in almost every tissue of the body<sup>[22]</sup> and 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> exhibits growth inhibitory effects on many different types of cancer cells, several studies have been performed to investigate the anticancer effect of vitamin D and its analogs on pancreatic cancer cells during the past two decades. For example, Kawa *et al*<sup>[56]</sup> reported that 22-oxa-1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, a 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> analog, caused growth inhibition in three pancreatic cancer cell lines and inhibited the growth of a BxPC-3 tumor in a xenograft nude mice model<sup>[58]</sup>. Colston *et al*<sup>[59]</sup> demonstrated that another 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> analog, EB 1089, exhibited more potent antitumor effects than 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> on the GER cell line, a human pancreatic cancer cell line, *in vitro* and *in vivo*. Pettersson *et al*<sup>[60]</sup> also reported that EB 1089 induced greater tumor growth inhibition than 9-cis-retinoic acid *in vitro*. These two pre-clinical studies therefore suggested that EB 1089 might be a promising vitamin D analog for further clinical trials in pancreatic cancer patients. However, in a phase II clinical trial, which is also the only clinical trial studying the use of vitamin D analogs against pancreatic cancer, EB 1089 given once daily orally failed to significantly improve patient survival, although most patients tolerated the daily orally dose of 10-15  $\mu$ g of the drug well, without causing hypercalcemia<sup>[61]</sup>.

With the knowledge that 19-nor-1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>2</sub> (Paricalcitol) is a Federal Drug Administration approved drug for the treatment and prevention of secondary hyperparathyroidism associated with chronic kidney disease<sup>[62]</sup>, and this analog has comparable growth inhibitory effects as 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> in human prostate cancer cells *in vitro*<sup>[63]</sup>, Schwartz *et al*<sup>[57]</sup> studied the same analog in human pancreatic cancer cells *in vitro* and demonstrated that it also inhibited the proliferation of these cells. Given the fact that Paricalcitol has been shown to be less calcemic than 1 $\alpha$ ,25(OH)<sub>2</sub>D and few therapeutic options for pancreatic cancer patients are available, it is worth further exploration as an anticancer drug for pancreatic cancer.

In addition to using synthetic 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> analogs to treat pancreatic cancer in an effort to avoid hypercalcemia, another strategy is the employment of inactive prohormone 25(OH)D<sub>3</sub> (Figure 1). This is based on the finding that many normal and cancerous cells, including prostate cell lines and primary cultures of prostate cells, possess 25(OH)D-1 $\alpha$ -hydroxylase (1 $\alpha$ -hydroxylase or CYP27b1), the enzyme responsible for the conversion of 25(OH)D<sub>3</sub> to 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub><sup>[26]</sup>, and CYP27b1 has been proposed as a tumor suppressor<sup>[64]</sup>. Furthermore, it has been demonstrated that 25(OH)D<sub>3</sub> had a comparable tumor growth inhibitory effect to 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> on primary cultures of prostate cells<sup>[63,65]</sup>. These findings led to a subsequent study demonstrating that CYP27b1 activity was also expressed in pancreatic cancer cells and the growth of these cancer cells was

inhibited in the presence of 25(OH)D<sub>3</sub><sup>[66]</sup>. Given the fact that the conversion of 25(OH)D<sub>3</sub> to 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> occurs within the cell, and the 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> formed acts in an autocrine/paracrine fashion<sup>[26,63-65]</sup>, systemic administration of 25(OH)D<sub>3</sub> might greatly minimize the risk of hypercalcemia. 25(OH)D<sub>3</sub> has been approved by the Federal Drug Administration to treat vitamin D deficiency in humans; therefore, 25(OH)D<sub>3</sub> could be another attractive candidate for clinical trials in pancreatic cancer patients.

## CONCLUSION

Abundant evidence indicates that 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> has antiproliferative, pro-differentiative, apoptotic and antiangiogenic activities in different types of tumor cells. In different cancer cell types, 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> can induce the expression of various molecular markers to regulate cell growth. Even in the same tissue, different cell types might exhibit heterogeneous responses to the addition of 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>. It is known that 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> and its analogs can inhibit pancreatic cancer cell growth *in vitro*<sup>[56,59]</sup> via activation of p21 and p27, which in turn influence cell cycle progression and arrest cells in G<sub>0</sub>/G<sub>1</sub>. So far, the only pancreatic cancer clinical trial using a 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> analog, EB 1089<sup>[61]</sup>, did not show significant prolongation of survival. However, a preclinical study with 19-nor-1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>2</sub> (Paricalcitol) in pancreatic cancer cells<sup>[60]</sup> has provided some encouraging results and might lead to further clinical trials. Moreover, given the fact that pancreatic cancer cells possess 1 $\alpha$ -hydroxylase (CYP27b1) activity and can convert the prohormone 25(OH)D, which has a low risk of inducing hypercalcemia even at high concentrations, into 1 $\alpha$ ,25(OH)<sub>2</sub>D within the cells and thereby inhibiting pancreatic cancer cell growth in an autocrine/paracrine fashion<sup>[26,63-65]</sup>, prohormone 25(OH)D-based therapy might be another attractive treatment strategy for pancreatic cancer in the future.

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# Insulin-like growth factor binding protein-5 influences pancreatic cancer cell growth

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PI3K or MEK1/2 abrogated IGFBP-5-mediated cell survival.

**CONCLUSION:** These results indicate that IGFBP-5 expression affects the cell cycle and survival signal pathways and thus it may be an important mediator of PaC cell growth.

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**Key words:** Insulin-like growth factor-binding protein 5; Extracellular signal-regulated mitogen activated protein kinases; Cyclin-dependent kinase inhibitor p27; Pancreatic neoplasms

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## Abstract

**AIM:** To investigate the functional significance of insulin-like growth factor binding protein-5 (IGFBP-5) overexpression in pancreatic cancer (PaC).

**METHODS:** The effects of IGFBP-5 on cell growth were assessed by stable transfection of BxPC-3 and PANC-1 cell lines and measuring cell number and DNA synthesis. Alterations in the cell cycle were assessed by flow cytometry and immunoblot analyses. Changes in cell survival and signal transduction were evaluated after mitogen activated protein kinase and phosphatidylinositol 3-kinase (PI3K) inhibitor treatment.

**RESULTS:** After serum deprivation, IGFBP-5 expression increased both cell number and DNA synthesis in BxPC-3 cells, but reduced cell number in PANC-1 cells. Consistent with this observation, cell cycle analysis of IGFBP-5-expressing cells revealed accelerated cell cycle progression in BxPC-3 and G2/M arrest of PANC-1 cells. Signal transduction analysis revealed that Akt activation was increased in BxPC-3, but reduced in PANC-1 cells that express IGFBP-5. Inhibition of PI3K with LY294002 suppressed extracellular signal-regulated kinase-1 and -2 (ERK1/2) activation in BxPC-3, but enhanced ERK1/2 activation in PANC-1 cells that express IGFBP-5. When MEK1/2 was blocked, Akt activation remained elevated in IGFBP-5 expressing PaC cells; however, inhibition of

## INTRODUCTION

Pancreatic cancer (PaC) is the fourth leading cause of cancer deaths in the United States. Annually, the incidence of PaC almost equals the number of deaths related to this disease, and these numbers have begun to increase<sup>[1]</sup>. Pancreatic tumors are highly chemoresistant, so chemotherapy rarely leads to eradication of the malignancy. Even with chemo- and radiation therapy, median survival for patients who have undergone resection is less than two years<sup>[1]</sup>. Therefore, a better understanding of aberrant signaling pathways that promote tumor growth is necessary to identify inhibitors to potentially enhance the response of current chemotherapeutics.

Insulin-like growth factors (IGFs) regulate cellular growth, differentiation, and apoptosis<sup>[2]</sup>. In non-malignant cells, IGF-binding proteins (IGFBPs) modulate the activity of IGF-I and -II, and their affinity for IGFs can be altered by proteolysis, phosphorylation, and adherence to cell-surface or extracellular matrix proteins<sup>[3]</sup>. Insulin-like growth factor

binding proteins (IGFBPs) can also act independently of IGF to either stimulate or inhibit growth of various cell types. Both IGF-I and the IGF-I receptor (IGF-I R) are expressed in pancreatic tumors and cell lines<sup>[4-6]</sup>, and IGFBP-1, -3, and -5 have been associated with pancreatic cancer<sup>[7-9]</sup>.

We found that IGFBP-5 is expressed at higher levels in pancreatic tumors than in non-malignant pancreatic tissues<sup>[9]</sup>. The role of IGFBP-5 in cancer cell growth and proliferation, however, is not fully understood and has not been studied in pancreatic cancer. In this study, therefore, we investigated the functional significance of IGFBP-5 overexpression in pancreatic cancer by stable transfection of the *IGFBP-5* cDNA into two pancreatic cancer cell lines to better represent the heterogeneous genetic background of pancreatic tumors. We examined the effects of IGFBP-5 on cell proliferation and on cell cycle distribution and the status of key cell cycle regulators. We also investigated the mechanism of IGFBP-5-mediated growth effects by assessing the activation status of Akt and extracellular signal-regulated kinase-1 and -2 (ERK1/2) and the effects of inhibition of the phosphatidylinositol 3-kinase (PI3K) and mitogen activated protein kinase (MAPK) pathways after serum deprivation. These studies show that IGFBP-5 can enhance pancreatic cancer cell growth by altering the expression and activity of cell cycle regulators and the activation of key signaling intermediates.

## MATERIALS AND METHODS

### *Cell lines, cloning, and stable transfection*

Human pancreatic cancer cell lines BxPC-3 and PANC-1 were obtained from the American Type Culture Collection (Manassas, VA). PANC-1 cells were grown in DMEM and BxPC-3 cells were grown in RPMI 1640, both media were supplemented with 100 mL/L fetal bovine serum.

The full-length cDNA encoding human *IGFBP-5* was synthesized by reverse transcription polymerase chain reaction from pancreatic tumor cDNA. The amplified product spanned 822 bp (nt 749-1570) of the published human *IGFBP-5* mRNA, covering the start (752) and stop codons (1568) (GenBank, NM\_000599). The primers used were as follows: 5'-CACCAAGATGGTGTGCTC-3' (sense) and 5'-TCACTCAACGTTGCTGCTGTCGAA-3' (antisense). The sense primer included sequences to facilitate TOPO cloning (underlined). The amplified product was cloned into the pENTR/SD TOPO vector (Invitrogen, Carlsbad, CA) and the sequence of the *IGFBP-5* insert was confirmed by sequencing.

The full-length human *IGFBP-5* cDNA was transferred into the expression vector pIRESpuro3GW<sup>[10]</sup> using Invitrogen's Gateway cloning technology and cells were stably transfected using LipofectAMINE (Invitrogen). IGFBP-5 transfectants (/IGFBP-5) and vector controls (/Vec) were selected in medium containing puromycin (2 µg/mL PANC-1 and 1.5 µg/mL BxPC-3). Individual clones were expanded and successful

transfection was confirmed by immunoblot analysis of conditioned medium concentrated using Microcon YM 10 filter devices after 24 h growth in serum-free medium (SFM) and detected with α-IGFBP-5 antibodies (R&D Systems, Minneapolis, MN). Two clones were selected per cell line, one that expressed low levels of IGFBP-5 (IGFBP-5L) and one that expressed high levels (IGFBP-5H).

### **Growth studies**

Stable transfectants were seeded ( $3.5 \times 10^4$  cells/well) in 24-well plates in the appropriate growth medium for 24 h. The medium was then removed, cells were washed with phosphate-buffered saline (PBS), and fresh growth medium or SFM was added to the cells. Cells were either cultured continuously in the same medium or SFM changed every 24 h. Growth was assessed based on cell number and [<sup>3</sup>H]-thymidine incorporation at various times in the above culture conditions.

### **Cell number**

The number of cells in each well was determined by harvesting the cells with trypsin-EDTA solution and counting cells in an aliquot using a Z1 Particle Counter (Beckman-Coulter) in duplicate.

### **[<sup>3</sup>H]-thymidine incorporation**

At the end of incubations, medium was removed, cells were washed with PBS, and then 2 µCi/mL [<sup>3</sup>H-methyl]-thymidine in SFM was added for the last 4 h of each growth period at 37°C. After the labeling period, cells were precipitated with ice-cold methanol: acetic acid (3:1, v/v) at 4°C overnight. The cells were washed with a solution of 800 mL/L methanol in water, and DNA was dissolved by adding 0.5 mol/L NaOH (300 µL) for 30 min. Radioactivity contained in 100 µL aliquots was determined by scintillation counting.

### **Cell cycle analysis**

Cells were seeded ( $3.5 \times 10^5$  PANC-1 cells/well or  $4 \times 10^5$  BxPC-3 cells/well) in 6-well plates in the appropriate growth medium for 24 h. The medium was removed, cells were washed with PBS, and either fresh growth medium or SFM was added. After 48 h, cells were trypsinized and aliquots of  $1 \times 10^6$  cells were fixed overnight in 700 mL/L ethanol at 4°C. Fixed cells were washed and suspended in 1 mL of 50 µg/mL propidium iodide, 1 mg/mL RNase A, and 1 g/L BSA in PBS for 30 min in the dark at room temperature. Using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA), cell cycle analysis was performed on 10000 cells for each sample. Quantitation of cell cycle distribution was performed using CellQuest software (Becton Dickinson).

### **Immunoblot analysis**

For immunoblots, equivalent amounts of protein were resolved by SDS-PAGE and blotted onto PVDF membrane (Invitrogen), followed by blocking with 50 g/L nonfat milk powder in 10 mmol/L Tris, pH 7.6,

100 mmol/L NaCl, and 1 mL/L Tween 20. Immunoblots were incubated with the following primary antibodies:  $\alpha$ -IGFBP-5 (R&D Systems);  $\alpha$ -phospho-ERK1/2,  $\alpha$ -ERK1/2,  $\alpha$ -phospho-Akt (Ser 473),  $\alpha$ -Akt (Cell Signaling Technology, Danvers, MA);  $\alpha$ -GAPDH (Ambion, Austin, TX); and  $\alpha$ -cyclin D1,  $\alpha$ -CDK4,  $\alpha$ -cyclin B1,  $\alpha$ -cdc2,  $\alpha$ -cyclin E,  $\alpha$ -CDK2,  $\alpha$ -p21,  $\alpha$ -p27,  $\alpha$ -actin (Santa Cruz Biotechnology, Santa Cruz, CA). Antigen-antibody complexes were detected using the appropriate secondary antibody linked to horseradish peroxidase and visualized using ECL Plus chemiluminescent reagent (Amersham Biosciences, Piscataway, NJ).

#### Cyclin-dependent kinase assays

CDK activity assays were performed essentially as described previously<sup>[11]</sup> using histone H1 and Rb<sub>773-928</sub> (Upstate Biotechnology) as substrates for CDK2 and CDK4, respectively. Reaction products were resolved by SDS-PAGE and visualized by autoradiography.

#### Cell viability and proliferation

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays (Roche, Indianapolis, IN) were performed to evaluate cell growth and viability of IGFBP-5 expressing cell lines after treatment with LY294002 or U0126 (Calbiochem, San Diego, CA). Cells were seeded ( $0.9 \times 10^4$  cells/well PANC-1 and  $1 \times 10^4$  cells/well BxPC-3) in 96-well plates in the appropriate growth medium. After 24 h the medium was removed, cells were washed with PBS, SFM was added, cells were incubated for 24 h, and inhibitors (LY294002, U0126, or vehicle) were then added to the medium. After 48 h, MTT assays were performed according to the manufacturer's protocol. The viable cells were expressed as a percentage of the optical absorbance relative to the optical absorbance of the corresponding control or untreated cells.

#### Analysis of effect of IGFBP-5-conditioned medium on signaling

IGFBP-5 transfected or vector transfected PANC-1 and BxPC-3 cells were seeded in 10 cm plates in the appropriate growth medium. After 24 h the medium was removed, cells were washed with PBS, SFM was added, and cells were incubated for 48 h. Conditioned medium was then collected and centrifuged at 1500 r/min for 10 min. PANC-1 and BxPC-3 cells were seeded in 6-well plates in the appropriate growth medium. After 24 h the medium was removed, cells were washed with PBS, SFM was added, and the cells were incubated for 24 h. PANC-1 and BxPC-3 cells were then treated for 15 min, 24, 48 and 72 h with conditioned medium from IGFBP-5 or vector-transfected controls. Cells were lysed and samples were prepared for immunoblot analysis.

#### Statistical analysis

Data are expressed as the mean  $\pm$  SE or a percentage. Statistical analyses of data were performed using a two-way analysis of variance (ANOVA) and Bonferroni post-

test (GraphPad Software; San Diego, CA). For data normalized to the vector transfected control, variables were log transformed prior to statistical analysis.  $P < 0.05$  was considered significant.

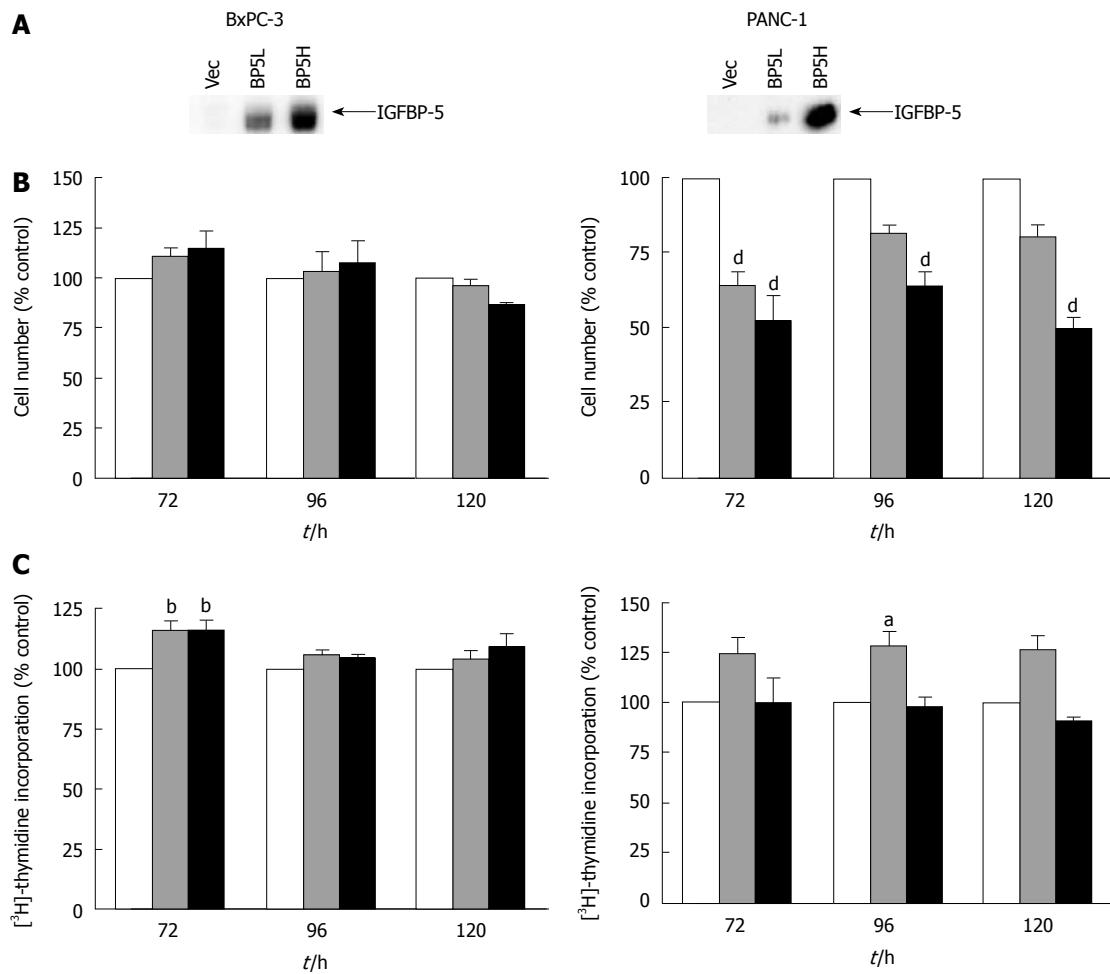
## RESULTS

#### *IGFBP-5 overexpression promotes BxPC-3 cell growth after serum deprivation*

The stable expression of IGFBP-5 in transfected PaC cells was verified by immunoblot analysis after 24 h growth in serum-free conditions and concentration of conditioned medium (Figure 1A). To examine dose-dependent effects and to obviate insertion effects resulting from the generation of the stable transfectants, growth effects were assessed by analyzing cell number and thymidine incorporation using cell lines expressing different levels of IGFBP-5 designated as low (IGFBP-5L) and high (IGFBP-5H). In serum-containing medium, cell numbers were significantly lower in PANC-1 cells expressing IGFBP-5 than in vector transfected control cells (Figure 1B). Although the decrease in PANC-1 cell number corresponded to the increase in IGFBP-5 expression, a similar association in DNA synthesis and IGFBP-5 expression was not observed (Figure 1C). These results suggest that IGFBP-5 inhibits growth of PANC-1 cells cultured in the presence of serum. In contrast, no growth effects were observed in BxPC-3 cells grown in serum-containing medium.

IGFBP-5 has been shown to have both growth factor-dependent and -independent growth and survival effects in both normal and cancer cells<sup>[3]</sup>. Therefore, we hypothesized that IGFBP-5 may aid growth of PaC cells when the bioavailability of growth factors is low. To investigate this, we examined IGFBP-5-mediated growth effects in the absence of exogenous growth factors. As IGFs are present in FBS, we performed these studies in the absence of serum as has been done previously to elucidate IGF-dependent and -independent effects<sup>[5,12-14]</sup>. Consistent with our hypothesis, both cell number and DNA synthesis increased significantly in BxPC-3 cells expressing high levels of IGFBP-5 in SFM (Figure 2A and B, respectively). In contrast, high levels of IGFBP-5 significantly reduced the number of PANC-1 cells (Figure 2A). Interestingly, low levels of IGFBP-5 significantly elevated thymidine incorporation in PANC-1 cells, similar to the effects observed in cells grown in serum-containing medium, though the increase was less dramatic in the presence of high levels of IGFBP-5 (Figure 2B). These data suggest that, in the absence of exogenous growth factors, IGFBP-5 promotes growth of BxPC-3 cells but inhibits growth of PANC-1 cells.

Growth factors and their receptors are upregulated and associated with increased tumorigenicity in pancreatic cancer<sup>[15]</sup>. Additionally, in PaC cells the overexpression of autocrine growth factors has been shown to allow cell proliferation in the absence of serum<sup>[16,17]</sup>. In the previous experiments, PaC cells were grown without replenishing SFM for 120 h. Thus,



**Figure 1 Effect of IGFBP-5 expression on PaC cells grown in the presence of serum.** Stable transfectants (empty vector /Vec-white bar, low /IGFBP-5L-gray bar, high/IGFBP-5H-black bar) were seeded in growth media. (A) IGFBP-5 expression was verified by immunoblot analysis of secreted IGFBP-5 after 24 h growth in SFM and concentration of conditioned medium. Cell counts (B) and thymidine incorporation (C) were determined at 72, 96 and 120 h post-seeding. Data shown represent the mean  $\pm$  SE of IGFBP-5-transfected cell growth relative to vector-transfected controls (100%) from 3-5 separate experiments performed in duplicate.  $^aP < 0.05$ ,  $^bP < 0.01$ ,  $^dP < 0.001$ .

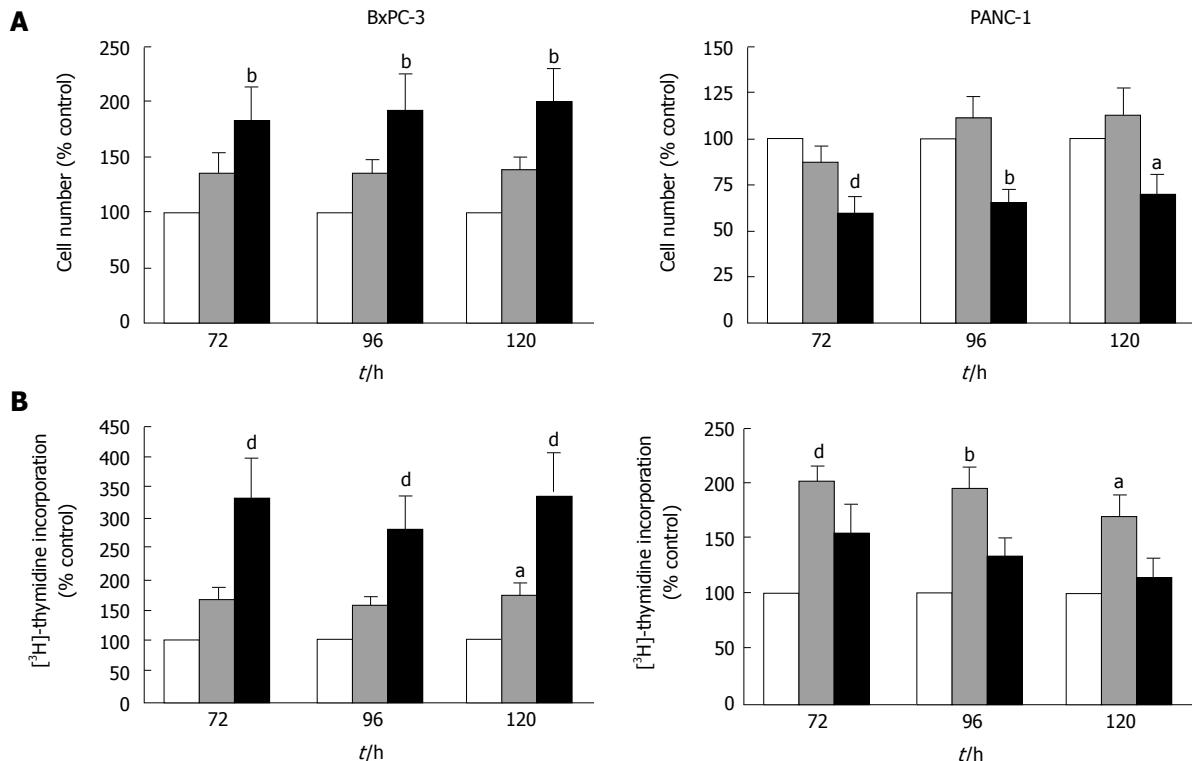
to determine whether IGFBP-5 confers a growth advantage in the absence of endogenously produced growth factors, cells were grown with SFM changed daily. In BxPC-3 cells cultured with frequent medium changes, IGFBP-5 expression was associated with significantly elevated thymidine incorporation and cell number (Figure 3). In contrast, significantly reduced cell numbers were observed in PANC-1 cells expressing high levels of IGFBP-5 grown with frequent changes of SFM (Figure 3A). Therefore, even in the absence of growth factors, IGFBP-5 enhanced BxPC-3 cell proliferation but inhibited PANC-1 cell growth, and these effects were exacerbated by frequent changes of SFM.

#### **IGFBP-5 expression is associated with cell cycle dysregulation in serum-containing growth conditions**

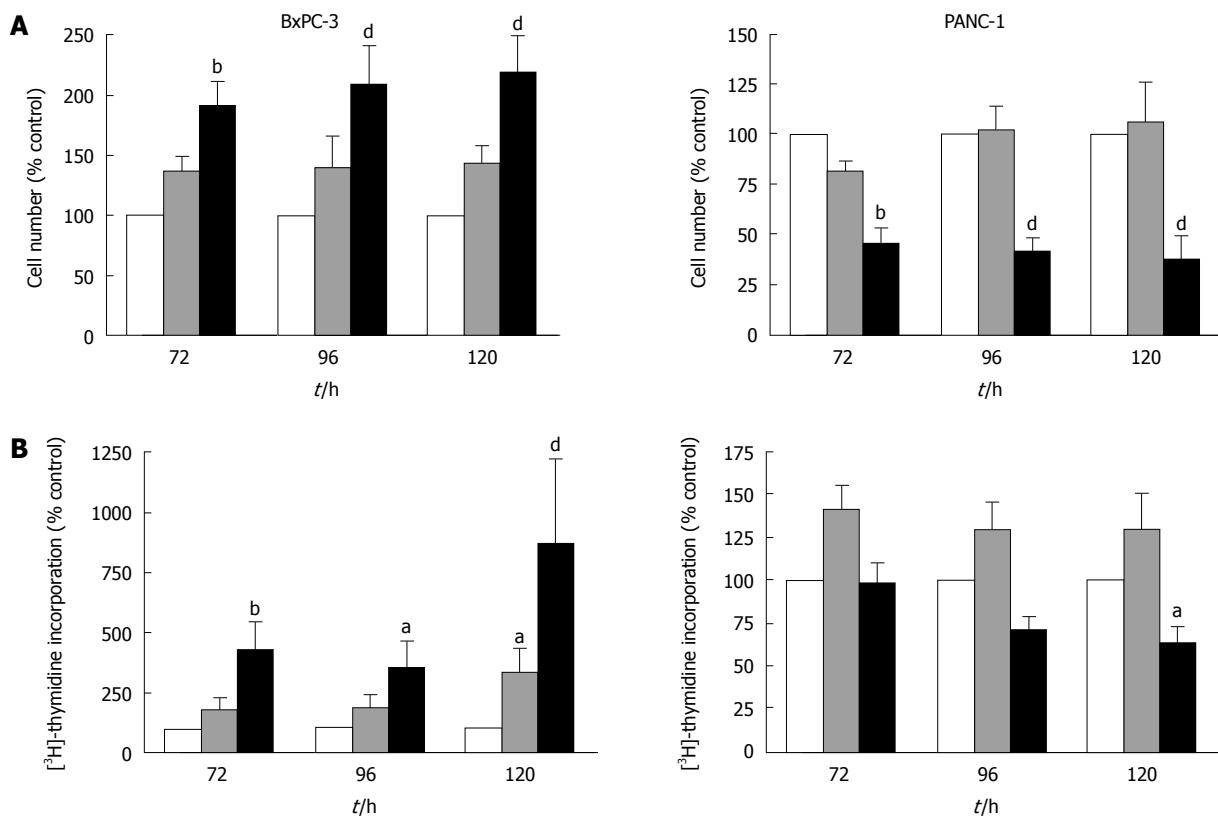
In the growth studies, PANC-1 cells that overexpress IGFBP-5 had lower cell numbers but more DNA synthesis than control cells. These data suggest a possible block in the cell cycle at the G2/M transition. In contrast, BxPC-3 cells expressing high levels of IGFBP-5 exhibited higher cell numbers and thymidine incorporation, indicating accelerated progression

through the cell cycle. While IGFBP-5 expression has different effects on growth of the two cell lines, these data indicate that the effects might result from a common overall process-cell cycle dysregulation.

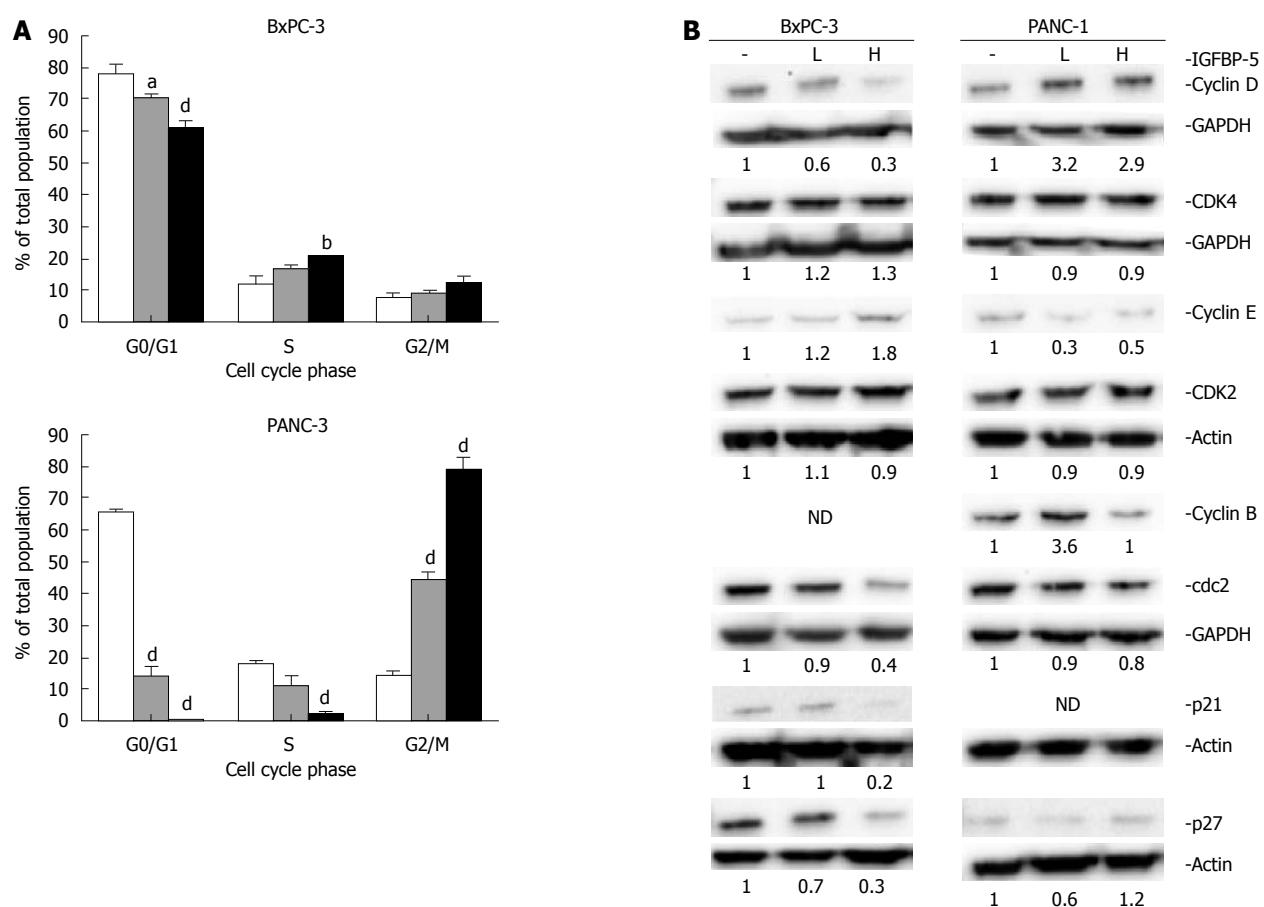
Therefore, we examined the cell cycle distribution and status of key cell cycle regulators of G1/S (cyclin D/CDK4, cyclin E/CDK2, p21<sup>Cip1</sup>, and p27<sup>Kip1</sup>) and G2/M (cyclin B/cdc2) transitions in transfected cell lines in the presence (Figure 4) and absence (Figure 5) of serum. The examination of cell cycle distribution by propidium iodide staining and FACS analysis revealed that IGFBP-5 expression in BxPC-3 cells grown in the presence of serum resulted in significantly fewer cells in G1 phase and an increased percentage of cells in S phase (Figure 4A). In contrast, in PANC-1 cells grown in the presence of serum, IGFBP-5 expression was associated with significantly fewer cells in G1 and S phases and an accumulation of cells in G2/M phase (Figure 4A). To identify the cell cycle components responsible for these effects, the expression of key cell cycle regulators was determined in the same growth conditions used for analyzing cell cycle distribution. In BxPC-3 cells, IGFBP-5 overexpression was associated with markedly



**Figure 2 Effect of IGFBP-5 expression on PaC cells grown in the absence of serum.** Stable transfectants (empty vector/Vec-white bar, low/IGFBP-5L-gray bar, high/IGFBP-5H-black bar) were seeded in growth media for 24 h then switched to serum-free medium (SFM) for the remainder of the growth period. Cell counts (A) or thymidine incorporation (B) were determined at 72, 96 and 120 h post-seeding. Data shown represent the mean  $\pm$  SE of IGFBP-5-transfected cell growth relative to vector-transfected controls (100%) from 3-5 separate experiments performed in duplicate.  $^aP < 0.05$ ,  $^bP < 0.01$ ,  $^dP < 0.001$ .



**Figure 3 Effect of IGFBP-5 expression on PaC cells grown with daily changes of SFM.** Stable transfectants (empty vector/Vec-white bar, low/IGFBP-5L-gray bar, high/IGFBP-5H-black bar) were seeded in growth media for 24 h and switched to SFM, which was replaced every 24 h for the remainder of the growth period. Cell counts (A) and thymidine incorporation (B) were determined at 72, 96 and 120 h post-seeding. Data shown represent the mean  $\pm$  SE of IGFBP-5-transfected cell growth relative to vector-transfected controls (100%) from 3-5 separate experiments performed in duplicate.  $^aP < 0.05$ ,  $^bP < 0.01$ ,  $^dP < 0.001$ .



**Figure 4 Cell cycle distribution of PaC cells that overexpress IGFBP-5 grown in the presence of serum.** Stable transfectants (empty vector/Vec-white bar, low/IGFBP-5L-gray bar, high /IGFBP-5H-black bar) were seeded in growth media. After 24 h, the medium was removed and replaced with fresh growth media for 48 h. (A) Cell cycle distribution was determined by propidium iodide staining and FACS analysis. Data shown represent the mean  $\pm$  SE from 3 experiments performed in duplicate. (B) Cell lysates were prepared for immunoblot analysis of cell cycle regulators and their expression was detected using the appropriate antisera. Membranes were subsequently stripped and reprobed for actin or GAPDH as a load control. Analyses of cyclin E/cdk2 and cyclin B/cdc2 expression were performed simultaneously, therefore, only a single load control, actin or GAPDH, respectively, is shown for each pair. Quantitation of band density represents the level of IGFBP-5-associated expression relative to vector-transfected controls (1.0) normalized to the expression level of actin. ND: Not detected. The results are representative of 3 experiments. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , <sup>d</sup> $P < 0.001$ .

lower levels of cyclin D, cdc2, p21<sup>Cip1</sup>, and p27<sup>Kip1</sup> but elevated levels of cyclin E (Figure 4B). In contrast, in PANC-1 cells overexpression of IGFBP-5 resulted in elevated levels of cyclin D but reduced levels of cyclin E (Figure 4B). Expression of cyclin B in BxPC-3 and p21 in PANC-1 cells was not detected.

#### IGFBP-5 expression is associated with cell cycle dysregulation during serum starvation

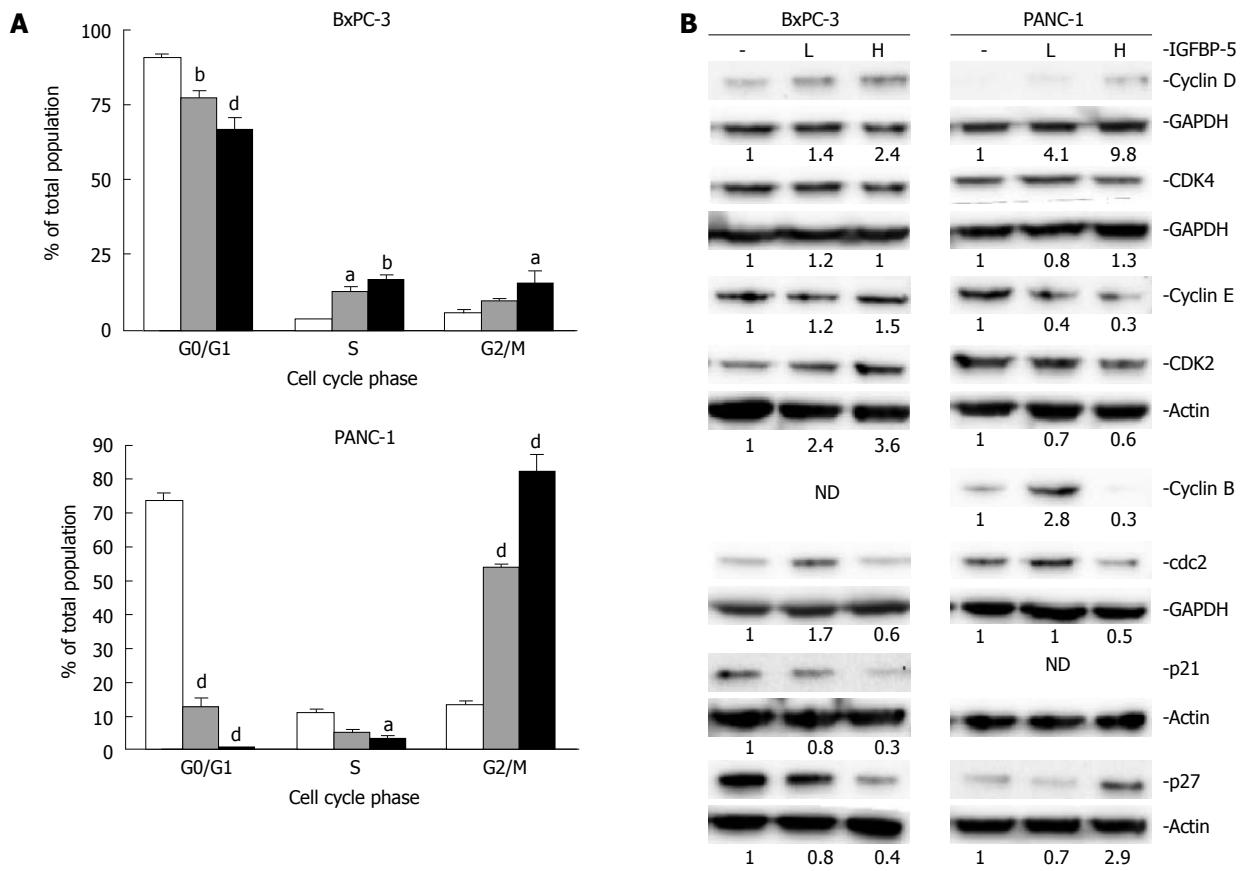
In the absence of serum, IGFBP-5 expression resulted in marked alterations in cell proliferation, particularly in BxPC-3 cells; therefore, the cell cycle was analyzed in serum-starved cells. Serum deprivation exacerbated the effects of IGFBP-5 expression on cell cycle distribution that were observed in serum-containing growth conditions. In BxPC-3 cells, the percentage of IGFBP-5-expressing cells was reduced in G1 phase but elevated in both S and G2/M phases (Figure 5A). In PANC-1 cells, IGFBP-5 expression was associated with a reduced percentage of cells in G1 and S phases and an increased percentage of cells in G2/M phase (Figure 5A).

To determine whether serum starvation changes

expression profiles of cell cycle regulators in cells that express IGFBP-5, we assessed the expression in cells grown in SFM. With IGFBP-5 expression, BxPC-3 cells showed prominent increases in the levels of cyclin D and CDK2, and to a lesser extent, cyclin E, which is consistent with enhanced cell proliferation. However, the levels of the G1/S transition inhibitors p21<sup>Cip1</sup> and p27<sup>Kip1</sup> were reduced (Figure 5B). IGFBP-5 expression in PANC-1 cells in the absence of serum produced increased levels of p27<sup>Kip1</sup> and cyclin D and reduced levels of cyclin E and CDK2 (Figure 5B), which reflects the observed growth arrest and accompanying enhanced thymidine incorporation (Figure 2). Similar to PaC cells grown in the presence of serum, cyclin B expression in BxPC-3 and p21 expression in PANC-1 cells was not detected.

#### PI3K signaling is required for IGFBP-5-enhanced growth of BxPC-3 cells

To investigate the mechanism of IGFBP-5-enhanced cell growth after serum deprivation, we examined the PI3K and MAPK pathways, which have been shown to



**Figure 5 Cell cycle distribution of PaC cells that overexpress IGFBP-5 grown in the absence of serum.** Stable transfectants (empty vector/Vec-white bar, low/IGFBP-5L-gray bar, high /IGFBP-5H-black bar) were seeded in growth media. After 24 h, the medium was changed to SFM for 48 h. A: Cell cycle distribution was determined by propidium iodide staining and FACS analysis. Data shown represent the mean  $\pm$  SE from 3 separate experiments performed in duplicate; B: Cell lysates were prepared for immunoblot analysis of cell cycle regulators and their expression was detected using the appropriate antisera. Membranes were subsequently stripped and reprobed for actin or GAPDH as a load control. Analyses of cyclin E/cdk2 and cyclin B/cdc2 expression were performed simultaneously, therefore, only a single load control, actin or GAPDH, respectively, is shown for each pair. Quantitation of band density represents the level of IGFBP-5 associated expression relative to vector-transfected controls (1.0) normalized to the expression level of actin. ND: Not detected. The results are representative of 3 experiments. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , <sup>c</sup> $P < 0.001$ .

enhance proliferation and survival of pancreatic cancer cells<sup>[18-21]</sup>. Immunoblot analysis of the PI3K signaling intermediate Akt revealed a 3.5-fold increase in the level of phosphorylated Akt in BxPC-3 cells expressing IGFBP-5, but Akt phosphorylation was reduced in PANC-1 cells expressing IGFBP-5 (Figure 6A). When cells were treated with a PI3K inhibitor, LY294002, IGFBP-5 no longer conferred a growth advantage in BxPC-3 cells (Figure 6B). These results indicate that PI3K signaling is necessary for IGFBP-5-enhanced growth of BxPC-3 cells.

#### MAPK signaling is required for IGFBP-5-enhanced growth of pancreatic cancer cells

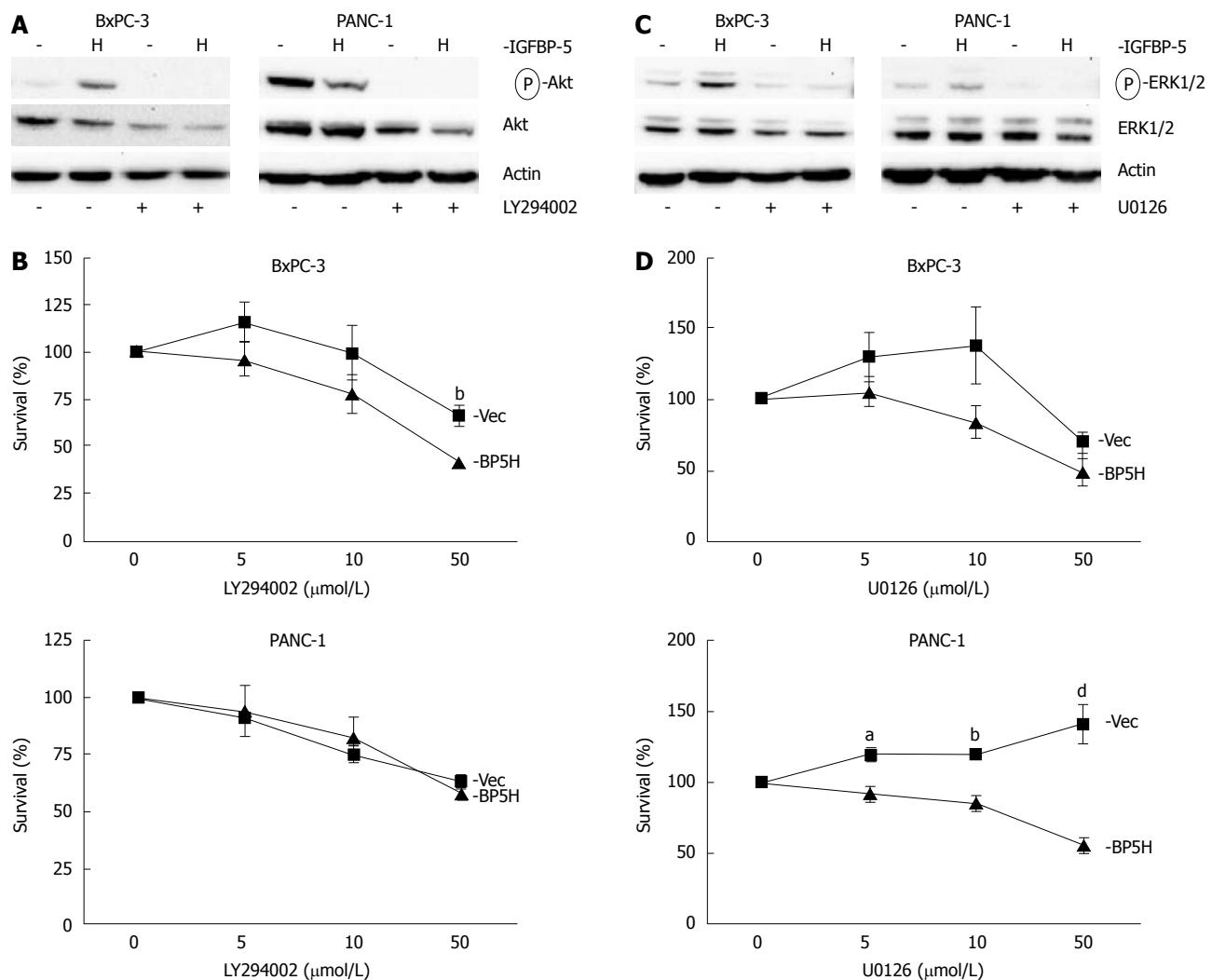
To determine whether IGFBP-5 mediates cell growth via MAPK signaling in PaC cells, the survival studies were repeated with U0126, a MEK1/2 inhibitor. Immunoblot analysis revealed that ERK1/2 activation (phosphorylation) is 2.6-fold greater in BxPC-3 cells expressing IGFBP-5 (Figure 6C) and IGFBP-5-mediated growth is abrogated when the MAPK pathway is blocked with U0126 treatment (Figure 6D). Similarly, ERK1/2 activation is 2-fold greater in PANC-1 cells that express IGFBP-5 (Figure 6C) and significantly fewer IGFBP-

5-expressing cells survive when the MAPK pathway is blocked (Figure 6D). These studies suggest that the MAPK pathway is necessary for IGFBP-5-enhanced cell growth after serum deprivation.

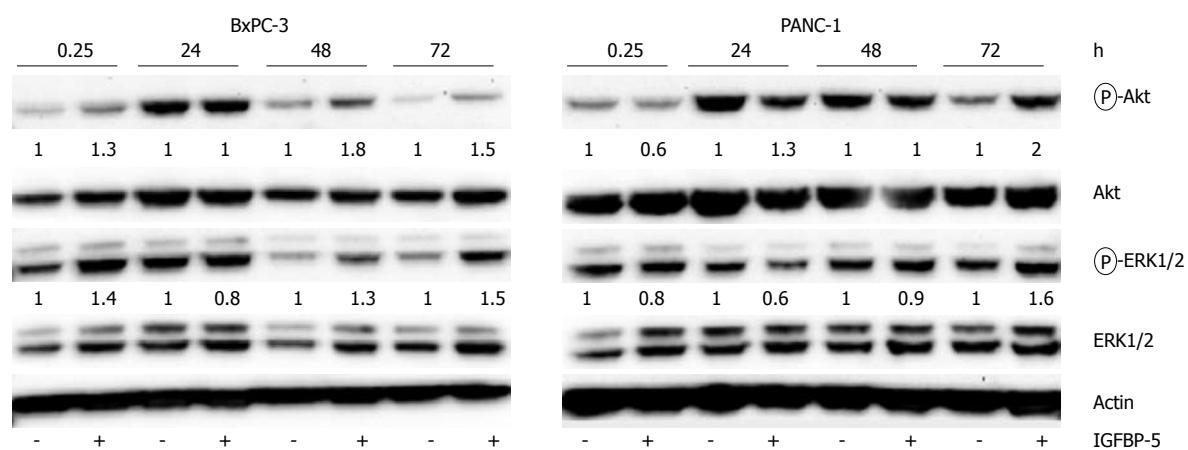
#### Effect of IGFBP-5-conditioned medium on signaling in PaC cells

To determine whether IGFBP-5 induced changes in Akt and ERK1/2 activation through an autocrine pathway, we applied conditioned medium from IGFBP-5-transfected cells to PaC cells and examined the effect on Akt and ERK1/2 activation. Consistent with our expression studies, BxPC-3 cells treated with IGFBP-5-conditioned medium increased activation of Akt and ERK1/2 compared with cells treated with conditioned medium from vector transfected cells (Figure 7). Similar levels of phosphorylated protein were observed in cells exposed to vector-transfected or IGFBP-5-conditioned medium at 24 h, however, only PaC cells exposed to IGFBP-5-conditioned medium had a prolonged induction of Akt and ERK1/2 activation (72 h).

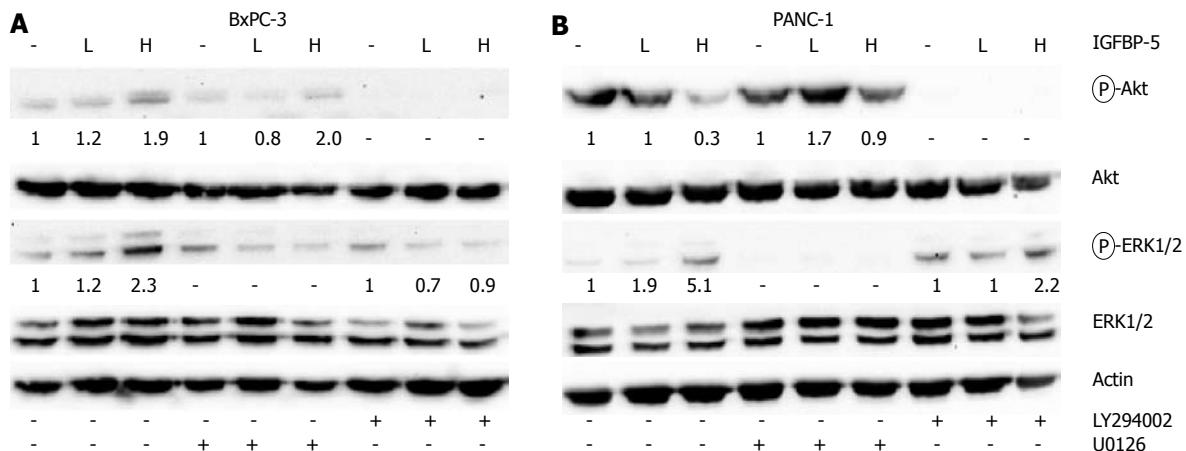
The effect of IGFBP-5 conditioned medium on ERK1/2 activation in PANC-1 cells was similar to the results observed in cells expressing IGFBP-5. After



**Figure 6** PI3K or MEK1/2 inhibition decreases IGFBP-5-enhanced growth of serum-starved PaC cells. Stable transfectants (empty vector/Vec-closed square or high /BP5H-closed triangle) were seeded in growth media. The serum-containing medium was changed to SFM 24 h post-seeding. After 24 h in serum-free conditions, a PI3K inhibitor (LY294002, A and B) or MEK1/2 inhibitor (U0126, C and D) was added to the medium. After 48 h, an MTT assay (B and D) was performed to assess cell viability. Cell lysates were prepared at this time for immunoblot analysis of Akt or ERK1/2 activation (A and C, respectively) using phospho-specific antibodies. For immunoblots, cells were treated with 25  $\mu$ mol/L LY294002 or 10  $\mu$ mol/L U0126, as indicated. Membranes were subsequently stripped and reprobed for total Akt or ERK1/2 (A and C, respectively). Actin level was monitored to normalize protein loading. Data shown represent the mean  $\pm$  SE from 4 separate experiments performed in triplicate. The percentage of viable cells was determined relative to control-treated cells (100%). <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , <sup>d</sup> $P < 0.001$ .



**Figure 7** IGFBP-5-conditioned medium enhances activation of Akt and ERK1/2 in PaC cells. IGFBP-5- (+) or vector-transfected (-) control cells were grown for 48 h in SFM. Conditioned medium was collected and applied to BxPC-3 or PANC-1 cells for indicated times. Cell lysates were prepared and immunoblot analysis of phosphorylated Akt or ERK1/2 was performed. Membranes were subsequently stripped and reprobed for total Akt or ERK1/2. Actin level was monitored to normalize protein loading. Quantitation of band density represents the level of IGFBP-5-associated expression relative to vector-transfected controls (1.0) normalized to the expression level of total Akt or ERK1/2. Results are representative of three independent experiments.



**Figure 8** Effect of IGFBP-5 on signaling in serum-starved PaC cells. BxPC-3 (A) and PANC-1 (B) stable transfectants [empty vector (-), low IGFBP-5-L, or high IGFBP-5-H] were deprived of serum for 24 h then incubated in the presence of vehicle (DMSO), LY294002, or U0126 (10  $\mu$ mol/L) for an additional 48 h. Cell lysates were prepared for immunoblot analysis of Akt or ERK1/2 activation using phospho-specific antibodies. Membranes were subsequently stripped and reprobed for total Akt or ERK1/2. Actin level was monitored to normalize protein loading. Quantitation of band density represents the level of IGFBP-5-associated expression relative to vector-transfected controls (1.0) normalized to the expression level of total Akt or ERK1/2. Results are representative of three independent experiments.

exposure of PANC-1 cells to IGFBP-5-conditioned medium, Akt and ERK1/2 activation levels were slightly reduced after 15 min, reached control levels by 48 h, and were elevated by 72 h (Figure 7). Interestingly, in PANC-1 cells that express IGFBP-5, activation of Akt was reduced whereas in cells exposed to IGFBP-5-conditioned medium Akt activation was elevated (2 fold). To determine if the IGFBP-5-mediated growth effects observed in PaC cells that express IGFBP-5 could be replicated by application of conditioned medium, PANC-1 and BxPC-3 cell growth was examined. The cell number and level of DNA synthesis observed in cells exposed to IGFBP-5-conditioned medium compared to controls in these growth conditions were not significantly different (data not shown). These results suggest that enhanced activation of Akt and ERK1/2 in IGFBP-5 expressing PaC cells is due to an autocrine pathway.

#### Effect of IGFBP-5 on Ras-mediated cross-talk

After serum deprivation of PANC-1 cells, Akt activation is reduced whereas ERK1/2 activation is elevated in cells expressing IGFBP-5. In BxPC-3 cells that express IGFBP-5, Akt and ERK1/2 phosphorylation is elevated. Previously, it has been shown that Ras- and PI3K-mediated pathways exhibit cross-talk in breast cancer cells<sup>[22]</sup>. Therefore, we sought to determine whether the differential effects observed between the PaC cells which express wild-type K-Ras (BxPC-3) or harbor mutated K-Ras (PANC-1) were a result of cross-talk between these pathways. Serum-deprived PaC cells were exposed to either U0126 or LY294002 and activation of ERK1/2 and Akt was examined by immunoblot analysis. After MEK1/2 inhibition in serum-starved BxPC-3 cells, Akt activation is decreased, however IGFBP-5 expressing cells maintain a level of Akt activation that is 2-fold greater than vector-transfected cells (Figure 8A). Inhibition of the PI3K pathway suppresses IGFBP-5-enhanced activation of ERK1/2 in serum-deprived

BxPC-3 cells. These results suggest that Akt activation is necessary for enhanced activation of ERK1/2 associated with IGFBP-5. In contrast, inhibition of MEK1/2 does not completely block IGFBP-5-enhanced Akt activation, therefore, ERK1/2-independent pathways must contribute to increased Akt activation associated with IGFBP-5.

In PANC-1 cells, inhibition of PI3K signaling is associated with elevated levels of ERK1/2 activation in the presence of IGFBP-5 compared to vector controls (Figure 8B). Interestingly, IGFBP-5 is associated with an elevated level of Akt activation (1.7 to 3 fold) when MAPK signaling is blocked compared to that observed in the absence of MAPK inhibition (Figure 8). These results suggest that inhibition of either the MAPK or PI3K pathway in IGFBP-5 expressing PANC-1 cells may shift signaling to the other pathway.

## DISCUSSION

Previously, we determined that IGFBP-5 was differentially expressed in pancreatic tumors compared to non-malignant pancreatic tissue<sup>[9]</sup>. IGFBP-5 has been implicated in cell survival in muscle, prostate cancer, and breast cancer cells<sup>[23-25]</sup>, however, the role of IGFBP-5 in pancreatic cancer has not been investigated. In this study we have examined the effects of IGFBP-5 expression on two cell lines, BxPC-3 and PANC-1, both of which express the IGF-1 receptor and are responsive to IGF-1<sup>[26]</sup>. Growth of the cell lines in the presence of serum, used as a surrogate for IGF-1 and other growth factors<sup>[12-14]</sup>, revealed that IGFBP-5 reduced PANC-1 cell growth thus opposing the cell proliferative effects of IGF-1 and other growth factors. This result is similar to the growth inhibitory effects of IGFBP-5 observed in breast cancer cells<sup>[27]</sup>.

When tumors reach a certain size, their growth is limited by a lack of available growth factors; therefore, mechanisms that circumvent growth factor deprivation

and allow tumor growth are utilized. Consistent with this notion, we found that IGFBP-5 promotes cell growth after serum starvation. In the presence of IGFBP-5 after serum starvation, cell number and DNA synthesis are significantly elevated in BxPC-3 cells, with a clear dose-dependency reflected by increased cell growth with increased IGFBP-5 expression. In contrast, high levels of IGFBP-5 in PANC-1 cells inhibited growth. Interestingly, low levels of IGFBP-5 appeared to enhance DNA synthesis as measured by thymidine incorporation, but this effect was lost with higher IGFBP-5 expression, though the reason for these differences remains unclear. Intriguingly, the growth promoting effects of IGFBP-5 on BxPC-3 cells parallel those observed in prostate cells<sup>[24]</sup>.

The upregulation of oncogenes and downregulation of tumor suppressor genes contributes to the uncontrolled growth and survival of pancreatic cancer cells<sup>[28]</sup>. To determine if IGFBP-5 overexpression contributes to the dysregulation of pancreatic cancer cell growth, we analyzed cell cycle distribution and found that IGFBP-5 promotes progression through the cell cycle in BxPC-3 cells but leads to a G2/M cell cycle arrest in PANC-1 cells. These results are consistent with observed changes in cell number and DNA synthesis associated with IGFBP-5 expression. Other studies have shown that serum-starved BxPC-3 cells undergo cellular arrest and increased apoptosis. However, after serum stimulation cellular proliferation proceeds and apoptosis is reduced; both responses are dependent on PI3K activation<sup>[29]</sup>. Our study shows that serum-deprived BxPC-3 cells that express IGFBP-5 continue to proliferate and thus IGFBP-5 may mimic the effect of serum exposure and provide the mitogenic stimulus necessary for tumor growth when the bioavailability of growth factors is low. Thus, in this context IGFBP-5 can act similarly to, and in the absence of, IGF-I to enhance proliferation of pancreatic cancer cells.

Neuroblastoma cells exposed to increasing doses of IGFBP-5 dramatically increased proliferation in the presence of low levels of IGFBP-5 but returned to control levels at a higher dose<sup>[30]</sup>. For this study, we chose clones which express IGFBP-5 at either a low or high level to determine if IGFBP-5 affects pancreatic cancer cell growth in a dose-dependent manner. Indeed, the effects of IGFBP-5 on the growth of BxPC-3 and PANC-1 cells appeared to be dose-dependent; however, the effect was additive rather than inverse as observed in neuroblastoma cells. Low levels of IGFBP-5 promote progression of BxPC-3 cells through the cell cycle and this effect is exacerbated by high levels of IGFBP-5. PANC-1 cells that expressed low levels of IGFBP-5 had cell numbers similar to the control but two-fold increases in DNA synthesis; whereas cells with high levels of IGFBP-5 had reduced cell numbers but maintained or slightly exceeded control levels of DNA synthesis which may be due to elevated Cyclin D1 levels observed in these conditions (Figure 4). Both low and high levels of IGFBP-5 in PANC-1 cells led to cell cycle arrest at G2/M; however, when cells expressed high levels of IGFBP-5 a greater percentage

accumulated in G2/M phase. Overexpression of IGFBP-5 in breast cancer cells also resulted in G2/M cell cycle arrest as well as induction of apoptosis<sup>[27]</sup>, though we did not observe an increase in the percentage of cells with subG1 DNA content.

Our studies show that changes resulting in IGFBP-5-enhanced BxPC-3 cell growth are reflected by elevations in cyclins D and E and accompanying reductions in p21<sup>Cip1</sup> and p27<sup>Kip1</sup> CDK inhibitors, which may allow BxPC-3 cells that express IGFBP-5 to rapidly progress through the G1/S transition and result in enhanced cell growth observed in cell growth studies. In a survey of pancreatic tumors, CDK2 and CDK4 upregulation was observed to directly correlate with progression of pancreatic cancer; in contrast cyclin E upregulation was observed in 25% of high-grade intraepithelial neoplasia and 75% of ductal carcinoma and cyclin D1 had the lowest frequency of upregulation in late stage disease further demonstrating the deregulation of the G1/S transition in the early stages of PaC<sup>[31]</sup>. The progressive loss of p27<sup>Kip1</sup> expression has been correlated with tumor grade and clinical stage (i.e. positive immunohistochemical staining in well-differentiated, early stage tumors) and thus may be involved in the malignant progression of PaC<sup>[32,33]</sup>. In contrast to the growth effects of IGFBP-5 observed in BxPC-3 cells, PANC-1 cells expressing IGFBP-5 exhibited reduced levels of cyclin E and CDK2 and an elevated level of p27<sup>Kip1</sup>, which may explain the distinct G2/M arrest observed for this cell line. This likely protects cells from unfavorable conditions and allows cells to rapidly re-enter the cell cycle when conditions are favorable.

IGFBP-5-mediated growth effects are often associated with activation of the PI3K and MAPK pathways<sup>[24,30]</sup>. In PaC cells, it has been observed that Akt is constitutively activated at a high level in PANC-1 cells but not in BxPC-3 cells<sup>[34]</sup>, which our study confirms; whereas MAPK activity is not constitutively active in PANC-1 or BxPC-3 cells<sup>[5,12]</sup>. In this study, overexpression of IGFBP-5 elicited increased phosphorylation of Akt in BxPC-3 cells but reduced phosphorylation in PANC-1. Both cell lines, however, exhibited a modest increase in ERK1/2 phosphorylation with IGFBP-5 expression. Upon inhibition of either the PI3K or MAPK pathway, IGFBP-5 no longer enhanced growth of BxPC-3 or PANC-1 cells after serum deprivation. These data suggest, therefore, that IGFBP-5 promotes cell growth in BxPC-3 cells by enhancing PI3K-Akt signaling. Consistent with our findings, a conditionally active form of Akt has been shown to transform cells by promoting progression through S and G2/M phases of the cell cycle, decreasing expression of p27<sup>Kip1</sup>, and increasing activity of CDK2<sup>[35]</sup>, similar to our observations in BxPC-3 cells overexpressing IGFBP-5.

In this study, we have demonstrated that in BxPC-3 cells IGFBP-5 promotes proliferation *via* activation of the PI3K pathway; whereas it promotes growth arrest *via* activation of the MAPK pathway in PANC-1 cells. Although both cell lines employed in these studies were derived from pancreatic adenocarcinomas, they represent

different genetic backgrounds and reflect differences observed in human tumors. For example, both cell lines express the IGF-1R and are responsive to IGF-1<sup>[26]</sup>, however, BxPC-3 cells have wild-type K-ras and express lower levels of PTEN mRNA while PANC-1 cells express higher levels of PTEN mRNA and possess an activating K-ras mutation. Thus, while the opposing effects of IGFBP-5 on these two cell lines may not be satisfying to those expecting a uniform response from cells originating from the same tumor type, these results highlight the clinical challenge of treating a disease that does not actually represent a single entity. Tumors arising from the same organ may exhibit a great diversity in the signal transduction pathways they utilize and treatments will continue to fail if they do not account for these differences. In both cell lines employed in this study the predominant pathway stimulated by IGFBP-5 (i.e. Akt in BxPC-3 and ERK1/2 in PANC-1) is maintained, although at a lower level, in the presence of pharmacological inhibition of the other pathway, which may represent a novel mechanism by which IGFBP-5 promotes PaC cell growth and survival.

Pancreatic tumors are highly chemoresistant, thus treatment rarely leads to eradication of the malignancy. Since tumors are comprised of cells with heterogeneous genetic backgrounds they often do not respond uniformly to any specific treatment. This cellular complexity is underscored by the divergent characteristics of the two PaC cell lines used in our study. Our results, therefore, highlight the need to identify multiple chemotherapeutic targets for treating pancreatic cancer. Further studies aimed at identifying signaling intermediates involved in IGFBP-5-enhanced cell growth and survival within the diverse cellular population of pancreatic tumors may therefore elucidate potentially important therapeutic targets.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Pancreatic cancer (PaC) is the fourth leading cause of cancer deaths in the United States. Annually, the incidence of PaC almost equals the number of deaths related to this disease, and these numbers have begun to increase. Previously, insulin-like growth factor binding protein-5 (IGFBP-5) was demonstrated to be differentially expressed in pancreatic tumors compared to non-malignant pancreatic tissue. IGFBP-5 has been implicated in cell survival in muscle, prostate cancer, and breast cancer cells; however, the role of IGFBP-5 in pancreatic cancer has not been investigated.

### Research frontiers

IGFPB-5 is expressed at higher levels in pancreatic tumors than in non-malignant pancreatic tissues. The role of IGFBP-5 in cancer cell growth and proliferation, however, is not fully understood and has not been studied in pancreatic cancer. In this study, the authors demonstrate that IGFBP-5 can enhance pancreatic cancer cell growth by altering the expression and activity of cell cycle regulators and the activation of key signaling intermediates.

### Innovations and breakthroughs

In this study, the effects of IGFBP-5 expression were examined on two cell

lines, BxPC-3 and PANC-1, both of which express the IGF-I receptor and are responsive to IGF-I. In BxPC-3 cells IGFBP-5 promotes proliferation via activation of the PI3K pathway; whereas it promotes growth arrest via activation of the MAPK pathway in PANC-1 cells. The opposing effects of IGFBP-5 on these two cell lines highlight the clinical challenge of treating a disease that does not actually represent a single entity. Tumors arising from the same organ may exhibit a great diversity in the signal transduction pathways they utilize and treatments will continue to fail if they do not account for these differences.

### Applications

Pancreatic tumors are highly chemoresistant, thus treatment rarely leads to eradication of the malignancy. Since tumors are comprised of cells with heterogeneous genetic backgrounds they often do not respond uniformly to any specific treatment. The results of this study highlight the need to identify multiple chemotherapeutic targets for treating pancreatic cancer. Further studies aimed at identifying signaling intermediates involved in IGFBP-5-enhanced cell growth and survival within the diverse cellular population of pancreatic tumors may therefore elucidate potentially important therapeutic targets.

### Terminology

Insulin-like growth factors (IGFs) regulate cellular growth, differentiation, and apoptosis and IGF-binding proteins (IGFBPs) modulate the activity of IGFs. IGFBPs can also act independently of IGF to either stimulate or inhibit growth of various cell types. Both IGF-I and the IGF-I receptor (IGF-I R) are expressed in pancreatic tumors and cell lines, and IGFBP-1, -3, and -5 have been associated with pancreatic cancer.

### Peer review

The authors investigate the functional significance of IGFBP-5 overexpression in pancreatic cancer. The manuscript is interesting, with some novel aspects of the IGFBP-5 biology in pancreatic cancer cells disclosed.

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# Correlation of MRI-determined small bowel Crohn's disease categories with medical response and surgical pathology

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findings of fibrosis and the severity of inflammation correlated with the MRI category in all cases.

**CONCLUSION:** Our findings suggest that SB CD can be grouped by the MRI findings and that these groups are associated with patients more likely to respond to continued medical therapy. The MRI categories also correlated with the presence and level of intestinal inflammation and fibrosis on surgical pathology, and may be of prognostic use in the management of CD patients.

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**Key words:** Magnetic resonance imaging; Crohn's disease; Fibrosis; Prognosis; Pathology

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## Abstract

**AIM:** To determine whether magnetic resonance imaging (MRI) can be used to categorize small bowel Crohn's disease (SB CD) into groups that correlate with response to medical therapy and surgical pathology.

**METHODS:** Data was collected from all patients with MRI evidence of SB CD without significant colonic disease over a 32-mo period. Two radiologists, blinded to clinical findings, evaluated each MRI and grouped them based on bowel wall thickness and wall enhancement. These categories were: (1) "fibrosis", (2) "mild segmental hyper-enhancement and mild wall thickening", (3) "mild segmental hyper-enhancement and marked wall thickening", (4) "marked segmental transmural hyper-enhancement". Patient response to additional medical therapy post-MRI was prospectively determined at 8-wk. Non-responders underwent endoscopy and were offered therapeutic endoscopy or surgery. Surgical pathology was assessed against the MRI category.

**RESULTS:** Fifty-five patients were included. Females and category "2" patients were more likely, and patients with luminal narrowing and hold-up less likely, to respond to medical therapy ( $P < 0.05$ ). Seventeen patients underwent surgery. The surgical pathological

## INTRODUCTION

Crohn's disease (CD) is a life-long, chronic, relapsing condition. Morbidity is high and its incidence and prevalence are increasing worldwide<sup>[1]</sup>. Despite the development of new medical therapies, there is little evidence that they alter the natural history of CD. Due to the transmural nature of CD, fibrosis, stenosis and obstruction often result<sup>[2]</sup>, with fibrosis and stricture formation still the most common reason for intestinal resection.

CD frequently involves the terminal ileum (TI) so investigation of the small bowel (SB) is of the utmost importance. Unfortunately, TI assessment by endoscopy is not optimal, with a significant proportion of colonoscopic examinations failing to reach the cecum<sup>[3]</sup>. The mainstay of SB investigation has been SB enterography or SB enteroclysis (SBE) that requires placement of a nasojejunal tube (NJT). More recently CT enteroclysis

(CTE), wireless capsule enteroscopy (WCE), double balloon enteroscopy, MR SB enteroclysis (MRE) and MR enterography have become available. Conventional radiology (SBE and SB enterography) only has a sensitivity of between 23% and 80% for the detection of typical CD radiological lesions<sup>[4-6]</sup>. It delivers ionising radiation and is suboptimal for the assessment of extra-intestinal involvement and complications. WCE on the other hand allows for excellent visualization of the SB mucosa, but its specificity is lower than other methods<sup>[6]</sup>, it does not clearly localise the lesions, histology cannot be taken, and there is a small but definite capsule retention rate contraindicating its use in SB strictures<sup>[7]</sup>.

CTE has been well described<sup>[8]</sup>, has good sensitivity (71%-95%) and impressive specificity (90%-98%) and is superior to conventional enteroclysis<sup>[9]</sup>. The use of ionising radiation in CTE in a generally young patient cohort, however, is not ideal. CTE also lacks functional information, has poor fluoroscopic control of SB filling and suboptimal soft tissue contrast<sup>[6,10-12]</sup>. MRI also has high sensitivity and specificity in the evaluation of CD<sup>[13]</sup> and while it does not provide as consistently good mucosal detail as conventional enteroclysis, it has a strong correlation with pathologic findings and does not use ionising radiation<sup>[14-16]</sup>. MRI also demonstrates good soft tissue contrast, subtle degrees of contrast enhancement and conveys functional information, while potentially differentiating active inflammation from fibrosis in preliminary studies<sup>[16,17]</sup>. A retrospective paper correlating CTE findings with surgical pathology in 36 patients accurately detected strictures, fistulas, abscesses and inflammatory masses in 94% of patients<sup>[18]</sup>. CTE was deemed to provide accurate preoperative diagnosis for SB CD. As MRI potentially has the ability to differentiate inflammation from fibrosis, the question arises as to whether MR imaging could be used to predict patient response to continued medical therapy and the need for surgery?

The hypothesis of this study was that SB enhancement, bowel wall thickness and other findings on MRI could categorise CD patients and that these categories could be used to identify patients who were more likely to respond to continuing medical therapy. It was also hypothesised that these categories would correlate with the presence and level of intestinal inflammation and fibrosis identified on surgical pathology.

## MATERIALS AND METHODS

All subjects were patients of the Centre for Inflammatory Bowel Diseases, Fremantle Hospital; a specialist inflammatory bowel disease (IBD) unit in a 450-bed tertiary institution. All patients had previously been confirmed as having CD on previous endoscopic examination with histological confirmation. Data was collected from all CD patients with SB disease undergoing MRI enterography or MRE over a 32-mo period. Any patient with a contraindication to the intravenous use of gadodiamide was excluded. Response to medical therapy was determined prospectively.

### ***MRI preparation***

All patients drank only clear fluids for 6 h prior to their MRI and were nil by mouth for 2 h. Patients either underwent MRE or MR enterography. Patients who underwent an MR enterography (including those patients where placement of the NJT failed for technical reasons or patient refusal) drank 1000 mL of the bowel contrast agent over 20 min. For an MRE, an NJT (Bilbao-Dotter, Cook, Australia) was placed under fluoroscopy. The bowel contrast agent used was a polyethylene glycol-water solution (Glycoprep-C, Pharmatek Fresenius Kabi, Australia), which was injected manually through the NJT (60 mL/min increasing to 120-150 mL/min). A total of 800-2000 mL was usually required to distend the SB to the TI. This varied depending on previous bowel resections.

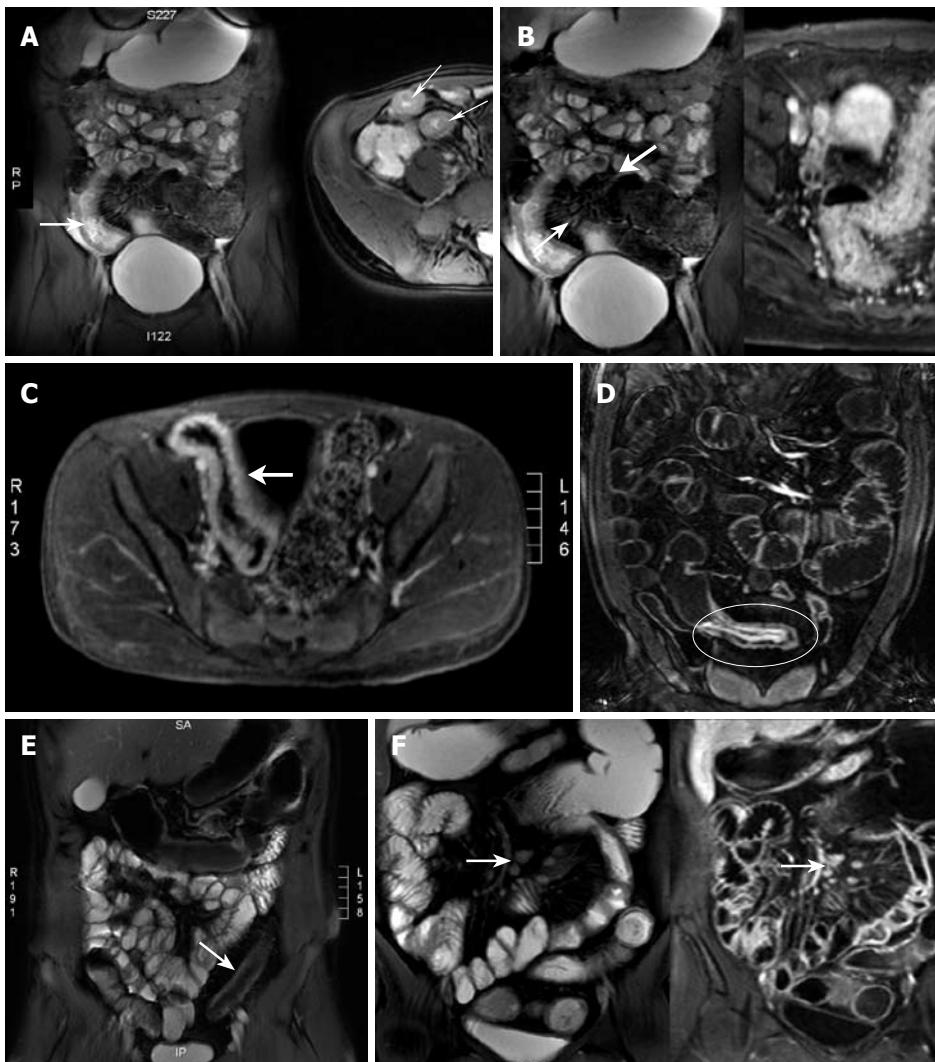
### ***MRI technique***

Patients were imaged supine using a 1.5T MRI system (Avanto SQ, Siemens Medical Solutions, Erlangen, Germany) with a surface array coil providing compression. In patients with an ileostomy, the stoma bag was emptied and a sponge placed between the surface coil and stoma.

A scout image was acquired to ensure adequate coverage and assess retrograde filling of the stomach. SB filling was dynamically assessed using a coronal 150 mm thick single slab T2-weighted (HASTE) fat saturated sequence acquired repeatedly without breath-holding to monitor for retrograde stomach filling and SB distension. These images were combined into a cine loop and used to assess stenotic lesions. If there was doubt as to TI contrast filling, a single breath-hold coronal T2-weighted sequence (HASTE) with a 6 mm slice thickness and 30% gap was obtained.

To reduce bowel peristalsis and prolong SB distension, 10 mg intravenous hyoscine butylbromide (Buscopan, Boehringer Ingelheim, Australia) was given if there were no contraindications. Once there was adequate SB filling, a coronal pre-contrast T1-weighted 3D gradient echo (VIBE) with a 2 mm slice thickness was obtained. Intravenous gadodiamide (Omniscan, Amersham, Australia) was injected (0.2 mL/kg) with post-contrast imaging commencing at 60 s. Post-contrast VIBE sequences were obtained in the coronal and axial planes. The axial plane required 2 overlapping sections covering the upper and lower abdomen.

Further imaging included a coronal steady-state free precession sequence (trueFISP) with a 6 mm slice thickness and 30% gap obtained with and without fat saturation, axial steady-state free precession sequence (trueFISP) with a 6 mm slice thickness and 30% gap obtained without fat saturation in 2 blocks of the upper and lower abdomen, coronal T2-weighted half Fourier single shot turbo spin echo (HASTE) sequence with 6 mm slice thickness and 30% gap, and axial T2-weighted HASTE sequence with a 5 mm slice thickness and 30% gap was obtained in 2 blocks of the upper and lower abdomen. If a site of pathology was identified at an overlap point on the axial images, then a further



**Figure 1 MRI characteristics of intestinal inflammation.** A: Bowel wall thickening. The distended bowel wall is normally 1-3 mm thick. An abnormal thickness is > 4 mm (arrowed); B: Mucosal hyperemia (thick arrow), engorged ileal vasa recta or the “comb sign” (thin arrow); C: Transmural enhancement (arrowed); D: Mucosal enhancement (circled); E: Fibrofatty infiltration (arrowed); F: Lymphadenopathy (arrowed).

set of targeted axial trueFISP and HASTE images were obtained.

For MR enterography, an initial single breath-hold coronal T2-weighted sequence (HASTE) with a 6 mm slice thickness and 30% gap was obtained. If there was adequate filling of the TI, buscopan was injected and routine imaging performed as above. With suboptimal filling of the TI, but sufficient bowel contrast proximally, reassessment occurred at 5-min intervals for 15 min. If there was still inadequate filling of SB loops, the patient drank a further 500 mL of contrast.

#### MRI assessment

Each MRI was evaluated by consensus of two radiologists (CW and PS) with experience in both gastrointestinal and MR imaging blinded to clinical findings. Image analysis was performed using a standardised worksheet. Diseased bowel was identified as abnormal bowel wall thickening and abnormal transmural or mucosal enhancement. Bowel wall thickness was assessed as normal (< 3 mm), mildly abnormal (3-6 mm) or markedly abnormal (> 6 mm)<sup>[19]</sup>. Mucosal enhancement was contrast enhancement localized to the inner layer of the intestinal wall and transmural enhancement was homogenous contrast enhancement of the whole wall. The degree of pathological bowel wall

contrast enhancement was assessed as none, mild (less than renal cortical enhancement) or marked (more than renal cortical enhancement) and classified as mucosal, transmural enhancement or both. The degree of enhancement was measured relative to the renal cortex on the coronal acquisition, and axial views were used for problem solving and cross-referencing of bowel loops.

Mesenteric hyperemia, fibrofatty proliferation, enlarged local lymph nodes and/or abnormally enhancing lymph nodes were assessed (Figure 1). Mesenteric nodes < 5 mm in short axis were considered physiological. Larger nodes, especially if clustered and contrast enhancing, were considered pathological<sup>[20]</sup>. The number of regional nodes and length of diseased segment were noted. Disease complications, including fistulas or abscesses, were recorded, as was free fluid, and stenoses with or without functional obstruction. Signs of functional obstruction were pre-stenotic dilatation and delayed contrast progression on the dynamic HASTE series.

#### MRI classification of fibrosis and inflammation

Based on the SB MRI findings, the CD patients were grouped into one of four categories; (1) “fibrosis”, (2) “mild segmental hyper-enhancement and mild wall thickening”, (3) “mild segmental hyper-enhancement

**Table 1 Grouping by MRI findings**

	Category 1	Category 2	Category 3	Category 4
Mucosal enhancement	Nil	Mild	Mild	Mild or marked
Transmural enhancement	Nil	Nil or mild	Nil or mild	Marked
Wall thickness (mm)	> 3	< 6	> 6	> 3

Patients were grouped on the basis of the SB wall thickness and mucosal or transmural enhancement. The 4 categories could also be described as (1) fibrosis, (2) mild segmental hyper-enhancement and mild wall thickening, (3) mild segmental hyper-enhancement and marked wall thickening, and (4) marked segmental transmural hyper-enhancement.

and marked wall thickening”, and (4) “marked segmental transmural hyper-enhancement” (Table 1). Studies were classified as “fibrosis” if there was bowel wall thickening without contrast enhancement. The diagnostic confidence of this was increased if the wall thickening had reduced signal intensity on T1- and T2-weighted images and if this region remained of constant calibre. “Marked segmental transmural hyper-enhancement” was diagnosed if there was marked transmural enhancement with increased bowel wall thickness.

“Mild segmental hyper-enhancement and mild wall thickening” was present if mucosal and/or transmural contrast enhancement was mild with only mild bowel wall thickening (3-6 mm). “Mild segmental hyper-enhancement and marked wall thickening” was present if there was mild mucosal and/or transmural contrast enhancement, but the bowel wall was > 6 mm.

### Response to medical therapy

Response to continued medical therapy was assessed prospectively. CD was diagnosed in accordance with previously established international criteria<sup>[21]</sup> exclusive of infective enterocolitis, Behcet’s disease and microscopic colitis. Response and remission to medical therapy was assessed by the Harvey Bradshaw index (HBI)<sup>[22]</sup>. A response was a reduction in the HBI of  $\geq 3$  and remission was defined as a HBI < 5. For patients who had undergone colectomy, physician assessment with endoscopy or imaging was used to assess response/remission. A response was determined after a further 8 wk of medical therapy. If medical therapy failed, then endoscopic or surgical options were offered. Correlation between the MRI findings and response to medical therapy was assessed, and for patients undergoing surgical resection the correlation between surgical pathology and the MRI was also assessed.

### Histological analysis

Specialist histopathologists reported on the macroscopic appearance and histology of the surgical specimens. They graded the degree of inflammation based on the bowel wall thickness, mesentery appearance, mesenteric lymph nodes, mucosal ulceration and the density and extent of both the acute and chronic inflammatory cell infiltrates, loss of goblet cells, crypt abscesses and

**Table 2 Characteristics of the CD patients prior to the MRI examination**

	CD (n = 52)
Gender: male	38.5% (20/52)
Age at diagnosis (yr)	38.9 ± 16.0 (14-79)
A1 ≤ 16	11.5% (6/52)
A2 17-40	55.8% (29/52)
A3 > 40	32.7% (17/52)
Crohn’s disease	
L1 Terminal ileum	78.8% (41/52)
L2 Colon	0% (0/52)
L3 Ileocolonic	19.2% (10/52)
L4 Upper GI	7.7% (4/52)
P Perianal	9.6% (5/52)
B1 Non-stricture	
Non-penetrating	75.0% (39/52)
B2 Stricture	19.2% (10/52)
B3 Penetrating	5.8% (3/52)
Colectomy	11.5% (6/52)
Terminal ileal resection	25.0% (13/52)
Proximal SB resection	1.9% (1/52)

architectural distortion. “Mild inflammatory infiltrates with scattered glandular architectural distortion and crypt branching” was termed mild inflammation. “Mucosal ulceration with marked glandular changes, dilated complex crypts, a transmural mixed cellular infiltrate and reactive changes in the mesentery” was termed severe. Fibrosis was determined by the presence of a stricture, the bowel wall thickness and assessment of collagen deposition in the bowel wall.

### Statistical analysis

Logistic regression was undertaken by a statistician (KM) and assessed wall enhancement, degree of enhancement, bowel wall thickness, abscess, fibro-fatty proliferation, fistula, free fluid, length of disease, mesenteric hyperemia, luminal narrowing with and without prestenotic dilatation, regional lymph nodes and enhancement, sex, and age. Variable selection was undertaken retaining effects significant at the 0.05 level, and the significant effects and odds ratios are presented, along with 95% confidence intervals.

## RESULTS

Data was collected over a 32-mo period on 55 CD patients who had abnormal SB-MRI findings consistent with SB CD without significant active colonic disease. Three of these patients were asymptomatic despite the abnormal MRI findings and were not included in any further analysis. Of the remaining 52 patients (Table 2), 38.5% were male (20/52) with an average age at diagnosis of 38.5 years (range 14-79 years). Forty-two patients (81%) had disease confined to the small intestine (one patient only suffered from proximal SB disease - L4) and 10 (19%) suffered from ileocolonic CD. Of these 10 patients, 6 had previously undergone a colectomy or proctocolectomy and had ongoing active disease confined to the SB. The remaining 4 patients had

**Table 3** MRI findings of patients according to the MRI category

MRI group (n)	Male (%)	Age (yr)	Bowel length involved (cm)	Wall thickness (mm)	Degree of SB wall enhancement	Mucosal enhancement (%)	Transmural enhancement (%)	Mesenteric hyperaemia (%)	Fibrofatty proliferation (%)	Regional lymph nodes (%)	Free fluid (%)	Narrowed lumen no hold-up (%)	Prestenotic dilation ± holdup (%)	Fistula (%)
1 (7)	28.6	37.9 ± 9.4 (23-54)	6.3 ± 7.9 (1-25)	< 3 0% 3-6 71.4% > 6 28.6%	None 100% Mild 0% Marked 0%	0	0	0	14.3	0	14.3	57.1	14.3	0
2 (9)	22.2	32.8 ± 9.2 (17-78)	13.8 ± 12.3 (3-30)	< 3 11.1% 3-6 88.9% > 6 0%	None 0% Mild 100% Marked 0%	100	88.9	33.3	33.3	66.6	33.3	44.4	0	22.2
3 (16)	43.8	40.1 ± 16.2 (14-79)	16.2 ± 12.0 (2-50)	< 3 0% 3-6 0% > 6 100%	None 0% Mild 100% Marked 0%	93.8	43.8	50.0	62.5	37.5	18.8	18.8	62.5	25.0
4 (20)	45.0	41 ± 19.2 (14-49)	19.2 ± 11.6 (2-45)	< 3 0% 3-6 10% > 6 90%	None 0% Mild 0% Marked 100%	100	90.0	75.0	75.0	85.0	35.0	35.0	35.0	20.0

**Table 4** MRI category, medical therapy and clinical response (%)

MRI group	Steroids	5ASA	Antibiotics	AZA/6MP	MTX	Anti-TNF α	Response
1 (n = 7)	57.10	57.10	28.50	85.70	0.00	42.90	14.30
2 (n = 9)	55.50	88.90	0.00	22.20	33.30	44.40	100.00
3 (n = 16)	81.30	68.80	6.30	50.00	0.00	12.50	43.80
4 (n = 20)	70.00	70.00	5.00	40.00	0.00	35.00	50.00
Responders (n = 27)	63.00	63.00	11.10	44.40	3.70	25.90	N/A
Non-responders (n = 25)	76.00	80.00	4.00	48.00	8.00	36.00	N/A

5ASA: 5 aminosalicylic acid; AZA/6MP: Azathioprine/6 mercaptopurine; MTX: Methotrexate; N/A: Not applicable.

ileal inflammation and colonic inflammation confined to the cecum or right colon. A further 14 patients had previously undergone surgery for their CD. Thirteen underwent a terminal ileal resection and a limited right hemicolectomy, with 3 of these patients each having had a further 2 resections of the neoterminal ileum for stricturing disease. The final patient had 3 resections of the mid SB for stenosing CD.

Utilising a combination of the maximal SB wall thickness and level of mucosal/mural enhancement, patients were allocated into 1 of 4 categories (Table 1). All the patients in category "4" also demonstrated 2 or more extra-intestinal changes of inflammation (i.e. mesenteric hyperemia, fatty proliferation, enlarged regional lymph nodes, pathological lymph node enhancement or free fluid) indicating a more severe level of inflammation. MRI detection of bowel wall enhancement and its degree, level of bowel wall thickness, as well as the presence of an abscess, fibro-fatty proliferation, mesenteric hyperemia, a fistula, free fluid or enlarged regional lymph nodes are detailed in Table 3. The length of bowel involved and the presence of luminal narrowing with and without prestenotic dilatation, patient age and sex are also presented.

The medical therapies received by the patients included the use of steroids (prednisone, budesonide), 5-aminosalicylic acid (5ASA) azathioprine/6-mercaptopurine (AZA/6MP), methotrexate (MTX), antibiotics and anti-TNF alpha therapy (Table 4). Medical therapy was not standardized between patients, but was tailored to the individual patient. Twenty-seven of the 52

patients responded to medical therapy and 2 of these patients responded to conservative non-IBD related medical therapy. One patient refused to take any medical therapy prior to an endoscopic examination. If any patient failed to respond to the continued medical therapy, an endoscopic examination was offered to determine whether there was ongoing active inflammatory disease or if the inflammation was controlled and any stricture was amenable to endoscopic dilatation.

Twenty-five patients failed to respond to medical therapy (2/4 patients with ileal and right sided colonic disease, 3/6 with active SB disease post colectomy and 20/42 with SB disease). No significant differences were detected between the medical therapy received and the response/remission rates overall, or between any of the MRI categories (Table 4), suggesting that despite non-standardized medical therapy, the differences in the medical therapies were not primarily responsible for differences observed in the response rates.

Six patients had previously undergone total colectomy, so clinical response after a further 8 wk of medical therapy was determined by physician assessment and either endoscopic or radiological assessment. Of the remaining 46 patients, the average HBI was 8.7 (range 6-20) prior to medical therapy. Logistic regression identified that females were more likely to respond to medical therapy than males (OR = 5.46, 95% CI = 1.295-23.00, P = 0.021). Patients with intestinal narrowing and prestenotic dilatation with or without hold up were less likely to respond to medical therapy (OR = 7.85, 95% CI 1.73-35.6, P = 0.008). This was primarily due to the lack

**Table 5 MRI category, response to medical therapy in relation to luminal narrowing with and without hold up**

<b>MRI group</b>	<b>No narrowed lumen</b>	<b>Narrowed lumen no hold up</b>	<b>Narrowed lumen with hold up</b>			
1 n = 7	Response No response	0% (0/2) 100% (2/2)	Response No response	25% (1/4) 75% (3/4)	Response No response	0% (0/1) 100% (1/1)
2 n = 9	Response No response	100% (5/5) 0% (0/5)	Response No response	100% (4/4) 0% (0/4)	Response No response	N/A N/A
3 n = 16	Response No response	66.7% (2/3) 33.3% (1/3)	Response No response	66.7% (2/3) 33.3% (1/3)	Response No response	40% (4/10) 60% (6/10)
4 n = 20	Response No response	83.3% (5/6) 16.7% (1/6)	Response No response	57.1% (4/7) 42.9% (3/7)	Response No response	14.3% (1/7) 85.7% (6/7)
Total n = 52	Response No response	75.0% (12/16) 25.0% (4/16)	Response No response	61.1% (11/18) 38.9% (7/18)	Response No response	27.8% (5/18) 72.2% (7/18)

of response in patients with luminal narrowing and hold up, who were less likely to respond to medical therapy than either patients without luminal narrowing, or those with narrowing and no hold up ( $P < 0.0001$  and  $P = 0.007$  respectively, Table 5). Luminal narrowing was observed in 44.4% (4/9), 81.3% (13/16) and 70% (14/20) of patients in category "2" "3" and "4" respectively, while narrowing also occurred in 71.4% (5/7) of category "1" patients, suggesting that luminal narrowing can occur without the presence of inflammation detected by MRI. No significant differences were detected between response rates and age, the presence of mesenteric hyperemia, fibrofatty proliferation, enlarged local lymph nodes, nodal enhancement, free fluid, length of involved segment, or the presence of fistulas or an abscess.

No statistical difference was detected in response to continued medical therapy related to bowel wall thickness (Figure 1A,  $\leq 6$  mm vs  $> 6$  mm). Patients with mild or marked SB intestinal wall enhancement, however, were more likely to respond than those with SB wall abnormalities without enhancement (OR 33.0, 95% CI 2.56-426.7 and OR 15.0, 95% CI 1.22-184, respectively). There was no difference in patient response related to enhancement localized to the mucosal or mural layers compared to enhancement in both layers.

Regarding a separate analysis, controlling for sex, patients in category "2" were more likely to respond to medical therapy when compared to any of the other categories (OR 39.69,  $P = 0.0014$  compared with category "1"; OR 11.56,  $P = 0.023$  compared with category "3"; OR 8.98,  $P = 0.040$  compared with category "4") and more likely to go into remission compared to patients in category "1" ( $P = 0.021$ ), with border-line significance for category "3" ( $P = 0.050$ ), but not when compared to category "4" ( $P = 0.24$ ). This lack of significance was most likely secondary to low patient numbers. There were no other statistically significant differences between the other 3 groups. Controlling for the category type, females were still more likely to respond than males (OR = 5.91,  $P = 0.0071$ ).

#### Failure of medical therapy

Twenty-five patients failed to respond to medical therapy and were offered colonoscopic examination to determine whether there was ongoing active inflammatory disease or if there was intestinal stricturing without in-

flammation that would be amenable to endoscopic dilatation. Six (85.7%) of 7 patients failed medical therapy in category "1"; 1 patient refused colonoscopy and 1 went to surgery due to uncontrolled intestinal bleeding. The remaining 4 patients demonstrated no active colonic or terminal ileal inflammation at colonoscopy, but 3 had an ileal/anastomotic stricture that was endoscopically dilated with resolution of their symptoms. All category "2" patients responded to continued medical therapy. Nine of 16 (56.3%) category "3" patients failed to respond to continued medical therapy. Two of these patients demonstrated no inflammation at colonoscopy and underwent dilatation of an ileal/anastomotic stricture with resolution of their symptoms. A further 4 patients demonstrated inflammation of the ileum alone without a stricture identified and 3 patients did not demonstrate inflammation or stricture in either the colon or terminal ileum. These patients had been noted to have proximal SB CD on MRI. Ten of 20 patients (50%) in category "4" failed to respond to medical therapy. Six patients underwent colonoscopy and all demonstrated severe ileal inflammation. Among those remaining, two patients elected to go straight to surgery without a colonoscopic examination, one was not physically fit for an endoscopic procedure and one died from other causes unrelated to his IBD.

Seventeen patients went to surgery (16 after failure of medical therapy and 1 who relapsed after responding to medical therapy; Table 6). Specialist histopathologists assessed the macroscopic appearance and histology of the surgical specimens. The 2 patients in category "1" demonstrated fibrosis macroscopically and microscopically with only minimal or no inflammation detected histopathologically. In conjunction with the endoscopic assessment, 5 of the 6 non-responders in category "1" did not demonstrate histological or endoscopic inflammation, consistent with the MRI findings of fibrosis without active inflammation. The single category "2" patient undergoing surgery demonstrated no fibrosis macroscopically or histologically. Macroscopic and histological fibrosis, however, was present in all 7 category "3" patients undergoing surgery. This MRI category included those patients considered to have mild inflammation but intestinal fibrosis on MRI, and the findings of the surgical pathology indicated that this classification highly correlated with intestinal fibrosis. Of the 8 patients with

**Table 6 Correlation of MRI findings with surgical pathology**

No.	Sex	Age	MRI assessment		Surgical pathology		
			Group	Fistula	Inflammation	Fibrosis	Fistula
1	F	23	1	No	Minimal	Yes	No
2	M	45	1	No	No	Yes	No
3	F	39	2	Yes	Mild	No	Yes
4	M	17	3	No	Mild	Yes	No
5	F	59	3	No	Mild	Yes	No
6	F	31	3	No	Mild	Yes	No
7	M	18	3	No	Moderate	Yes	No
8	M	24	3	Yes	Mild	Yes	Yes
9	F	37	3	No	Mild	Yes	No
10	M	41	3	No	Mild	Yes	No
11	F	62	4	No	Florid	Yes	No
12	M	19	4	No	Florid	Yes	No
13	M	61	4	No	Florid	Yes	No
14	F	21	4	Yes	Florid	No	Yes
15	M	43	4	Yes	Florid	Yes	Yes
16	M	14	4	No	Florid	No	No
17	F	56	4	No	Florid	No	No

mild segmental hyper-enhancement on MRI (categories “2” and “3”), 7 demonstrated mild and 1 moderate SB inflammation histologically. Of the 7 category “4” patients undergoing surgery, 3 demonstrated fibrosis macroscopically and histologically. All 7 demonstrated florid SB inflammation. In one patient, the pathology described severe inflammation and fistulas, in combination with submucosal fibrosis without macroscopic fibrosis. All fistulas ( $n = 4$ ) identified by surgical pathology had previously been identified on SB MRI.

In summary, the surgical pathology confirmed the MRI assessment as to the presence or absence of fibrosis in all the category “1”, “2” and “3” patients (10/10) while confirming the presence of moderate to severe intestinal inflammation with or without fibrosis in all 7 of the category “4” patients. This suggests that the MRI categories are accurate in the classification of both inflammation severity and the presence of fibrosis.

## DISCUSSION

CD is a transmural disease that may result in obstructive symptoms secondary to intestinal inflammation and/or fibrosis. Fibrosis is not effectively treated by pharmaceutical agents and the presence of fibrotic strictures is one reason behind the high rate of surgical intervention<sup>[23]</sup>. Radiological investigations have undergone a revolution with both CTE and MRE demonstrating impressive specificity and sensitivity in the assessment of SB CD. CTE, however, delivers ionising radiation with concerns that even a single abdominal CT may increase the lifetime risk of malignancy<sup>[24]</sup>. This risk is even greater in the younger population<sup>[25]</sup>. MR, however, provides good soft tissue contrast that may differentiate between intestinal inflammation and fibrosis, and appears to be superior to CT scanning<sup>[16,17]</sup>. For long-term safety issues, as well as potentially better diagnostic capabilities, MR examination of the SB would appear to be preferable to CT.

TI attenuation is associated with histologically active

CD on CTE<sup>[26]</sup>. There are limited studies that compare MRI findings with SB inflammation, but they suggest that contrast enhancement intensity correlates with intestinal inflammation<sup>[27]</sup>. The level of bowel wall thickening on CTE, however, was not an independent risk factor for disease activity once attenuation was considered, similar to our MRI findings. MRI data that combines both bowel wall thickening and signal intensity has previously demonstrated greater accuracy in assessing disease severity<sup>[26,27]</sup>. In view of these observations patients in our study were classified on the basis of intestinal wall enhancement and bowel wall thickening on MRI.

Our findings suggest that the SB MRI findings can be used to categorise CD patients, and that these categories correlate with patient response to ongoing medical therapy and the histological findings at surgery. No significant differences were detected between the medical therapy regime used and the response/remission rates overall, or between any of the MRI categories, suggesting that the medical therapy was not primarily responsible for differences observed in the response rates. Overall females were more likely to respond to medical therapy than males. This may potentially be impacted by 7 of the 9 patients in category “2” being female, but reasons behind this observation are unclear. Controlling for sex, however, patients in category “2” were still more likely to respond to continued medical therapy than any of the other categories.

Macroscopic/histological fibrosis was present on surgical pathology in all the category “1” and “3” patients undergoing surgery. All category “4” patients undergoing surgery demonstrated florid inflammation on the surgical pathology. In contrast, no category “2” or “3” patients demonstrated florid inflammation on surgical pathology with all but one having only mild inflammation. These findings may explain why category “2” patients were more likely to respond to medical therapy as they only had mild inflammation and were yet to develop fibrotic changes, unlike patients with marked wall thickening which is associated with fibrosis and/or florid inflammation.

A further finding from this work was that patients with intestinal narrowing and prestenotic dilatation were less likely to respond to medical therapy. It has been suggested that narrowing can be differentiated by CTE into inflammatory and fibrostenotic lesions<sup>[28]</sup>. In our study it was noted that there was a similar percentage of patients with luminal narrowing with severe inflammation (70%, 14/20) compared to patients with fibrosis and mild inflammation (81.3%, 13/16) or fibrosis and no inflammation (71.4%, 5/7). Patients with narrowing and inflammation, however, were more likely to have narrowing with hold up (47.2% 17/36) than patients without inflammation (14.3%, 1/7), suggesting that MRI may also be able to differentiate inflammatory from fibrostenotic lesions.

MRI findings of marked wall thickening without bowel wall enhancement were observed to be negatively associated with a response to medical therapy, while minimal bowel wall thickening with mild wall enhance-

ment predicted a good response to continued medical therapy. As patient MR categories based on SB wall thickness and luminal wall enhancement appear to correlate with the response to continuing medical therapy as well as with the surgical pathology, our findings would support the use of SB MRI as a diagnostic and prognostic tool in the management of patients with SB CD.

## COMMENTS

### Background

Small bowel (SB) involvement is common in Crohn's disease (CD). The study aimed to determine whether magnetic resonance imaging (MRI) could predict response to medical therapy and correlate with surgical pathology in these patients.

### Research frontiers

Utilising a combination of the maximal SB wall thickness and level of mucosal/mural enhancement detected on MRI, patients were able to be grouped into 1 of 4 categories; (1) "fibrosis", (2) "mild segmental hyper-enhancement and mild wall thickening", (3) "mild segmental hyper-enhancement and marked wall thickening", and (4) "marked segmental transmural hyper-enhancement". This categorization was then used to determine a prognostic role for SB MRI in the management of CD.

### Innovations and breakthroughs

Category "2" patients were more likely to respond to medical therapy than any other category, as were females, while patients with intestinal narrowing and prestenotic dilatation with or without hold up were less likely to respond. The presence of marked wall thickening without bowel wall enhancement was negatively associated with a medical response. Surgical pathology confirmed the MRI assessment of inflammation severity and presence of fibrosis.

### Applications

The proposed MR categories appeared to correlate with response to continuing medical therapy and the surgical pathology, thereby supporting the use of SB MRI as a diagnostic and prognostic tool in the management of patients with SB CD.

### Terminology

CD is a chronic inflammatory condition of the intestine that frequently involves the terminal ileum with narrowing and fibrosis. Harvey Bradshaw index (HBI) is a means of measuring a clinical response in CD with a reduction in the HBI of  $\geq 3$  being a clinical response and remission defined as a HBI < 5 terminal ileum. MRI is a cross sectional imaging technique that does not utilize radiation and provides excellent tissue differentiation.

### Peer review

This manuscript can be accepted. This paper has new useful information for the readers of *World J Gastroenterol*.

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ORIGINAL ARTICLES

## Is it possible to differentiate gastric GISTs from gastric leiomyomas by EUS?

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### Abstract

**AIM:** To evaluate the ultrasonography (EUS) features of gastric gastrointestinal stromal tumors (GISTs) as compared with gastric leiomyomas and then to determine the EUS features that could predict malignant GISTs.

**METHODS:** We evaluated the endoscopic EUS features in 53 patients with gastric mesenchymal tumors confirmed by histopathologic diagnosis. The GISTs were classified into benign and malignant groups according to the histological risk classification.

**RESULTS:** Immunohistochemical analyses demonstrated 7 leiomyomas and 46 GISTs. Inhomogeneity, hyperechogenic spots, a marginal halo and higher echogenicity as compared with the surrounding muscle layer appeared more frequently in the GISTs than in

the leiomyomas ( $P < 0.05$ ). The presence of at least two of these four features had a sensitivity of 89.1% and a specificity of 85.7% for predicting GISTs. Except for tumor size and irregularity of the border, most of the EUS features were not helpful for predicting the malignant potential of GISTs. On multivariate analysis, only the maximal diameter of the GISTs was an independent predictor. The optimal size for predicting malignant GISTs was 35 mm. The sensitivity and specificity using this value were 92.3% and 78.8%, respectively.

**CONCLUSION:** EUS may help to differentiate gastric GISTs from gastric leiomyomas. Once GISTs are suspected, surgery should be considered if the size is greater than 3.5 cm.

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**Key words:** Endoscopic ultrasonography; Gastrointestinal stromal tumor; Stomach

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### INTRODUCTION

Mesenchymal tumors of the gastrointestinal tract are usually incidentally discovered as a firm, protruding submucosal lesion during upper gastrointestinal examinations for unrelated conditions, although the larger tumors may occasionally cause bleeding<sup>[1]</sup>. Pathologically, most of these tumors are composed of spindle cells and display smooth muscle differentiation. In recent years, with the advance of immunohistochemistry, it is known that most gastric and small bowel mesenchymal tumors are gastrointestinal stromal tumors (GISTs) that

are derived from the interstitial cells of Cajal<sup>[1-3]</sup>. The incidence of GISTs is 10-20 per million with the stomach being the most common location as 60% to 70% of GISTs arise in this organ<sup>[4]</sup>.

Endoscopic ultrasonography (EUS) is a valuable imaging tool for the diagnosis and evaluation of gastric GISTs. Gastric GISTs generally appear as round, hypoechoic lesions with a ground-glass echo texture, and these lesions are contiguous with the fourth layer of the stomach<sup>[5-7]</sup>. Several studies have attempted to differentiate benign and malignant stromal tumors on the basis of their EUS features<sup>[6-11]</sup>. However, most of these studies were performed before the concept of GISTs was introduced and they included non-gastric tumors in their study samples. In addition, little is known about differentiating GISTs and leiomyomas by EUS<sup>[11]</sup>.

Therefore, the aim of this study was to evaluate the EUS features of gastric GISTs in comparison with gastric leiomyomas, and we wanted to determine if the EUS features could predict the malignant potential of gastric GISTs according to the histological risk classification.

## MATERIALS AND METHODS

### Patients

From July 2005 to June 2008, the medical records of all patients with histopathologically proven gastric leiomyomas or GISTs who underwent EUS examination at our endoscopic unit were retrospectively reviewed. There were 53 patients (22 men and 31 women), with a mean age of 59 years (range 29-75 years). The histologic specimens were obtained by surgical resection in 50 patients (94.3%), by needle biopsy in two patients (3.8%), and by endoscopic resection in 1 patient (1.9%). This study was reviewed and approved by the Institutional Review Board at Pusan National University Hospital.

### Histopathology

The tumors were histopathologically proved to be gastric mesenchymal tumors and they were classified immunohistochemically as leiomyomas or GISTs<sup>[5]</sup>. In particular, the leiomyomas were defined as being desmin positive and c-kit (CD117) negative tumors and the GISTs were defined as being c-kit positive tumors. The GISTs were divided into 4 groups in accordance with the consensus meeting report at the National Institute of Health (Table 1)<sup>[12]</sup>. Then, the GISTs with a very low risk or low risk were defined as benign GISTs, and the GISTs with an intermediate risk or high risk were defined as malignant GISTs.

### EUS

EUS was performed with a radial scanning ultrasound endoscope (GF-UM2000; Olympus, Tokyo, Japan) using scanning frequencies of 7.5 and 12 MHz. All the examinations were performed under intravenous conscious sedation (midazolam with or without meperidine). Scanning of the tumor was performed after filling the stomach with 400-600 mL of deaerated water. About

**Table 1 Proposed approach for defining the risk of aggressive behavior in GISTs**

	<b>Size (cm)</b>	<b>Mitotic count</b>
Very low risk	< 2	< 5/50 HPF
Low risk	2-5	< 5/50 HPF
Intermediate risk	< 5 5-10	6-10/50 HPF < 5/50 HPF
High risk	> 5 > 10 Any size	> 5/50 HPF Any mitotic rate > 10/50 HPF

HPF: High-power field.

10-20 endosonograms were recorded for each patient, and these images were stored on magneto-optical disks. A review of the EUS photos was performed by a single experienced endosonographer (Kim GH) who was kept "blinded" to the final diagnosis, and this endosonography had previously performed more than 1000 examinations. The following EUS features were recorded for all the tumors: (a) the maximal diameter, (b) the presence of mucosal ulceration on endoscopy and/or EUS, (c) the echogenicity in comparison with the surrounding normal proper muscle layer (hyperechoic or isoechoic), (d) the homogeneity (homogenous or heterogenous), (e) the presence of a marginal halo and lobulation, (f) the presence of cystic spaces, hyperechogenic spots and calcification, (g) the regularity of the marginal border (regular or irregular) and (h) the pattern of tumor growth (inside or outside the gastric wall).

### Statistical analysis

The differences in gender and the EUS findings between leiomyomas and GISTs were assessed using the  $\chi^2$  test or Fisher's exact test, and the patient age and tumor size were assessed using the Student *t*-test. Calculation of the sensitivity, specificity and the positive and negative predictive values of each EUS feature and combinations of these features for differentiating GISTs from leiomyomas were carried out manually.

Univariate analyses using the  $\chi^2$  test or Fisher's exact test were performed to identify the EUS features that could predict malignant GISTs. Multivariate logistic regression analyses were performed to identify the independent predictors of malignant GISTs. The odds ratios and their 95% confidence intervals were used to predict malignant GISTs. Receiver operating characteristic (ROC) curve was applied to find the best sensitivity and specificity cut-off value of the tumor size for predicting malignant GISTs. A *P* value < 0.05 was considered statistically significant. The statistical calculations were performed using the SPSS version 12.0 for Windows software (SPSS Inc, Chicago, IL, USA).

## RESULTS

### EUS features differentiating GISTs from leiomyomas

Immunohistochemical analyses demonstrated that 7 cases were leiomyomas and 46 cases were GISTs. The baseline characteristics and endosonographic features are shown in

**Table 2 Baseline characteristics and EUS features of the patients with leiomyomas and GISTs of the stomach n (%)**

Variables	Leiomyomas (n = 7)	GISTs (n = 46)	P-value
Gender			0.686
Male	2 (28.6)	20 (43.5)	
Female	5 (71.4)	26 (56.5)	
Age (yr, mean ± SD)	52.6 ± 13.5	57.5 ± 8.4	0.193
Location			0.272
Upper	6 (85.7)	29 (63.0)	
Middle	0 (0)	13 (28.3)	
Lower	1 (14.3)	4 (8.7)	
Originating layer			0.644
Second layer	0	1 (2.2)	
Third layer	2 (28.6)	7 (15.2)	
Fourth layer	5 (71.4)	38 (82.6)	
Size (cm, mean ± SD)	3.6 ± 2.6	3.5 ± 2.3	0.967
Ulcer			0.172
Absent	7 (100)	31 (67.4)	
Present	0 (0)	15 (32.6)	
Growth			0.660
In	6 (85.7)	33 (71.7)	
Out	1 (14.3)	13 (28.3)	
Border			0.082
Regular	7 (100)	29 (63.0)	
Irregular	0 (0)	17 (37.0)	
Lobulation			0.426
Absent	5 (71.4)	23 (50.0)	
Present	2 (28.6)	23 (50.0)	
Marginal halo			0.002
Absent	6 (85.7)	10 (21.7)	
Present	1 (14.3)	36 (78.3)	
Echogenicity in comparison with the surrounding muscle echo			0.004
Isoechoic	7 (100)	19 (41.3)	
Hyperechoic	0 (0)	27 (58.7)	
Homogeneity			0.001
Homogenous	6 (85.7)	9 (19.6)	
Inhomogeneous	1 (14.3)	37 (80.4)	
Cystic change			0.661
Absent	6 (85.7)	31 (67.4)	
Present	1 (14.3)	15 (32.6)	
Hyperechogenic spots			0.012
Absent	4 (57.1)	5 (10.9)	
Present	3 (42.9)	41 (89.1)	
Calcification			1.000
Absent	6 (85.7)	39 (84.8)	
Present	1 (14.3)	7 (15.2)	

Table 2. The tumor size and presence of ulceration were not different between the leiomyomas and the GISTs. A marginal halo appeared more frequently in the GISTs than in the leiomyomas ( $P = 0.002$ ). The echogenicity of all the leiomyomas was nearly similar to that of the surrounding normal proper muscle layer, but more than half of the GISTs showed higher echogenicity than that of the surrounding normal muscle layer (Figure 1) ( $P = 0.004$ ). Inhomogeneity of the tumor and hyperechogenic spots were observed more frequently in the GISTs than in the leiomyomas ( $P < 0.05$ ).

Table 3 shows the value of each EUS feature for differentiating GISTs from leiomyomas. Each criterion had a high positive predictive value, but limited sensitivity or specificity. The presence of at least two of these four features in a given tumor had a sensitivity of 89.1%, a specificity of 85.7%, a positive predictive value

**Table 3 Sensitivity, specificity and positive and negative predictive values of the EUS features that differentiate GISTs from leiomyomas in the stomach (%)**

EUS features	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Echogenicity in comparison with the surrounding muscle echo	58.7	100	100	26.9
Homogeneity	80.4	85.7	97.4	40.0
Echogenic foci	89.1	57.1	93.2	44.4
Marginal halo	78.3	85.7	97.3	37.5
Of the above 4 features				
≥ 1	97.8	57.1	93.8	80.0
≥ 2	89.1	85.7	97.6	54.5
≥ 3	84.8	85.7	97.5	46.2
All	34.8	100	100	18.9

Values are expressed as percentages.

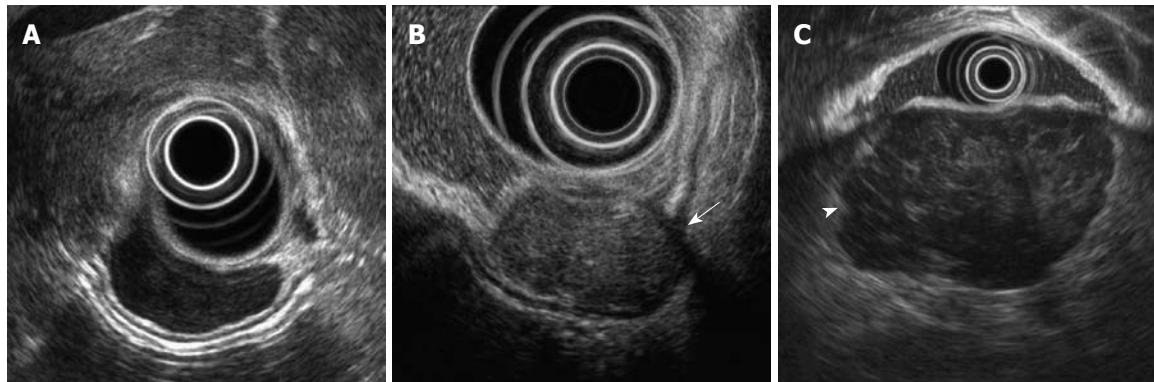
of 97.6% and a negative predictive value of 54.5% for predicting GISTs.

### EUS features predicting malignant potential of GISTs

When the GISTs were classified into benign and malignant groups according to the histological risk classification, 33 cases were grouped as benign GISTs (very low risk, 11 cases; low risk, 22 cases) and 13 cases as malignant GISTs (intermediate risk, 8 cases; high risk, 5 cases). Except for the size and irregularity of the tumor border, most of the endosonographic features were not helpful in predicting the malignant potential of GISTs (Figure 1, Table 4). On the multivariate logistic regression analysis, only the maximal diameter of the GISTs was an independent predictor (OR, 9.3; 95% CI, 1.6-53.6) (Table 5). A ROC curve was created to identify the discriminative value of size for predicting the malignant potential of GISTs (Figure 2). The sensitivity was almost optimized when the critical value of the size was 35 mm. Of the 19 patients with a tumor size  $\geq 35$  mm, 12/19 (63.2%) were malignant GISTs. However, of the 27 patients with a size  $< 35$  mm, 26 (96.3%) were benign GISTs and 1 (3.7%) was a malignant GIST. Therefore, this model of prediction for malignant GISTs had a positive predictive value of 63.2% and a negative predictive value of 96.3%. This resulted in a sensitivity of 92.3% and a specificity of 78.8%.

## DISCUSSION

GISTs are rare neoplasms that account for less than 1% of all gastrointestinal malignancies. GISTs have the capability to become malignant and then metastasize, whereas leiomyomas are almost invariably benign<sup>[4]</sup>. In clinical practice, preoperative differentiation between GISTs and leiomyomas is usually difficult, even if EUS-guided fine-needle aspiration or trucut biopsy is performed<sup>[13-15]</sup>. Thus, if it were possible to differentiate GISTs from leiomyomas and then to predict the malignant potential of GISTs by EUS imaging, then this would be essential in the clinical management of gastrointestinal mesenchymal tumors. There have been



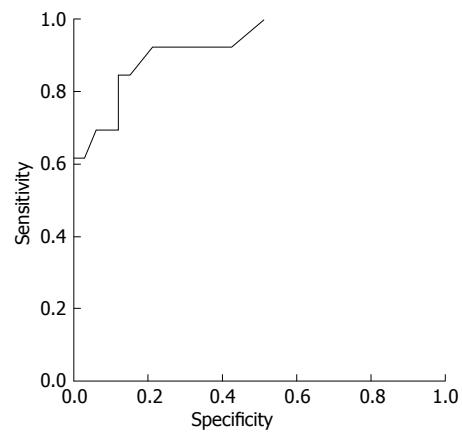
**Figure 1 EUS features of gastric mesenchymal tumors.** A: A gastric leiomyoma. The mass is homogenous and its echogenicity is similar to that of the surrounding normal muscle layer. It is 35 mm × 13 mm in size and a marginal halo is not observed; B: A gastric GIST with low risk potential. The mass is inhomogenous and its echogenicity is somewhat higher than that of the surrounding muscle layer. It is 25 mm × 18 mm in size. A marginal halo (arrow) and hyperechogenic spots are seen; C: A gastric GIST with high risk potential. The mass is inhomogenous and 82 mm × 76 mm in size. A marginal halo, hyperechogenic spots and irregular border (arrow head) are seen.

**Table 4 Univariate analysis of EUS features between benign and malignant GISTs of the stomach n (%)**

Variables	Benign GIST (n = 33)	Malignant GIST (n = 13)	P-value
Size (cm, mean ± SD)	2.5 ± 1.0	6.0 ± 2.7	0.001
Ulcer			0.299
Absent	24 (72.7)	7 (53.8)	
Present	9 (27.3)	6 (46.2)	
Growth			0.145
In	26 (78.8)	7 (53.8)	
Out	7 (21.2)	6 (46.2)	
Border			0.044
Regular	24 (72.7)	5 (38.5)	
Irregular	9 (27.3)	8 (61.5)	
Lobulation			0.743
Absent	17 (51.5)	6 (46.2)	
Present	16 (48.5)	7 (53.8)	
Marginal halo			0.240
Absent	9 (27.3)	1 (7.7)	
Present	24 (72.7)	12 (92.3)	
Echogenicity in comparison with the surrounding muscle echo			0.115
Isoechoic	16 (48.5)	3 (23.1)	
Hyperechoic	17 (51.5)	10 (76.9)	
Homogeneity			0.199
Homogenous	8 (24.2)	1 (7.7)	
Inhomogenous	25 (75.8)	12 (92.3)	
Cystic changes			0.082
Absent	25 (75.8)	6 (46.2)	
Present	8 (24.2)	7 (53.8)	
Hyperechogenic spots			1.000
Absent	4 (12.1)	1 (7.7)	
Present	29 (87.9)	12 (92.3)	
Calcification			0.385
Absent	29 (87.9)	10 (76.9)	
Present	4 (12.1)	3 (23.1)	

several studies that have attempted to differentiate benign from malignant stromal tumors based on their EUS features<sup>[2,5-11]</sup>. However, most of these studies did not differentiate the EUS features of GISTs and leiomyomas, and they did not characterize the EUS features of GISTs according to the histological risk classification. In addition, they did not restrict the study subjects to those with the gastric mesenchymal tumors.

Initially, we tried to find the EUS features that could



**Figure 2 Receiver operating characteristic (ROC) curve of the tumor size for predicting malignant GISTs in the stomach.**

**Table 5 Multivariate analysis of EUS features between benign and malignant GISTs of the stomach**

Variables	Odds ratio (95% CI)	P value
Size	9.3 (1.6-53.6)	0.013
Growth	8.7 (0.6-119.8)	0.105
Border	2.3 (0.2-22.7)	0.490
Homogenicity	2.2 (0.1-48.0)	0.606
Cystic change	1.4 (0.1-19.5)	0.800

differentiate GISTs from leiomyomas. In a previous study<sup>[9]</sup>, the EUS features such as size greater than 4 cm, ulceration or cystic foci were almost exclusively seen in CD-117 positive tumors as compared with CD-117 negative tumors. In that study, immunohistochemical staining such as for desmin or S-100 was not performed, so the CD-117 negative tumors were not homogenous; i.e. they may have been leiomyomas or schwannomas. In the present study, we restricted the subjects to compare GISTs with only leiomyomas, and the latter are almost invariably benign. As a result, tumor size and the presence of ulceration and cystic changes in the tumor were not helpful for differentiating GISTs from leiomyomas in this study. Instead, inhomogeneity, hyperechogenic spots, a

marginal halo and higher echogenicity in comparison with the surrounding muscle layer were helpful for predicting GISTs. Especially, if at least two of these four features are present, then the sensitivity and specificity for predicting GISTs were 89.1% and 85.7%, respectively. This result is similar to the results of a previous report<sup>[11]</sup> that a marginal halo and the relatively higher echogenicity, as compared to the adjacent normal muscular layer on EUS, might suggest GISTs. In addition, the previous reports, which were carried out before the concept of GISTs was introduced, have suggested that inhomogeneity and hyperechogenic spots are the EUS features that are predictive of malignancy<sup>[6,7]</sup>.

A hypoechoic halo is a well known characteristic ultrasonographic sign of malignant liver tumors such as hepatocellular carcinoma<sup>[16]</sup>. A pseudocapsule of the collapsed surrounding tissues, which is the result of an expansively growing tumor, is thought to be the cause of the halo in malignant liver tumors<sup>[16]</sup>. A previous study showed that on pathologic examination, the tumor cells of GISTs were partially or completely circumscribed by the residual muscle tissues of the surrounding muscular propria and this formed a capsule-like structure<sup>[11]</sup>. In the present study, a marginal halo was found in 78.3% of GISTs, whereas this was seen in only 14.3% of leiomyomas.

In this study, higher echogenicity in comparison with the surrounding muscle layer was found in more than half of GISTs, whereas the echogenicity of the leiomyomas was nearly equal to that of the surrounding muscle layer. Pathologically, it is well known that the cellularity of leiomyomas is normal to moderate with eosinophilic cytoplasm, whereas GISTs show higher overall cellularity, which creates a basophilic appearance on hematoxylin and eosin staining<sup>[1-3]</sup>. It is assumed that the difference in echogenicity between GISTs and leiomyomas might reflect these pathologic differences of cellularity and the structural components of the tumors.

Second, we tried to find the EUS features that could predict the malignant potential of GISTs after dividing the GISTs into 2 groups (benign and malignant) according to the histological risk classification. Previous studies have suggested that a large size, exogastric growth, ulceration, cystic changes, hyperechogenic foci and irregularity of the margin favored a diagnosis of malignant gastrointestinal mesenchymal tumors<sup>[6,7,17,18]</sup>, but these studies were performed before the concept of GISTs had been introduced, as was mentioned earlier. In the present study, only tumor size and irregularity of the border were helpful in predicting the malignant potential of GISTs. Multivariate logistic regression analysis showed that only size was an independent predictor, which is consistent with a previous report that conducted multivariate analysis according to the histological risk classification of GISTs<sup>[10]</sup>. With the critical size of 35 mm, the sensitivity and specificity were 92.3% and 78.8%, respectively. This could be explained by the fact that tumor size, together with the mitotic count, is used to determine the histological classification system. Therefore, once we discriminate GISTs from leiomyomas, the size of the tumor ( $> 35$  mm) might be

the most reliable indicator of malignancy.

This study had several limitations. First, this was a retrospective study that compared the EUS features of gastric GISTs and leiomyomas. In addition, there might have been a potential bias when retrospectively reviewing the endosonographic photos. During the EUS examination, we took at least 10-20 endosonographic photos to determine EUS characteristics of gastric mesenchymal tumors. Therefore, this would compensate, to some degree, the limitation of this retrospective study. Second, although EUS examinations were performed, patients were selected for surgery or biopsy according to the clinical opinions and decisions of the medical doctors. Third, the number of leiomyomas included in this study was small relative to the number of GISTs. This limitation might be due to the fact that the most common mesenchymal tumors of the stomach are GISTs and other tumors, such as leiomyomas and schwannomas, are rarely encountered in clinics.

Gastric mesenchymal tumors are often asymptomatic, and they are usually incidentally discovered during upper gastrointestinal endoscopy for unrelated conditions. The main problem in the asymptomatic patient is to determine whether or not the tumors have a malignant potential. Because GISTs have a malignant potential, the gastric mesenchymal tumors, even if they are small, should not be ignored if EUS features are suggestive of GISTs. Further large prospective long-term studies are needed to validate our results for gastric mesenchymal tumors.

In conclusion, EUS is a useful method to diagnose gastric mesenchymal tumors and to predict the malignant potential of GISTs. The EUS features such as inhomogeneity, hyperechogenic spots, a marginal halo and higher echogenicity as compared with the surrounding muscle layer may help to differentiate GISTs from leiomyomas. Once GISTs are suspected by EUS, surgical treatment should be considered if the size of the tumor is greater than 3.5 cm.

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## COMMENTS

### Background

Endoscopic ultrasonography (EUS) is a valuable imaging tool for the diagnosis and evaluation of gastric gastrointestinal stromal tumors (GISTs). Several studies have attempted to differentiate benign and malignant stromal tumors on the basis of their EUS features. However, most of these studies were performed before the concept of GISTs was introduced and they included non-gastric tumors in their study samples.

### Research frontiers

Most previous studies did not differentiate the EUS features of GISTs and leiomyomas, and they did not characterize the EUS features of GISTs according to the histological risk classification. In addition, they did not restrict the study subjects to those with gastric mesenchymal tumors. Therefore, we evaluated the EUS features of gastric GISTs in comparison with gastric leiomyomas, and tried to determine the EUS features that could predict the malignant potential of gastric GISTs according to the histological risk classification.

### Innovations and breakthroughs

To differentiate GISTs from leiomyomas by EUS, the following four features were helpful; inhomogeneity, hyperechogenic spots, a marginal halo and higher echogenicity as compared with the surrounding muscle layer. These features appeared more frequently in GISTs than in leiomyomas. The presence of at least two of these four features had a sensitivity of 89.1% and a specificity of 85.7% for predicting GISTs. Except for tumor size and irregularity of the border, most of the EUS features were not helpful in predicting the malignant potential of GISTs. On multivariate analysis, only the maximal diameter of the GISTs was an independent predictor. The optimal size for predicting malignant GISTs was 35 mm. The sensitivity and specificity using this value were 92.3% and 78.8%, respectively.

### Applications

EUS is a useful method to diagnose gastric mesenchymal tumors and to predict the malignant potential of GISTs. The EUS features such as inhomogeneity, hyperechogenic spots, a marginal halo and higher echogenicity as compared with the surrounding muscle layer may help to differentiate GISTs from leiomyomas. Once GISTs are suspected by EUS, surgical treatment should be considered if the size of the tumor is greater than 3.5 cm.

### Terminology

GISTs are mesenchymal tumors derived from the interstitial cells of Cajal. The incidence of GISTs is 10-20 per million and the stomach is the most common location of GISTs (60%-70%).

### Peer review

This is a relatively large single-center study aiming to evaluate the EUS features of gastric GISTs vs leiomyomas initially and subsequently focusing on GISTs to determine EUS-features associated with malignancy.

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ORIGINAL ARTICLES

## Peripheral T-lymphocyte subpopulations in different clinical stages of chronic HBV infection correlate with HBV load

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HBV DNA load was assessed by quantitative real-time polymerase chain reaction.

**RESULTS:** CD8<sup>+</sup> T-cells were significantly higher in patients at the immune-tolerant stage than in patients at the immune-active and -inactive carrier stages ( $36.87 \pm 7.58$  vs  $34.37 \pm 9.07$ ,  $36.87 \pm 7.58$  vs  $28.09 \pm 5.64$ ,  $P < 0.001$ ). The peripheral blood in patients at the immune-tolerant and immune active stages contained more CD8<sup>+</sup> T-cells than CD4<sup>+</sup> T-cells ( $36.87 \pm 7.58$  vs  $30.23 \pm 6.35$ ,  $34.37 \pm 9.07$  vs  $30.92 \pm 7.40$ ,  $P < 0.01$ ), whereas the peripheral blood in patients at the immune-inactive carrier stage and in normal controls contained less CD8<sup>+</sup> T-cells than CD4<sup>+</sup> T-cells ( $28.09 \pm 5.64$  vs  $36.85 \pm 6.06$ ,  $24.02 \pm 4.35$  vs  $38.94 \pm 3.39$ ,  $P < 0.01$ ). ANOVA linear trend test showed that CD8<sup>+</sup> T-cells were significantly increased in patients with a high viral load ( $39.41 \pm 7.36$ ,  $33.83 \pm 7.50$ ,  $31.81 \pm 5.95$  and  $26.89 \pm 5.71$ ,  $P < 0.001$ ), while CD4<sup>+</sup> T-cells were significantly increased in patients with a low HBV DNA load ( $37.45 \pm 6.14$ ,  $33.33 \pm 5.61$ ,  $31.58 \pm 6.99$  and  $27.56 \pm 5.49$ ,  $P < 0.001$ ). Multiple regression analysis displayed that log copies of HBV DNA still maintained its highly significant coefficients for T-cell subpopulations, and was the strongest predictors for variations in CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> cells and CD4<sup>+</sup>/CD8<sup>+</sup> ratio after adjustment for age at HBV-infection, maternal HBV-infection status, presence of hepatitis B e antigen and HBV mutation.

**CONCLUSION:** Differences in peripheral T-cell subpopulation profiles can be found in different clinical stages of chronic HBV infection. T-cell impairment is significantly associated with HBV load.

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**Key words:** Hepatitis B virus; Chronic hepatitis B virus infection; Clinical stages; Hepatitis B virus DNA; T lymphocyte subpopulation

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## INTRODUCTION

Hepatitis B virus (HBV) infection is a dynamic process with variable biochemical, virological and histological profiles at different stages of chronic HBV infection depending on host and viral factors. Furthermore, the profiles may change at a variable pace over time<sup>[1-5]</sup>. Based on the virological and biochemical parameters, chronic HBV infection can be divided into three stages: immune-tolerant stage, immune active stage and immune-inactive carrier stage<sup>[5-7]</sup>. Patients at the immune-tolerant stage have no symptoms, but their serum hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) are detectable, and HBV DNA levels are high, while their serum aminotransferase level is normal or minimally elevated, and their histological activity is minimal. This stage often lasts decades in perinatal HBV infection patients. The serum HBV DNA level decreases, and the serum aminotransferase level increases, symptoms may appear and flares of aminotransferase may be observed in patients at the immune active stage. The time of this stage ranges months-years in patients with chronic HBV infection. Flares of aminotransferase are followed by HBeAg seroconversion in some patients. The immune-inactive carrier stage is characterized by a low HBV DNA level and a normal aminotransferase concentration. HBV clearance is closely associated with the appearance of a vigorous virus-specific T-cell response<sup>[3,4]</sup>. In contrast, HBV persistence and chronic hepatitis are associated with a markedly diminished HBV-specific T-cell response<sup>[4,8-11]</sup>. Interestingly, different profiles of chronic hepatitis B are also associated with different magnitudes of HBV-specific immune response<sup>[12]</sup>. Episodes of acute flares during chronic HBV infection are associated with the recovery of HBV-specific CD4<sup>+</sup> T cell response<sup>[13,14]</sup>. Increased HBV-specific CD8<sup>+</sup> T cell response has only been demonstrated in patients with a low HBV replication, irrespective of the inflammation degree<sup>[15-17]</sup>. These data have led to the hypothesis that the composition of T cell subpopulations is different in different clinical stages of chronic HBV infection, which may be related to the HBV load. Thus, characterization of T cell profiles is relevant to the improved understanding of chronic HBV infection and the design of antiviral therapy. This study was to characterize the different stages of chronic HBV infection by analyzing the composition of T cell subpopulations in peripheral blood and its correlation with HBV replication.

## MATERIALS AND METHODS

### Subjects

Four hundred and twenty-two consecutive chronic HBV infection patients were recruited from Department of Infectious Diseases of First Affiliated Hospital of

Kunming Medical University, Third Kunming People's Hospital, and Yunnan General Hospital of Chinese People's Armed Police Forces, between January 2006 and May 2007. The patients were diagnosed according to the criteria for viral hepatitis<sup>[5-7]</sup>.

All patients fulfilled the following criteria: positive HBsAg for at least 12 mo, no other concomitant causes of liver disease (hepatitis C and D, HIV infection and alcohol consumption of more than 60 g/d), relatively rare liver diseases (autoimmune hepatitis and metabolic liver disease), on immunosuppressive therapy or antiviral therapy for HBV-infection within the recent 12 mo before entry. None of the patients was a drug user, or exposed to hepatotoxin. All patients gave their informed consent to participate in the study. The study protocol, conforming to the guidelines of Declaration of Helsinki, was approved by the Ethics Committees of the Faculty of Medicine of Prince of Songkla University and the First Affiliated Hospital of Kunming Medical University.

One hundred healthy individuals (61 males, 39 females), with a mean age of 33.24 ± 10.28 years, served as controls.

### **Serological test of liver function and evaluation of HBVs markers**

Serum alanine aminotransferase (ALT), aspartate transaminase (AST) and total bilirubin levels were measured with routine automated techniques (upper limit: 40 U/L, 40 U/L and 17.1 μmol/mL, respectively). HBV markers (HBsAg, HBsAb, HBeAg, HBeAb, HBcAb, and anti-HBcAb IgM) were detected by enzyme-linked immunosorbent assay.

### **Detection of HBV DNA and HBV pre-core mutant**

Serum HBV DNA load in individuals was detected by real-time polymerase chain reaction (RT-PCR) using a Lightcycler PCR system (FQD-33A, Bioer) with a lower detection limit of approximately 1000 viral genome copies/mL with a reagent kit package insert (Shenzhen PG Biotech Co. Ltd.) following its manufacturer's instructions. The primer was provided with the kit, the reaction volume was 40 μL. PCR was performed at 37°C for 5 min and at 94°C for 1 min, followed by 40 cycles at 95°C for 5 s and at 60°C for 30 s. HBV pre-core A1896 mutant was detected by PCR (PE9600, Perkin Elmer Co., Ltd. USA) with the reagent kit package insert (Shanghai Haoyuan Biotech Co. Ltd) following its manufacturer's instructions.

### **Detection of peripheral blood T lymphocyte subsets**

The key compositions of cellular immune are T-lymphocytes and their subpopulations. CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> cells are the major function subgroups of T cells and play an important role in response to HBV infection, which can reflect the cellular immune function and immunoregulation and are usually regarded as a valuable index of the changes in immunity of patients<sup>[3,4]</sup>. This index was used in our study to evaluate the cellular immune function of chronic HBV infection patients.

Blood samples were collected into heparinized vacuum tubes. Whole blood samples were analyzed using a Muti-Q-Prep processor (Coulter, USA) and an Epics-XL flow cytometer (Coulter, USA). Lymphocytes were analyzed using a gate set on a forward scatter *vs* a side scatter, and a three-color flow cytometry was used to combine CD3, CD4 and CD8. Anti-human monoclonal antibodies, CD3-PE-CY5/CD4-FITC/CD8-PE, were purchased from Immunotech, Ltd, USA. The detected peripheral blood T lymphocyte subsets in each sample were analyzed using the CellQuest software (Coulter, USA). The results were expressed as percentages of CD3<sup>+</sup>, CD3<sup>+</sup>/CD4<sup>+</sup> (short for CD4<sup>+</sup> below) and CD3<sup>+</sup>/CD8<sup>+</sup> (short for CD8<sup>+</sup> below) cells which were positive for the marker antigen in the total T cell population. The procedures were performed with the reagent kit package insert following its manufacturer's instructions.

#### **Maternal HBV infection status**

All medical records of mothers of the subjects were reviewed for previous HBV infection and those who were infected with HBV were identified. In addition, all of them were invited to undergo HBV-marker tests. For those with a positive result, a second set of tests were conducted three months after the first test to confirm their chronic HBV carrier status. If the mother was dead, the cause of death was investigated based on the medical records of HBV-related liver diseases, such as chronic hepatitis B, liver cirrhosis or hepatocellular carcinoma. If yes, the maternal HBV infection status was classified as positive.

#### **Age at HBV infection**

In the recent three decades, all children in China have been obligated to have a test for HBV markers when they first go to kindergarten and elementary school. Subsequent obligatory tests are made when they apply for entrance examination of university or for a job. Based on the results of these tests obtained from medical records and interview, we classified the age at the first positive test as < 8 years, between 8-20 years and > 20 years, respectively.

#### **Statistical analysis**

Initial calculation came up with a sample size of 50 patients with positive HBV DNA and 50 patients with negative HBV DNA, which could provide the study with a statistical power of 80% at the 0.025 level of significance to detect the difference in T-cell variation values of 33 and 38. However, to cover the potentially confounded problem due to other variables and to have enough subjects for stratifying the HBV DNA load to examine the dose-response relationship, we recruited 422 chronic HBV infection patients and 100 healthy controls.

Descriptive statistical analysis was used to examine the age, gender, serum HBV load, HBeAg status, age at HBV-infection and maternal HBV infection status.

The number of T-lymphocyte subpopulations in normal individuals (HBsAg-negative), which was expressed as mean  $\pm$  SD, served as a control reference. Effects of different independent demographic, clinical and serological variables on T-cell profiles were analyzed only in HBsAg-positive patients. Univariate analysis of these profiles broken down by individual independent variables was carried out. Independent *t* test was done for 2-level independent variables and one-way ANOVA for more than 2-level variables. The relationship between HBV replication level and peripheral T-lymphocyte subpopulation was analyzed by correlation analysis and ANOVA linear trend test. Finally, multiple linear regression models were employed in multivariate analysis for the assessment of independent effects of variables on peripheral blood T lymphocytes. Variables yielding a *P* value  $\leq 0.2$  in the univariate analysis were included in multivariate analysis, and the models were refined by backward elimination guided by the change in log likelihood of successive models. *P* < 0.05 was considered statistically significant. Computation was carried out with the aid of R Software version 2.5.1<sup>[18]</sup>.

## **RESULTS**

#### **Characteristics of patients at different stages of chronic HBV infection**

The demographic, virological, serological, and clinical characteristics of patients are summarized in Table 1. The mean age of 422 chronic HBV infection patients (males accounted for 63.5%) was 30.59  $\pm$  10.40 years. Over half of the patients' mothers were HBV positive. Two fifths of the patients were infected with HBV before the age of 8 years. Two thirds of the patients had detectable HBV DNA load and the majority of them (62.8%) had over  $10^7$  copies of HBV DNA per milliliter, more than half of them were HBeAg positive (51.7%) and nearly one third of them were infected with HBV pre-core region mutant.

Based on the predefined virological and biochemical parameters, all patients were divided into different stages of chronic HBV infection. A total of 112 patients were assigned to the immune-tolerant stage with a mean ALT level of 25.37  $\pm$  12.56 IU/L, a mean viral load of 7.88  $\pm$  1.54 log<sub>10</sub> copies of HBV DNA/mL, while 106 patients had more than  $10^5$  copies of HBV DNA/mL, six patients with positive HBeAg had  $10^5$  copies of HBV DNA/mL. Of the 112 patients, all with HBeAg positive and 37 had HBV DNA pre-C mutations. The patients at the immune-tolerant stage had a higher maternal HBV infection rate (83.0%) and a younger age (< 8 years) at HBV infection (55.4%). Two hundred and twenty-two patients were assigned to the immune active stage with a mean ALT level of 161.42  $\pm$  139.26 IU/L, a mean HBV load of 5.94  $\pm$  2.21 log<sub>10</sub> copies/mL. Of the 222 patients, 96 were HBeAg positive, 71 had HBV DNA pre-C mutations, and 57 had a serum HBV load of less than  $10^3$  copies/mL, but no other reasons for the elevated aminotransferase level. Eighty-eight patients at the immune-inactive carrier stage had a mean

**Table 1** Characteristics of patients with chronic HBV infection (mean  $\pm$  SD), n (%)

Characteristics	Patients (n = 422)	Clinical stage of chronic HBV infection			P
		Tolerant (n = 112)	Active (n = 222)	Inactive carrier (n = 88)	
Sex (Male/female)	268/154	67/45	156/66	45/43	< 0.05 <sup>1</sup>
Mean age (yr)	30.59 $\pm$ 10.40	29.45 $\pm$ 11.72	29.69 $\pm$ 9.30	34.34 $\pm$ 10.53	< 0.001 <sup>2</sup>
Patients with MH	233 (55.2)	93 (83.0)	115 (51.8)	25 (28.4)	< 0.001 <sup>1</sup>
Age at HBV-infection (yr)					< 0.001 <sup>1</sup>
< 8	166 (39.3)	62 (55.4)	92 (41.4)	12 (13.6)	
8-20	127 (30.1)	33 (29.5)	80 (36.0)	14 (15.9)	
> 20	103 (24.4)	9 (8.0)	46 (20.7)	48 (54.5)	
Unknown	26 (6.2)	8 (6.3)	4 (1.8)	14 (15.9)	
Serum ALT (IU/L)	116.52 $\pm$ 82.66	25.37 $\pm$ 12.56	161.42 $\pm$ 139.26	31.15 $\pm$ 19.67	< 0.001 <sup>2</sup>
Serum AST (IU/L)	82.61 $\pm$ 79.89	22.20 $\pm$ 14.63	116.63 $\pm$ 107.84	25.29 $\pm$ 12.52	< 0.001 <sup>2</sup>
Serum TBIL ( $\mu$ mol/mL)	15.66 $\pm$ 9.22	15.08 $\pm$ 2.55	20.78 $\pm$ 14.25	13.55 $\pm$ 11.24	< 0.001 <sup>2</sup>
HBV DNA positive	285 (67.5)	112(100)	165 (74.3)	8 (9.1)	< 0.001 <sup>1</sup>
Serum HBV DNA (copies/mL)					< 0.001 <sup>1</sup>
$\leq 1.0 \times 10^3$	137 (32.5)	0	57 (25.7)	80 (90.9)	
$> 1.0 \times 10^3$ - $1.0 \times 10^5$	46 (10.9)	6 (5.4)	32 (14.4)	8 (9.1)	
$> 1.0 \times 10^5$ - $1.0 \times 10^7$	60 (14.2)	22 (19.6)	38 (17.1)	0	
$> 1.0 \times 10^7$	179 (42.4)	84 (75.0)	95 (42.8)	0	
HBV DNA (Log, copies/mL)	5.87 $\pm$ 2.43	7.88 $\pm$ 1.54	5.94 $\pm$ 2.21	3.11 $\pm$ 0.37	< 0.001 <sup>2</sup>
HBeAg positive	218 (51.7)	112 (100)	96 (43.2)	10 (11.4)	< 0.001 <sup>1</sup>
HBV pre-C region mutation positive	122 (28.9)	37 (33.0)	71 (32.0)	14 (15.9)	< 0.01 <sup>1</sup>

<sup>1</sup> $\chi^2$  test; <sup>2</sup>t-test. HBV: Hepatitis B virus; MH: Maternal HBV-infection status.

**Table 2** Characteristics of chronic HBV infection patients at the immune-tolerant and immune-active stages (mean  $\pm$  SD), n (%)

Characteristics	Immune-tolerant stage (n = 112)			Immune-active stage (n = 222)		
	HBV DNA (< 1.0 $\times$ 10 <sup>7</sup> copies/mL) (n = 28)	HBV DNA (> 1.0 $\times$ 10 <sup>7</sup> copies/mL) (n = 84)	P	HBV DNA (< 1.0 $\times$ 10 <sup>7</sup> copies/mL) (n = 127)	HBV DNA (> 1.0 $\times$ 10 <sup>7</sup> copies/mL) (n = 95)	P
Sex (Male/female)	13/15	54/30	> 0.05 <sup>1</sup>	87/40	69/26	> 0.05 <sup>1</sup>
Mean age (yr)	30.80 $\pm$ 10.20	30.61 $\pm$ 10.90	> 0.05 <sup>2</sup>	31.17 $\pm$ 9.82	27.71 $\pm$ 8.18	< 0.01 <sup>2</sup>
Patients with MH	16 (57.1)	77 (91.7)	< 0.001 <sup>1</sup>	48 (37.8)	67 (70.5)	< 0.001 <sup>1</sup>
Age at HBV-infection (yr)			> 0.05 <sup>1</sup>			< 0.001 <sup>1</sup>
< 8	14 (50.0)	48 (54.8)		34 (26.8)	58 (61.0)	
8-20	10 (35.7)	23 (31.2)		54 (42.5)	26 (27.4)	
> 20	2 (7.1)	7 (7.5)		38 (29.9)	8 (8.4)	
Unknown	2 (7.1)	6 (6.5)		1 (0.8)	3 (3.2)	
Serum ALT (IU/L)	25.32 $\pm$ 12.6	29.54 $\pm$ 11.28	> 0.05 <sup>2</sup>	111.36 $\pm$ 86.5	211.33 $\pm$ 173.20	< 0.001 <sup>2</sup>
Serum AST (IU/L)	24.54 $\pm$ 16.33	24.59 $\pm$ 18.31	> 0.05 <sup>2</sup>	79.08 $\pm$ 60.87	151.84 $\pm$ 136.65	< 0.001 <sup>2</sup>
Serum total bilirubin (μmol/mL)	12.55 $\pm$ 6.28	11.83 $\pm$ 8.59	> 0.05 <sup>2</sup>	17.68 $\pm$ 8.28	23.80 $\pm$ 17.97	< 0.001 <sup>2</sup>
HBV DNA positive	28 (100)	84 (100)	> 0.05 <sup>1</sup>	70 (55.1)	95 (100)	< 0.001 <sup>1</sup>
HBV DNA (Log, copies/mL)	5.62 $\pm$ 0.81	8.63 $\pm$ 0.82	< 0.001 <sup>2</sup>	4.31 $\pm$ 1.36	8.13 $\pm$ 0.74	< 0.001 <sup>2</sup>
HBeAg positive	28 (100.0)	84 (100.0)	> 0.05 <sup>1</sup>	30 (23.6)	66 (69.5)	< 0.001 <sup>1</sup>
HBV pre-C region mutation positive	1 (3.6)	36 (42.9)	< 0.001 <sup>1</sup>	29 (22.8)	42 (44.2)	< 0.01 <sup>1</sup>

<sup>1</sup> $\chi^2$  test; <sup>2</sup>t-test.

ALT level of 31.15  $\pm$  19.67 IU/L, a mean HBV load of 3.11  $\pm$  0.37 log<sub>10</sub> copies/mL. Eighty patients had undetectable serum HBV DNA, and 8 patients with negative HBeAg had a serum HBV load of less than 10<sup>5</sup> copies/mL. Ten patients had positive HBeAg and undetectable HBV DNA, and 14 patients had HBV pre-core mutant infection. Patients at the immune-inactive carrier stage also had a low maternal HBV infection rate (28.4%) and an older age (> 20 years) at HBV-infection (54.5%). All parameters had a statistically significant difference in patients at the three stages of chronic HBV infection. Profess from immune tolerance to chronic

HBV infection was significantly associated with HBV replication status including a higher HBV load, HBeAg expression, and HBV pre-core mutation ( $P < 0.001$ ).

The characteristics of patients with a high HBV load (HBV DNA  $>$  10<sup>7</sup> copies/mL) and a low HBV load (HBV DNA  $<$  10<sup>7</sup> copies/mL) at the immune-tolerant and -active stages are summarized in Table 2. Patients with a high HBV load had a higher maternal HBV infection rate (91.7%) and a higher positive HBV pre-C mutation rate (42.9%) than those with a lower HBV load at the immune-tolerant stage ( $P < 0.001$ ). A similar pattern was observed in patients at the immune active

**Table 3** Peripheral T-cell subsets broken down by various factors in normal controls and chronic HBV infection patients (mean  $\pm$  SD)

Groups	Patients (n)	CD3 <sup>+</sup>	CD4 <sup>+</sup>	CD8 <sup>+</sup>	CD4 <sup>+</sup> /CD8 <sup>+</sup> ratio
HBV status <sup>b</sup>					
Negative (normal control)	100	71.07 $\pm$ 4.76	38.94 $\pm$ 3.39	24.02 $\pm$ 4.35	1.67 $\pm$ 0.33
Positive	422	56.42 $\pm$ 13.16	31.97 $\pm$ 7.30	33.73 $\pm$ 8.63	1.04 $\pm$ 0.45
Clinical stages of HBV infection <sup>c</sup>					
Tolerant	112	50.78 $\pm$ 13.26	30.23 $\pm$ 6.35	36.87 $\pm$ 7.58	0.86 $\pm$ 0.29
Active	222	55.51 $\pm$ 12.50	30.92 $\pm$ 7.40	34.37 $\pm$ 9.07	1.00 $\pm$ 0.47
Inactive carrier	88	65.89 $\pm$ 9.09	36.85 $\pm$ 6.06	28.09 $\pm$ 5.64	1.37 $\pm$ 0.39
HBV DNA loads (copies/mL) <sup>d</sup>					
$\leq 1.0 \times 10^3$	137	65.96 $\pm$ 8.58	37.45 $\pm$ 6.14	26.89 $\pm$ 5.71	1.46 $\pm$ 0.42
$> 1.0 \times 10^3$ - $1.0 \times 10^5$	46	62.28 $\pm$ 7.50	33.33 $\pm$ 5.61	31.81 $\pm$ 5.95	1.10 $\pm$ 0.36
$> 1.0 \times 10^5$ - $1.0 \times 10^7$	60	60.05 $\pm$ 12.97	31.58 $\pm$ 6.99	33.83 $\pm$ 7.50	0.97 $\pm$ 0.26
$> 1.0 \times 10^7$	179	46.39 $\pm$ 9.93	27.56 $\pm$ 5.49	39.41 $\pm$ 7.36	0.73 $\pm$ 0.23
HBeAg status <sup>b</sup>					
Negative	204	62.62 $\pm$ 10.67	34.83 $\pm$ 7.16	29.66 $\pm$ 7.35	1.27 $\pm$ 0.48
Positive	218	50.61 $\pm$ 12.62	29.29 $\pm$ 6.37	37.53 $\pm$ 7.98	0.83 $\pm$ 0.29
HBV pre-C region mutation status <sup>b</sup>					
Negative	300	58.40 $\pm$ 12.89	33.63 $\pm$ 6.94	31.73 $\pm$ 8.04	1.15 $\pm$ 0.44
Positive	122	51.55 $\pm$ 12.57	27.89 $\pm$ 6.56	38.63 $\pm$ 8.06	0.78 $\pm$ 0.37
Maternal HBV-infection status <sup>b</sup>					
Negative	189	62.34 $\pm$ 10.89	35.31 $\pm$ 6.32	29.64 $\pm$ 6.87	1.27 $\pm$ 0.43
Positive	233	51.61 $\pm$ 12.89	29.27 $\pm$ 6.93	37.04 $\pm$ 8.49	0.85 $\pm$ 0.37
Age at HBV infection <sup>c</sup> (yr)					
< 8	166	51.83 $\pm$ 12.86	28.17 $\pm$ 6.61	37.31 $\pm$ 8.16	0.80 $\pm$ 0.29
8-20	127	57.17 $\pm$ 12.27	33.05 $\pm$ 5.90	33.39 $\pm$ 7.91	1.07 $\pm$ 0.41
> 20	103	61.42 $\pm$ 11.89	36.02 $\pm$ 7.27	28.86 $\pm$ 7.89	1.35 $\pm$ 0.49
Unknown	26	62.25 $\pm$ 14.39	34.93 $\pm$ 6.34	31.78 $\pm$ 7.58	1.19 $\pm$ 0.45

Effects of various independent demographic, clinical and serological variables on T-cell profile of HBsAg positive individuals were analyzed. <sup>b</sup>P < 0.001 vs measurement of each T-cell parameter; <sup>c</sup>P < 0.01, <sup>d</sup>P < 0.001 vs ANOVA linear trend test.

stage. Furthermore, patients with a high HBV load had a younger age at HBV infection and a higher HBeAg expression level than those with a low HBV load at the immune active stage.

#### Composition of peripheral blood T lymphocyte subpopulations

The composition of T lymphocytes in peripheral blood of all chronic HBV infection patients is summarized in Table 3. Univariate analyses showed that T-cell impairment was significantly associated with both clinical stages of chronic HBV infection and higher HBV loads, serum HBeAg expression, HBV pre-C region mutation, history of maternal HBV infection, and younger age at HBV-infection. The linear dose-response relationship between T-lymphocyte subpopulations and HBV DNA copies was highly significant (linear trend test,  $P < 0.001$ ). The number of CD3<sup>+</sup> and CD4<sup>+</sup> cells and CD4<sup>+</sup>/CD8<sup>+</sup> ratio were negatively correlated with the serum HBV load in chronic HBV infection patients ( $r = -0.67, -0.58, -0.69, P < 0.0001$ ), while the number of CD8<sup>+</sup> cells was positively correlated with the HBV load ( $r = 0.64, P < 0.0001$ ).

The composition of peripheral blood T-lymphocytes in patients at different immune stages of chronic HBV infection is shown in Figure 1. The number of peripheral blood CD8<sup>+</sup> T-cells was higher in patients at the immune-tolerant stage than in patients at the immune-active and -inactive carrier stages, whereas the number of CD4<sup>+</sup> T cells was lower in patients at the immune-tolerant stage than in patients at the immune-inactive carrier stage ( $P < 0.001$ ), suggesting that patients

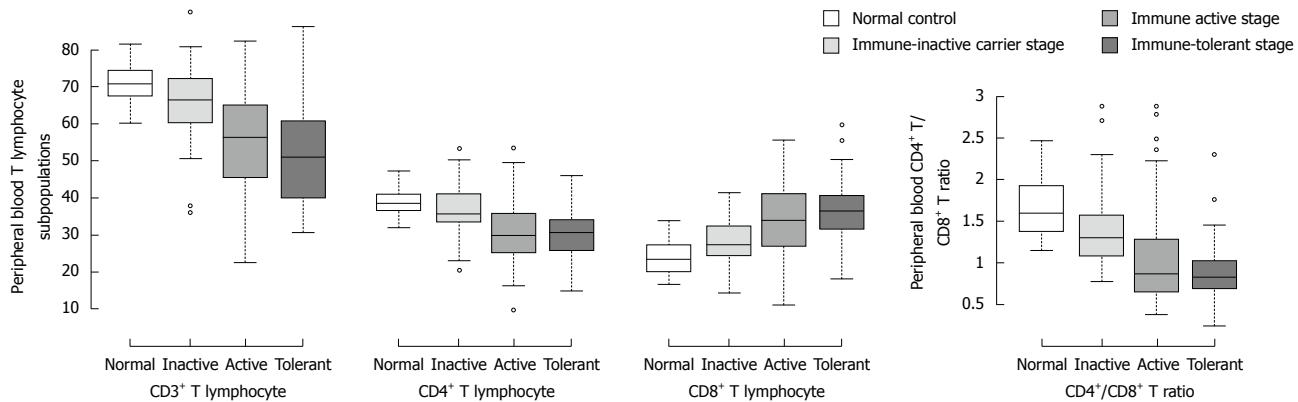
at the immune-tolerant stage have the highest number of peripheral blood CD8<sup>+</sup> T-cells and the lowest number of peripheral blood CD4<sup>+</sup> T-cells and ratio between CD4<sup>+</sup>/CD8<sup>+</sup> cells than those at the immune-active and -inactive carrier stages. The peripheral blood contained more CD8<sup>+</sup> T-cells than CD4<sup>+</sup> T-cells in patients at the immune-tolerant and -active stages, whereas the peripheral blood contained more CD4<sup>+</sup> T-cells than CD8<sup>+</sup> T-cells in patients at the immune-inactive carrier stage and normal controls ( $P < 0.01$ ).

The proportion of CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> T-cells in peripheral blood of patients at the immune-tolerant stage is summarized in Figure 2A. The mean percentage of CD3<sup>+</sup> and CD4<sup>+</sup> T-cells was higher, whereas the mean percentage of CD8<sup>+</sup> T-cells was lower in patients with a low HBV load than in patients with a high HBV load ( $P < 0.001$ ). A similar pattern was also observed between patients with a high HBV load and those with a low HBV load at the immune active stage ( $P < 0.001$ , Figure 2B).

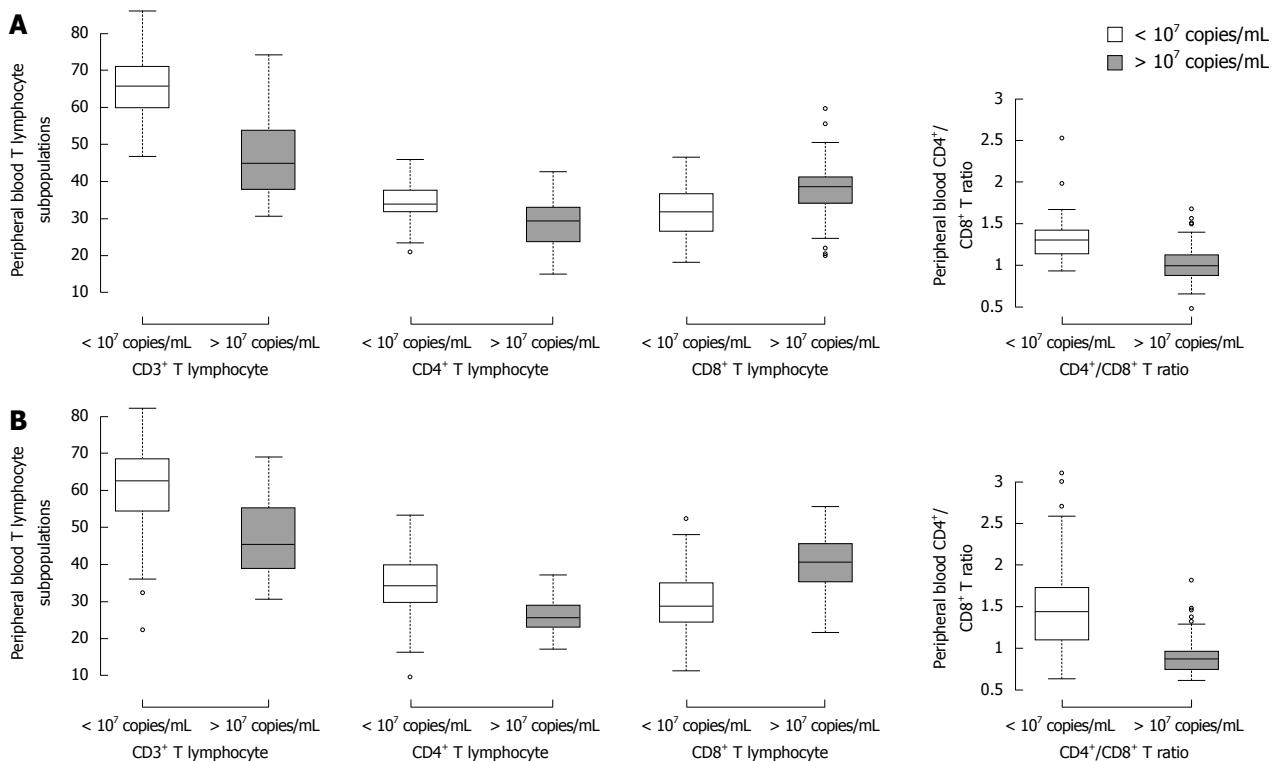
The mean percentage of CD3<sup>+</sup> and CD4<sup>+</sup> T-cells was higher in patients with negative HBeAg than in those with positive HBeAg at the immune active stage, whereas CD8<sup>+</sup> T-cells was lower in patients with negative HBeAg than in those with positive HBeAg ( $P < 0.001$ , Figure 3B). A similar pattern was observed between patients with negative HBeAg and those with positive HBeAg at the immune-tolerant stage ( $P > 0.05$ , Figure 3A).

#### Linear regression predicting peripheral blood T lymphocyte subpopulation

Linear regression models of CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup>



**Figure 1** Peripheral blood T-lymphocyte subpopulations in patients at different clinical stages of chronic HBV infection. The mean percentages of CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> T cells and CD4<sup>+</sup>/CD8<sup>+</sup> ratio were shown in patients at the different stages and control. Top of the box represents the 75%, the bottom of the box represents the 25%, the solid line in the middle of the box represents the median. Whiskers above and below the box indicate the 90% and 10%, while filled circles represent outliers. A statistically significant difference in T cells but not in CD4<sup>+</sup> cells was observed between chronic HBV infection patients and normal control ( $P < 0.01$ ). In the peripheral blood of patients at the immune-tolerant and immune-active stages, CD8<sup>+</sup> T-cells were the dominant lymphocytes compared to CD4<sup>+</sup> T-cells, whereas in the peripheral blood of patients at the immune-inactive carrier stage and normal controls, CD4<sup>+</sup> T-cells were the dominant lymphocytes compared to CD8<sup>+</sup> T-cells.

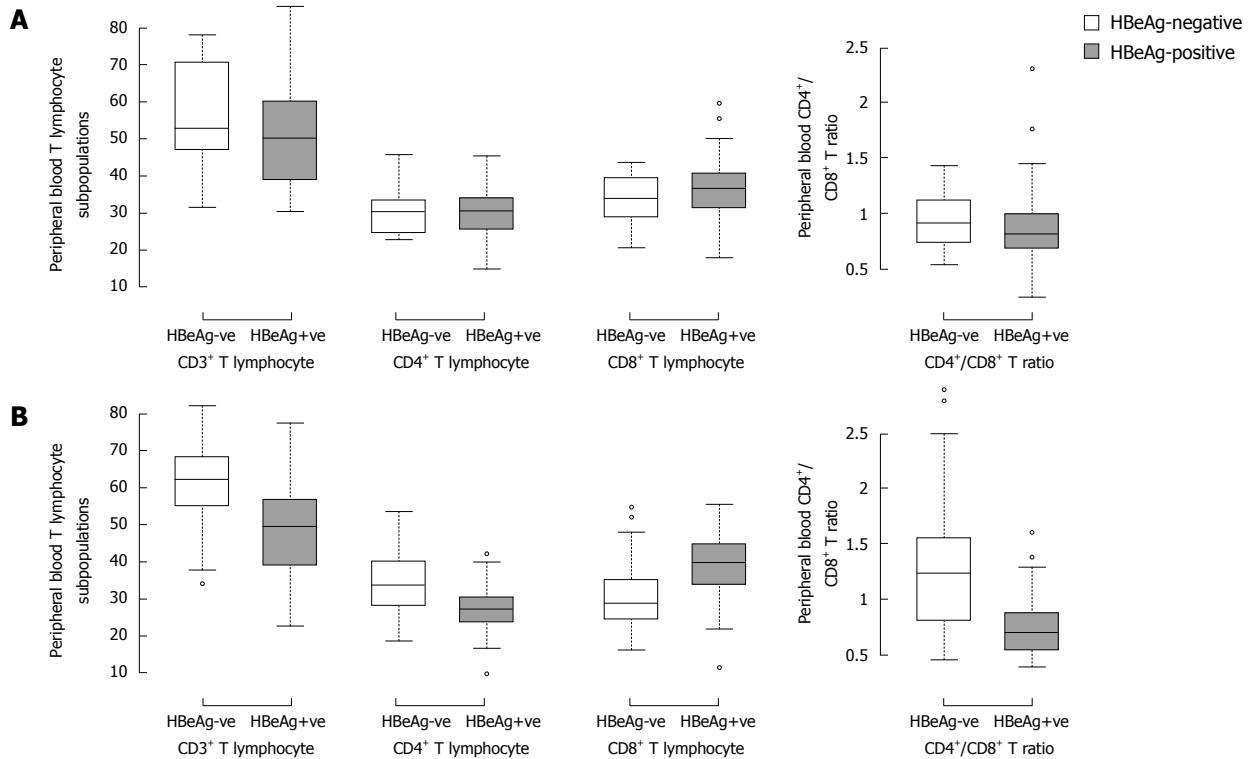


**Figure 2** Mean percentages of CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> T-cells in patients at immune-tolerant stage (A) and immune active stage (B) of chronic HBV infection. Patients were divided into two groups based upon HBV DNA load. The proportion of CD8<sup>+</sup> T-cells was significantly higher in patients with a high HBV load than in patients with a low HBV load at the immune-tolerant stage ( $38.66 \pm 7.11$  vs  $31.50 \pm 6.40$ ,  $P < 0.001$ ) and at the immune active stage ( $40.07 \pm 7.55$  vs  $30.11 \pm 7.69$ ,  $P < 0.001$ ). The percentage of CD4<sup>+</sup> T-cells was significantly higher in patients with a low HBV load than in patients with a high HBV load at the immune-tolerant stage ( $33.77 \pm 5.81$  vs  $29.05 \pm 6.11$ ,  $P < 0.001$ ) and at the immune active stage ( $34.41 \pm 7.23$  vs  $26.25 \pm 4.52$ ,  $P < 0.001$ ). Significant differences were found in CD3<sup>+</sup> T-cells and CD4<sup>+</sup>/CD8<sup>+</sup> ratio between patients with a high HBV load and a low HBV load at the immune-tolerant stage ( $45.90 \pm 10.69$  vs  $65.40 \pm 8.70$ ,  $0.78 \pm 0.21$  vs  $1.11 \pm 0.32$ ,  $P < 0.001$ ) and at the immune active stage ( $46.82 \pm 9.25$  vs  $62.01 \pm 10.52$ ,  $0.69 \pm 0.23$  vs  $1.24 \pm 0.47$ ,  $P < 0.001$ ). CD8<sup>+</sup> T-cells were predominant compared with CD4<sup>+</sup> T-cells in patients with a high HBV load, whereas CD4<sup>+</sup> T-cells were predominant compared with CD8<sup>+</sup> T-cells in patients with a low HBV load.

cells and CD4<sup>+</sup>/CD8<sup>+</sup> ratio as dependent variables are listed in Table 4. After adjustment for all independent variables, serum HBV load was the key predictor for T-cell profiles. The number of CD3<sup>+</sup> and CD4<sup>+</sup> T lymphocytes was significantly reduced, the number of CD8<sup>+</sup> T cells was increased, and the CD4<sup>+</sup>/CD8<sup>+</sup> ratio was decreased in patients at the immune-tolerant stage. Those with chronic HBV infection at a younger age

had less CD4<sup>+</sup> T cells and a lower CD4<sup>+</sup>/CD8<sup>+</sup> ratio than those with chronic HBV infection at an older age. HBeAg expression, HBV pre-C mutation, and maternal HBV-infection status also had partial independent effects on T-lymphocyte profiles.

The relationship between T-lymphocyte subpopulations and HBV load stratified by age at HBV infection is shown in Figure 4A. No significant difference of T-cell



**Figure 3 Mean percentages of CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> T-cells in peripheral blood of patients at the immune-tolerant stage (A) and immune active stage (B).** Patients were divided into two groups based upon the HBeAg status. The proportion of CD8<sup>+</sup> T-cells was significantly higher in HBeAg positive patients than in HBeAg negative patients at the immune active stage ( $39.24 \pm 8.05$  vs  $30.66 \pm 8.01$ ,  $P < 0.001$ ). The percentage of CD4<sup>+</sup> T-cells was significantly higher in HBeAg negative patients than in HBeAg positive patients at the immune active stage ( $33.60 \pm 7.41$  vs  $27.39 \pm 5.75$ ,  $P < 0.001$ ). Significant differences were found in CD3<sup>+</sup> T-cells and CD4<sup>+</sup>/CD8<sup>+</sup> ratio between HBeAg positive and negative patients ( $48.99 \pm 11.06$  vs  $60.48 \pm 11.22$ ,  $0.74 \pm 0.25$  vs  $1.21 \pm 0.50$ ,  $P < 0.001$ ). CD8<sup>+</sup> T-cells were predominant compared with CD4<sup>+</sup> T-cells in patients with a high HBeAg expression level, whereas CD4<sup>+</sup> T-cells were predominant compared with CD8<sup>+</sup> T-cells in patients with a low HBeAg expression level. In immune-tolerant-patients, no significant difference was observed in parameters of T-cell profile between HBeAg negative and positive patients ( $P > 0.05$ ).

**Table 4 Multiple linear regression predicting peripheral blood T lymphocyte subpopulations ( $n = 422$ )**

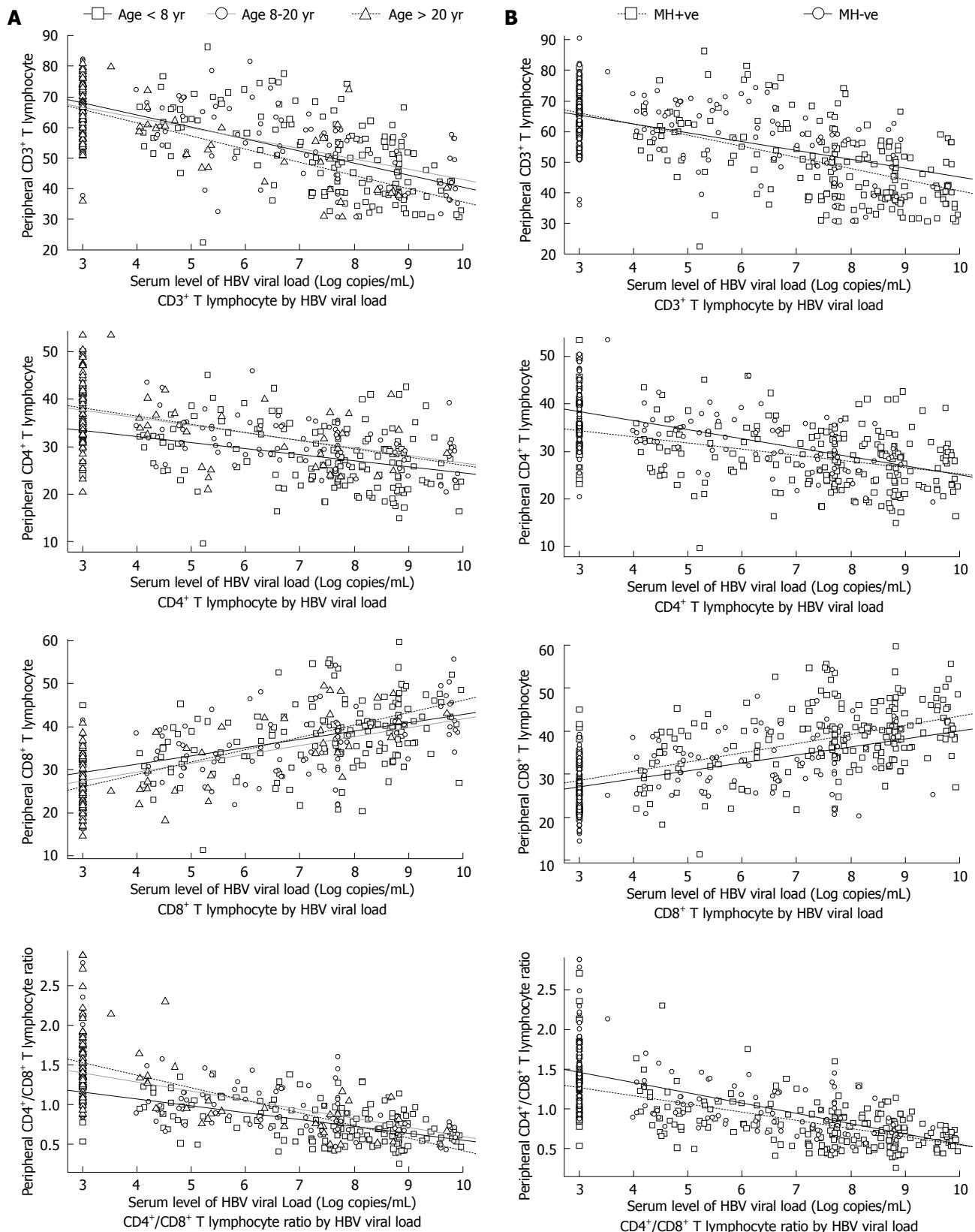
	CD3 <sup>+</sup> T lymphocytes			CD4 <sup>+</sup> T lymphocytes			CD8 <sup>+</sup> T lymphocytes			CD4 <sup>+</sup> /CD8 <sup>+</sup> ratio		
	$\beta$	SE	$P$	$\beta$	SE	$P$	$\beta$	SE	$P$	$\beta$	SE	$P$
Intercept	85.35	2.64		43.32	1.53		16.48	1.73		1.89	0.08	
Serum HBV load (Log, copies/mL) <sup>1</sup>	-3.62	0.34	< 0.0001	-1.29	0.19	< 0.0001	1.80	0.22	< 0.0001	-0.1	0.01	< 0.0001
HBeAg <sup>2</sup>	-2.74	1.40	0.06	-1.95	0.81	0.01	3.42	0.92	< 0.001	-0.16	0.04	< 0.01
HBV pre-C region mutation <sup>3</sup>	-0.67	1.13	0.55	-2.79	0.66	< 0.0001	3.38	0.74	< 0.0001	-0.15	0.04	< 0.0001
Clinical stages of HBV infection <sup>4</sup>			0.014			< 0.0001			< 0.001			< 0.0001
Active	-4.19	1.34		-3.64	0.77		3.63	0.88		-0.19	0.04	
Inactive carrier	-5.17	1.98		-3.55	1.15		4.93	1.30		-0.26	0.06	
Age at HBV infection <sup>5</sup> (yr)			0.038			< 0.001			0.37			< 0.001
8-20	-1.07	1.25		2.21	0.73		0.14	0.82		0.07	0.04	
> 20	-3.55	1.49		2.35	0.86		-0.55	0.98		0.15	0.05	
Unknown	2.51	2.17		3.18	1.25		-0.94	1.42		0.16	0.07	
Maternal HBV infection status <sup>6</sup>	-3.23	1.21	< 0.01	-1.35	0.70	0.06	2.14	0.79	< 0.01	-0.08	0.04	0.03

The linear regression model was employed in multivariate analysis to assess the independent effects of variables on peripheral blood T lymphocyte subpopulation.  $\beta$ : Coefficients from the model; SE: Standard error; <sup>1</sup>Continuous variable; <sup>2</sup>Reference group with positive HBeAg; <sup>3</sup>Reference group without pre-C mutation; <sup>4</sup>Reference group at tolerant stage; <sup>5</sup>Reference group with the age < 8 years at HBV infection; <sup>6</sup>Reference group without maternal HBV infection status.

subsets was found in patients at different HBV infection ages after adjustment for serum HBV load. A similar pattern of the relationship between T-lymphocyte subpopulations and HBV load stratified by maternal HBV carrier status was also observed in patients with chronic HBV infection (Figure 4B). No significant difference was found in T-cell subsets between patients in maternal HBV carrier status after adjustment for serum HBV load.

## DISCUSSION

This study demonstrated that there were clear differences of peripheral T cell subpopulation profile in patients at different clinical stages of chronic HBV infection. An impaired balance of T-cell subsets was related to an increased proportion of CD8<sup>+</sup> T lymphocytes, a decreased proportion of CD4<sup>+</sup> T lymphocytes and a



**Figure 4 Correlation between T-cell subsets and HBV load stratified by age at HBV infection (A) and maternal HBV infection status (B).** Three regression lines (with different slopes) were drawn for patients at different ages of HBV infection. The coefficient of the interaction term "HBVDNA: age-at-HBV-infection" was not statistically significant for each parameter of T lymphocyte subpopulations ( $P > 0.05$ ). A similar pattern was seen when stratified by maternal HBV infection status.

decreased ratio between CD4<sup>+</sup> and CD8<sup>+</sup> cells. T-cell impairment had a linear dose-response relationship with the HBV DNA load.

The composition of T-cell subpopulations in

peripheral blood was significantly different in patients at different clinical stages of chronic HBV infection, indicating that patients at the immune-tolerant stage have the highest number of CD8<sup>+</sup> T-cells, the lowest number

of CD4<sup>+</sup> T-cells, and lowest ratio between CD4<sup>+</sup> and CD8<sup>+</sup> cells than those at the immune-active and inactive carrier stages. It has been reported that the composition of peripheral blood lymphocyte populations in patients at different stages of chronic HBV infection with research subject sample sizes ranging from 29 to 47<sup>[19,20]</sup>, which are different from our study and is, thus, unable to detect any significant difference of T-cell subsets in patients at different clinical stages of chronic HBV infection. Using a larger sample size that covered a wide range of different stages of chronic HBV infection in this study, we showed the discrimination power of the composition of T cell profile in peripheral blood.

An impaired balance of T-cell subsets is related to an increased proportion of CD8<sup>+</sup> T lymphocytes, a decreased proportion of CD4<sup>+</sup> T lymphocytes, and decreased ratio between CD4<sup>+</sup> and CD8<sup>+</sup> cells during chronic HBV infection<sup>[21-27]</sup>. However, the results of previous studies are controversial results<sup>[28-32]</sup>. The fact that chronic hepatitis B represents a dynamic disease state may explain such divergent results. Therefore, any analysis of the composition of peripheral blood lymphocyte populations needs to be viewed within the context of disease progression. In this study, we attempted to correlate the trend in HBV infection, diseases progression, and stages of HBV infection with the T-cell phenotypes based on the virological and biochemical profiles (Figure 1). Our results revealed that T-cell impairment was significantly associated with the HBV replication level, indicating that serum HBV load is a strong predicting factor for T-lymphocyte subpopulations. Interestingly, our results are consistent with previous findings<sup>[19]</sup>. It has been reported that the proportion of CD8<sup>+</sup> T-cells and CD4<sup>+</sup> T-cells in liver is increased, respectively, in patients with a high HBV load and in those with a low HBV load at the immune-clearance stage<sup>[19]</sup>. Pham *et al*<sup>[33]</sup> also reported that the CD4<sup>+</sup>/CD8<sup>+</sup> ratio in liver-derived lymphocytes is correlated with HBV replication in patients with chronic hepatitis B. The decreased proportion of CD4<sup>+</sup> T-cells and CD4<sup>+</sup>/CD8<sup>+</sup> ratio in chronic HBV infection patients with a high HBV load and HBeAg expression may be a consequence but not the cause of a high HBV DNA load<sup>[3-5,12]</sup>. The impact of HBV load on antiviral T-cell responses has been precisely characterized in animal models of virus infections (like LCMV), showing that the sustained presence of viral antigens leads to a progressive functional decline of virus-specific CD8<sup>+</sup> T-cell responses and ultimately virus-specific T-cell deletion<sup>[34-36]</sup>. Similarly, the frequency and function of circulating and intrahepatic HBV-specific CD8<sup>+</sup> T cells are inversely proportional to the HBV DNA load in HBV-infected patients<sup>[37-39]</sup>. High HBV loads are associated with the ablation of CD8<sup>+</sup> T cell responses and IL-2 production impaired by virus-specific CD4<sup>+</sup> T cells<sup>[35]</sup>. Matloubian *et al*<sup>[40]</sup> demonstrated that CD4<sup>+</sup> T cells are required to sustain the CD8<sup>+</sup> T cell responses during chronic HBV infection. Furthermore, Lamivudine treatment can transiently restore the efficient antiviral T cell responses in patients

with chronic hepatitis B and alleviate their viremia<sup>[41-44]</sup>, indicating that HBV load plays an important role in the pathogenesis of T cell hypo-response in chronic HBV infection patients.

Although no information on the proportion of T-cell profile in liver was provided in this study, Pernollet *et al*<sup>[45]</sup> demonstrated that intrahepatic lymphocyte subsets are correlated with peripheral lymphocyte subsets and there is an exchange between intrahepatic and peripheral compartments. Similarly, two recent works have shown that the same specific T lymphocytes can be isolated from either the liver compartment or the PBL of chronically HCV-infected patients<sup>[46,47]</sup>. For this reason, the authors of one of these works proposed a characterization of the lymphocyte response to HCV in the peripheral blood instead of in the liver compartment<sup>[46]</sup>. We can also hypothesize that T lymphocytes leave the liver after encountering antigen there, although it has recently been shown that the majority of activated CD8<sup>+</sup> T cells entering the liver undergo apoptosis within the liver<sup>[48,49]</sup>. On the other hand, it has been reported that peripheral blood mononuclear cells can be infected with HBV<sup>[50,51]</sup>. Nuti *et al*<sup>[52]</sup> suggested that intrahepatic lymphocytes do not undergo clonal expansion within the liver but migrate from extrahepatic sites to the chronically infected liver, where they display the function of effector cells and subsequently die, implying that maintenance of the intrahepatic lymphocyte pool depends on continuous immigration of lymphocytes.

Multivariate multiple regression analysis in our study demonstrated a partial independent effect of HBeAg and pre-core mutant infection on T-cell profile, which is supported by previous studies<sup>[53-69]</sup>. It is well known that factors including HBeAg<sup>[53-59]</sup> and viral mutations<sup>[60-69]</sup> may contribute to the outcome and pathogenesis of HBV infection by inducing tolerance to specific T cells, reducing their potential to kill cells, inhibiting antigen processing and presentation, thereby decreasing the visibility of infected hepatocytes in the immune system. In addition, our results indicate that there was no significant difference of the proportion of T-cell subsets in patients at different ages of HBV infection and in patients of maternal HBV carrier status after adjustment for serum HBV load. In our study, patients with a maternal carrier history were usually infected with HBV at a younger age (Table 1) and a higher HBV load was detected in most of them, suggesting that HBV infection from the mother and/or at a younger age predisposes to tolerance to HBV infection and, thus, has a higher BHV load.

The differences of peripheral T cell subpopulation profile in patients at different clinical stages of chronic HBV infection and the strong relationship between peripheral T-lymphocyte subpopulations with a HBV load were observed in this study. Unfortunately, no information on the proportion of virus-specific T-cell subpopulations was provided in this study. Whether the increased CD8<sup>+</sup> T cells contain an increment of virus-specific CD8<sup>+</sup> T cells and the decreased CD4<sup>+</sup> T cells include a decrement of HBV-specific CD4<sup>+</sup> T cells

remains unclear. Further studies are needed to confirm their relationship and the correlation between intrahepatic and peripheral lymphocyte subsets.

In conclusion, HBV viral load in patients at the immune-tolerant and immune active stages contributes to the variations in peripheral T cell subpopulation profile, which is relevant to the design of individualized new anti-viral strategies. Further study is required to better understand the complex host-virus interaction that determines the persistence and outcome of HBV infection.

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## COMMENTS

### Background

Hepatitis B virus (HBV) infection is a serious public health problem worldwide and a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. The pathogenesis of persistent HBV infection and hepatitis B is very complex and has not been clarified until now. HBV infection, a dynamic process with variable biochemical, virological and histological profiles at its different stages, depends on host and viral factors. Furthermore, the profiles may change at a variable pace over time. Based on the virological and biochemical parameters, chronic HBV infection patients can divided into immune-tolerant stage, immune active stage, and immune-inactive carrier stage.

### Research frontiers

Outcome of HBV infection and the pathogenesis of liver disease are determined by immune-mediated host-virus interaction, which is difficult to fully elucidate because the host range of HBV is limited to human beings and chimpanzees. The pathogenesis of liver disease and interaction between virus and host remain the hotspots in this field.

### Innovations and breakthroughs

The pathogenesis of cellular immune disorder and viral replication remains unknown. In the present study, peripheral T-lymphocyte subpopulations in chronic HBV infection patients were detected by flow cytometry and HBV load was determined by real-time polymerase chain reaction. The results suggest that differences in the peripheral T cell subpopulation profile were observed in patients at different clinical stages of chronic HBV infection. HBV load in patients at the immune-tolerant and immune active stages contributes to the variations in peripheral T cell subpopulation profile. The substantial linear dose-response relationship and strong independent predictive effect of HBV load on T-lymphocyte subpopulations suggested a close proximity of the causal pathway between them, indicating that HBV load plays an important role in the pathogenesis of T-cell impairment in chronic HBV infection patients.

### Applications

The results, which suggest that a high HBV load contributes to the impaired balance of T-cells in chronic HBV infection patients, have practical implications

because understanding of the immune response to HBV infection is useful in developing appropriate therapeutic strategies for controlling viral hepatitis and disease progression, improving current knowledge about the prognosis of persistent HBV infection. In addition, a high HBV load can predict the variations in peripheral blood T-lymphocyte subpopulations of chronic HBV infection patients.

### Terminology

CD4<sup>+</sup> T cells, classically referred to helper T cells that are required for the efficient effector cytotoxic/suppressor CD8<sup>+</sup> T- and B-cell antibody production, play an important role in HBV infection by secreting Th1 cytokines that down-regulate HBV replication, and by promoting CD8<sup>+</sup> T- and B-cell responses. CD8<sup>+</sup> T cells go on to clear HBV-infected hepatocytes through cytolytic and non-cytolytic mechanisms, thus reducing the circulating virus level, while B-cell antibody production neutralizes free viral particles, thus preventing (re) infection. CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> cells are the major function subgroups of T cells and play an important role in the response to HBV infection, which can reflect the cellular immune function and immunoregulation and are usually regarded as a valuable index of the changes in immunity of patients.

### Peer review

This is a descriptive study of the peripheral T cell sub-populations in chronic hepatitis B based in a large cohort of patients.

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BRIEF ARTICLES

## Topical negative pressure in managing severe peritonitis: A positive contribution?

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are needed to assess its value in managing these difficult cases.

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### Abstract

**AIM:** To assess the use of topical negative pressure (TNP) in the management of severe peritonitis.

**METHODS:** This is a four-year prospective analysis from January 2005 to December 2008 of 20 patients requiring TNP following laparotomy for severe peritonitis.

**RESULTS:** There were 11 males with an average age of  $(59.3 \pm 3.95)$  years. Nine had a perforated viscus, five had anastomotic leaks, three had iatrogenic bowel injury, and a further three had severe pelvic inflammatory disease. TNP and the VAC® Abdominal Dressing System were initially used. These were changed every two to three days. Abdominal closure was achieved in 15/20 patients within  $4.53 \pm 1.64$  d. One patient required relaparotomy due to residual sepsis. Two patients with severe faecal peritonitis due to perforated diverticular disease received primary anastomosis at second look laparotomy, as sepsis and their general condition improved. In the remaining 5/20 cases, the abdomen was left open due to bowel oedema and or abdominal wall oedema. Dressing was switched to TNP and VAC® GranuFoam®. Three of the five patients returned a few months later for abdominal wall reconstruction and restoration of intestinal continuity. Two patients developed intestinal fistulae. All 20 patients survived.

**CONCLUSION:** The use of TNP is safe. Further studies

### INTRODUCTION

The principles of managing peritonitis have not changed for decades. They include elimination of the septic focus, removal of necrotic tissue, and drainage of purulent material<sup>[1]</sup>. Despite advances in antibiotics therapy and intensive care support, mortality and morbidity remain high<sup>[2]</sup>. In certain cases of severe peritonitis, the surgeon may feel that a further look is required to achieve these goals<sup>[1]</sup>. This can be achieved by leaving the abdomen open at the index operation<sup>[3]</sup>. While this concept of a planned second look following major abdominal trauma is well recognised<sup>[4]</sup>, its use following abdominal catastrophe due to severe peritonitis remains controversial<sup>[3]</sup>. Several different techniques have been tried to facilitate a second look<sup>[4-6]</sup>. Recently, topical negative pressure (TNP) with the VAC® Abdominal Dressing System was introduced<sup>[3,7]</sup>. However, concerns were raised about its safety<sup>[8-10]</sup>. We report our experience using TNP in managing patients with advanced peritonitis.

### MATERIALS AND METHODS

This is a four-year prospective analysis from January 2005 to December 2008 of 20 patients who developed severe peritonitis and whose abdomens were left open requiring TNP to allow a second look laparotomy. This

decision was taken independently by the operating surgeon. Patients' demographics, type and cause of peritonitis were noted. APACHE II scoring<sup>[11]</sup> and hospital mortality probability were calculated independently by critical care staff. Morbidity, mortality and delayed abdominal closure were recorded. TNP and the VAC® Abdominal Dressing System (Kinetic Concepts Inc., San Antonio, Texas, USA) were initially used to allow further exploration of the abdomen every 48 to 72 h. The TNP was set at 125 mmHg continuous. Once the abdomen was judged to be clean, and if delayed abdominal closure was not possible due to bowel oedema and or abdominal wall oedema, the dressing was switched to TNP and a VAC® GranuFoam® dressing with a non-adherent silicone dressing underneath. Tissue viability staff could carry this out in the ward. Dressing techniques are described elsewhere<sup>[3,7]</sup>. All data are presented as mean  $\pm$  SE.

## RESULTS

There were 11 males and 9 females with an average age of ( $59.3 \pm 3.95$ ) years. Seven patients developed severe peritonitis following elective surgery and 13 were initially admitted as emergencies. 10/20 patients required TNP from the onset and the remaining half after they developed a complication requiring further surgical intervention. Nine patients developed faecal peritonitis, four had a perforated stomach or duodenum, another four developed severe peritonitis due to small bowel perforation, and the remaining three patients had severe pelvic sepsis. Peritonitis was due to perforated viscus in nine patients, five had an anastomotic leak, three sustained an iatrogenic bowel injury, and in a further three patients peritonitis was due to severe pelvic inflammatory disease. The APACHE II scoring was ( $16.7 \pm 1.9$ ) and the hospital mortality probability was ( $37.2\% \pm 5.2\%$ ). The length of stay in the critical care unit (level III and level II) was ( $19.8 \pm 4.48$ ) d. The VAC® Abdominal Dressing System and TNP were initially used in all patients, with a change of dressing every two to three days in theatre (Table 1).

Abdominal closure was achieved in 15/20 patients within ( $4.53 \pm 1.64$ ) d. This required ( $2.35 \pm 1.81$ ) dressings. Two of those fifteen patients required a biological mesh to assist in abdominal wall closure. One patient required relaparotomy due to residual sepsis. In two patients who had severe faecal peritonitis due to perforated diverticular disease, primary anastomosis was possible at second look laparotomy because sepsis and their general condition improved. No covering stoma was required and they made an uneventful recovery.

For the remaining five cases, the abdomen was left open due to bowel and or abdominal wall oedema and the dressing was switched to TNP and a VAC® GranuFoam® dressing with a non-adherent silicone dressing underneath. This was carried out in the ward. Two patients developed intestinal fistulae. In the first patient, a small bowel anastomosis broke down on the fifth postoperative day, which was converted to an end ileostomy. The second patient had multiple enterotomies,

**Table 1** Details of the patients included in this study (mean  $\pm$  SE)

Male/female	11/09
Age (yr)	$59.3 \pm 3.95$
APACHE II scoring	$16.7 \pm 1.9$
4-9	3 patients
10-25	14 patients
26-35	3 patients
Hospital mortality probability (%)	$37.2 \pm 5.25$
Peritonitis: type	
Faecal peritonitis	9 patients
Perforated stomach/duodenum	4 patients
Small bowel perforation	4 patients
Pelvic sepsis	3 patients
Underlying problem	
Perforated viscus	9 patients
Anastomotic leak	5 patients
Iatrogenic bowel injury	3 patients
Pelvic sepsis	3 patients
No. of changes of dressings in theatre	$2.35 \pm 1.81$
Delayed primary closure	15/20
Complications	
Fistulae	2/20
Residual collection	1/20
Mortality	0/20

of which two leaked on the ninth postoperative day and these were treated conservatively. These two patients and a further third patient returned a few months later for abdominal wall reconstruction and restoration of intestinal continuity. One patient underwent surgery after 6 mo and the remaining two around 12 mo from their initial surgery. One patient is awaiting reversal of a stoma and abdominal wall reconstruction.

## DISCUSSION

Surgery is the cornerstone of treatment of abdominal sepsis<sup>[12]</sup>. It is every surgeon's desire to achieve this goal with a single operation, but this is not always possible<sup>[11]</sup>. The concept of a planned relaparotomy was suggested<sup>[12]</sup>. When some authors found that one extra laparotomy was not enough the concept of the open abdomen was developed<sup>[13]</sup>. A randomised study of 232 patients from the Dutch peritonitis study group<sup>[14]</sup> failed to show any advantage of "planned relaparotomy" compared to "laparotomy on demand". A further randomised study by Robledo *et al*<sup>[11]</sup> comparing open with closed "on demand" management of severe peritonitis was terminated after the inclusion of 40 patients because of a mortality disadvantage for the open abdomen group (55 vs 30%). However in this study, the "open abdomen" was managed with a non-absorbable polypropylene mesh.

Billing *et al*<sup>[15]</sup> in a case control study found no advantage of planned relaparotomy, but for a subgroup analysis of patients in whom source control was not achieved during the index operation, a lower mortality rate was reported with planned relaparotomy. Furthermore, Koperna *et al*<sup>[16]</sup> have shown that patients reoperated on after 48 h had a higher mortality rate than those operated on earlier.

Barker *et al*<sup>[4]</sup> reported their 15 years experience using

a locally designed vacuum-pack system in 258 patients (116 trauma and 142 general and vascular surgery). Primary closure was achieved in 68% and they reported a 5% fistula rate. Perez *et al*<sup>[3]</sup> reported similar results using TNP and the VAC® Abdominal Dressing System in a series of 37 patients following severe abdominal sepsis and compartment syndrome.

The use of TNP in the management of severe peritonitis was never subjected to a randomised study. Bee *et al*<sup>[17]</sup>, after damage control surgery following abdominal trauma, compared temporary abdominal closure of the open abdomen (using polyglactin mesh) with TNP and the VAC® Abdominal Dressing System or a locally designed vacuum-pack system. They failed to show a clear benefit of the latter. Furthermore, the fistula rate in the second group was 21%, but this was not statistically different from the 5% rate for the mesh group. These fistulae were related to feeding enterostomies or anastomotic dehiscences, and they all occurred with the vacuum-pack system. Adkins *et al*<sup>[18]</sup> compared the outcome of 81 patients with severe peritonitis managed with the vacuum-pack system with a historical control group. Hospital mortality was 33% and 25%, respectively. This did not reach statistical significance. A fistula rate of 14.8% was demonstrated in the open group.

TNP facilitates temporary abdominal wall closure<sup>[7]</sup>, controlled collection of exudate<sup>[2]</sup> and the small bowel " cocoons" inside the abdominal cavity, preventing interloop abscesses<sup>[19]</sup>. Furthermore the Abdominal Dressing System allows easy access for a second look<sup>[3]</sup>. While the Bogota bag<sup>[3]</sup> is a cheap alternative in managing the open abdomen; it does not control exudate and the bowel can adhere to the abdominal wall, making re-exploration difficult.

It was suggested<sup>[2]</sup> that a second look to be beneficial for patients with an APACHE II score of 10 to 25. A score greater than 26 has a high mortality rate regardless of the strategy used, and a score less than 10 has a good prognosis whatever the approach used. In our study, the operating surgeon was not aware of the APACHE II score, which was calculated independently by the intensive care staff. 14/20 had an APACHE II score of 10 to 25 and 3/20 had a score greater than 26. In addition, half of the patients had a hospital mortality probability greater than 30%. All patients in this study survived their surgical admission. Delayed abdominal wall closure was achieved in 75% of patients and intestinal continuity was restored in two patients when conditions became favourable; this is known as delayed primary anastomosis<sup>[2]</sup>.

There are very few reports of the use of TNP and the Abdominal Dressing System in peritonitis<sup>[7]</sup>. Most reports include patients with compartment syndrome<sup>[13]</sup> or trauma patients. Others include both TNP and the abdominal dressing that we used in this study, which was developed by the Wake Forest group or the vacuum-pack system that was popularized by the Chattanooga group<sup>[17]</sup>. Trevelyan and Carlson<sup>[9]</sup> suggested that the results of TNP use following sepsis should be evaluated separately from trauma cases. Its safety in the open

septic abdomen should not be assumed simply because of data gathered in trauma patients and they reported a high fistula rate<sup>[8-10]</sup>. However, it is not always clear in these reports what type of dressings were used. Over the last five years we used TNP and the Abdominal Dressing System in the open abdomen in 42 patients, of whom 20 had severe peritonitis. Two patients (2/20) developed intestinal fistulae. This is an acceptable figure in these difficult cases.

We believe patients who would benefit most from the use of this technique are: (1) Patients with anastomotic leak and iatrogenic bowel injuries; (2) Damage control for unstable patients, particularly in the presence of hypothermia, severe acidosis or coagulopathy<sup>[20]</sup>; (3) Bowel and/or abdominal wall oedema making closure difficult<sup>[19]</sup>; (4) Source of sepsis not identified<sup>[15]</sup>; (5) Sepsis not fully controlled<sup>[4]</sup>; (6) Doubtful tissue viability requiring a further look<sup>[20]</sup>; and (7) Severe faecal peritonitis, particularly if delayed primary anastomosis can be considered at a second look<sup>[2]</sup>.

Leaving an abdomen open is a decision that should not be taken lightly. It can have a negative impact on quality of life if used inappropriately.

In conclusion, the management of patients with abdominal catastrophe requires the close cooperation of surgeon, intensivist and microbiologist. In 1991 Schein<sup>[21]</sup> suggested that the open abdomen technique in the management of peritonitis generated great interest and hope. Fifteen years later, Wild *et al*<sup>[7]</sup> declared that the Abdominal Dressing System set a new standard in the management of the open abdomen following peritonitis. In our study, TNP appears to be safe and we feel its use had a positive contribution to a group of patients who were severely ill. However, used unwisely, it might lead to unnecessary morbidity. Further studies are needed to identify patients for whom this technique would be beneficial.

## COMMENTS

### Background

Surgery is the cornerstone in the management of severe peritonitis, which can be life threatening. A planned second look operation might be required. This can be achieved by leaving the abdomen open at the index operation. Over the years, different techniques have been suggested to facilitate this. Recently topical negative pressure (TNP) with VAC® Abdominal Dressing (Kinetic Concepts Inc., San Antonio, Texas, USA) were introduced. Some studies have raised concerns about its value and safety.

### Research frontiers

TNP is a wound management system that was modified to assist in the management of the open abdomen following major abdominal trauma. The research hot spot is its efficacy and safety in the management of the open abdomen following severe peritonitis.

### Innovations and breakthroughs

The management of the open abdomen is challenging. Various methods have been tried but none proved to be optimal. An ideal temporary dressing allows easy access for further surgery, controls exudate from the abdomen, reduces bowel oedema and ultimately makes abdominal closure possible when conditions are favourable. TNP with the VAC® Abdominal Dressing appears to fit these criteria.

### Applications

This study suggests that the use of TNP with the VAC® Abdominal Dressing System in patients with severe peritonitis is safe, with acceptable morbidity. Delayed closure can be achieved if conditions become favourable.

## Terminology

Peritonitis is an inflammatory process of the peritoneum caused by any irritant agent such as bacteria, fungi, virus, talc, drugs, granulomas, and foreign bodies. TNP and the VAC® Abdominal Dressing System: Encapsulated foam with a non-adherent fenestrated layer is placed between the abdominal wall and the exposed abdominal content. On top of this, a layer of perforated Granufoam® is placed; this is covered by an adhesive drape to create an airtight seal. A Pad lies on top of the foam and is connected to a fluid collection canister contained in a programmable computer controlled vacuum pump creating negative pressure at the wound surface. The dressing is changed every 48-72 h.

## Peer review

It is an interesting prospective non-comparative analysis of 20 patients with severe peritonitis and open abdomen. Fifteen patients underwent primary closure of the abdomen using VAC and TPN. In the remaining five cases, the abdomen was left open with TNP and VAC.

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BRIEF ARTICLES

## Applicability and variability of liver stiffness measurements according to probe position

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### Abstract

**AIM:** To investigate the liver stiffness measurement (LSM) applicability and variability with reference to three probe positions according to the region of liver biopsy.

**METHODS:** The applicability for LSM was defined as at least 10 valid measurements with a success rate greater than 60% and an interquartile range/median LSM < 30%. The LSM variability compared the inter-position concordance and the concordance with FibroTest.

**RESULTS:** Four hundred and forty two consecutive patients were included. The applicability of the anterior position (81%) was significantly higher than that of the reference (69%) and lower positions (68%), (both  $P = 0.0001$ ). There was a significant difference (0.5 kPa, 95% CI 0.13-0.89;  $P < 0.0001$ ) between mean LSM estimated at the reference position (9.3 kPa) vs the anterior position (8.8 kPa). Discordance between positions was associated with thoracic fold ( $P = 0.008$ ). The discordance rate between the reference position result and FibroTest was higher when the 7.1 kPa cutoff was used to define advanced fibrosis instead of 8.8 kPa (33.6% vs 23.5%,  $P = 0.03$ ).

**CONCLUSION:** The anterior position of the probe should be the first choice for LSM using Fibroscan, as it has a higher applicability without higher variability compared to the usual liver biopsy position.

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**Key words:** Fibroscan; Fibrotest; Liver fibrosis; Variability; Concordance

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### INTRODUCTION

A major clinical challenge is to find the best method to evaluate and to manage the increasing numbers of patients with chronic liver disease<sup>[1-4]</sup>. Liver biopsy, due to its risks and limitations, is no longer considered mandatory as the first-line indicator of liver injury, and several markers have been developed as non-invasive alternatives<sup>[1-4]</sup>.

The assessment of liver fibrosis by non-invasive techniques such as biomarkers, [FibroTest® (FT)]<sup>[5]</sup> and liver stiffness measurement (LSM) by Fibroscan®<sup>[6,7]</sup>, is now widely performed in countries where these techniques are available and approved<sup>[8,9]</sup>. It is therefore essential to identify factors associated with a variability of the results of these techniques to reduce the risk of false positives or false negatives. There are no published procedures for the most accurate position of the probe in LSM. In almost all

publications<sup>[6,7,9-10]</sup>, the described method is copied from the original description by Sandrin *et al*<sup>[13]</sup>: "Because liver biopsies are performed on the right lobe of the liver, so were the elasticity measurements. During the acquisition, patients were lying on their backs with their right arms behind their heads. The physician first proceeded to a sonographic examination to localize the best ultrasonic imaging window between the rib bones. Additionally, regions with large vessels were avoided and a minimal liver parenchyma thickness of 6 cm was sought".

Few studies have examined the variability possibly associated with different positions in the rather vaguely defined area called "the liver biopsy zone". The variability associated with position could be part of the interobserver effect. Only two published studies have estimated the interobserver effect: Sandrin *et al*<sup>[13]</sup> studied 10 patients involving 3 operators (standardized CV 3.3%) and Coco *et al*<sup>[14]</sup> compared 2 operators in 40 patients using correlation coefficients (0.92) and paired *t*-tests. Tanne *et al*<sup>[17]</sup> also observed a significant discordance (25%) between predicted fibrosis stages according to three different positions of the probe and suggested using three different positions, to reduce the "sampling error".

We previously compared 9 different positions in the right lobe in 35 healthy subjects with the same operator and observed a very significant variability<sup>[18]</sup>. Three positions were therefore selected according to their applicability: the reference position, an anterior position and a lower position.

The aims of this study were to compare the applicability of these three positions, their inter-position concordance, and their concordance alone and relative to FT, a reference biomarker of fibrosis.

## MATERIALS AND METHODS

Consecutive patients with chronic liver disease seen in the Hepatology Department of the Pitié-Salpêtrière Hospital in Paris, France were pre-included to undergo LSM and FT. Patients were not included if they did not accept the protocol, or if the quality requirements for FT were not achieved. All patients gave informed consent for the use of data and serum for research purposes in this non-interventional clinical study, which was approved by the local institutional review board. The study protocol was in accordance with the ethical guidelines of the Declaration of Helsinki.

### Biochemical markers

FibroTest, and ActiTest (Biopredictive, Paris, France) were performed according to published recommendations<sup>[5,19,20]</sup>.

### Liver stiffness measurements

LSM was performed with the non-invasive method of transient elastography (FibroScan, Echosens, Paris, France). The stiffness results were expressed in kilopascal (kPa). The technique was performed by two trained (more than 100 measurements) senior hepatologists, blinded to all other characteristics, and according to the manufacturers' recommendations. During the acquisition,

patients were lying on their backs with their right arms behind their heads. The operator firstly proceeded to a sonographic examination to localize the best ultrasonic imaging window between the rib bones in the liver biopsy area. Regions with large vessels were avoided and a minimal liver parenchyma thickness of 6 cm was sought.

The reference position was the region usually recommended for biopsy located at the intersection between the xiphoid line and the median axillary line, where the operator would have performed the biopsy. The second position (lower position) was a more posterior position 2-3 cm in the next intercostal space on the same xiphoid line as the reference position and the third position (anterior position) was an anterior position 2-3 cm ahead of the reference position in the same intercostal space.

Two of the most commonly recommended cutoffs for advanced fibrosis (F2, F3 and F4 in METAVIR staging)<sup>[21]</sup> vs non-advanced fibrosis (F0 and F1) were used: 7.1 kPa<sup>[6]</sup> and 8.8 kPa<sup>[12]</sup>.

### Applicability

The applicability for LSM was defined as: a success rate greater than 60% (SR60)<sup>[7,9,12,13]</sup>, at least 10 valid liver stiffness measurements (V10)<sup>[7,9,12,13]</sup> and an interquartile range/median LSM < 30% (IQR30)<sup>[7,9,14-19]</sup>.

The applicability for FT was defined as: a security algorithm profile excluding Gilbert's disease, hemolysis, acute inflammation profiles and extreme values of FT components, leading to a change of at least 0.30 in the FT result if the median value of each component was used<sup>[5,20]</sup>.

### Statistical analysis

The two main endpoints were the applicability rate and the discordance rate with FT, for the two new positions vs the reference position. Because of the number of statistical comparisons for these two endpoints for two positions, a *P* value lower than 0.01 has been taken for a significant difference.

The strength of concordance between each LSM, or their combinations, and FT was assessed using three methods, the kappa reliability test (K) for 2 fibrosis stages (advanced vs non-advanced fibrosis), the Spearman rank correlation coefficient (R), and the intraclass coefficient of correlation (ICC)<sup>[22]</sup>.

Applicabilities were compared using Chi square and Fisher's exact tests, quantitative variables were compared using Mann-Whitney test, Wilcoxon signed rank test for paired comparisons, and multivariate analysis using logistic regression analysis. All comparisons were performed separately with subpopulations of operator 1 and 2, as well as with the population of patients with all positions applicable and populations with at least one position applicable. Analyses were performed with NCSS software (Kaysville, Utah, USA)<sup>[23]</sup>.

## RESULTS

A total of 468 consecutive patients were pre-included

**Table 1 Characteristics of included and non-included patients n (%)**

Characteristics	Included	Non-included
Number of patients	442	26
Mean age (SE)	49 (1)	50 (2)
Male	293 (66)	17 (65)
Ethnic origin		
Caucasian	285 (65)	17 (65)
Asian	36 (8)	2 (8)
North African	43 (10)	1 (4)
Other African	78 (17)	6 (23)
Anthropometric data		
Height (m)	1.71 (0.05)	1.70 (1.91)
Weight (kg)	71.7 (0.7)	69.7 (2.4)
BMI (kg/m <sup>2</sup> )	24.4 (0.2)	24.1 (0.8)
Abdominal fold mm	21.7 (0.5)	26.1 (3.2)
Thoracic fold mm	13.1 (0.3)	13.6 (1.1)
Waist circumference cm	84 (1)	83 (2)
Daily alcohol ≥ 30 g/d	18 (7)	2 (11)
Diagnosis		
HCV	200 (45)	13 (50)
HBV	79 (18)	6 (23)
NAFLD	59 (13)	1 (4)
HIV coinfection	50 (11)	4 (15)
ALD	11 (3)	0 (0)
Other/unknown	93 (21)	2 (8)
Biochemistry		
ALT (IU/L)	58 (3)	151 (100)
AST (IU/L)	48 (2)	45 (6)
n = 416	n = 16	
Cholesterol (mmol/L)	4.70 (0.05)	4.50 (0.34)
n = 381	n = 14	
Glucose (mmol/L)	5.34 (0.07)	5.28 (0.16)
n = 404	n = 16	
Triglycerides (mmol/L)	1.21 (0.06)	1.10 (0.19)
n = 381	n = 14	
FibroTest	0.40 (0.01)	0.51 (0.06)
n = 442	n = 18	
ActiTTest	0.32 (0.01)	0.39 (0.07)
n = 442	n = 18	
SteatoTest	0.33 (0.01)	0.27 (0.06)
n = 378	n = 12	

between April and September 2007. Twenty six patients were not included and 442 patients were included (Table 1). There was no difference between included and non-included patient characteristics.

### Applicability

The applicability of LSM according to position is described in Table 2. The applicability of the anterior position (81%) was significantly higher than that of the reference (69%) and lower positions (68%), (both  $P = 0.0001$ ). These differences in applicability were mainly due to an IQR30 obtained more often with the anterior position than with the reference or lower positions for both operator 1 and operator 2 respectively: 82% vs 67%,  $P < 0.0001$ ; 82% vs 73%,  $P = 0.004$  and 91% vs 87%,  $P = 0.40$ ; 91% vs 76%;  $P = 0.004$ .

### Liver stiffness measurements between positions

Among 268 patients with both anterior and reference positions applicable, the mean LSM estimated at the reference position [9.0 kPa; (0.5)] was significantly higher in comparison to the anterior position [8.5 kPa (0.5);  $P < 0.0001$ ].

**Table 2 Liver stiffness measurements applicability**

Position, operator and quality criteria	Applicability (%)	Presumed fibrosis stages among applicable patients FOF1/F2/F3/F4 n (%) <sup>c</sup>
Reference position	306/442 (69) <sup>a</sup>	167 (55)/59 (19)/51 (17)/29 (9) <sup>d</sup>
Operator 1	208/329 (63)	117 (56)/40 (19)/32 (15)/19 (9)
Valid10	312/329 (95)	
SR60	300/329 (91)	
IQR30	220/329 (67)	
Operator 2	98/113 (87)	50 (51)/19 (19)/19 (19)/10 (10)
Valid10	113/113 (100)	
SR60	113/113 (100)	
IQR30	98/113 (87)	
Anterior position	357/442 (81) <sup>a</sup>	223 (62)/52 (15)/49 (14)/33 (9) <sup>d</sup>
Operator 1	255/329 (78)	166 (65)/34 (13)/31 (12)/24 (9)
Valid10	300/329 (91)	
SR60	296/329 (90)	
IQR30	271/329 (82) <sup>b</sup>	
Operator 2	102/113 (90)	57 (56)/18 (18)/18 (18)/9 (9)
Valid10	111/113 (98)	
SR60	111/113 (98)	
IQR30	103/113 (91) <sup>b</sup>	
Lower position	302/442 (68) <sup>a</sup>	170 (56)/49 (16)/47 (16)/36 (12)
Operator 1	224/329 (68)	126 (56)/36 (16)/36 (16)/26 (12)
Valid10	299/329 (91)	
SR60	291/329 (88)	
IQR30	240/329 (73) <sup>b</sup>	
Operator 2	78/113 (69)	44 (56)/13 (17)/11 (14)/10 (13)
Valid10	96/113 (85)	
SR60	94/113 (83)	
IQR30	86/113 (76) <sup>b</sup>	

<sup>a</sup>Applicability of the anterior position was significantly higher than that of the reference and the lower positions (both  $P = 0.0001$ ); <sup>b</sup>IQR30 was obtained more frequently with the anterior position than with the reference and lower positions for operator 1 (82% vs 67%  $P < 0.0001$ ; 82% vs 73%  $P = 0.004$ ) and for operator 2 (91% vs 87%  $P = 0.40$ ; 91% vs 76%  $P = 0.004$ ); <sup>c</sup>Presumed fibrosis stage (METAVIR scoring system) using 7.7 kPa for F2, 8.8 kPa for F3 and 14.5 kPa for F4; <sup>d</sup>Presumed prevalence of non-advanced fibrosis was lower using the reference position than the anterior position ( $P = 0.04$ ).

There was no significant difference between LSM measured at the reference in comparison to the lower position [9.5 kPa (0.5) vs 9.3 kPa (0.5),  $n = 253$ ,  $P = 0.36$ ].

### Presumed prevalence of fibrosis

Among 268 patients with both applicable anterior and reference positions, using a 7.1 kPa cutoff, 121/268 (45%) of patients had advanced fibrosis using the reference position vs 102/268 (38%) using the anterior position ( $P = 0.10$ ). Using an 8.8 kPa cutoff, 73/268 (27%) of patients had advanced fibrosis using the reference position vs 58/268 (24%) using the anterior position ( $P = 0.40$ ).

When the prevalence of presumed fibrosis stages was compared according to the probe position of all applicable patients, prevalence of non-advanced fibrosis (7.1 kPa cutoff) was lower using the reference position (55%) than the anterior position (62%,  $P = 0.04$ ) (Table 2).

**Table 3 Strength of concordance between stiffness measurements assessed in three positions in the biopsy area**

Position (No. of patients)	Method assessing concordance	
Quantitative concordance	Spearman mean (95% CI)	Intra class coefficient mean (95% CI)
Reference vs anterior (196)	0.81 (0.75-0.85)	0.90 (0.86-0.94)
Reference vs lower (196)	0.77 (0.70-0.82)	0.86 (0.79-0.93)
Anterior vs lower (196)	0.77 (0.70-0.82)	0.87 (0.80-0.94)
Two classes concordance	Discordance rate (%)	Kappa mean (SE)
Advanced vs non advanced fibrosis		
7.1 cutoff		
Reference vs anterior (196)	35 (17.9) <sup>a</sup>	0.63 (0.07)
Reference vs lower (196)	33 (16.8) <sup>b</sup>	0.66 (0.07)
Anterior vs lower (196)	34 (17.3) <sup>c</sup>	0.64 (0.07)
8.8 cutoff		
Reference vs anterior (196)	22 (11.2)	0.71 (0.07)
Reference vs lower (196)	28 (14.3)	0.65 (0.07)
Anterior vs lower (196)	20 (10.2)	0.74 (0.07)

<sup>a</sup>P = 0.06 vs 8.8 kPa cutoff; <sup>b</sup>P = 0.50 vs 8.8 kPa cutoff; <sup>c</sup>P = 0.04 vs 7.1 kPa cutoff.

Using an 8.8 kPa cutoff, there was no difference between the prevalence of non-advanced fibrosis using the reference position (226/306, 74%) than the anterior position (275/357, 77%; P = 0.34).

### Concordance between positions

The discordance rates and strength of concordance between LSM assessed in three positions are detailed in Table 3. The discordance rate between the anterior and the lower probe positions was higher (17.3%) when the 7.1 kPa cutoff was used to define advanced fibrosis, instead of 8.8 kPa (10.2%; P = 0.04), and for the anterior vs the reference position (17.9% vs 11.3%; P = 0.06). There was no significant operator effect.

The factors significantly associated with discordance between the reference and the anterior positions were thoracic fold (P = 0.0008) thickness and non-alcoholic fatty liver disease (NAFLD) as the cause of liver disease (P = 0.008) (Table 4). BMI (P = 0.02), abdominal (P = 0.03) and waist circumference (0.047), and SteatoTest (P = 0.04) were not significantly associated when protected for multiple statistical comparisons (Table 4). In multivariate analysis, only thoracic fold was significantly associated with position discordance (regression coefficient beta= 0.07; 95% CI 0.02-0.13; P = 0.01). Same results were observed in the population with three positions applicable.

### Concordance with FT

Discordance rates and strength of concordance between LSM assessed in three positions and FT are detailed in Table 5. There were no significant differences between the discordances rates and the strength of concordance between the three probe positions at a sufficient P value protected for multiple testing.

The discordances rates between probe positions and FT were higher when the 7.1 kPa cutoff was used to define advanced fibrosis instead of 8.8 kPa for the

**Table 4 Factors associated with discordance between reference and anterior positions n (%)**

Characteristics	Concordant	Discordant	P
Number of patients	221	47	
Mean age (SE)	47 (1)	49 (2)	0.16
Male	151 (68)	34 (72)	0.59
Ethnic origin			
Caucasian	134 (61)	30 (64)	0.69
Asian	16 (7)	4 (8)	0.68
North African	27 (12)	6 (13)	0.87
Other African	44 (20)	7 (15)	0.38
Anthropometric data			
Height (m)	1.71 (0.01)	1.71 (0.10)	0.96
Weight (kg)	69.8 (0.9)	73.6 (2.2)	0.12
BMI (kg/m <sup>2</sup> )	23.7 (0.2)	25.0 (0.5)	0.02
Abdominal fold (mm)	19.2 (0.6)	23.0 (1.6)	0.03
Thoracic fold (mm)	11.4 (0.4)	14.5 (0.9)	0.001
Waist circumference (cm)	82 (1)	86 (2)	0.047
Daily alcohol ≥ 30 g/d	18/137 (13)	2/23 (9)	0.55
Diagnosis			
HCV	102 (46)	18 (38)	0.29
HBV	44 (20)	9 (19)	0.88
NAFLD	21 (10)	11 (23)	0.002
HIV coinfection	29 (13)	5 (11)	0.63
ALD	8 (4)	0 (0)	0.16
Other	46 (20)	9 (19)	0.88
Biochemistry			
ALT (IU/L)	55 (3) n = 221	81 (19) n = 47	0.56
AST (IU/L)	47 (2) n = 221	57 (6) n = 47	0.54
Cholesterol (mmol/L)	4.63 (0.08) n = 186	4.85 (0.13) n = 42	0.06
Glucose (mmol/L)	5.26 (0.09) n = 197	5.42 (0.17) n = 46	0.15
Triglycerides (mmol/L)	1.15 (0.06) n = 186	1.37 (0.19) n = 42	0.10
FibroTest	0.40 (0.01) n = 221	0.41 (0.06) n = 47	0.76
ActiTTest	0.32 (0.02) n = 221	0.34 (0.04) n = 18	0.59
SteatoTest	0.30 (0.01) n = 183	0.38 (0.04) n = 42	0.04

reference position (33.6% vs 23.5%, P = 0.03) in the 196 patients with all 3 positions applicable and also among the 306 patients with only the reference position applicable (34.9% vs 26.8%, P = 0.03).

The mean of the 3 positions (a total of 30 LSM), did not increase the strength of concordance with FT.

## DISCUSSION

This study provides an improved assessment of the variability of LSM due to the position of the probe in the right liver lobe. We confirmed the preliminary results we had observed in 35 healthy subjects, in whom 9 different positions had been assessed<sup>[18]</sup>.

The diagnostic value of LSM and FT has been validated in the most common chronic liver diseases and FT has shown at least a similar prognostic value as liver biopsy (which is also an imperfect gold-standard<sup>[24]</sup>) in patients with chronic hepatitis C<sup>[25]</sup> and B<sup>[26]</sup>. We demonstrated previously that the strength of concordance between LSM and FT could be used to identify LSM

**Table 5 Strength of concordance between LSM and FibroTest (FT) according to positions**

Position (No. of patients)	Method assessing		Kappa
	Discordance rate (%)		
Quantitative concordance	Spearman mean (95% CI)	Intra class coefficient mean (95% CI)	
All positions applicable			
Reference (196)	0.46 (0.34-0.56)	0.55 (0.33-0.67)	
Anterior (196)	0.46 (0.34-0.56)	0.56 (0.34-0.68)	
Lower (196)	0.40 (0.27-0.51)	0.50 (0.38-0.62)	
Mean of positions (196)	0.47 (0.35-0.57)	0.56 (0.34-0.68)	
At least one position applicable			
Reference (306)	0.44 (0.35-0.53)	0.51 (0.39-0.63)	
Anterior (357)	0.46 (0.38-0.54)	0.54 (0.32-0.66)	
Lower (302)	0.39 (0.29-0.49)	0.50 (0.38-0.62)	
Two classes concordance	Discordance rate n (%)	Kappa mean (SE)	
Cutoff 7.1 kPa			
All positions applicable			
Reference (196)	66 (33.6) <sup>a</sup>	0.30 (0.07)	
Anterior (196)	61 (31.1)	0.32 (0.07)	
Lower (196)	71 (36.2)	0.24 (0.07)	
Mean of positions (196)	67 (34.2)	0.28 (0.07)	
At least one position applicable			
Reference (306)	107 (34.9) <sup>b</sup>	0.28 (0.06)	
Anterior (357)	112 (31.6)	0.33 (0.05)	
Lower (302)	109 (36.1) <sup>c</sup>	0.24 (0.06)	
Cutoff 8.8 kPa			
All positions applicable			
Reference (196)	46 (23.5) <sup>a</sup>	0.45 (0.07)	
Anterior (196)	54 (27.6)	0.33 (0.07)	
Lower (196)	56 (28.6)	0.34 (0.07)	
Mean of positions (196)	52 (26.5)	0.37 (0.07)	
At least one position applicable			
Reference (306)	82 (26.8) <sup>b</sup>	0.38 (0.06)	
Anterior (357)	108 (30.2)	0.30 (0.05)	
Lower (302)	84 (27.8) <sup>c</sup>	0.34 (0.06)	

<sup>a,b,c</sup>P = 0.03 between 7.1 and 8.8 kPa.

variability factors<sup>[27]</sup>.

The results strongly suggest that the reference position for LSM has two weaknesses in comparison with a more anterior position: a significantly lower applicability and a possible higher variability for the diagnosis of advanced fibrosis using the 7.1 kPa cutoff. The third position analyzed at a lower level compared to the reference position had no advantage either in terms of applicability or in strength of concordance with FT.

The main significant weakness of the reference position in this population was the low applicability rate: 69% compared to 81% in the anterior position. There may be several explanations for the difference in this rate compared to the rates observed in the largest series already published.

Firstly, most Fibroscan validation studies do not apply the strict recommendations for applicability. Foucher *et al*<sup>[11]</sup> achieved in 758 patients a 93.8% applicability rate but used weak criteria: less than 5 valid measurements and a success rate lower than 30%, without taking into account the IQR/median percentage. Kettaneh *et al*<sup>[12]</sup> obtained, in 935 patients, 10 LSM in 91.6%, and did not

specify the rate of patients with a success rate lower than 60% and with an IQR/median higher than 30%. In applying only the criteria of 10 valid measurements, we also observed in the present study 95% and 100% applicability for the two operators. There is a major risk of false positive or false negative conclusions for the diagnosis of advanced fibrosis if LSM results with a low success rate or a high dispersion (IQR) are interpreted<sup>[19]</sup>.

Secondly, the design of the present study was to start at the usual position for liver biopsy and then move to a more anterior and then to a lower position. Skilled operators probably automatically make the small change of position, when they are not satisfied with the first LSM results. Our results suggest that they must probably start with the anterior position first.

The mean LSM was significantly lower (0.5 kPa) at the anterior position *vs* the reference position. This difference was also clinically significant. When using the anterior position instead of the reference position, 7% of patients changed status from advanced fibrosis to non-advanced fibrosis when a cutoff of 7.1 kPa was chosen. The difference of 0.5 kPa is particularly clinically relevant in the zone of 7 to 9 kPa for the risk of a false negative/positive diagnosis of advanced fibrosis; it is less relevant for the diagnosis of cirrhosis as LSM cutoffs are usually recommended at a 12.5 kPa or 14 kPa cutoff with a range to 75 kPa. From these data it is possible to say that the reference position using 7.7 kPa cutoff for F2 and 8.8 kPa for F3 increases the risk of false positive conclusions in comparison with 8.8 and 12.5 kPa cutoffs, respectively.

Several anthropometric factors were associated with discordance between the reference and the anterior positions but the most significant factor was the thoracic skin fold thickness. More studies must now be conducted to better understand the role of these anthropometric factors both with regard to the applicability and to the variability of LSM.

Improved knowledge of LSM variability is also important for the definition of normal values of LSM. In contrast to FT, very few studies have assessed the normal range of LSM with biopsy without fibrosis (F0 in the METAVIR scoring system). Roulot *et al*<sup>[10]</sup> proposed the 95% percentile of a healthy non-obese population as the upper normal limit; 7.8 for females and 8.0 kPa for males. If these definitions of normal range are widely validated, the usual recommended cutoffs values of 7.1 and even 8.8 kPa for stage F2 must be re-assessed, as well the performance of Fibroscan to identify the F1 stage.

Before attributing the observed variability to a specific position, the following confounding factors must be discussed: an order effect, an operator effect, and another factor associated with LSM variability such as skin fold thickness or steatosis.

The order of LSM measurements began with the reference position first, followed by the lower and finally the anterior position. There was no systematic order effect for applicability rates or strength of concordance estimates and this bias can be excluded.

Two operators participated in the present study. The study was not designed as an inter-operator study and

therefore the two operators measured LSM in different patients. There was a difference between operators for the applicability rate of the reference position due to a lower IQR30 percentage. This was not a systematic operator effect, as this lower IQR30 percentage was not observed for the anterior or the lower positions. Operator 1 had twice as many NAFLD patients [50/329 (15.2%)] as operator 2 [9/113 (8%)]  $P = 0.05$ , which could explain the greater variability of LSM and lower applicability in comparison to operator 2. As with other authors<sup>[7,12,15,16]</sup>, we previously observed that the non-applicability and the variability of LSM at the reference position were higher in patients with NAFLD vs non-NAFLD patients.

We acknowledge that the number of comparisons increased the risk of false positives. The comparison between the strength of concordance anterior position-FT and the strength of concordance reference position-FT did not reach a high statistical significance ( $P < 0.01$ ).

However, all the comparisons indicated the same direction and at least a lower concordance of the anterior position with FT in comparison with the reference position can be excluded.

In conclusion, our results suggest that the anterior position of the probe, 2-3 cm ahead of the usual position of liver biopsy, should be the first choice for LSM using FibroScan for liver fibrosis estimates. Compared with the reference position, the anterior position improved the applicability of FibroScan without decreasing its concordance with FibroTest.

## COMMENTS

### Background

Liver fibrosis describes the phenomenon of scarification of the liver tissue in chronic liver diseases. The common final path of chronic liver damage is liver cirrhosis with a high morbidity and mortality and the risk of developing liver cancer. Liver biopsy has always been the traditional gold-standard to "measure" liver fibrosis. Recently, new non-invasive methods of measurement such as serum markers (i.e. FibroTest®) or elastometry (FibroScan®) have emerged to replace liver biopsy in the clinical setting.

### Research frontiers

Liver stiffness measurement (LSM) by transient elastometry is now widely used in countries where the method is accessible and approved. However, the best location to perform the liver elastometry has not been identified and recommendations are derived from an estimated "best spot" defined in an earlier study. In this study, the authors showed that applicability rates may change in a significant way depending where the measurements are done and that results tend to vary.

### Innovations and breakthroughs

This is the first systematic research to identify applicability and variability limits of transient elastometry. The study has shown that applicability rates are higher when a more anterior position is chosen compared to the recommended position. A trend towards higher fibrosis rates measured in the recommended position compared to the anterior position has also been seen in this study. Moreover, the LSM results were compared with a well established biomarker (FibroTest®) and the anterior position was not inferior with regard to concordance rates.

### Applications

This study implies that FibroScan® examinations reach higher applicability rates when performed at a more anterior position than the hitherto recommended position.

### Terminology

Liver fibrosis is the process of collagen septa production in the hepatic tissue.

Liver biopsy gives a pathologist the possibility to examine the liver tissue but samples are potentially too small. Transient elastometry uses the change in liver stiffness due to collagen content to estimate the liver damage. Biomarkers use direct or indirect blood markers associated with liver fibrosis.

### Peer review

This study arouses interest for readers and provides an important clue to evaluate liver stiffness by using non-invasive probe techniques. Liver biopsy is day-after-day decreasing in importance when dealing with patients suffering from HCV-related chronic hepatitis mainly because all in all the therapeutic approach does not change. In this light the elastography plays a new extraordinary role and the present study contributes to its better applicability, defining rigorously the positions of the probe.

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# Peptic ulcer and childhood adversities experienced by working-aged people

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varied between < 0.001 and 0.003). Age- and sex-adjusted odds ratios (OR) of childhood adversities in the multivariate logistic analysis for self-reported peptic ulcer varied between 1.45 and 2.01. Adjusting for smoking, heavy drinking, stress and nonsteroidal anti-inflammatory drug use had no further influence (ORs between 1.22 and 1.73).

**CONCLUSION:** Our findings suggest that childhood adversities maintain a connection with and have a predictive role in the development of peptic ulcer.

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**Key words:** Peptic ulcer; Working-aged; Childhood adversities; Stress factors; Predictive role

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## Abstract

**AIM:** To study the association between self-reported peptic ulcer and childhood adversities.

**METHODS:** The Health and Social Support Study (HeSSup) population consisted of a stratified random sample drawn from the Finnish Population Register in four age groups: 20-24, 30-34, 40-44 and 50-54. The survey was carried out by postal questionnaire during 1998, with a response rate of 40.0%. A follow-up questionnaire was sent during 2003 to all those who responded to the first. Altogether 19626 individuals returned the follow-up questionnaire; a response rate of 75.8%. The subjects were asked whether a doctor had told them that they have or have had peptic ulcer. The analyses covered those who responded affirmatively to both the baseline and the follow-up enquiries ( $n = 718$ ). Those not reporting a peptic ulcer in either of the two questionnaires ( $n = 17677$ ) were taken as controls. The subjects were further requested (through six questions) to think about their childhood adversities.

**RESULTS:** The most common adversities mentioned were long-lasting financial difficulties in the family, serious conflicts in the family, and a family member seriously or chronically ill. All the adversities reported, except parental divorce, were more common among peptic ulcer patients than among controls ( $P$  values

## INTRODUCTION

*Helicobacter pylori* (*H pylori*), nonsteroidal anti-inflammatory drugs (NSAID) and smoking are the most important risk factors for peptic ulcer<sup>[1]</sup>. Infection with *H pylori* causes most duodenal ulcers (95%) and gastric ulcers (70%)<sup>[2]</sup>. Between 89% and 95% of peptic ulcer-related serious upper gastrointestinal events may be attributed to NSAID use, *H pylori* infection and cigarette smoking. Moreover, there is synergism for the development of peptic ulcer and ulcer bleeding between *H pylori* infection and NSAID use<sup>[3]</sup>. Alcohol intake may also play a role in the development of gastric ulcers<sup>[4]</sup>.

Between 5% and 20% of patients with gastric or duodenal ulcers lack an identifiable organic etiology<sup>[5]</sup>. Psychological stress may also have an impact on the onset and course of ulcer disease<sup>[6]</sup>. Moreover, various types of stress may play a role in the onset and modulation of acute or chronic peptic ulcer disease<sup>[7]</sup>. Persons who perceive their lives as stressful may run an increased risk of

developing peptic ulcer disease<sup>[8]</sup>. In low socioeconomic populations psychological stress and health risk behaviours contribute to an increased risk of ulcer<sup>[9]</sup>. However, familial aggregation of the disease is modest, according to a population-based twin cohort study<sup>[10]</sup>.

Childhood is an important and vulnerable period in individual development. Early life experiences are particularly important in determining how the individual responds over the life course<sup>[11]</sup>. However, very little is known as to how childhood adversities concerning financial problems, conflicts in the family, problems with alcohol, and matters of personal security are associated with peptic ulcer. We now examine this aspect in a nationwide sample in Finland, enquiring whether there has been something particular in the social circumstances of peptic ulcer patients during childhood. Do childhood adversities have a connection with the development of peptic ulcer in adulthood?

## MATERIALS AND METHODS

The Health and Social Support Study (HeSSup) is a prospective etiological follow-up study of the psychosocial health of the Finnish working-aged population. The HeSSup population is comprised of a stratified random sample drawn from the Finnish Population Register in four age groups: 20-24, 30-34, 40-44 and 50-54. The survey was carried out by postal questionnaire during 1998. Forms were returned by 25 898 individuals; a response rate of 40.0%. A follow-up was sent during 2003 to all those who responded to the first questionnaire. Altogether 19 626 individuals returned the follow-up questionnaire; a response rate of 75.8%. The Turku University Central Hospital Ethics Committee had approved the study.

The sample in 1998 was subjected to a thorough analysis of non-response<sup>[12]</sup>. The analysis was made using the official statistics of the Finnish population for the respective age groups in 1998 to assess whether the study population adequately represented the Finnish population. The major reason for refusal to participate was suspicion as to the purpose behind the request for written consent. Less educated, divorced, widowed and unemployed recipients and those on disability pension were least willing to participate. Differences in physical condition between the study participants and the general population were, however, small.

The participants were asked whether or not a doctor had told them that they have or have had a peptic ulcer. The analyses focused on those who responded affirmatively to both the baseline and the follow-up questionnaire ( $n = 718$ ). Those who did not report a peptic ulcer in either questionnaire ( $n = 17677$ ) were taken as the control group. It is noteworthy that those administered a new peptic ulcer during the five-year period were not included in the analyses.

The participants were asked to think about their childhood adversities in terms of the following questions, most of them successfully used in a nationwide interview

**Table 1 Kappa coefficients of responses between questionnaires in 1998 and 2003**

	$\kappa$ (95% CI)	
	Peptic ulcer	No peptic ulcer
Parents divorced	0.94 (0.91-0.98)	0.90 (0.89-0.91)
Long-lasting financial difficulties in the family	0.72 (0.64-0.79)	0.74 (0.73-0.76)
Serious conflicts in the family	0.71 (0.64-0.78)	0.72 (0.71-0.74)
Often afraid of some member of the family	0.64 (0.55-0.73)	0.71 (0.69-0.72)
Someone in the family seriously or chronically ill	0.55 (0.47-0.63)	0.62 (0.61-0.64)
Someone in the family had problems with alcohol	0.82 (0.76-0.87)	0.84 (0.83-0.85)

survey on living conditions<sup>[13]</sup>: (1) "Did your parents divorce?"; (2) "Did your family have long-lasting financial difficulties?"; (3) "Did serious conflicts arise in your family?"; (4) "Were you often afraid of some member of your family?"; (5) "Was someone in the family seriously or chronically ill?"; (6) "Did someone in the family have problems with alcohol?". The alternative answers were Yes, No or I do not know. Only the first two options were included in the statistical analyses. The number of childhood adversities per individual was determined. Childhood in the questionnaires, however, was not defined according to age. To confirm the reliability of the answers concerning childhood adversities, the kappa coefficient was used to assess associations between the questionnaires in 1998 and 2003. The coefficient varied between 0.55 and 0.94 among those reporting peptic ulcer and between 0.62 and 0.90 among controls (Table 1).

Participants were asked whether or not they had ever smoked. Smokers were considered those who currently smoked at least five cigarettes daily. Doses and frequency of alcohol use were also asked about and the total amount of alcohol consumed was calculated. Consumption of alcohol drinking was calculated, and at least 175 g alcohol during a week among women and at least 263 g among men was rated as heavy drinking. These limits were set because women achieve higher blood alcohol concentrations than men after drinking equivalent amounts of alcohol.

The Reeder stress inventory<sup>[14]</sup>, a 4-item questionnaire instrument previously widely used, was applied to measure the general sense of stressfulness in daily life. The inventory comprises the following four statements: (1) "In general I am usually tense or nervous"; (2) "There is a great amount of nervous strain connected with my daily activities"; (3) "At the end of the day I am completely exhausted mentally and physically"; and (4) "My daily activities are extremely trying and stressful." Participants indicate the extent to which each statement applies to them using a 5-point Likert scale (0-4). The mean value was calculated and multiplied by four, the computed value thus indicating the amount of stress. The proportion of those having a stress point higher than the upper quartile ( $\geq 12$ ) was calculated.

**Table 2** Occurrence of childhood adversities among peptic ulcer patients and controls

	Peptic ulcer (n = 604-673, %)	No peptic ulcer (n = 15 545-17 188, %)	Difference (%-units)	P value
Long-lasting financial difficulties in the family	45.2	26.8	18.4	< 0.001
Someone in the family seriously or chronically ill	42.3	26.6	15.7	< 0.001
Serious conflicts in the family	32.7	27.2	5.5	0.003
Someone in the family had problems with alcohol	30.2	25.1	5.1	0.003
Often afraid of some member of the family	19.6	13.4	6.2	< 0.001
Parents divorced	18.0	16.0	2.0	0.173

**Table 3** Age distribution and occurrence of risk factors among peptic ulcer patients and controls

	Women		P value	Men		P value
	Peptic ulcer (n = 344-362, %)	No peptic ulcer (n = 10 233-10 975, %)		Peptic ulcer (n = 326-350, %)	No peptic ulcer (n = 5 970-6 667, %)	
Age group (yr)						
25-29	8.5	28.7		4.0	22.6	
35-39	19.5	23.4		8.5	23.2	
45-49	25.3	24.3		26.8	25.2	
55-59	46.7	23.6		60.7	29.0	
Smoking						
Current smoking, ≥ 5 cigarettes daily	29.7	16.6	< 0.001	37.7	22.8	< 0.001
Heavy drinking						
> 175 g alcohol/wk	5.0	3.8	0.253	10.3	7.7	0.080
> 263 g alcohol/wk						
Stress						
Reeder points ≥ 12	32.9	20.9	< 0.001	32.8	22.8	< 0.001
Use of nonsteroidal anti-inflammatory drugs						
At least 2 mo during the last year	33.2	16.2	< 0.001	21.9	9.3	< 0.001

Regarding the matter of NSAID use, participants were asked how often they had used them during the last year. The alternative answers were "not at all", "less than 10 d", "10-59 d", "2-6 mo" and "more than 6 mo". In the analyses at least two months' consumption during the last year was taken to indicate NSAID use.

The analyses were made using the SAS System for Windows, release 9.1. Statistical significance for the occurrence of adversities was tested with  $\chi^2$ -test. The results were considered significant at  $P < 0.05$ . Age- and sex-adjusted odds ratios (OR) in the logistic regression analysis were calculated (Model 1). The same analyses were carried out with adjustment for both age and sex and smoking (Model 2), for age and sex, smoking and heavy drinking (Model 3), for age and sex, smoking, heavy drinking and stress (Model 4), and also for age and sex, smoking, heavy drinking, stress and current NSAID use (Model 5).

## RESULTS

The most common childhood adversities to emerge were long-lasting financial difficulties in the family, serious conflicts in the family and someone in the family having been seriously or chronically ill (Table 2). All adversities reported were more common among peptic ulcer patients than among controls. Alcohol problems in the family and

fear of some member of the family were also more common among peptic ulcer patients than among controls. With regard to parental divorce, there was no statistically significant difference between the two groups.

Among both genders the life-time prevalence of peptic ulcer increased with age, the tendency being, however, more apparent among men than among women. In the oldest age group the life-time prevalence was over 7% among men and nearly 5% among women. Among men, more than half, and among women, almost half, of the peptic ulcer patients belonged to the oldest age group (Table 3).

Current smoking was more common among peptic ulcer patients than among controls (Table 3). With regard to heavy drinking, there were no differences between ulcer patients and controls. The proportion of subjects scoring Reeder's stress points more than the upper quartile was greater among patients than among controls, the difference being ten percentage units among both genders. NSAID use was twice as common among patients as among controls.

The total number of childhood adversities per individual was greater among peptic ulcer patients than among controls. At least two adversities had been experienced by 46% of patients vs 33% of controls. No adversity had been experienced by 28% of patients vs 43% of controls.

**Table 4** Childhood adversities in the multivariate logistic analysis for peptic ulcer

	OR (95% CI)				
	Model 1	Model 2	Model 3	Model 4	Model 5
Long-lasting financial difficulties in the family	2.01 (1.71-2.38)	1.90 (1.60-2.26)	1.88 (1.58-2.23)	1.77 (1.48-2.11)	1.73 (1.45-2.07)
Someone in the family seriously or chronically ill	1.67 (1.42-1.96)	1.66 (1.41-1.96)	1.64 (1.39-1.94)	1.59 (1.34-1.88)	1.53 (1.29-1.82)
Serious conflicts in the family	1.47 (1.23-1.75)	1.33 (1.11-1.60)	1.33 (1.11-1.60)	1.24 (1.03-1.50)	1.22 (1.01-1.47)
Someone in the family had problems with alcohol	1.45 (1.22-1.72)	1.37 (1.14-1.63)	1.36 (1.14-1.62)	1.30 (1.09-1.56)	1.27 (1.06-1.52)
Often afraid of some member of the family	1.61 (1.32-1.97)	1.46 (1.18-1.81)	1.44 (1.16-1.78)	1.31 (1.05-1.62)	1.26 (1.01-1.57)
Parents divorced	1.60 (1.30-1.97)	1.40 (1.13-1.74)	1.40 (1.13-1.74)	1.37 (1.10-1.71)	1.37 (1.10-1.71)

Model 1: Age- and sex-adjusted; Model 2: Age- and sex-adjusted and also adjusted for smoking; Model 3: Age- and sex-adjusted and also adjusted for smoking and heavy drinking; Model 4: Age- and sex-adjusted and also adjusted for smoking, heavy drinking and stress; Model 5: Age- and sex-adjusted and also adjusted for smoking, heavy drinking, stress and NSAID use (at least 2 mo during the last year).

Age- and sex-adjusted ORs of childhood adversities for peptic ulcer were statistically significant, indicating that participants with childhood adversities had a higher proportional risk of developing peptic ulcer (Table 4). Adjusting also for smoking, heavy drinking, stress and current NSAID use had no further influence. Long-lasting financial difficulties in the family had the greatest influence.

## DISCUSSION

The principal finding in this study was that individuals reporting peptic ulcer have experienced significantly more childhood adversities than the general population. Life events include not only family problems but also financial problems and those concerning personal security. All of the childhood adversities investigated were statistically significant after adjustment for age. Adjusting for lifestyle issues such as smoking and heavy drinking had a slight influence. It is noteworthy that stress and current NSAID use had no further influence.

Findings in a previous Norwegian study did not support the concept that peptic ulcer is a psychosomatic disorder<sup>[15]</sup>. However, childhood adversities have been found in association with other diseases, for example coronary heart disease<sup>[16]</sup> and migraine<sup>[17]</sup>, and also with disability pensions<sup>[18]</sup>. According to our findings, childhood adversities are also associated with self-reported peptic ulcer. Self-reported childhood trauma has been found to be associated with an increased risk of a range of physical illnesses during adulthood, including peptic ulcer<sup>[19]</sup>. In a Danish study poor socio-economic status emerged as an important risk factor for peptic ulcer disease which exerts its effect independently of *H pylori* infection<sup>[20]</sup>. In functional dyspepsia the role of psychosocial factors continues to be the subject of debate<sup>[21]</sup>. In this respect our findings support those previous studies.

The study material may be considered representative of the Finnish working-aged population, although the response rate in 1998 was only 40%. Careful non-response analysis indicated that respondents and non-respondents were comparable with respect to the most important demographic variables<sup>[12]</sup>. Moreover, the rate of response to the follow-up questionnaire was high.

As it is a question of self-reported peptic ulcer, we

cannot be completely sure whether the subjects really have a pathological condition meeting the diagnostic criteria. Some of the subjects reporting peptic ulcer may only have non-ulcer dyspepsia. However, we regarded as peptic ulcer patients only those reporting the disease in two questionnaires carried out at 5-year intervals.

There was no way of knowing whether the ulcer reported was a duodenal or a stomach ulcer. We did not enquire whether or not the respondents had undergone gastroscopies, and have no data on the possible occurrence of complications. In Finland most uncomplicated peptic ulcers are treated in primary health care. Neither did we ask whether or not the participants had received antibiotic therapy for *H pylori* infection.

It is possible that ulcer patients reported childhood adversities more readily than did controls. Perhaps individuals with psychological stress or distress also tend to report on a peptic ulcer more easily than other people. Although retrospective studies of adverse childhood experiences have a worthwhile place in research<sup>[22]</sup>, we must concede that there are unanswered questions with regard to selective recall, and this may be considered a weakness of our study. The reports of childhood adversities are subjective, based upon memories where the time span from childhood to adulthood is not further defined. This notwithstanding, the kappa coefficients confirming the reliability of the answers may be considered quite high and also alike for the ulcer and non-ulcer groups, indicating that peptic ulcer patients had not remembered childhood adversities more readily than controls.

With regard to lifestyle issues and NSAID use there are some unknown concerns. Although we could calculate the total amount of alcohol consumed, we do not know the amount of cigarettes the respondents had smoked. Neither do we know the daily doses of NSAID use. We therefore considered it reasonable to dichotomize these variables. This may be a confounding factor. In our opinion, however, it hardly had an impact on our findings.

Peptic ulcer has been considered a valuable model for understanding the complex interactions among psychosocial, socioeconomic and infectious factors in causing organic disease<sup>[23]</sup>. Childhood adversities may be associated with lower socioeconomic level and modest living conditions, which may in part increase the risk

of infection by *H pylori*<sup>[24]</sup>. Moreover, children living in the most crowded conditions are at the greatest risk for *H pylori* acquisition<sup>[25]</sup>. Unfortunately our data did not contain details about the respondents' socioeconomic status and housing conditions.

What then are the reasons for the associations between childhood adversities and self-reported peptic ulcer? In this respect childhood adversities may predispose to *H pylori* infection. It is also possible that childhood adversities may predispose to a variety of aches and pains which are alleviated by NSAIDs. We must also admit that we do not know the possible role of genetic factors concerning e.g. the vulnerability of the gastric mucosa. Moreover, vigilant concern and anxiety might affect chronic automatized coping mechanisms and thus stress responses later in life.

Although the occurrence of peptic ulcer has declined due to widespread empirical treatment of dyspepsia, the peptic ulcer patient is still a common client in doctors' offices. Only a few decades ago peptic ulcer was considered almost exclusively a psychosomatic disease. When the association with *H pylori* was discovered in the 1980s the disease was regarded mainly as an infection, and the stress theory fell into oblivion. According to our findings there is reason to believe that stress factors during childhood still have a connection with the development of peptic ulcers. We thus venture to state that there are also social factors in the background. Childhood adversities are not necessarily true risk factors for peptic ulcer, but may play a predictive role in the development of the disease. A more comprehensive understanding of peptic ulcer patients is worth aspiring to.

## COMMENTS

### Background

*Helicobacter pylori* (*H pylori*), nonsteroidal anti-inflammatory drugs (NSAID) and smoking are the most important risk factors for peptic ulcer. However, between 5% and 20% of patients with gastric or duodenal ulcers lack an identifiable organic etiology. Psychological stress may have an impact on the onset and course of ulcer disease. Very little is known as to whether childhood adversities involving financial problems, conflicts in the family, problems with alcohol, and matters of personal security are associated with peptic ulcer.

### Research frontiers

Childhood is an important and vulnerable period in individual development. The research hotspot is whether there has been something particular in the social circumstances of peptic ulcer patients during childhood. Do childhood adversities have a connection with the development of peptic ulcer in adulthood?

### Innovations and breakthroughs

The most common childhood adversities to emerge were long-lasting financial difficulties in the family, serious conflicts in the family and someone in the family having been seriously or chronically ill. Alcohol problems in the family and fear of some member of the family were also more common among peptic ulcer patients than among controls. All of the childhood adversities investigated were statistically significant after adjustment for age. Adjusting for life style issues such as smoking and heavy drinking had only a slight influence.

### Applications

The study results suggest that stress factors during childhood have a connection with the development of peptic ulcers. Childhood adversities may play a predictive role in the development of the disease. A more comprehensive understanding of peptic ulcer patients is worth aspiring to.

### Terminology

Peptic ulcer: An ulcer on the mucous membrane of stomach (gastric ulcer) or

duodenum (duodenal ulcer) that is usually acidic and thus extremely painful. Childhood adversities: Negative life experiences during childhood such as long-lasting financial difficulties, serious conflicts in the family, fear of some family member, someone in the family being seriously or chronically ill, and someone in the family having had problems with alcohol.

### Peer review

This work is well conducted. The results from this work shows that there is a reason to believe that *H pylori* is not the only etiological factor regarding peptic ulcer but others factors like childhood adversities may play a predictive role in the development of peptic ulcer. According to this survey, it is reasonable to believe that stress factors during childhood maintain a connection with the development of peptic ulcers.

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BRIEF ARTICLES

# Cytomegalovirus frequency in neonatal intrahepatic cholestasis determined by serology, histology, immunohistochemistry and PCR

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## Abstract

**AIM:** To determine cytomegalovirus (CMV) frequency in neonatal intrahepatic cholestasis by serology, histological revision (searching for cytomegalic cells), immunohistochemistry, and polymerase chain reaction (PCR), and to verify the relationships among these methods.

**METHODS:** The study comprised 101 non-consecutive infants submitted for hepatic biopsy between March

1982 and December 2005. Serological results were obtained from the patient's files and the other methods were performed on paraffin-embedded liver samples from hepatic biopsies. The following statistical measures were calculated: frequency, sensibility, specific positive predictive value, negative predictive value, and accuracy.

**RESULTS:** The frequencies of positive results were as follows: serology, 7/64 (11%); histological revision, 0/84; immunohistochemistry, 1/44 (2%), and PCR, 6/77 (8%). Only one patient had positive immunohistochemical findings and a positive PCR. The following statistical measures were calculated between PCR and serology: sensitivity, 33.3%; specificity, 88.89%; positive predictive value, 28.57%; negative predictive value, 90.91%; and accuracy, 82.35%.

**CONCLUSION:** The frequency of positive CMV varied among the tests. Serology presented the highest positive frequency. When compared to PCR, the sensitivity and positive predictive value of serology were low.

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**Key words:** Cytomegalovirus; Hepatitis; Neonatal; Cholestasis; Liver; Children; Immunohistochemistry; Polymerase chain reaction

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## INTRODUCTION

The frequency of cholestatic jaundice is difficult to determine, varying between 1:2500 and 1:5000

newborns<sup>[1-3]</sup>. The initial objective in the management of neonatal cholestatic jaundice is to distinguish between intrahepatic and extrahepatic causes, as the latter requires urgent surgical intervention<sup>[4]</sup>. Neonatal intrahepatic cholestasis (NIHC) represents 2/3 of the cases of neonatal cholestasis<sup>[5-9]</sup>. The most common causes of the disease are infection, including cytomegalovirus (CMV)<sup>[10-12]</sup>. Data on the prevalence of CMV in NIHC vary greatly depending on the diagnostic method used (5%-46%)<sup>[2,12-14]</sup>. Congenital CMV infection is assessed by viral isolation detected within 2-3 wk after birth<sup>[15]</sup>. If CMV is detected three weeks after birth, diagnosis of congenital infection should be supported by clinical and epidemiologic features<sup>[12,14,16]</sup>. Congenital disease might occur due to maternal primary infection (where the vertical transmission rate ranges from 40%-50%), or as a recurrence (where the vertical transmission rate ranges from 0.5%-2%). The clinical manifestations are essentially limited to neonates of mothers presenting with a primary infection during pregnancy and include: purpura, intracerebral calcifications, retinitis, ventriculitis, hepatosplenomegaly, microcephaly, intrauterine growth retardation, and jaundice<sup>[15,17-22]</sup>. The general prevalence of congenital CMV infection in Brazil is similar to that reported in studies on highly immune populations<sup>[16,23-27]</sup>. There is a higher rate of congenital infection, but fewer clinical manifestations<sup>[28,29]</sup>.

The purpose of the present study was to establish the frequency of CMV infection in patients with NIHC based on serology (IgM-ELISA), histological revision (searching for cytomegalic cells), immunohistochemistry (IHC), and polymerase chain reaction (PCR) and to verify the relationships among these methods.

## MATERIALS AND METHODS

Data from NIHC patients were evaluated at two tertiary centers between March 1982 and December 2005: the Pediatric Department of the State University of Campinas (Unicamp) Teaching Hospital and at the Children's Institute of the Medical School of the University of São Paulo (USP). A uniform diagnostic approach was followed throughout the observation period.

Cholestasis was defined by laboratory criteria as suggested by Moyer *et al*<sup>[4]</sup>. Inclusion criteria were: jaundice appearing up to three months of age and hepatic biopsy performed during the investigation.

To establish the etiology of NIHC, one author (Bellomo-Brandao) collected the following data: neonate's identification, symptoms, history, clinical findings, physical examination, and results of laboratory testing [alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AlkPhos),  $\gamma$ -glutamyltransferase (GGT), international normalized ratio (INR), direct bilirubin (DB), albumin, liver biopsy, serum  $\alpha$ -1-antitrypsin, sweat sodium and chloride, innate metabolism errors in urine, polymerase chain reaction (PCR), CMV antigenemia, and serology of: CMV, HIV, EBV, rubella, toxoplasmosis, and syphilis].

## CMV serology

An Enzyme-linked immunosorbent assay (ELISA) was employed using commercial kits from Sorin Biomedica (Italy) and Vidas Biomerieux (France) at the Unicamp Teaching Hospital. Cobas Half Core, Roche (Germany) and MEIA, Abbott AxSYM® system (USA) kits were used at USP.

## Histological revision

Histological revision was carried out by one hepatopathologist (CAFE). When necessary, new slides were created and examined. Among the 84 liver biopsies, five were surgical and 79 were percutaneous. The presence of at least one cytomegalic cell was considered suggestive of CMV.

## IHC

Four- $\mu$ m-thick histological sections of the liver biopsies were placed on silanized slides, deparaffinized in xylene, and rehydrated. For antigen retrieval, the slides were immersed in 10 mmol/L citrate buffer (pH 6.0) in a steamer at 90°C for 30 min. A commercially available cocktail of two mouse monoclonal antibodies to CMV was applied to the sections (clones DDG9/CCH2 [prediluted]; Cell Marque, Hot Springs, AR, USA) for 1 h at 37°C and subsequently overnight (18 h) at 4°C. The reaction was amplified using the peroxidase-conjugated Novolink polymer (Novocastra, Newcastle, UK). Staining was achieved using 3,3-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA) and counterstaining with Mayer's hematoxylin. All reactions were performed with appropriate positive and negative controls. The positive control corresponded to a lung fragment from a patient who had died due to a generalized CMV infection. The negative control corresponded to the same specimen, in which a specific anti-CMV antibody was replaced by saline buffer. Positive results consisting of brown-colored nuclei were evaluated using a conventional optical microscope.

## PCR

The recommendations of Kwok and Higuchi<sup>[30]</sup> were followed to prevent sample contamination.

DNA was extracted according Saiki *et al*<sup>[31]</sup>, Shibata *et al*<sup>[32]</sup> and Demmler *et al*<sup>[33]</sup> from formalin-fixed, paraffin-embedded fragments using a commercial kit (Dneasy, Qiagen, Germany). The reaction mixture consisted of 0.5-1  $\mu$ g sample DNA in a total volume of 20  $\mu$ L, containing 50 mmol/L KCl, 10 mmol/L Tris (pH 8.4), 2.5 mmol/L MgCl<sub>2</sub>, 0.1 mmol/L of each primer, 200 mmol/L of deoxyribonucleotide triphosphates (dATP, dCTP, dGTP, and dTTP) and 2 U of *Taq* DNA polymerase. Water was used to complete the total reaction volume. The mixture was covered with a drop of mineral oil.

Amplifications were carried out in a DNA thermocycler (PTC100; MJ Research, Inc., Watertown, MA, USA) using 30-35 cycles for each sample (94°C for 45 s, 55°C for 45 s, and 72°C for 1 min). The cycles were preceded by an initial denaturation at 94°C for 5 min and

were followed by a final extension for 7 min at 72°C.

The human β-globin gene was amplified as an internal control for the reaction<sup>[32]</sup>. Primers for detection of the β-globin gene were as follows: PCO3 (5' CTCTGACAC AACTGTGTTCACTAGC 3') and PCO4 (5' TCACCAC CAACTTCATCCACGTTCAC 3').

CMV was detected by PCR and nested-PCR<sup>[32-35]</sup>. External primers were as follows: MIE4 (CCAAGCGGC CTCTGATAACCAAGGCC) and MIE5 (CAGCACCA TCCTCCTCTCCTCTGG); internal primers were as follows: IE1 (CCACCCGTGGTGCCAGCTCC) and IE2 (CCCGCTCCTCCTGAGCACCC). AD169 DNA strain was used as a positive control and water was used as a negative control.

Both β-globin and nested PCR products were visualized on ethidium bromide-stained, 2% agarose gels, after electrophoresis.

### Ethical aspects

The present research study was approved by the Medical Research Ethics Committees of both institutions. Informed consent was not needed, because serologies and liver biopsies had been done during the investigation process.

### Statistical analysis

The frequencies of CMV positive results of each test were calculated. Sensitivity, specificity, positive predictive value, negative predictive value, and accuracy were calculated between the results obtained with PCR and serology<sup>[36]</sup>.

## RESULTS

One hundred one non-consecutive patients with NIHC were included (84 patients from Unicamp and 17 patients from USP). Sixty-nine patients were males and 32 patients were females. The median age at the time of the biopsy was two months and 14 d.

The etiologies of NIHC are presented in Table 1. Most of them had an idiopathic etiology (58%). Five patients were previously diagnosed with CMV infection based on serology, PCR (plasma) and antigenemia.

This was a retrospective study and there was a paucity of liver biopsy fragments (most of the biopsies were obtained percutaneously); therefore, it was not possible to perform all four tests in all patients. In 17 patients, histological revision was not done because a shortage of paraffinized sample material did not permit making new slides. In the remaining 84 cases, the pathologist did not observe the presence of cytomegalic cells. Only one of 44 patients had positive IHC, 6/77 had positive PCR and 7/64 were IgM-ELISA positive. Table 2 shows the results of IgM-ELISA, histological revision, IHC, and PCR and the number of patients submitted to each diagnostic method.

Table 3 presents the clinical and laboratory data of PCR-positive patients. Five of six patients birth weights > 2500 g. Two of them had positive IgM-ELISA: one had a previous diagnosis of cystic fibrosis and the other had

**Table 1 Etiologic diagnoses of neonatal intrahepatic cholestasis (NIHC)**

Etiology	Number of cases
Neonatal sepsis	9
Cytomegalovirus	5
Urinary infection tract	4
Syphilis	2
Toxoplasmosis	2
α1-antitripsin deficiency	5
Other metabolic diseases	5
Galactosemia	4
Allagile's syndrome	2
Byler's disease	1
Cystic fibrosis	1
Secondary to use of parenteral nutrition	1
Down's syndrome	1
Panhypopituitarism	1
Idiopathic	58
Total	101

**Table 2 Serological results (IgM-ELISA), presence of cytomegalic cells (histological analysis), IHC, and PCR**

Methods	Positive	n <sup>1</sup> (%)
IgM-ELISA	7	64 (11)
Histological analysis	0	84 (0)
IHC	1	44 (2)
PCR	6	77 (8)

<sup>1</sup>Number of patients submitted to each diagnostic method.

Byler's Disease. Figure 1 shows the immunostaining of the liver in a positive case, consisting of brown colored nuclei, and Figure 2 exemplifies the PCR for CMV.

Compared to PCR, serology was highly accurate (82.35%), specific (88.89%) and had a high negative predictive value (90.01) for detecting CMV infection. However, its sensitivity was 33.3% and its positive predictive value was 28.57%. Table 4 shows the results of PCR and serology (IgM-ELISA) and the number of patients tested. Table 5 shows the results of sensitivity, specificity, positive predictive value (PV+), negative predictive value (PV-), and the accuracy between PCR and serology.

## DISCUSSION

The ubiquitous nature of CMV makes for difficulties in establishing direct evidence of the actual role of CMV in neonatal cholestasis, as stated in the editorial of Persing and Rakela<sup>[34]</sup>: "When does the detection of an infectious agent become unequivocally the etiologic agent of a specific condition?" Detection of the virus from the liver provides strong evidence that neonatal cholestasis is caused by CMV infection<sup>[15]</sup>.

Histological searching for cytomegalic cells was negative in 84 patients. Lurie *et al*<sup>[35]</sup>, after studying four fatal cases of NIHC from CMV, described the presence of cytomegalic cells in the liver from two of them. None were detected in the present study or observed in cases of extrahepatic cholestasis (EHC)<sup>[36-38]</sup>. In CMV hepatitis following orthotopic liver transplantation

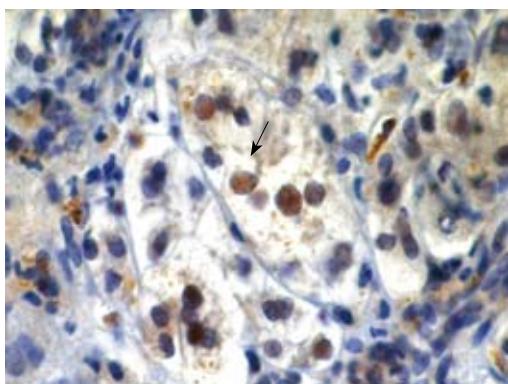
**Table 3 Clinical and laboratory data of CMV-positive patients by PCR: gender, diagnosis, weight at birth, serology (IgM-ELISA), IHC, and presence of cytomegalic cells**

Patients	Gender	Diagnosis	Weight at birth (g)	IgM-ELISA	IHC	Cytomegalic cell
1	M	Cystic fibrosis	3460	Positive	-	Absent
2	F	Idiopathic	2500	Negative	Negative	Absent
3	M	Byler's disease	3320	Positive	Positive	Absent
4	M	Idiopathic	2700	Negative	-	Absent
5	F	Idiopathic	1500	Negative	Negative	Absent
6	M	Neonatal sepsis	2570	Negative	-	Absent

-: Not performed.

**Table 4 Results of PCR and serology (IgM-ELISA) and number of patients tested**

IgM-ELISA	PCR		
	+	-	Total
+	2	5	7
-	4	40	44
Total	6	45	51

**Figure 1 Immunohistochemistry of a positive case, consisting of brown colored nuclei (arrow), using a conventional optical microscope ( $\times 640$ ).**

(immunosuppressed patients), demonstration of cytomegalic inclusion bodies in hematoxylin and eosin sections is sufficient for a diagnosis of CMV hepatitis<sup>[39]</sup>.

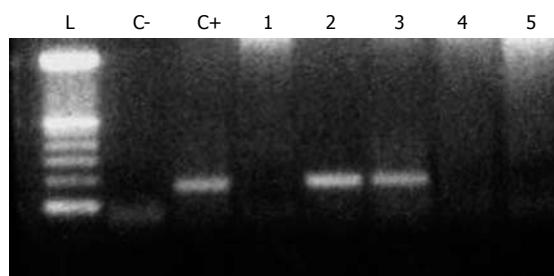
Only one patient had positive immunostaining and a positive PCR, despite encouraging data in immunosuppressed patients<sup>[40,41]</sup>. There are no reports in the medical literature in which the IHC method has been used to identify CMV in NIHC.

In the literature, the frequency of positive CMV in NIHC by PCR techniques varies between 5% and 46%<sup>[14,42,43]</sup>. Different techniques and samples make it difficult to establish a correlation between the data. In the present study, PCR was positive in 8% of the patients (6/77). Three patients had been previously diagnosed with other defined etiologies, indicating that concomitant infection by CMV and other agents is possible. This finding suggests that multiple agents must be investigated in the search for a diagnosis of NIHC. Four of these six cases presented with a negative IgM-ELISA, probably due to immaturity of the neonatal immune system<sup>[17,44]</sup>.

Few studies have been carried out comparing the different diagnostic methods of CMV infection in

**Table 5 Results of positive predictive value (PV+), negative predictive value (PV-), accuracy, sensitivity, and specificity between PCR and serology with 95% confidence intervals (CI)**

	Percent	CI (95%)	
		Inferior limit	Superior limit
Sensitivity	33.3	6.00	75.89
Specificity	88.89	75.15	95.84
PV+	28.57	5.11	69.74
PV-	90.91	77.42	97.05
Accuracy	82.35	68.64	91.13

**Figure 2 HCMV DNA results of 2% agarose gel electrophoresis under ultraviolet light (PCR). L: Ladder; C+: Positive control; C-: Negative control; 1, 4 and 5: Negative samples; 2 and 3: Positive samples.**

neonatal cholestasis. Fischler *et al*<sup>[45]</sup> found positive serology for IgM-CMV in 32% of cases tested. In another study<sup>[6]</sup>, the same author found IgM-CMV and/or the presence of positive CMV in the urinary samples of 19/54 (35%) patients with NIHC and positive results in 4/11 carriers of  $\alpha$ -1-antitrypsin deficiency. A retrospective analysis of 39 patients (who presented with CMV in blood or urine cultures, or positive PCR in liver biopsies collected between 30 and 220 d of life) showed the presence of CMV by PCR-CMV on filter papers collected during the first three days of life in two neonates, suggesting that congenital infection was not the cause of cholestasis<sup>[42]</sup>.

Chang *et al*<sup>[14]</sup> studied 50 children diagnosed with neonatal hepatitis by PCR-CMV of liver samples. Twenty-three children had positive PCR, 13 of them had serology suggestive of an acute infection, and nine children had negative serologies; in one case serology was unknown. Twenty-one of the 27 patients with a negative PCR presented with negative serologies and six had positive serologies.

Shibata *et al*<sup>[43]</sup> evaluated 26 non-cholestatic infants

(1-24 mo old), who presented with an increase in ALT, through a quantitative PCR technique on their plasma. CMV was positive in four patients (15.4%). Three patients who presented with positive IgM-CMV did not have a positive PCR.

These studies were conducted in areas that were considered to have a low prevalence of CMV<sup>[15,46]</sup>. In Brazil, infection due to CMV is highly prevalent in the population and occurs early in the first year of life<sup>[25,26,27]</sup>. In populations where the majority of women of reproductive age have antibodies against CMV, there is a higher rate of congenital infection (as compared to populations with a low prevalence of antibodies against CMV), but without clinical disease. Although the presence of maternal antibodies does not prevent CMV transmission, it appears to confer protection or may be a marker of another factor that protects the child<sup>[28,29]</sup>.

In the present study, compared to PCR, the sensitivity and positive predictive value of IgM-ELISA serology were low, whereas specificity, negative predictive value and accuracy were high.

In conclusion, the frequencies of positive results for CMV varied from 0 (searching for cytomegalic cells) to 11% (IgM-ELISA). Disparities in serology and PCR were observed in the present study, as well as in other studies using different PCR techniques. Although our data are not encouraging, it will be necessary to conduct new studies to establish the role of IHC in the diagnosis of CMV in neonatal cholestasis. Even if there was a previous diagnosis, the involvement of CMV should be determined. All results should be interpreted considering the sum of clinical and epidemiologic features.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

The ubiquitous nature of cytomegalovirus (CMV) makes for difficulties in establishing direct evidence of the actual role of CMV in neonatal cholestasis. The aim of this study was to determine the CMV frequency in neonatal cholestasis and to compare results of different diagnostic tests: serology, histology, immunohistochemistry, and polymerase chain reaction.

### Research frontiers

CMV is one of the most common causes of neonatal intrahepatic cholestasis, but the CMV best diagnostic criteria are not yet established because the positivity of different diagnostic tests varies considerably.

### Innovations and breakthroughs

In the present study, compared to polymerase chain reaction (PCR), the sensitivity and positive predictive value of IgM-ELISA serology were low, whereas specificity, negative predictive value and accuracy were high. Only one patient had positive immunostaining and a positive PCR. There are no previous reports in the medical literature on the use of immunohistochemistry to identify CMV in neonatal hepatitis.

### Applications

Even if there was a previous diagnosis, the involvement of CMV should be determined. It will be helpful to establish the role of CMV in neonatal cholestasis.

### Terminology

Serology, histology, immunohistochemistry, and polymerase chain reaction could be used in the differential diagnosis of CMV in neonatal cholestasis; however, the results should be interpreted considering the sum of clinical and epidemiologic features.

### Peer review

The study by Bellomo-Branda et al investigated the relationship between the infection with cytomegalovirus and the appearance of neonatal intrahepatic cholestasis. The aims of this study are very appealing. The study is well designed and the methods appropriate.

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# Hepatic angiomyolipoma: Dynamic computed tomography features and clinical correlation

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## Abstract

**AIM:** To study the dynamic computed tomography (CT) features of hepatic angiomyolipoma (AML) in patients with or without tuberous sclerosis complex (TSC).

**METHODS:** The clinical information, CT findings and histopathological results of hepatic AML were analyzed retrospectively in 10 patients.

**RESULTS:** Hepatic AML was prone to occur in female patients (7/10), and most of the patients (8/10) had no specific symptoms. All tumors presented as well-defined, unenveloped nodules in the liver. Six patients with sporadic hepatic AML had a solitary hepatic nodule with a definite fat component. Non-fat components of the hepatic lesions were enhanced earlier and persistently. Prominent central vessels were noted in the portal venous phase in three patients. In four patients with hepatic AML and TSC, most of the nodules were within the peripheral liver. Seven fat-deficient nodules were found with earlier contrast enhancement and rapid contrast material washout in two patients. Lymphangioleiomyomatosis was found in one patient.

**CONCLUSION:** Imaging features of hepatic AML are characteristic. Correct diagnosis preoperatively can be made in combination with clinical features.

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## INTRODUCTION

Hepatic angiomyolipoma (AML) is a rare, benign mesenchymal neoplasm that consists of varying numbers of smooth muscle cells, thick-walled blood vessels, and mature adipose tissue<sup>[1]</sup>. It can occur sporadically or in conjunction with tuberous sclerosis. It is often asymptomatic and found incidentally on imaging studies for unrelated disease. The imaging features of hepatic AML are diverse as a result of the various proportions of its three components<sup>[1,2]</sup>. It should not be treated surgically because of its benign nature. However, at present, definitive preoperative diagnosis of hepatic AML is a challenge for radiologists and clinicians. We studied the imaging features of hepatic AML in patients with or without tuberous sclerosis complex (TSC).

## MATERIALS AND METHODS

### Patients and clinical data

We studied retrospectively the clinical, radiological and pathological records of 10 patients with hepatic AML, who were referred to our hospital between 2004 and 2007. There were three male and seven female patients with a mean age of 55.1 years (range, 19-78 years). Four were asymptomatic and were found incidentally upon general health examination. Two had complained of upper abdominal pain. Four had symptoms of unilateral flank pain. Previous medical history showed nephrectomy for renal AML in three patients, and recurrent pneumothorax and hydrothorax in one. Physical examination presented multiple miliary russet

papilla on the face and several periungual fibromas in four patients. Renal ultrasound demonstrated unilateral renal AML in three patients who had a previous history of nephrectomy, bilateral multiple renal AML in one, and intratumoral hemorrhage in three. Laboratory analysis showed normal liver function and serum  $\alpha$ -fetoprotein in all 10 patients.

#### **Multi-slice computed tomography (MSCT)**

MSCT was adopted for all 10 patients, using a 16-slice computed tomography (CT) scanner (Lightspeed, GE Healthcare, USA). The scan parameters included a section thickness of 5 mm, pitch of 1.375:1, reconstruction thickness of 1.25 mm, and field of view of 248 mm  $\times$  330 mm. Intravenous contrast material [60-120 mL iopamidol (Isovue 370); Bracco Diagnostics, Princeton, NJ, USA] was used in all patients. Enhanced CT was performed in the arterial phase, portal venous phase, and delayed phase with a delay time of 26, 70 and 120 s since the injection of intravenous contrast material, respectively. All imaging studies were reviewed retrospectively by the same radiologist with over 15 years experience in abdominal CT studies.

#### **Histopathological and immunohistochemical examination**

Ultrasound-guided biopsy of hepatic nodules was adopted in six patients, with an 18-gauge needle. Left hemihepatectomy was performed in two patients. Microscopical examination of the specimens was performed with routine HE staining. Immunohistochemical studies were carried out on the specimens.

## **RESULTS**

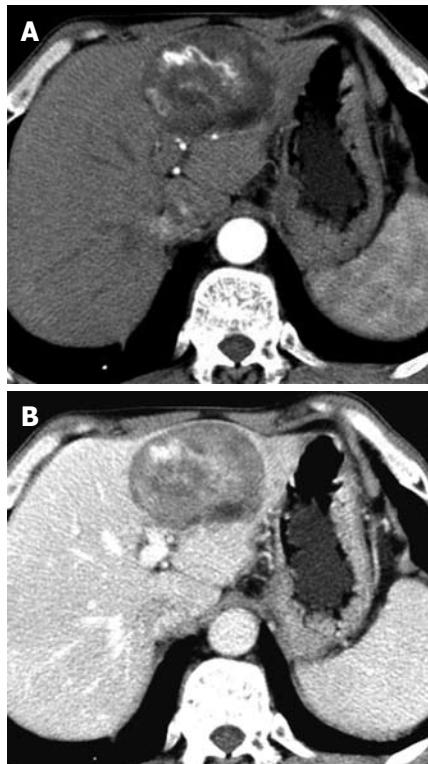
#### **Clinical information**

There were six patients with sporadic hepatic AML. The diagnosis of hepatic AML was confirmed by liver biopsy in four patients and partial liver resection in two.

Four patients with hepatic AML had associated TSC, the diagnosis of which was based on its revised criteria<sup>[3]</sup>. The diagnosis of hepatic AML was established by biopsy of the hepatic fat-deficient nodules in two patients. In another two case, the diagnosis of hepatic AML was based on the detection of fatty density in multiple hepatic nodules<sup>[4]</sup>. In one patient with hepatic AML and TSC, the diagnosis of lymphangioleiomyomatosis (LAM) was based on the typical imaging features of multiple, thin-walled lung cysts, hydrothorax and enlarged periaortic lymph nodes.

#### **MSCT findings**

Six patients with sporadic hepatic AML had solitary fat-containing masses with a mean size of 45 mm (size range, 10-80 mm) in the liver. On dynamic contrast-enhanced CT, the non-fat component of the lesions showed heterogeneous hyperdensity in the arterial phase and portal venous phase, and heterogeneous isodensity in the delayed phase, in comparison to the surrounding liver in five patients, (Figure 1A and B). Three had marked intratumoral



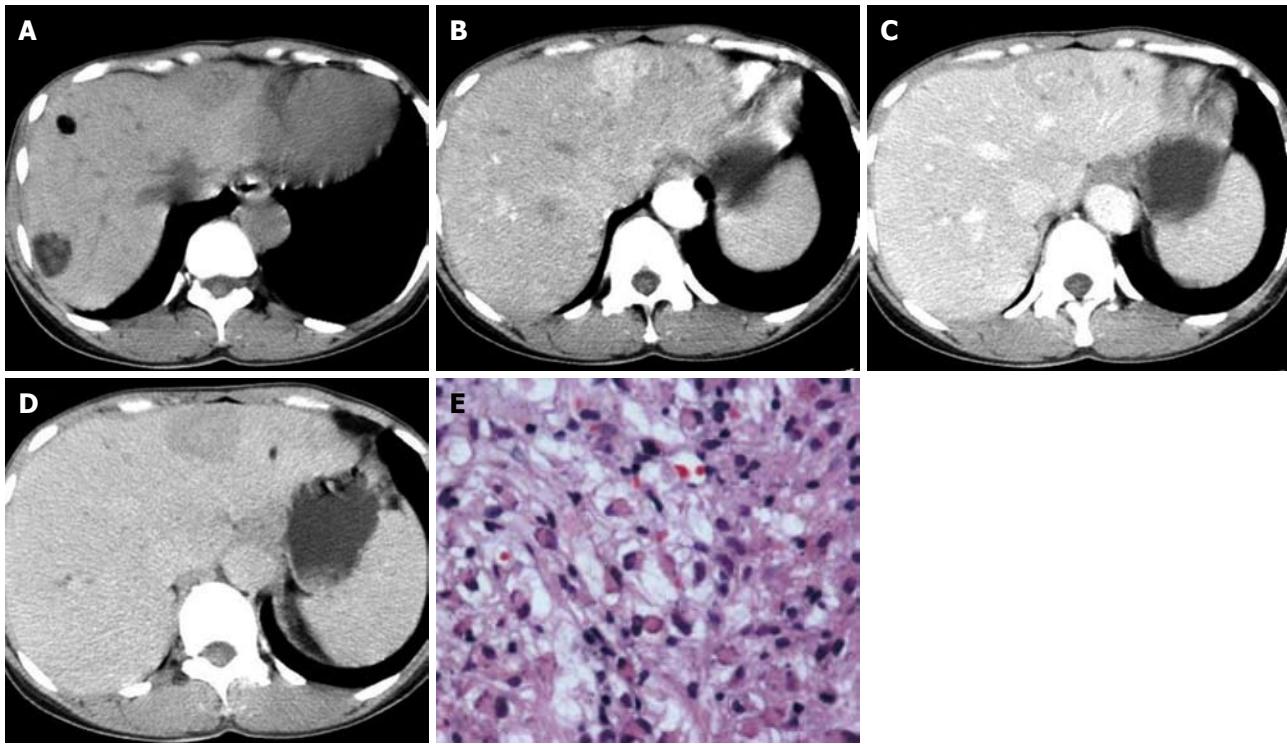
**Figure 1 Patient with sporadic hepatic angiomyolipoma.** A: Enhanced CT showed one well-defined, fat-containing nodule with prominent central vessels in the arterial phase; B: Enhanced CT showed sustained contrast enhancement of the intratumoral non-fat component in the portal venous phase.

vessels in the arterial and portal venous phases.

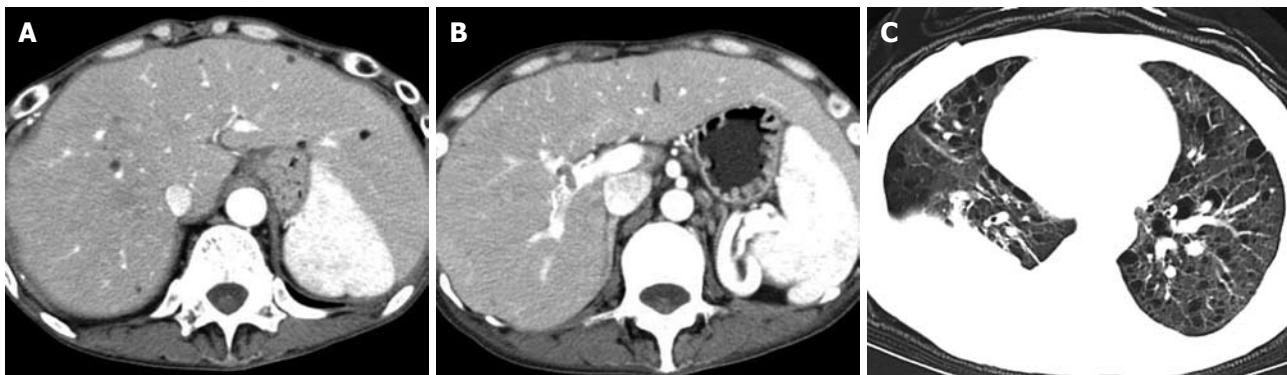
Four patients with hepatic AML and TSC had multiple, well-circumscribed hepatic nodules ranging from 2 to 40 mm (mean size, 8 mm) in the peripheral liver. Most of the nodules contained definite fatty density, and their non-fat components were enhanced mildly to markedly after administration of intravenous contrast material. Seven fat-deficient nodules were found in two patients (Figure 2A). On dynamic contrast-enhanced CT, the non-fat-containing nodules presented with heterogeneous high density in the arterial phase, mild hypodensity in the portal venous phase, and hypodensity in the delayed phase. Distorted intratumoral vessels and dilated feeding arteries were found in two of the seven nodules (Figure 2B-D). Except for multiple fat-containing nodules in the liver parenchyma, one sheet-like lesion in the right portal vein and several enlarged periaortic lymph nodes were noted in one patient (Figure 3A and B). Multiple thin-walled lung cysts and hydrothorax had been demonstrated previously by chest CT (Figure 3C).

#### **Histopathological and immunohistochemical findings**

Histopathological examination of the specimens showed various amounts of vacuolated lipocytes, blood vessels and scattered epithelioid cells with eosinophilic cytoplasm in all eight patients with liver biopsy or partial liver resection (Figure 2E). Immunohistochemistry demonstrated tumor cells with cytoplasm positive for HMB-45 in all eight patients.



**Figure 2** Patient with hepatic angiomyolipoma and TSC. A: Plain CT showed two fat-containing nodules in the right liver lobe, and one unenveloped fat-deficient nodule in the left liver lobe; B: Enhanced CT showed a fat-deficient nodule with marked hyperdensity, with intratumoral vessels in the arterial phase; C: Enhanced CT showed a fat-deficient nodule with mild hypodensity, with prominent central vessels in the portal venous phase; D: Enhanced CT showed a fat-deficient nodule with homogeneous hypodensity in the delayed phase; E: Microscopic examination demonstrated vacuolated lipocytes, blood vessels and scattered epithelioid cells with eosinophilic cytoplasm and peripherally located nuclei (HE, original magnification  $\times 40$ ).



**Figure 3** Patient with TSC and hepatic angiomyolipoma and LAM. A: Enhanced CT showed multiple fat-containing nodules in the liver; B: Enhanced CT showed an intra-portal-venous lesion and several enlarged periaortic lymph nodes; C: Chest CT showed multiple, thin-walled lung cysts and right hydrothorax.

## DISCUSSION

Hepatic AML is an unusual mesenchymal tumor that has no specific symptoms<sup>[5]</sup>. It often presents as well-demarcated hyperechoic nodules, and can be misinterpreted easily as hepatic cavernous hemangioma upon ultrasound examination<sup>[5]</sup>. It is demonstrated typically as a well-defined, unencapsulated mass with a fat component upon CT examination. However, fat-deficient hepatic AML has been reported with higher frequency than ever, and is difficult to be differentiated from hepatocellular carcinoma<sup>[6]</sup>. It is very important to recognize hepatic AML before surgery, because its natural history is benign, and it should not be removed surgically, except for patients who suffer spontaneous rupture<sup>[4,7]</sup>.

Hepatic AML can occur sporadically or in conjunction with TSC. It has been reported with a frequency of about 13% in patient with TSC<sup>[4]</sup>. Our study showed that hepatic AML often presents as a solitary mass, with a larger size in sporadic cases, but multiple smaller nodules in patient with TSC. This may be because it is asymptomatic, but growing with time. A long period before discovery may explain the relatively larger size of hepatic AML in patients without TSC. Dynamic contrast-enhanced CT showed sporadic hepatic AML as a hypervascular fat-containing mass with prominent central vessels in the arterial and portal venous phases. This was in accordance with previous studies<sup>[1,5]</sup>. In the setting of TSC, hepatic AML often presents as multiple nodules

with or without a fat component in the peripheral liver. The enhancement pattern of hepatic AML in TSC varies, and dilated intratumoral vessels are found less often than in sporadic cases. This may be related to their small size and variation in number.

Generally speaking, diagnosis of hepatic AML can be made upon detection of an unenveloped nodule with intratumoral fat, prominent central vessel and no capsule<sup>[8,9]</sup>. However, fat-deficient hepatic AML has been reported with increasing frequency in recent years<sup>[6,8]</sup>. Our study showed that fat-deficient hepatic AML has the propensity to occur in patients with TSC. Dynamic contrast-enhanced CT showed fat-deficient hepatic AML with earlier contrast enhancement and rapid contrast material washout in our two patients with TSC. The enhancement pattern was similar to that of hepatocellular carcinoma. Although it has been reported that non-fatty hepatic lesions in TSC can be regarded safely as hepatic AML<sup>[8]</sup>. However, we have reported previously a case of coexisting hepatocellular carcinoma and hepatic AML in a hepatitis B carrier with TSC<sup>[10]</sup>. This suggests that hepatocellular carcinoma should be considered upon detection of hepatic fat-deficient nodules in hepatitis B carriers with TSC.

LAM has been reported with a frequency of 1% in patients with TSC<sup>[11]</sup>. It is characterized by disorderly smooth muscle proliferation throughout the bronchioles, perivascular spaces and lymphatic system. Its diagnosis can be made on the detection of multiple, thin-walled lung cysts, pneumothorax, pleural effusion, or lymphadenopathy<sup>[12]</sup>. In our one TSC patient with hepatic AML and LAM, malignant hepatic AML could not be excluded completely because of the coexistence of intravascular lesions. This patient had an uneventful 2 years follow-up. Therefore, the intravascular lesions may be explained by invasion of LAM that originated from the perivascular space.

In summary, imaging features of hepatic AML are related closely to its clinical setting. Sporadic hepatic AML usually presents as a solitary large nodule with a varying fat component. In the setting of TSC, hepatic AML often presents as multiple small nodules, with or without a fat component, in the peripheral liver. Dynamic CT features of hepatic AML are very characteristic, and correct diagnosis can be made in combination with clinical features. When coexisting intravascular lesions and hepatic AML are found in patients with TSC, LAM should be considered.

## COMMENTS

### Background

Hepatic angiomyolipoma (AML) is a rare benign mesenchymal neoplasm. It can occur sporadically or in conjunction with tuberous sclerosis complex (TSC). Definitive preoperative diagnosis of hepatic AML is important because of its benign nature and it should not be resected. It is still a difficult condition for the radiologist and clinician.

### Research frontiers

The correlation between hepatic AML and its clinical setting has not been reported often in the literature. The imaging features of hepatic AML vary. Fat-

deficient hepatic AML has been reported with increasing frequency in recent times. It is difficult to differentiate from hepatocellular carcinoma.

### Innovations and breakthroughs

This study emphasized that imaging features of hepatic AML are related closely to its clinical setting. Multiple hepatic AMLs, with or without a fat component, are characteristic of TSC. When coexisting intravascular lesions and hepatic AML are detected in patients with TSC, lymphangioleiomyomatosis (LAM) should be considered. Hepatocellular carcinoma must be suspected when hepatic non-fatty nodules are detected in hepatitis B carriers with TSC.

### Applications

This study serves as a reminder of the imaging features of hepatic AML in patient with or without TSC. This study is limited by its retrospective nature and small number of patients, and a large sample study should be undertaken in the future.

### Terminology

TSC is an autosomal dominant genetic disease characterized by the growth of hematomas in multiple organs. LAM is a rare disease that is characterized by disorderly smooth muscle proliferation throughout the bronchioles, perivascular spaces and lymphatic system.

### Peer review

The authors studied the dynamic computed tomography features of hepatic AML in patients with or without TSC. This study was well performed and will be of interest to readers.

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## Laparoscopic and open splenectomy and azygoportal disconnection for portal hypertension

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of patients after OSD ( $6.5 \pm 2.3$  d vs  $11.7 \pm 4.5$  d,  $P < 0.05$ ). The pain requiring medication was less severe in patients after LSD than in those after OSD (7.69% vs 73.08%,  $P < 0.001$ ). The overall complication rate was lower in patients after LSD than in those after OSD (19.23% vs 42.31%,  $P < 0.05$ ).

**CONCLUSION:** Laparoscopic splenectomy and azygoportal disconnection are the feasible, effective, and safe surgical procedure, and are advantageous over minimally invasive surgery for bleeding portal hypertension and hypersplenism.

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**Key words:** Liver cirrhosis; Portal hypertension; Hypersplenism; Laparoscopy; Devascularization

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### Abstract

**AIM:** To compare the outcomes of laparoscopic and open splenectomy and azygoportal devascularization for portal hypertension.

**METHODS:** From June 2006 to March 2009, laparoscopic splenectomy and azygoportal disconnection (LSD) were performed on 28 patients with cirrhosis, bleeding due to portal hypertension, and secondary hypersplenism. Success was achieved in 26 patients. Demographic, intraoperative, and postoperative variables of the patients were compared.

**RESULTS:** Success of laparoscopic splenectomy and azygoportal disconnection was achieved in all but two patients (7.14%) who required open splenectomy and azygoportal devascularization (OSD). The operation time was significantly longer in patients undergoing LSD than in those undergoing OSD ( $235 \pm 36$  min vs  $178 \pm 47$  min,  $P < 0.05$ ). The estimated intraoperative blood loss was much more in patients receiving OSD than in those receiving LSD ( $420 \pm 50$  mL vs  $200 \pm 30$  mL,  $P < 0.01$ ). The proportion of patients undergoing laparoscopic and open splenectomy and azygoportal disconnection who received transfusion of packed red blood cells during or after the operation was 23.08% and 38.46%, respectively ( $P < 0.05$ ). The time of first oral intake was faster in patients after LSD than in those after OSD ( $1.5 \pm 0.7$  d vs  $3.5 \pm 1.6$  d,  $P < 0.05$ ). The hospital stay of patients after LSD was shorter than that

### INTRODUCTION

Liver cirrhosis leads frequently to the development of ascites and formation of varicose veins in esophagus and proximal stomach. Variceal bleeding is the most severe complication of portal hypertension, which may occur in 30% of patients with cirrhosis with a 30-d mortality of 20% when the portal-systemic gradient reaches above 12 mmHg<sup>[1]</sup>. Cirrhotic patients with variceal bleeding occasionally require surgical intervention. The ideal surgical procedure is to effectively control bleeding with a little impairment of liver function and a low rate of encephalopathy<sup>[2]</sup>. The morbidity of traditional open splenectomy and azygoportal devascularization (OSD) developed first by Sugiura in the 1960s<sup>[3]</sup> is a

consequence of a large upper abdominal incision rather than removal of the spleen itself. Recent development in technical skills has extended the indications for laparoscopic removal of the spleen<sup>[4]</sup>, and significant progress in laparoscopic technology has enabled splenectomy and devascularization less invasive of the proximal stomach and distal esophagus.

Compared to open splenectomy, the advantages of laparoscopic splenectomy include decreased use of anesthetic agents, earlier initiation of oral diet, short hospital stay, and fewer complications<sup>[5-7]</sup>. However, few studies comparing the outcomes of open and laparoscopic splenectomy and azygoportal devascularization for bleeding varices are available. This study was to assess the in-hospital results of open and laparoscopic splenectomy and devascularization for portal hypertension.

## MATERIALS AND METHODS

### **Patients**

From June 2006 to March 2009, laparoscopic splenectomy and azygoportal disconnection were performed on 28 patients with cirrhosis, bleeding portal hypertension, and secondary hypersplenism and success was achieved in 26 patients (19 males, 7 females). The patients at the age 29-65 years (average  $41.5 \pm 21.8$  years) were admitted due to repeated variceal bleeding. The liver cirrhosis stage was Child-Pugh A in 17 patients, Child-Pugh B in 8 patients, and Child-Pugh C in 1 patient, respectively.

Of the 26 patients undergoing laparoscopic splenectomy and azygoportal disconnection (LSD), 23 had liver damage caused by chronic hepatitis B, 1 had alcoholic cirrhosis, and 2 had no history of hepatitis or chronic alcohol consumption and were diagnosed with cryptogenic cirrhosis. All patients had splenomegaly, and the diameter of spleen ranged 14-21 cm as shown at ultrasound, CT scan, and MRI before surgery.

The results of traditional OSD performed in January 2004 to June 2006 on the 26 patients were assessed retrospectively.

Demographic data of the patients undergoing LSD and OSD are shown in Table 1.

### **Operation procedures**

Laparoscopic splenectomy was performed with the patient placed at the right lateral semidecubitus position and the operating table slightly tilted to the reverse Trendelenburg position. The surgeon stood at the right side of operating table and 4 operative ports were used. A 10-mm trocar was placed at the right upper umbilicus for a 10-mm 30-degree telescope camera, and another 10-mm operating trocar was inserted into the left midclavicular line just below the border of spleen for passing the ultrasonic dissector or LigaSure vessel-sealing equipment. A 5-mm trocar was placed in the subxiphoid space, allowing for the use of a supplementary retractor or grasper. A 12-mm trocar was placed in the left axillary line halfway between the costal margin and iliac crest, for the application of an endoscopic linear vascular stapling

**Table 1 Characteristics of patients undergoing LSD and OSD (mean  $\pm$  SD), n (%)**

Variable	LSD	OSD	P value
Patients (n)	26	26	-
Average age (yr)	$41.5 \pm 21.8$	$44.6 \pm 19.6$	NS
Gender (M/F)	19/7	21/5	NS
Etiology			
HBV + cirrhosis	23 (88.46)	21 (80.77)	NS
Alcoholic cirrhosis	1 (3.85)	2 (7.69)	NS
Cryptogenic	2 (7.69)	3 (11.54)	NS
Child-Pugh score			
A	17 (65.38)	20 (76.92)	NS
B	8 (30.77)	5 (19.23)	NS
C	1 (3.85)	1 (3.85)	NS
Variceal bleeding events	$3.2 \pm 1.8$	$3.7 \pm 1.6$	NS

LSD: Laparoscopic splenectomy and azygoportal devascularization; OSD: Open splenectomy and azygoportal devascularization; HBV: Hepatitis B virus; NS: Not significant.

device and other supplementary instruments.

The spleen was mobilized starting at the lateroposterior side by dividing the splenocolic and splenodiaphragmatic attachments. Then, the splenogastric ligament (including short gastric vessels) and the splenorenal ligament were divided with the ultrasonic dissector or LigaSure vessel-sealing equipment. Splenectomy was performed by carefully dissecting the splenic hilum to protect the pancreas and en bloc transecting the splenic artery and vein using the linear laparoscopic vascular stapler (EndoGIA).

After splenectomy, dissection was begun by approaching to the left crus with the ultrasonic dissector or LigaSure vessel-sealing equipment, then the gastrohepatic ligament was cut open to identify the right crus. The gastric coronary vein was visualized, and all branches toward the esophagus and proximal stomach were divided with the ultrasonic dissector and LigaSure vessel-sealing equipment. At least 6 cm of the distal esophagus was dissected through the hiatus with the paraesophageal venous collaterals divided.

Finally, the spleen was put into an impermeable retrieval bag and morcellated for removal.

OSD was performed either through a midline laparotomy or a left subcostal incision using traditional methods.

Operation time, estimated blood loss, intraoperative blood transfusion, time (d) of postoperative oral diet intake, pain requiring medication, postoperative complications, and hospital stay were compared.

### **Statistical analysis**

Data were compared using the *t*-test, chi-square analysis, or Fisher's exact test, where applicable, and expressed as mean  $\pm$  SD. All statistical analyses were performed using the SPSS 13.0 for Windows.  $P < 0.05$  was considered statistically significant.

## RESULTS

The demographic data included in this study are listed

**Table 2 Results and complications of LSD and OSD (mean  $\pm$  SD), n (%)**

Variable	LSD	OSD	P value
Operative time (min)	235 $\pm$ 36	178 $\pm$ 47	< 0.05
Estimated blood loss (mL)	200 $\pm$ 30	420 $\pm$ 50	< 0.01
PRBC transfusion	6 (23.08)	10 (38.46)	< 0.05
Time of oral intake (d)	1.5 $\pm$ 0.7	3.5 $\pm$ 1.6	< 0.05
Pain requiring medication	2 (7.69)	19 (73.08)	< 0.001
Postoperative hospital stay (d)	6.5 $\pm$ 2.3	11.7 $\pm$ 4.5	< 0.05
Overall complication rate	5 (19.23)	11 (42.31)	< 0.05

PRBC: Packed red blood cells.

in Table 1. No difference was found in preoperative data between the two groups of patients.

The surgical results are shown in Table 2. Success of laparoscopic splenectomy and azygoportal disconnection was achieved in all but 2 patients (7.14%) who required open laparotomy because of intraoperative bleeding from the hilar vessels which was impossible to control by laparoscopic splenectomy. All the patients survived the surgery.

The operation time was significantly longer in patients undergoing LSD than in those undergoing OSD (235  $\pm$  36 min vs 178  $\pm$  47 min,  $P < 0.05$ ). The estimated intraoperative blood loss was much more in patients undergoing OSD than in those undergoing LSD (420  $\pm$  50 mL vs 200  $\pm$  30 mL,  $P < 0.01$ ). The proportion of patients undergoing LSD and OSD who received transfusion of packed red blood cells during or after the operation was 23.08% and 38.46%, respectively ( $P < 0.05$ ).

The time of first oral intake was faster in patients after LSD than in those after OSD (1.5  $\pm$  0.7 d vs 3.5  $\pm$  1.6 d,  $P < 0.05$ ). The hospital stay of patients after LSD was shorter than that of those after OSD (6.5  $\pm$  2.3 d vs 11.7  $\pm$  4.5 d,  $P < 0.05$ ). The pain requiring medication was less severe in patients after LSD in those after OSD (7.69% vs 73.08%,  $P < 0.001$ ).

As shown in Table 2, the overall complication rate was lower in patients after LSD than in those after OSD (19.23% vs 42.31%  $P < 0.05$ ). Wound seroma, pancreatic fistula, pleural effusion, and asymptomatic portal vein thrombosis were found in 1, 1, 2, and 1 patient, respectively, after LSD. Amylase elevation with effusion around the pancreatic tail was drained under ultrasound guidance. Of the patients with pleural effusion, one underwent percutaneous drainage, the other received conservative treatment. Of the patients undergoing OSD, 2 had urinary complications, 4 had wound complications, 1 had pleural effusion, 2 had pneumonia, and 1 had incisional hernia. Emergency laparotomy reintervention for bleeding was not necessary following LSD, but bleeding occurred in 1 patient after OSD. No pancreatic fistula or postoperative portal vein thrombosis was observed after OSD.

During a postoperative follow-up period of 1-34 mo for the patients after LSD, liver failure or encephalopathy or esophagus variceal rebleeding did no recur, and all the patients had an improved quality of life.

## DISCUSSION

The portal gradient exceeding 12 mmHg in patients with portal hypertension due to liver cirrhosis may result in portosystemic collaterals, in which the esophageal and proximal stomach varices present a most important connection between portal and systemic blood circulation. Unfortunately, their tendency to bleed due to rupture of esophageal and proximal varices is the most life-threatening complication in patients with portal hypertension, which can significantly increase the mortality rate of patients with liver cirrhosis. The bleeding event occurs in 10%-30% of patients with liver cirrhosis within 1 year, and reoccurs in 40% of patients within 6 wk. The mortality of bleeding varicose veins increases up to 30% depending on the liver insufficiency<sup>[8-10]</sup>. After acute treatment of bleeding, subsequent bleeding should be prevented.

Surgery for portal hypertension and variceal bleeding has evolved widely in terms of techniques and indications. Many treatment modalities available suggest that no single therapy is entirely satisfactory for all patients or for all clinical situations. Currently, shunt and devascularization surgeries are two basic surgical methods, but shunt procedures are more commonly used in Western countries and devascularization procedures are more commonly used in China<sup>[11,12]</sup>. The ideal surgical procedure for patients with bleeding portal hypertension and secondary hypersplenism should control bleeding, correct hypersplenism, avoid recurrence of bleeding, with little impairment of liver function and a low rate of encephalopathy.

The laparoscopic splenectomy has become the gold standard for removal of normal to moderately enlarged spleens. However, it is really more challenging when laparoscopic splenectomy is performed on patients with cirrhosis, portal hypertension, and splenomegaly, because portal hypertension and the severity of liver cirrhosis are the risk factors for high intraoperative blood loss during laparoscopic splenectomy<sup>[13]</sup>. In addition, it is difficult to remove the extremely enlarged spleen because of its higher conversion rate<sup>[14]</sup>.

Less invasive laparoscopic devascularization of the lower esophagus and upper stomach was performed partly through experimentation<sup>[15]</sup> or minilaparotomy<sup>[16-21]</sup>.

In this study, success of laparoscopic splenectomy and azygoportal disconnection was achieved in all but 2 patients. The patients recovered uneventfully after operation. The indications for laparoscopic splenectomy and azygoportal disconnection are similar to those for OSD. We prefer to perform laparoscopic splenectomy and azygoportal disconnection for patients with liver cirrhosis at Child-Pugh A and B. The absolute contraindications of laparoscopic splenectomy and azygoportal disconnection are patients who are not able to tolerate the general anesthesia, intractable coagulopathy, and/or unfitted to laparoscopy.

The most important intraoperative complication during laparoscopic splenectomy and azygoportal disconnection is bleeding, which is the most common

reason for conversion to open surgical procedure. Furthermore, since conversion is usually done due to lack of working space or bleeding, the operation time of converted patients is longer, leading to more blood loss than open splenectomy. However, laparoscopic splenectomy cannot effectively control massive hemorrhage from the major vessels or capsule fracture. In many cases, bleeding interferes with the dissection and does not result in any other event. Therefore, the crucial point of devascularization technique is to prevent the brisk bleeding from dilated vessels like enlarged azygoportal collaterals. Capsule or small vessel laceration may cause oozing, which contaminates the operating field and makes the surgical procedure more difficult. We recommend that complete mobilization of the spleen before operation is a safe option for adequately controlling the hilar vessels and any possible bleeding during the procedure. The splenic artery should be dissected and ligated whenever it is found superior to the pancreatic tail during the operation for splenomegaly. This maneuver can reduce the size and bleeding of spleen with a certain autotransfusion effect. Although vessels within the splenic hilum can be dissected and divided individually with vascular clips in some cases, the manipulation is quite tedious and not encouraged. In addition, if bleeding occurs in the hilum, vascular staplers should be used to control it. Use of clips should be limited since it may interfere with the use of vascular staplers.

Technical advances that have facilitated laparoscopic splenectomy and azygoportal disconnection are the availability of LigaSure vessel-sealing equipment and ultrasonic dissector, which facilitate the performance of very difficult laparoscopic procedures. LigaSure vessel-sealing equipment is safer than ultrasonic dissector in dividing the surrounding tissues of spleen, distal esophagus and proximal stomach, including paraesophageal venous collaterals, and can thus be used for mobilization of the spleen and esophagogastric devascularization. As use of clips may interfere with the use of LigaSure vessel-sealing equipment to stop the bleeding, it is wiser not to use any clips during esophagogastric devascularization.

The LigaSure vessel-sealing sealing system has been developed for the safe closure of arteries up to 7 mm<sup>[22]</sup>. Although the data about venous closure are lacking, this system can be used to close veins up to 12 mm<sup>[23]</sup>. Kwok *et al*<sup>[24]</sup> showed that the LigaSure sealing system can treat grades 3 and 4 of hemorrhoids.

In our series, intraoperative bleeding during LSD was a rare event, leading to conversion to OSD in only 2 cases (7.14%). The estimated blood loss was significantly lower in patients undergoing LSD than in those undergoing OSD.

The overall complication rate was lower in patients after LSD than in those after OSD. The main complications of LSD included pancreatic fistula and thromboembolic event, which did not occur after OSD. Meanwhile, incisional hernia was uncommon after LSD but occurred in 1 patient after OSD.

In summary, LSD can alleviate postoperative pain, earlier recovery bowel function and feeding, has

better cosmetic results, reduce the hospital stay and recovery time, and is thus advantageous over minimally invasive surgery and OSD. We believe that laparoscopic splenectomy and azygoportal disconnection is a technically feasible, safe, and preferred procedure for bleeding varices due to hypersplenism. LSD should primarily be attempted in all patients with portal hypertension, if judged technically feasible and the general condition of the patient allows laparoscopy.

## COMMENTS

### Background

Liver cirrhosis leads frequently to the development of ascites and formation of varicose veins in esophagus and proximal stomach. Variceal bleeding is the most severe complication of portal hypertension, which occurs in 30% of patients with cirrhosis. Cirrhotic patients with variceal bleeding occasionally require surgical intervention. Traditional open splenectomy and azygoportal devascularization (OSD) are performed due to large upper abdominal incision rather than removal of the spleen itself. Recent development in laparoscopic technology has enabled splenectomy and devascularization of the proximal stomach and distal esophagus less invasive.

### Research frontiers

Less invasive laparoscopic devascularization of the lower esophagus and upper stomach is performed partly through minilaparotomy. However, few studies are available on the outcomes of open and laparoscopic splenectomy and azygoportal devascularization for bleeding varices due to portal hypertension.

### Innovations and breakthroughs

Few studies are available on the outcomes of open and laparoscopic splenectomy and azygoportal devascularization (LSD) for bleeding varices due to portal hypertension. The aim of the present study, was, therefore, to assess the in-hospital results of open and laparoscopic splenectomy and azygoportal devascularization for portal hypertension. The results show that LSD can alleviate postoperative pain, earlier recovery bowel function and feeding, has better cosmetic results, reduce hospital stay and recovery time, and is thus advantageous over minimally invasive surgery and OSD.

### Applications

Laparoscopic splenectomy and azygoportal disconnection is a technically feasible, safe, and preferable procedure for bleeding varices due to portal hypersplenism. LSD should primarily be attempted in all patients with portal hypertension, if judged technically feasible and if the general condition of the patient allows laparoscopy.

### Terminology

Portal hypertension: Increased pressure in the portal vein. When the blood flow in the liver is obstructed, the blood can become backed up into the intersections of the portal venous system and the systemic venous system. The intersections of these two systems are small, fragile blood vessels known as capillaries. These vessels are not able to withstand the increased blood pressure and become engorged or dilated. Such vessels can be seen on the surface of esophagus or stomach during endoscopy. They are fragile and at a risk of bleeding. Portal hypertension can be managed through diet, medications, endoscopic therapy, surgery, or radiology. Once the bleeding episode is stabilized, treatment options should be prescribed based on the severity of symptoms and the function of liver.

### Peer review

Although laparoscopic splenectomy and azygoportal disconnection are not easy to perform, they represent a new and alternative procedure for portal hypertension and are advantageous over open splenectomy and azygoportal disconnection.

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CASE REPORT

## Early plasmapheresis and rituximab for acute humoral rejection after ABO-compatible liver transplantation

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steroid pulses, and OKT3. Because of persisting signs of biopsy-proven AHR at day 26, she was treated by plasmapheresis and rituximab. Liver enzyme levels did not improve, and she died on day 41. Patient 2 experienced a biopsy-proven AHR on day 10 post-transplant. She was treated by steroid pulses, plasmapheresis, and rituximab. Liver enzymes returned to within normal range 18 d after diagnosis. Liver biopsies, at 3 and 9 mo post-transplant, showed complete resolution of AHR. We conclude that plasmapheresis should be started as soon as AHR is diagnosed, and be associated with a B-cell depleting agent. Rituximab may be considered as a first-line therapy.

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**Key words:** Acute humoral rejection; Liver transplantation; Donor-specific antibodies; Rituximab; Plasmapheresis

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## INTRODUCTION

Acute humoral rejection (AHR) is well described in ABO-incompatible orthotopic liver transplant (OLT) patients; however, its occurrence in ABO-compatible OLTs is still an uncommon phenomenon. Acute humoral or antibody-mediated rejection in ABO-identical transplants is usually related to the presence of preformed or acquired anti-human leukocyte antigen (HLA) donor-specific antibodies (DSA)<sup>[1]</sup>. The diagnosis of AHR relies on the presence

## Abstract

Acute humoral rejection (AHR) is uncommon after ABO-compatible liver transplantation. Herein, we report two cases of AHR treated with plasmapheresis and rituximab in two ABO-compatible liver-transplant patients with preformed anti-human leukocyte antigen donor-specific antibodies. Patient 1 experienced a biopsy-proven AHR at day 10 post-transplant. She was treated by

of DSA, C4d deposition, tissue pathology, and evidence of organ dysfunction<sup>[2]</sup>. Although the liver has long been regarded as resistant to antibody-mediated rejection, and despite conflicting published data, it is now thought that preformed DSA and a positive cross-match in ABO-compatible OLTs is associated with rejection and graft loss<sup>[3-6]</sup>. Recently, using a modern multiple-bead assay, i.e. Luminex, Castillo-Rama *et al*<sup>[7]</sup> have shown that Luminex-detected antibodies, as well as positive complement-dependent cytotoxicity T crossmatches, were associated with shorter graft survival within the first year post-transplant. In addition, they observed a correlation between the presence of preformed Luminex-detected class II or Luminex I and II antibodies and allograft rejection<sup>[7]</sup>. The treatment of these anti-HLA-mediated acute humoral rejections is still not well established. Herein, we report on two ABO-compatible liver-transplant patients with AHR, caused by specific anti-donor HLA antibodies (Table 1), who were treated with plasmapheresis and rituximab.

## CASE REPORT

### Case 1

A 49-year-old woman, who was ABO compatible, underwent 4 antigen mismatch orthotopic liver transplantation in July 2005 because of end-stage liver disease related to hepatitis C virus infection with hepatopulmonary syndrome. Her Child-Pugh stage was C10. Anti-viral therapy had failed to clear HCV before transplantation; and her HCV RNA concentration was 5.9 log copies/mL at transplantation. On the day of transplantation, her panel-reactive antibody rate (PRA) was 30% and she had circulating DSA directed against class II HLA antigens (anti-DR7, detected by Luminex). At the time of transplantation, crossmatches were positive for both T and B cells. The immediate postoperative period was marked by the occurrence of acute respiratory-distress syndrome, which required mechanical ventilation, and acute renal failure, which required continuous veno-venous haemodiafiltration. The initial immunosuppressive therapy was based on induction therapy of anti-thymocyte globulins (1.25 mg/kg on days 1, 3, 5 and 6; Thymoglobulin, Genzyme), plus tacrolimus monotherapy, which was introduced on day 1 at a targeted trough level of between 10 and 15 ng/mL. Liver enzyme levels improved until day 6, but then gamma glutamyl transpeptidase ( $\gamma$ GT), alkaline phosphate (AP), and total bilirubin levels increased again, while transaminases remained within the normal ranges (Figure 1). By day 10,  $\gamma$ GT, AP, total bilirubin, and direct bilirubin levels were, 251 IU/L, 808 IU/L, 258  $\mu$ mol/L, and 136  $\mu$ mol/L, respectively. Biliary tract and vascular complications were ruled out by abdominal ultrasonography and a liver CT scan. A Doppler ultrasound confirmed good blood flow in the hepatic artery, portal vein, and hepatic veins. A liver biopsy performed on day 10 revealed acute rejection with a Banff activity index of 7, mixed inflammatory cells in the portal triad, and significant cholangitis and endothelitis. Unfortunately, immunostaining for C4d

**Table 1** Immunological data regarding donors and recipients

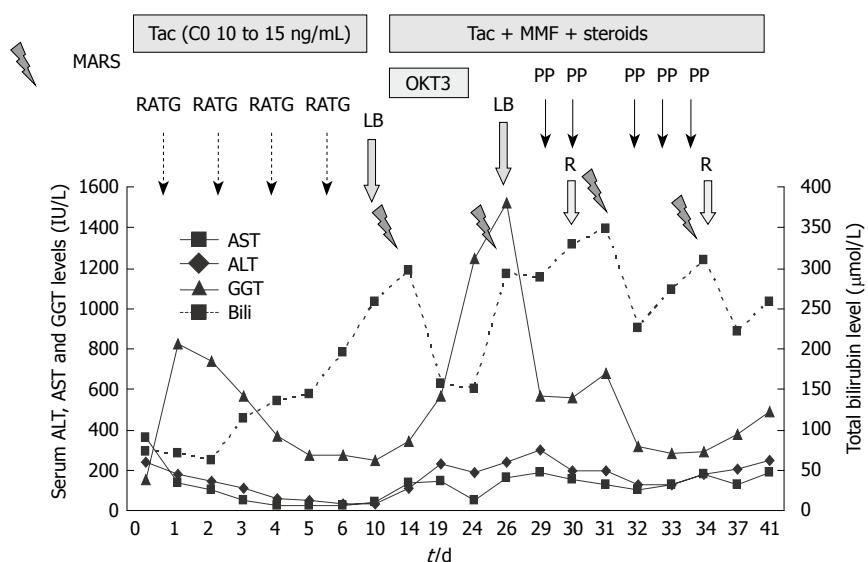
	HLA typing		CDC cross-match pre-OLT	DSA	
	Donor	Recipient		Pre-OLT	At the time of AR
Case 1	A2 A3	A3 A36	Positive on T and B cells	Anti-A2	Anti-A2
	B35 B44	B7 B35		Anti-DR7	Anti-DR7
	DR7 DR11	DR15 -		Anti-B17	
Case 2	A2 A24	A3 A29	Positive on T and B cells	Anti-A2	Anti-A2
	B27 B38	B7 B60		Anti-A24	Anti-A24
	DR4 DR11	DR7 DR13		Anti-B27	Anti-B27
					Anti-DR4

HLA: Human leukocyte antigen; CDC: Complement-dependent cytotoxicity; OLT: Orthotopic liver transplantation; DSA: Donor-specific alloantibody; AR: Acute rejection.

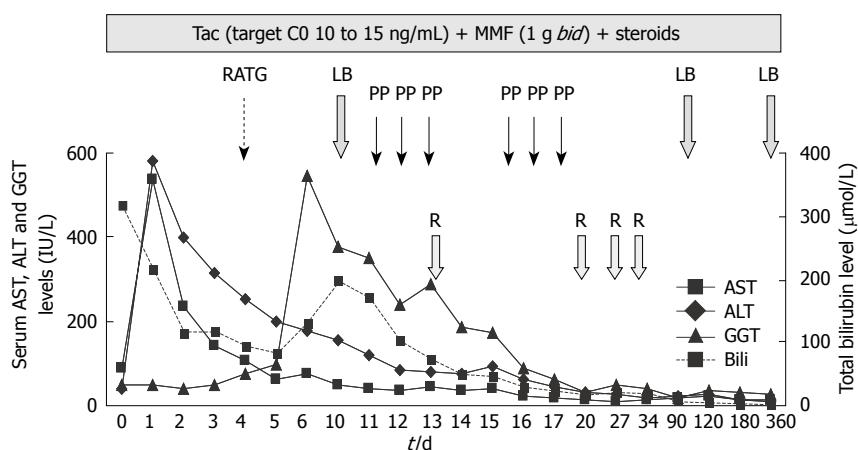
was not performed. Retrospective immunostaining of liver biopsy for the presence of T and B lymphocytes showed a high proportion of B cells, i.e. 60% of total cells. Screening for anti-HLA antibodies confirmed the presence of anti-class I (anti-A2) and anti-class II (anti-DR7) HLA antibodies directed against the donor. Because of this, the patient was treated with steroid pulses (10 mg/kg per day for 3 d, and then gradually tapered), OKT3 (10 mg/d for 10 d), plus mycophenolate mofetil was introduced at a daily dose of 2 g. In the absence of any improvement in liver enzyme levels, she underwent a second liver biopsy 16 d after the first, on day 26 post-transplant. This showed the presence of inflammatory infiltration by lymphocytes, histiocytes and plasmacytes, persistent cholangitis, venulitis, hepatocanalicular cholestasis, biliary thrombi, and hepatocyte necrosis. Retrospective immunostaining of the liver biopsy for the presence of T and B lymphocytes showed a high proportion of B cells with 30% of B cells. At that time, we decided to treat the humoral part component. She was treated with plasmapheresis (5 sessions using fresh frozen plasma or 50% fresh frozen plasma and 50% albumin) and two rituximab infusions (375 mg/m<sup>2</sup> per week for 2 wk). Unfortunately, her liver enzyme levels did not improve. She required liver support that utilized four sessions of molecular adsorbents recirculating system (MARS). Her acute respiratory distress syndrome did not improve within this postoperative period. She experienced pulmonary infections and died on day 41, i.e. 4 d after the second rituximab infusion. Before rituximab infusion, her circulating CD4-, CD8-, and CD19-positive cell counts were, respectively, 47, 49 and 85/mm<sup>3</sup>.

### Case 2

A 39-year-old woman underwent six antigen mismatch, ABO-compatible orthotopic liver transplantation in January 2008 because of end-stage liver disease related to alcohol with hepatopulmonary and hepatorenal syndromes. Her Child-Pugh stage was C10. On the day of transplantation, her PRA was 44% and she had circulating DSA directed against both class I (anti-A2, anti-A24, anti-B27) and class II (anti-DR4) HLA antigens, which were detected by Luminex. At the time of transplantation, crossmatches were positive for T and B cells. Surgery



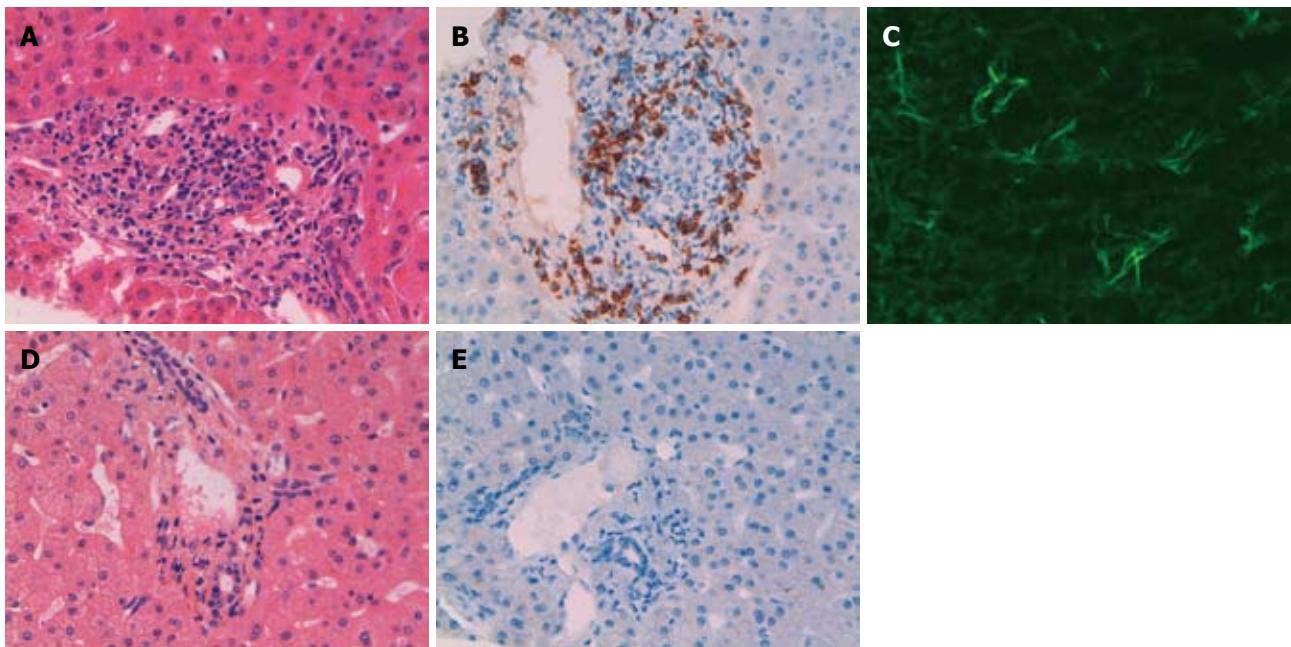
**Figure 1 Postoperative clinical course of patient 1.** Tac: Tacrolimus; MMF: Mycophenolate mofetil; RATG: Rabbit anti-thymocyte globulins; MARS: Molecular adsorbents recirculating system; LB: Liver biopsy; PP: Plasmapheresis; R: Rituximab; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma glutamyl transpeptidase.



**Figure 2 Postoperative clinical course of patient 2.**

was uneventful, and the immediate postoperative course was unremarkable with a regular decrease in liver test parameters. The initial immunosuppressive therapy was based on tacrolimus (targeting a trough level of between 10 and 15 ng/mL), mycophenolate mofetil (1 g *bid*), and steroids (10 mg/kg before transplantation, 5 mg/kg on day 1, 2.5 mg/kg on day 2, and then 1 mg/kg). By day 4, because of significant renal function impairment (serum creatinine level rose from 130 to 280 μmol/L) caused by the high tacrolimus trough level (21 ng/mL), tacrolimus was discontinued for 2 d. The patient then received one infusion of anti-thymocyte globulins (1.25 mg/kg; Thymoglobulin, Genzyme). Liver enzyme levels improved until day 5 but, on day 6, γGT, AP, and total bilirubin levels increased significantly (Figure 2). Biliary tract complications were ruled out by abdominal ultrasonography and magnetic resonance imaging. Doppler ultrasound confirmed good blood flow in the hepatic artery, portal vein, and hepatic veins. A liver biopsy performed on day 10 revealed acute rejection, with a Banff activity index of 7, diffuse cholangitis and endothelitis, periportal edema, inflammatory infiltration by lymphocytes, histiocytes and neutrophils (Figure 3A). Immunostaining for C4d was positive (Figure 3B). Immunostaining of liver biopsy for the presence of T and

B lymphocytes showed a high proportion of T cells. B cells represented 30% of total cells (Figure 3C). Consequently, she received three pulses of methylprednisolone (10 mg/kg per day), plus four infusions of rituximab (375 mg/m<sup>2</sup> per week for 4 wk). In addition, six plasmapheresis sessions using fresh frozen plasma were realized. Her liver enzyme levels improved rapidly and were within normal range 18 d after AHR diagnosis. At 3 mo post-transplant, she underwent a second liver biopsy, which showed complete resolution of the acute rejection episode (Figure 3D). Immunostaining of liver biopsy for the presence of T and B lymphocytes showed no T and B cells (Figure 3E). All previous features of acute rejection had disappeared. At 12 mo post-transplant, her liver enzyme levels were still within the normal range, DSA directed against class I HLA antigens were still detected, while the DSA directed against the class II HLA antigen was not detected anymore. A third liver biopsy was performed and was considered normal. During the 12 mo post-transplant, she did not present with any infection episodes. Her circulating CD4-, CD8-, and CD19-positive cell counts, which were 364, 165 and 45, respectively, before the first rituximab infusion, became 980, 527 and 0/mm<sup>3</sup> by month 3 and 673, 374 and 0/mm<sup>3</sup> by 12 mo post-transplant.



**Figure 3** Liver biopsies performed in patient 2. A: Liver biopsy performed on day 10. Acute rejection with mixed inflammatory cells in the portal triad, significant cholangitis and endothelitis. Banff activity index of 7 (HE,  $\times 400$ ); B: Immunostaining with L26 antibody (anti-CD20) showed moderate proportion of B cells ( $\times 400$ ); C: Liver biopsy performed on day 10: Immunostaining for C4d in liver biopsy ( $\times 400$ ); D: Liver biopsy performed at 3 mo post-transplant showed complete resolution of acute rejection (HE,  $\times 400$ ); E: Immunostaining with L26 antibody (anti-CD20) showed no B cell infiltration ( $\times 400$ ).

## DISCUSSION

Anti-HLA antibody-induced acute humoral rejection is uncommon after ABO-compatible liver transplantation. Herein, we report two cases of acute humoral rejection that occurred within the first week after an ABO-compatible liver transplantation in two patients with preformed donor-specific anti-HLA antibodies. In both cases, an acute rejection episode occurred very early after transplantation, and induced significant cholestasis, while transaminases were only moderately elevated. Liver biopsies revealed acute rejection, the presence of inflammatory infiltration and, more specifically, cholangitis, endothelitis, and periportal edema. Immunostaining for C4d was found to be positive, and immunostaining of liver biopsies for the presence of T and B lymphocytes showed a relative high proportion of B cells. To the best of our knowledge, our second case report is the first to show that early treatment of AHR with plasmapheresis and rituximab without using intravenous hyperimmune globulins (IVIg) is effective in this setting, especially in a patient with four different DSAs.

Whatever the transplanted organ may be, until recently, the treatment of AHR has been based on plasmapheresis, immunoabsorption, IVIg, and tacrolimus, mycophenolate mofetil, with or without T-cell depleting agents<sup>[8-10]</sup>. Because of B-cell involvement in humoral rejection, the use of monoclonal anti-CD20 antibodies (rituximab) has been used in this setting. For ABO-incompatible kidney and liver transplantation, as well as historical and/or current positive crossmatch pre-transplantation highly sensitized patients, and in desensitization protocols in kidney transplantation, rituximab has been successfully used in association with

splenectomy and plasmapheresis<sup>[11-15]</sup>. In kidney transplant patients, Becker *et al*<sup>[16]</sup> have shown that vascular rejection with a possible humoral component responds to a single dose of rituximab therapy when associated with plasmapheresis and steroid pulses, with or without anti-thymocyte globulins. More recently, we found that, despite a high rate of infection, the association of plasmapheresis and rituximab was efficient in treating acute humoral kidney rejection<sup>[17]</sup>. These interesting results obtained in kidney transplant patients prompted us to use the same strategy in liver transplant patients who were experiencing acute humoral rejection. Previously, Watson *et al*<sup>[6]</sup> treated a liver transplant patient with preformed anti-HLA antibodies and who experienced an early acute humoral rejection with IVIg, plasmapheresis, and one infusion of rituximab. In Watson *et al*<sup>[6]</sup> plasmapheresis and rituximab therapies were started late after AHR diagnosis, i.e. on post-transplant day 34 for rituximab, and post-transplant day 70 for plasmapheresis. This is similar to our first case report, where we treated the cellular component before the humoral component. Unfortunately, this was not effective in treating the humoral acute rejection.

In contrast, starting plasmapheresis and medications that target B cells as soon as AHR is diagnosed seems to be more effective. In our second case report, plasmapheresis and rituximab therapy were started as soon as AHR was diagnosed, i.e. on day 11 post-transplant. Similarly, Rostron *et al*<sup>[18]</sup> had previously reported a case of severe AHR in an ABO compatible liver transplant patient successfully treated with plasmapheresis, IVIg and conversion from azathioprine to mycophenolate mofetil. In the latter case, plasmapheresis was started at day 7 post-transplant. In another study on a combined kidney-liver transplant patient who experienced both cellular and humoral

rejection, early plasmapheresis associated with steroid pulses, anti-thymocyte globulins, and IVIg effectively treated both rejections<sup>[19]</sup>. Finally, Wilson *et al*<sup>[20]</sup> reported a late AHR in an ABO-compatible liver transplant patient that was successfully treated with plasmapheresis, one rituximab infusion, and anti-thymocyte globulins. However, in the latter case, we wonder whether it was a late AHR or a cellular rejection that occurred in a patient with a chronic humoral rejection. Indeed, it is unknown how many anti-HLA antibodies were present in this patient. Positive immunostaining for C4d may be related to long-term silent chronic humoral rejection. In addition, in the Wilson *et al* case report, the acute rejection that occurred very late after transplantation, which is very uncommon, may be a steroid-resistant acute cellular rejection, especially since liver enzyme levels decreased after anti-thymocyte globulin therapy.

In summary, plasmapheresis should be started as soon as AHR is diagnosed in order to remove circulating antibodies. It should be associated with either IVIg or rituximab, both of which can be effective in this setting. However, because of rituximab's capacity to deplete B-cells, and the good results obtained in ABO-incompatible liver transplant patients, rituximab could be the preferred first-line therapy. Further controlled studies to compare rituximab and IVIg are needed.

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# Combined goblet cell carcinoid and mucinous cystadenoma of the veriform appendix

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## INTRODUCTION

Goblet cell carcinoid (GCC) of the veriform appendix is an uncommon neoplasm. It is characterized by dual endocrine and glandular differentiation, a feature that has led to confusion regarding nomenclature, histogenesis, and clinical management. The origin and histogenesis of GCC are still controversial, and whether GCC represents a histological variant of appendiceal classical carcinoid or a distinct morphological subtype of appendiceal adenocarcinoma with endocrine differentiation is still a matter of debate. Rare cases of GCC combined with other benign and malignant epithelial appendiceal neoplasms have been reported; the relationship between GCC and these neoplasms is not clear. Herein, we report an unusual and rare case of combined GCC and mucinous cystadenoma (MCA) of the veriform appendix and discuss the possible related histopathogenesis.

## Abstract

Goblet cell carcinoid is an uncommon primary tumor of the veriform appendix, characterized by dual endocrine and glandular differentiation. Whether goblet cell carcinoid represents a morphological variant of appendiceal classical carcinoid or a mucin-producing adenocarcinoma is a matter of conjecture. Rare cases of goblet cell carcinoid with other concomitant appendiceal epithelial neoplasms have been documented. In this report, we describe a rare case of combined appendiceal goblet cell carcinoid and mucinous cystadenoma, and discuss the possible histopathogenesis of this combination.

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**Key words:** Goblet cell carcinoid; Mucinous cystadenoma; Vermiform appendix

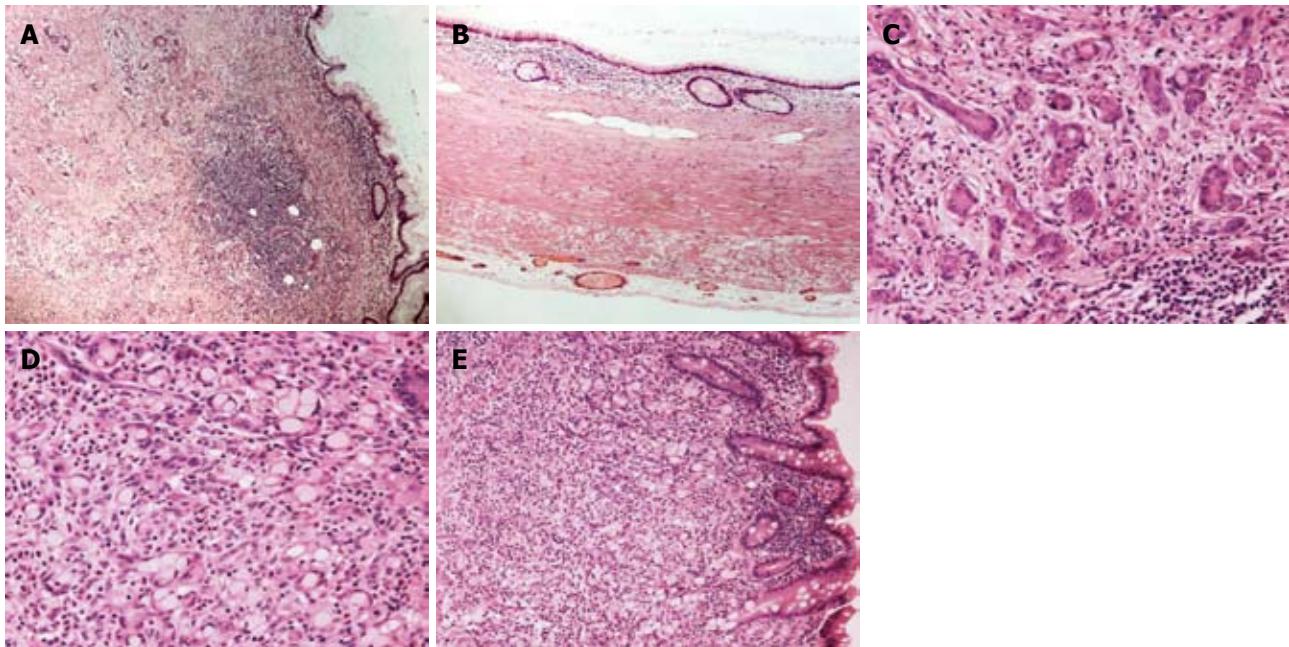
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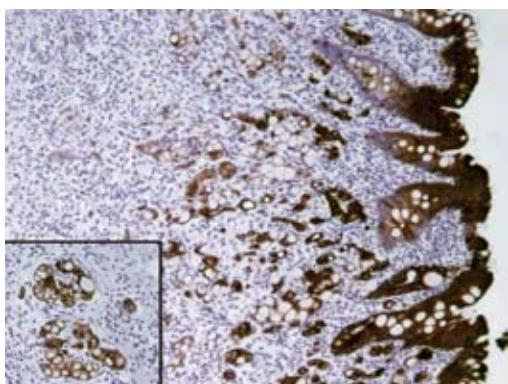
## CASE REPORT

A 46-year-old woman presented with severe acute pain in the right iliac fossa and perumbilical region. Ultrasound and a computed tomography scan revealed a mucocele in the veriform appendix, with a well defined lesion located at the mid zone of the appendix. The patient underwent right hemicolectomy, and her postoperative clinical course was uneventful. Gross examination of the surgical specimen showed an enlarged appendix, which was filled with thick mucinous material. A distinct lesion which involved the appendiceal wall, and measured 1.5 cm maximally, was identified in the mid-portion of the appendix. There was no evidence of perforation, extravasation of mucin into the periappendiceal tissue, or pseudomyxoma peritonei during surgery.

Histopathological examination showed combined GCC and MCA of the veriform appendix (Figure 1A). The appendiceal lumen was dilated and lined by mucin-containing columnar epithelial cells (Figure 1B). There was no significant cytologic atypia, and no mitotic figures were identified. Focal papillary configurations of the lining epithelium, and mild epithelial pseudostratification were present. In addition, the appendiceal wall was infiltrated by glandular structures of various sizes which



**Figure 1 Histopathological examination.** A: Mucinous cystadenoma of the appendix combined with infiltrating goblet cell carcinoid (HE,  $\times 50$ ); B: The mucinous cystadenoma of the appendix showing lumen lined by mucous-containing bland epithelial cells (HE,  $\times 100$ ); C: Cells with neuroendocrine cytonuclear features in goblet cell carcinoid (HE,  $\times 200$ ); D: Mucin-filled neoplastic cells of goblet cell carcinoid (HE,  $\times 200$ ); E: The infiltrating nests of goblet cell carcinoid appear to arise from the basiglandular region of the intestinal crypts in close proximity to the mucinous cystadenoma (HE,  $\times 100$ ).



**Figure 2 Immunohistochemically.** The neoplastic cells of the goblet cell carcinoid expressed strong and diffuse positivity for cytokeratin 20 ( $\times 100$ ).

were arranged in nests and tubules. These glandular structures comprised 2 distinct types of cells: (1) small to intermediate sized monotonous neuroendocrine cells with a small amount of finely granular eosinophilic cytoplasm, and mild cytonuclear atypia (Figure 1C); (2) mucin-filled intermediate sized cells (goblet cells), with peripherally located small, crescent-like hyperchromatic nuclei, and indistinct nucleoli (Figure 1D). Scattered infiltrating single goblet neoplastic cells were focally present. As previously described<sup>[1]</sup> the tumor nests appeared to arise from the basiglandular region of the intestinal crypts in close proximity to the MCA (Figure 1E). There was no lymphovascular invasion, although perineural and intraneuronal invasion was present. The tumor infiltrated the full thickness of the appendiceal wall and extended to the mesoappendix. Ten lymph nodes were histologically identified, of which all

**Table 1 Immunohistochemical panel used in this study**

Antibody	Clone	Dilution	Source
CK7	OV-TL 12/30	1:2000	Dako corporation, carpinteria, CA
CK19	B170	1:50	Vector laboratories, burlingame CA
CK20	KS20.8	1:100	Dako
CD99	O13	1:100	Signet laboratories, dedham, MA
Ki67	MIB1	1:100	Dako
Chromogranin	DAK-A3	1:200	Dako
Synaptophysin	SY38	1:200	Dako
Serotonin	Polyclonal	1:200	Dako

were negative for malignancy.

Immunohistochemically (Table 1), the tumor cells of the GCC were positive for chromogranin, synaptophysin, and serotonin, which are neuroendocrine markers. Diffuse staining for cytokeratin (CK) 20 (Figure 2), CK19, and CD99 was also present. The Ki67 proliferating index revealed nuclear staining in approximately 15% of the tumor cells. There was no staining for CK7.

## DISCUSSION

GCC is an uncommon neoplasm of the veriform appendix with uncertain histopathogenesis and biological behaviour. It is believed that GCC represents an amphicrine tumor, which originates from a single undifferentiated pluripotent intestinal stem cell with divergent neuroendocrine and mucinous differentiation<sup>[2]</sup>, resulting in a composite biphasic neoplasm of 2 distinct populations of endoderm-derived cells. Whether this

makes GCC a variant of carcinoid tumor or a subtype of appendiceal adenocarcinoma which exhibits morphological and immunophenotypical features of neuroendocrine differentiation is still a subject of debate. Molecular studies have not elucidated the exact nature of GCC. There is a substantial overlap between GCC and classical carcinoid from a molecular standpoint<sup>[3,4]</sup>. Morphological features such as minimal cytologic atypia, presence of non-goblet cells, tubuloacinar neuroendocrine elements, continuity with the basiglandular crypt cells of the mucosal membrane, and the lack of continuity and involvement with the luminal surface mucosa, favour the GCC being related to carcinoid tumor. However, compared to classical carcinoid, the positive immunostaining for CK20<sup>[5]</sup>, the demonstration of IgA staining that is typical of intestinal crypt cells<sup>[6]</sup>, the tendency for regional lymph node and distant metastasis, and tendency for recurrence and more aggressive clinical behaviour are common features for GCC. This suggests that GCC is histologically a form of "crypt cell carcinoma" or more accurately an "amphicrine carcinoma" rather than a variant of appendiceal carcinoid.

Rare cases of GCC coexisting with conventional appendiceal mucinous tumors were reported<sup>[1,7,8]</sup>, of which 2 cases were MCA<sup>[1]</sup>. Similar to our case, both patients were women, aged 54 and 64 years, who presented with clinical features of acute appendicitis and dull ache in the right iliac fossa. Histological examination revealed combined GCC and MCA. If GCC is a true subtype of carcinoid tumor, its coexistence with an appendiceal mucinous neoplasm would support the theory that GCC is derived from a single undifferentiated pluripotent intestinal stem cell with divergent dual neuroendocrine and mucinous differentiation (unitary stem cell hypothesis). The concomitant appendiceal mucinous neoplasm may be considered a coincidental occurrence and raises the possibility of a common etiological factor for both GCC

and appendiceal epithelial neoplasms<sup>[9]</sup>. If GCC is to be considered as an adenocarcinoma of crypt cell origin rather than a carcinoid, then the occurrence of combined GCC and appendiceal mucinous neoplasms may represent an example of adenoma-carcinoma sequence<sup>[11]</sup>. The nature of GCC and its relation to other neuroendocrine and non-neuroendocrine epithelial tumors of the appendix needs further examination, and more cases of GCC with concomitant appendiceal epithelial neoplasms need to be recorded, which may help in explaining this rare association.

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CASE REPORT

## Metastatic melanoma of the gallbladder: An unusual clinical presentation of acute cholecystitis

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**Author contributions:** Vernadakis S and Legakis N designed the research; Vernadakis S, Rallis G, Serafimidis C and Legakis N performed the research; Danias N, Christodoulou E and Troullinakis M performed data analysis; Vernadakis S and Peros G contributed to data interpretation and made an important intellectual contribution to the study.

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### INTRODUCTION

Malignant cutaneous melanoma can metastasize to virtually any organ. The most common sites of metastatic disease, apart from the regional lymph nodes, are the lungs, liver, brain and colon<sup>[1,2]</sup>. Metastases of the biliary tract are rare. Autopsy studies have shown that gallbladder metastases are found in 15% of patients with metastatic melanoma of the gastrointestinal tract, but are reported rarely during the lifetime of the patient because involvement of the gallbladder seldom produces symptoms<sup>[3]</sup>.

We report a case of metastatic melanoma of the gallbladder in a patient who developed symptomatic acute cholecystitis. The patient underwent explorative laparotomy and open cholecystectomy. Despite appropriate therapy, the diagnosis of this condition portends a poor prognosis, with very few patients surviving more than 12 mo<sup>[4,5]</sup>.

### CASE REPORT

A 58-year-old man was admitted to our clinic with abdominal pain in his right upper quadrant, and associated nausea and vomiting. On examination, he was febrile and there was tenderness upon palpation of the right upper abdominal quadrant and the epigastrium. Laboratory tests were within normal limits (white blood cell count 10 600/mm<sup>3</sup>, normal liver enzymes and normal serum amylase). An ultrasound scan demonstrated sludge in the gallbladder and thickening of the gallbladder wall, without dilatation of the intra- and extrahepatic biliary tree. The patient had undergone a Billroth II operation via median laparotomy for gastric ulcer disease 15 years previously.

A presumptive diagnosis of acute cholecystitis was made. An exploratory laparotomy and cholecystectomy was performed. Intraoperatively, the gallbladder was shown macroscopically to be acutely inflamed and

### Abstract

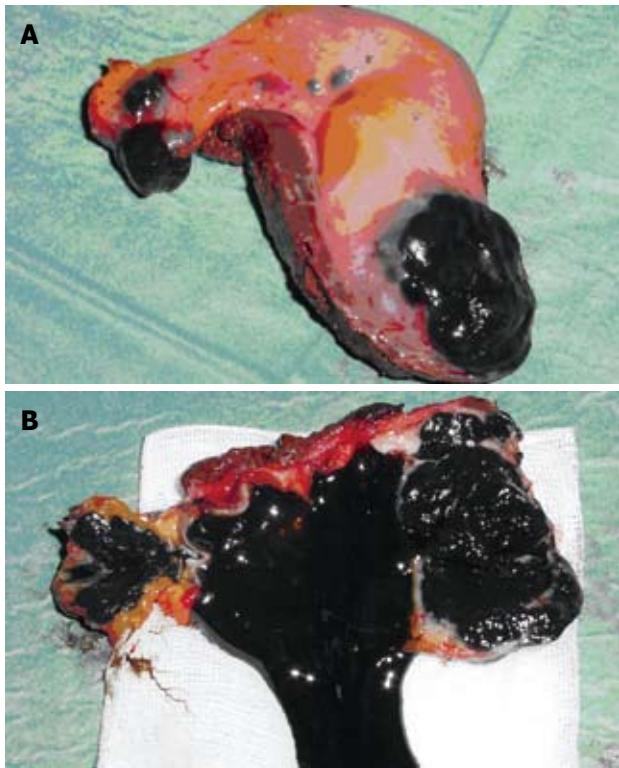
Metastatic disease from cutaneous melanoma can affect all organs of the body, and varies in its biological behavior and clinical presentation. We present the case of a 58-year-old man who arrived at our clinic with acute abdominal pain, which, after investigation, was diagnosed as acute cholecystitis. The patient underwent laparotomy and cholecystectomy. Two years ago, he underwent surgical removal of a primary cutaneous melanoma on his right upper back. Pathological examination revealed the presence of malignant melanoma with a metastatic lesion of the gallbladder.

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**Key words:** Metastatic melanoma; Gallbladder metastasis; Cholecystectomy; Prognosis

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Vernadakis S, Rallis G, Danias N, Serafimidis C, Christodoulou E, Troullinakis M, Legakis N, Peros G. Metastatic melanoma of the gallbladder: An unusual clinical presenta-



**Figure 1** Surgical specimen. A: Ulcerated black mass in the fundus and the cystic duct of the resected gallbladder; B: Gallbladder filled with black sludge and necrotic material.

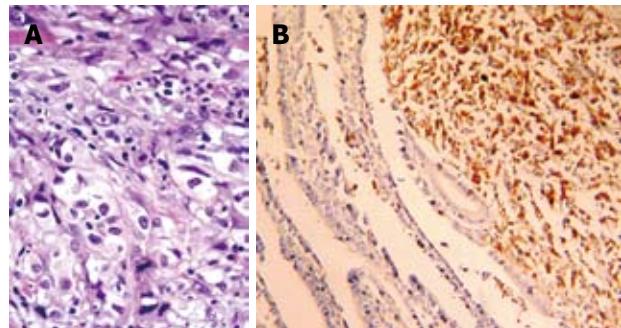
thickened. Examination of the surgical specimen showed an ulcerated black mass in the fundus and the cystic duct of the gallbladder, and the lumen was filled with black sludge and necrotic material (Figure 1).

Pathological examination revealed acute cholecystitis and the presence of a malignant tumor invading through the gallbladder wall, with extensive invasion of lymphatic vessels and fat adipose tissue around the gallbladder. Its morphologic morphological appearance and immunohistological profile<sup>[6]</sup>, corresponded to a metastatic focus of a cutaneous malignant melanoma with high cellular polymorphism and melanin production (Figure 2).

The patient had undergone surgical resection of a malignant melanoma (superficial spreading type, Clark anatomical level III, with a depth of infiltration of 3.8 mm by Breslow) 2 years ago on his right upper back. Adjuvant immunological therapy with interferon was performed at that time.

On postoperative day 7, the patient was discharged home in good condition. Four weeks later, his course was complicated by the development of increasing neurological symptoms. On computer tomography investigation, he was found to have brain metastases. The primary tumor was probably the cutaneous malignant melanoma. He received two courses of combination chemotherapy with a dacarbazine (DTIC) regimen, but the disease continued to progress. He received two cycles of chemotherapy with temozolomide, with no success.

The patient died of widespread disease 5 mo after the initial diagnosis of the metastatic disease.



**Figure 2** Pathological examination. A: Microscopic picture of metastatic melanoma in the gallbladder (HE,  $\times 20$ ); B: Neoplastic cells immunostained with anti-S-100 antibodies ( $\times 400$ ).

## DISCUSSION

Metastases of cutaneous melanoma to the gallbladder and biliary tree are uncommon and usually clinically asymptomatic. The most common sites of distant metastases are the skin, lungs, liver and brain<sup>[2,7,8]</sup>. According to autopsy results, such asymptomatic metastases to the gallbladder and bile ducts occur in 4%-20% of patients with metastatic melanoma<sup>[3,8,9]</sup>, and constitute up to 50% of the metastatic neoplasms of these organs<sup>[2,4]</sup>.

Despite these statistical data, it is rare for metastatic gallbladder melanoma to be present with symptoms during the patient's life time<sup>[4,8,9]</sup>, as demonstrated by the discrepancy in published case reports and the rate of detection at autopsy<sup>[3,4,8,10]</sup>.

The most common presentation of symptomatic metastatic disease to the gallbladder is acute cholecystitis followed by jaundice caused by obstruction of the common bile duct<sup>[4,8]</sup>. Also hematobilia and biliary fistulae may develop<sup>[4,8,11]</sup>.

The examination of choice for the assessment of gallbladder metastatic tumors is ultrasound scanning, an examination with high specificity and sensitivity<sup>[10]</sup>. In particular, the use of color Doppler ultrasound can support a preoperative differential diagnosis of malignancy, because of the presence of pathological blood flow within the lesion in patients with a past medical history of cutaneous malignant melanoma<sup>[2,4,12]</sup>.

The role of surgical treatment of metastatic melanoma of the gallbladder remains unclear because of the lack of experience<sup>[2]</sup>. Goals of therapy include palliation of symptoms, minimization of complications and improvement in overall survival. Recently, cholecystectomy has been indicated for patients with isolated, resectable gallbladder metastases, and can achieve longer survival<sup>[2,4]</sup>. As an alternative to open surgery, the laparoscopic approach has arisen as a minimally invasive option<sup>[5,8,9]</sup>.

From the literature, the prognosis of metastatic melanoma to the gallbladder is dismal, with a median survival of 6-9 mo<sup>[2,4,8]</sup> and long-term disease-free survival is achieved in only 1%-2% of patients<sup>[2]</sup>. Once involvement of the gallbladder is documented, it is probable that the metastases are widespread.

Despite the advances made in chemotherapy<sup>[13]</sup> and

immunotherapy<sup>[14,15]</sup> for metastatic melanoma, the results are still poor and disappointing<sup>[2,4,8]</sup>.

In conclusion, we report this case to make clear the necessity of meticulous investigation of lesions of the hepatobiliary system when the patient has a past history of cutaneous malignant melanoma. The best chance for achieving the longest survival is active screening and early detection. In addition, diligence and vigilance are required to ensure that patients adhere to current screening guidelines. Active follow-up is necessary to identify metachronous lesions early.

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S- Editor Tian L L- Editor Kerr C E- Editor Ma WH



# Right trisegmentectomy with thoracoabdominal approach after transarterial embolization for giant hepatic hemangioma

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## INTRODUCTION

Liver hemangiomas are the most common benign tumors occurring in the liver, and are believed to be slowly growing hamartomatous lesions or true vascular neoplasms<sup>[1]</sup>. They occur more frequently in women than in men, and are believed to be related to levels of female hormones because their size increases during pregnancy<sup>[2]</sup>. In most cases, the hemangioma is small in size and asymptomatic, and thus follow-up is considered enough, without treatment. However, a giant hemangioma, which is defined as a hemangioma over 4 cm in diameter, can cause symptoms and require intervention. Preoperative diagnosis is possible using ultrasound or helical CT, and the indications for surgical resection are the presence of symptoms, a high risk of rupture, and being indistinguishable from malignancy. Here, we report the successful removal of a giant hemangioma (over 30 cm) from the right side of the liver through a J-shaped incision, using a thoracoabdominal approach, after transarterial embolization (TAE).

## CASE REPORT

A 41-year-old female patient visited a secondary hospital for abdominal girth (which had not decreased since delivery a year ago), intermittent abdominal pain, and indigestion, as the chief complaints. Ultrasonography, performed during the visit, detected huge hyperechoic lesions, and under the diagnosis of giant hemangioma, she was recommended for surgery and transferred to the present hospital. During a physical examination upon admission, an abdominal mass was palpated from the right upper quadrant to the pelvic cavity. Preoperative serum biochemical studies revealed no abnormal findings, except for an increase of total bilirubin to 2.5 (normal < 1.20 mg/dL). Using helical CT, a non-contrast image showed a homogenous hypodense lesion

## Abstract

Hepatic hemangiomas need to be treated surgically in cases where they are accompanied with symptoms, have a risk of rupture, or are hardly distinguishable from malignancy. The present authors conducted embolization of the right hepatic artery one day before an operation for a huge hemangioma accompanied with symptoms and confirmed a decrease in its size. The authors performed a right trisegmentectomy through a J-shape incision, using a thoracoabdominal approach, and safely removed a giant hemangioma of 32.0 cm × 26.5 cm × 8.0 cm in size and 2300 g in weight. Even for inexperienced surgeons, a J-shape incision with a thoracoabdominal approach is considered a safe and useful method when right-side hepatectomy is required for a large mass in the right liver.

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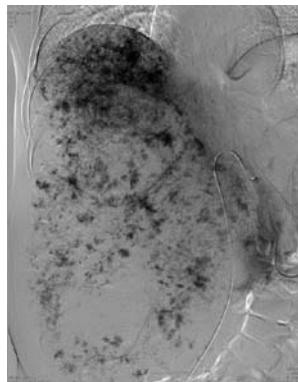
**Key words:** Hemangioma; Transarterial embolization; Thoracoabdominal approach

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Seo HI, Jo HJ, Sim MS, Kim S. Right trisegmentectomy with



**Figure 1** Coronal reformatted CT scan obtained for the portal venous phase shows an ill-defined heterogeneous enhancing mass lesion in the right lobe of the liver.



**Figure 2** Angiography obtained at the delayed phase, before embolization, shows displacement of vessels and pooling of contrast medium in the right lobe of the liver.

contrasted with the surrounding liver parenchyma, the arterial image showed peripheral nodular enhancement, and the portal venous image showed progressive centripetal enhancement. These findings indicated a giant hemangioma (Figure 1). The indocyanine green clearance rate at 15 min was 13% (normal range < 15%). TAE of the right hepatic artery was conducted one day before the operation (Figure 2), and thereafter the size decreased by around 5 cm and no more.

Through a J-shaped skin incision using a thoracoabdominal approach, the incision was extended to the 10th intercostal space and the adjacent pleura and diaphragm were opened. Through the incision, the right liver was fully mobilized, and through the hanging method and Glissonian approach, a right trisegmentectomy was safely conducted. The duration of operation was 310 min and intraoperative blood loss was 250 mL. The resected tumor was 32.0 cm × 26.5 cm × 8.0 cm in size and 2300 g in weight. Upon histological examination, it was diagnosed as a cavernous-type hemangioma. The patient's postoperative course was stable. The chest tube was removed on the fifth day and the patient was discharged on the 10th day. Follow-up has been continued for eight months without any particular findings.

## DISCUSSION

Hemangiomas show specific features in imaging diagnosis, therefore most cases can be diagnosed preoperatively. As a hemangioma increases in size, it can cause congestion, bleeding, thrombosis and infarction (and consequent stomachache), Kasabach-Merritt syndrome, and spontaneous rupture. Out of the mass effect, it might show symptoms such as obstructive jaundice, biliary colic, and gastric outlet obstruction<sup>[3]</sup>. In our case, the patient complained of only non-specific symptoms such as mild abdominal pain and dyspepsia. Total bilirubin increased slightly but direct bilirubin was normal, which findings did not indicate obstructive jaundice. For the treatment of a giant hemangioma accompanied with symptoms, surgical resection is primarily recommended. Various other treatment methods have been reported but their long-term results have been poor<sup>[4]</sup>. TAE is suggested as an excellent therapy for easing the symptoms of a giant hemangioma, but recurrence is common because

of vascular recanalization. However, there is a report that TAE for a giant hemangioma, performed prior to surgical resection, facilitated the mobilization of the right liver by shrinking the hemangioma and, consequently, decreased intraoperative hemorrhage<sup>[5]</sup>. The authors also performed TAE one day before the operation, and experienced a decrease in the size of hemangioma, as in the previous report. Various complications can occur after embolization and they could postpone the operation and result in the loss of an opportunity for surgery due to vascular recanalization. Accordingly, it is desirable to perform the operation soon after embolization. Thus, the present authors performed the laparotomy within 24 h, and observed the shrinkage of hemangioma during the operation, as in the previous report. This helped minimize bleeding from the hepatic raw surface during the mobilization of the liver.

Preoperative TAE can decrease the risk of bleeding during resection of a hemangioma, but in the liver resection of a 30 cm large giant hemangioma, conventional approaches might cause difficulties. Lai *et al*<sup>[6]</sup> reported successful resection of a giant hemangioma using the anterior approach, but they recommended that it should be done by skillful and experienced surgeons because it can cause excessive bleeding from the middle hepatic vein during parenchymal resection. The thoracoabdominal approach is advantageous in that it provides a sufficiently large field of view in a partial hepatectomy or the resection of Couinaud's Segment 7 and 8, and it has been reported as a useful method for right side hepatectomy by Japanese and European researchers since the 1990s<sup>[7]</sup>. Compared to conventional approaches, the thoracoabdominal approach is just as safe as a right-sided hepatectomy, but it seems not to be used frequently because the chest has to be opened and an intra-thoracic drainage tube has to be inserted. We think that pulmonary complications are caused by thoracotomy but they are not common in the thoracoabdominal approach, and the problem of pleural effusion can be controlled by the use of an intra-thoracic drainage tube and diuretics. The present authors inserted a postoperative intra-thoracic drainage tube, and did not observe intercostal pain or neurogenic pain caused by the insertion. In addition, the intra-thoracic drainage tube was removed early; therefore it did not affect the postoperative course. The thoracoabdominal approach

is obviously a useful method for the safe resection of the right triangular ligament and mobilization of the right liver from the inferior vena cava. Accordingly, when a right-side hepatectomy is performed for a huge mass, such as a giant hemangioma in the right liver, a J-shaped incision using a thoracoabdominal approach is considered a safe and useful method. The addition of "sternotomy" to this incision might be considered of clinical value if the tumor is even larger than in this case.

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CASE REPORT

## Metachronous bile duct cancer nine years after resection of gallbladder cancer

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## INTRODUCTION

The occurrence of two or more primary malignant tumors in a patient is regarded as uncommon. In 1889, Billroth documented several such cases as rare events. However, in recent years, several studies have noted multiple primary cancers frequently due to the prolonged survival of patients who have previously been cured of a primary cancer<sup>[1]</sup>. Multiple primary cancers are defined as either synchronous tumors or metachronous tumors, according to whether the diagnostic intervals of the lesions are shorter or longer than six months, respectively<sup>[2,3]</sup>. Synchronous multiple biliary cancers occur in 3.7%–7.4% of all surgically resected biliary tumors. Most of them develop in the gallbladder and bile duct<sup>[4-6]</sup>. On the other hand, metachronous multiple biliary cancers are rare, with only 11 cases reported in the world literature<sup>[7-17]</sup>. In Korea, one case was reported in 1997 when a 61-year-old man underwent curative resection for bile duct cancer 30 mo after a radical cholecystectomy to cure gallbladder cancer<sup>[17]</sup>.

We report the case of a 74-year-old man with metachronous gallbladder cancer and hilar bile duct cancer, who underwent curative resection twice, with an interval of nine years separating the operations. We also refer to 12 cases of metachronous biliary tract cancer published in the world literature, including the present case.

## CASE REPORT

A 74-year-old man was admitted for further evaluation



**Figure 1 Radiologic finding of hilar bile duct cancer.** A: Dynamic abdominal CT during arterial phase of axial image showed a low-density mass, 1.7 cm in diameter at the hilum of the liver, extending to the right intrahepatic bile duct (arrow) and intrahepatic bile duct dilatation in the right lobe (arrow head); B: Dynamic abdominal CT during venous phase of coronal image showed the mass extending to the second branch of the intrahepatic bile duct in right hepatic lobe (arrow); C: T2WI, axial image of MRI showed the mass located in the perihilar bile duct with the same signs of liver parenchyma (arrow), and extending to the second branch of the intrahepatic bile duct in the right hepatic lobe.



**Figure 2 Gross specimen of bile duct excision and right hepatic lobectomy.**

of elevated serum alkaline phosphatase level. Nine years previously, he had undergone a cholecystectomy and resection of his liver bed for gallbladder cancer. The pathologic specimen revealed a well-differentiated adenocarcinoma with muscular invasion, no hepatic invasion, and with negative resection margins (T2N0M0, stage I B). He did not receive adjuvant therapy and was followed up periodically without evidence of tumor recurrence. However, nine years later, blood tests showed an elevated serum alkaline phosphatase level. He had been taking antihypertensive agents and oral hypoglycemic agents for ten years. He underwent splenectomy for idiopathic thrombocytopenic purpura 15 years ago. He is a nonsmoker and a farmer.

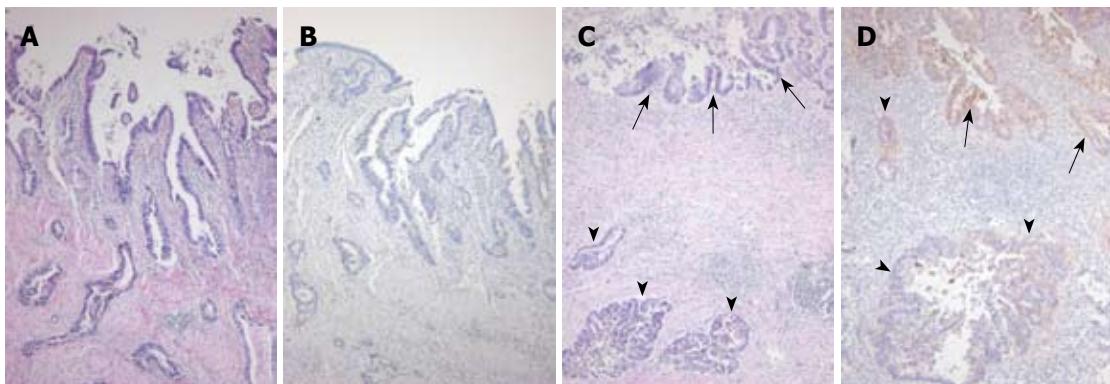
On admission, the patient complained of dyspepsia but physical examination revealed no abnormalities. Laboratory test results showed WBC  $9010/\text{mm}^3$ , hemoglobin 13.3 g/dL, hematocrit 41.3%, platelet  $312 \times 10^9/\text{L}$ , AST 44 U/L, ALT 43 U/L, total bilirubin 0.5 mg/dL, alkaline phosphatase 975 IU/L, CEA 5.2 ng/mL ( $< 5 \text{ ng/mL}$ ), and CA 19-9 77.4 ng/mL ( $< 37 \text{ ng/mL}$ ). Abdominal CT and magnetic resonance imaging (MRI) revealed an enhanced 1.7 cm mass in the hilum that extended to the second branch of the intrahepatic bile duct on the right side of the liver, as well as diffuse dilated intrahepatic bile ducts (Figure 1). These findings suggested hilar bile duct cancer, Bismuth type IIIa. There was no evidence of peritoneal metastasis or enlarged lymph nodes. The

patient underwent curative surgery comprising bile duct excision, right hepatic lobectomy and Roux-en-Y hepaticojejunostomy (Figure 2). Pathologic specimens revealed a well-differentiated adenocarcinoma invading the entire thickness of the bile duct; one of seven lymph nodes was positive for malignant cells; and there was a high degree of dysplasia around the main lesion (T1N1M0, stage II B). We used genetic alterations in CA19-9, p53 gene, and k-ras as a means of distinguishing multi-centric primary cancers from primary cancer metastases. The tissues of the gallbladder cancer and the bile duct cancer were both positive for the p53 and k-ras gene. However, CA19-9 was negative in the tissue of the gallbladder cancer, but positive in the tissue of the bile duct cancer (Figure 3). Now, 12 mo after the second operation, the patient is doing well with no evidence of recurrence. These two cancers apparently occurred independently and each surgical treatment was successful.

## DISCUSSION

Synchronous multiple primary cancers of the biliary system occur in up to 7% of resected bile duct cancers. Most are found on removing the gallbladder during a resection for bile duct cancer<sup>[4-6]</sup>. However, metachronous multiple bile duct cancer is very rare. Because biliary tract cancers are usually advanced by the time of diagnosis, and surgery involves a broad bile duct resection with curative intent, the chances of developing a metachronous double biliary cancer are slim<sup>[7-17]</sup>.

In 1932, Warren and Gates proposed the three still generally accepted criteria that had to be met in order to consider malignant tumors as multiple primary tumors rather than recurrences: first, each of the tumors must present a definite picture of malignancy; second, each tumor must have a different histological appearance; third, the possibility that one might be a metastatic lesion from a former lesion must be excluded<sup>[18]</sup>. Gertsh also stated the criteria for double primary cancer in the biliary tract: first, each tumor must be distinct; second, each tumor must be separated from normal tissue; third, there should be no evidence of invasion to the submucosal layer and lymph nodes around the bile duct<sup>[19]</sup>. Bile duct



**Figure 3 Histopathological finding of hilar bile duct cancer.** A: Histologic section of the gallbladder shows well-differentiated biliary-type adenocarcinoma infiltrating the entire thickness of the gallbladder wall (HE,  $\times 100$ ); B: None of the tumor cells expressed CA19-9 (immunoperoxidase method,  $\times 100$ ); C: Histologic section of the hepatic duct demonstrates severe epithelial dysplasia of the mucosa in the upper field (arrows) and invasive adenocarcinoma in the lower field (arrow heads) (hematoxylin and eosin,  $\times 100$ ); D: Most of the dysplastic epithelial cells in the upper field (arrows) and some of invasive tumor cells in the lower field express cytoplasmic CA19-9 (arrow heads). An invasive tumor gland is noted in the mid-left field (immunoperoxidase method,  $\times 100$ ).

**Table 1 Clinicopathological characteristics of the 12 reported cases with metachronous cancers of the biliary tract**

Yr	Age (yr)	Sex	Interval (yr) (1st-2nd cancers)	1st cancer			2nd cancer			Ref.
				Site	Pathology	Type of surgery	Site	Pathology	Type of surgery	
1987	73	F	3.5	GB	Tub, ss	Chole	CBD	Pap, ss	PD <sup>2</sup>	[7]
1997	66	M	4	GB	Pap, mp	Chole	HBD	Tub, ss	Excision HBD	[8]
1999	75	F	7	LHBD	Tub, mp, WD	LH extended	CBD	Tub, MD-PD <sup>1</sup> , si	PD <sup>2</sup>	[9]
1999	21	M	7	GB	Pap, m	Chole	CBD	Pap, fm	PD <sup>2</sup>	[10]
2001	49	F	4	CBD	Tub, ss	PD <sup>2</sup>	HBD	Tub, si	LH extended	[11]
2001	65	F	7	GB	Muc, mp	Chole + liver resection + resection extrahepatic BD	LHBD	muc	Liver resection	[12]
2004	63	M	4	GB	Pap,mp	Chole	CBD	Tub, ss	HPD	[13]
2003	64	F	4	GB	CIS with adenoma	Chole	CBD	CIS	Excision CBD	[14]
2004	83	F	10	GB	Ss, WD	Chole+ liver resection	CBD	WD	PD <sup>2</sup>	[15]
2007	65	M	2.3	CBD	fm	Resection middle third extrahepatic BD + chole	CBD	fm	PD <sup>2</sup>	[16]
1997	61	M	2.5	GB	Ss, MD-PD <sup>1</sup>	Chole+ liver resection	CBD	Ss, MD	PD <sup>2</sup>	[17]
2009	74	M	9	GB	mp	Chole+ liver resection	HBD	ss	RH	This case

<sup>1</sup>PD: Poorly differentiated; <sup>2</sup>PD: Pancreaticoduodenectomy; CIS: Carcinoma *in situ*; CBD: Common bile duct; Chole: Cholecystectomy; fm: Fibromuscular layer; GB: Gallbladder; HBD: Hilar bile duct; HPD: Hepaticopancreatic duodenectomy; LH: Left hepatectomy; m: Mucosal layer; LHBD: Left hepatic bile duct; MD: Moderately differentiated; mp: Proper muscle layer; muc: Mucinous adenocarcinoma; pap: Papillary adenocarcinoma; RH: Right hepatectomy; si: Serosal invasion; ss: Subserosal layer; tub: Tubular adenocarcinoma; WD: Well differentiated.

cancer often exhibits metastasis to the lymph node nearest the mass, or *via* the bile duct; therefore, spreading of micro-vessels and nerve system, depth of tumors and the possibility of peri-lymph node invasion must all be considered in discriminating between metastatic cancer and double primary cancer. When multiple cancers are identified, the distinction between multi-centric primary cancers and primary cancer metastases is often clinically difficult. Recent studies reported that analysis of loss of heterozygosity, point mutations of k-ras oncogene<sup>[20]</sup>, overexpression of the tumor suppressor gene p53<sup>[20]</sup> and tumor marker CA19-9, and CEA in the cancers, may all play an important role in diagnosing the second cancer as primary or metastatic tumors<sup>[4,19]</sup>. Hori *et al*<sup>[4]</sup> diagnosed four cases of double primary cancer of gallbladder and bile duct cancer by point mutation of k-ras and overexpression of p53, CEA, and CA19-9 by immunohistochemistry. Ogawa *et al*<sup>[19]</sup> reported metastatic bile duct cancer, located in both

the upper and lower extrahepatic bile duct, which met the criteria of double primary tumors; then he revealed metastasis by analyzing the loss of heterozygosity, using microsatellite genetic markers on five arms. Therefore, it is not sufficient to diagnose double cancer only by means of pathologic examination. New diagnostic technology, such as genetic analysis, may be needed.

This case was diagnosed as metachronous double biliary cancer, which developed nine years after resection for gallbladder cancer, and was cured surgically. This case shows evidence to support a diagnosis of double primary cancers. First, both the gallbladder cancer and the bile duct cancer were malignant; however, there was no evidence of malignancy in the surgical stump, including the neck of the gallbladder, indicating there was no link between them. Second, advanced gallbladder cancers usually recur within five years of resection. The second bile duct cancer was detected nine years after the first operation, and so it would be extremely

unlikely for the second cancer to be a remnant tumor from the first operation. Third, although both tumors of the gallbladder and the bile duct showed the same histological differentiation and positive k-ras and p53 immunohistochemistry, the expressions of CA19-9 were different: CA19-9 expression was negative in the gallbladder cancer and positive in the bile duct cancer. Lastly, the bile duct cancer resembled a primary tumor from evidence of dysplastic changes near the main mass which would not be found in metastatic cancer.

The etiology of double biliary tract cancer has not been defined, but it is generally accepted that the anomalous union of the pancreaticobiliary duct (AUPBD) plays an important role in the development of multiple biliary tract cancers. Kaneko *et al*<sup>[8]</sup> and Fujii *et al*<sup>[13]</sup> reported that 50%-62.5% of cases of synchronous double biliary tract cancers were associated with AUPBD. A close relationship is known to exist between cancer of the bile duct system and AUPBD. In most patients with AUPBD, there is a long common channel between the junction of the pancreatic and common bile ducts and the sphincter of Oddi. We also know that the risk of bile duct carcinoma increases with age. Continuous reflux of the pancreatic juice into the bile duct induces chronic inflammation and metaplastic epithelial changes in the biliary tree<sup>[20]</sup>. Despite more than half of synchronous double biliary cancers having AUPBD as a risk factor, none of the twelve metachronous cases was associated with AUPBD (Table 1). Also, bile duct cancer is associated with AUPBD, almost middle and inferior regions of the bile duct and none of superior part of the bile duct cancer are associated with AUPBD<sup>[8]</sup>. This would reinforce the idea, suggested by other investigators, that upper biliary tract cancers and metachronous double biliary cancers have a strong genetic predisposition, or oncogenic susceptibility, irrespective of the presence of AUPBD. Although most metachronous double cancers have no obvious cause, several predisposing factors have been identified including: previous treatment of cancer, environmental factors, race, genetic factors, and long-term survival<sup>[1]</sup>. Although this case shows no evidence of AUPBD, double cancer of the gallbladder and bile duct is associated with expression of k-ras oncogene and the p53 tumor suppressor gene. Thus, we can guess which oncogenes were responsible for both biliary systems forming a malignant lesion simultaneously.

Table 1 illustrates all twelve cases of metachronous biliary tract cancer (including this case) previously described in the literature. There were six male and six female patients, ranging in age from 12-83 years (mean, 63 years). One patient developed metachronous biliary double cancer aged 21 years<sup>[10]</sup>. The three cases of a second cancer in the upper bile duct and six cases of a second cancer in the lower bile duct were detected at a mean age of 5.7 years after a first operation for gallbladder resection. Three second cancers developed in the remnant bile duct at 7, 4, and 2.3 years after the first operation for bile duct resection. There was no definite cause identified in the 12 cases of metachronous bile duct cancer, including this case. None of them has AUPBD as

etiology. Also, the criteria for double primary cancer are based solely on pathologic evidence: with the exception of this case, no previous cases have used gene expression as a diagnostic tool.

There is no clear evidence about follow-up intervals and diagnostic methods during postsurgical monitoring. Takai *et al*<sup>[12]</sup> reported that magnetic resonance cholangiography and gadolinium enhanced dynamic MRI were useful in the diagnosis of a bile duct cancer that developed seven years after resection for gallbladder cancer, in the anatomically rearranged structure following gallbladder resection. Merenda *et al*<sup>[16]</sup> suggested a potential role for PET-CT to detect metachronous bile duct cancer in the postsurgical monitoring of bile duct cancer when abdominal CT did not reveal any lesions. However, metachronous bile duct cancer is very rare, so it is not cost-effective to routinely provide expensive procedures, such as MRI or PET-CT, during the postoperative period. Further studies are needed to facilitate the cost-effective early detection of metachronous double cancers.

In conclusion, we present a rare case of advanced metachronous double cancer of the gallbladder and bile duct cancer, separated by a period of nine years. We enhance our report with a review of the relevant literature.

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## Fulminant amoebic colitis during chemotherapy for advanced gastric cancer

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### INTRODUCTION

Amoebic colitis, the disease caused by the intestinal protozoan parasite *Entamoeba histolytica*, is common and sometimes fatal<sup>[1]</sup>. The manifestations of intestinal amoebiasis vary from no symptoms to severe ulceration with perforation and peritonitis. There are many reports on fulminant amoebic colitis<sup>[2,3]</sup>, but the development of fulminant amoebic colitis during chemotherapy is very rare. We describe our experience with a patient in whom fulminant amoebic colitis occurred while receiving chemotherapy for advanced gastric cancer.

### CASE REPORT

A 52-year-old man presented at our hospital suffering from epigastric pain. An upper gastrointestinal endoscopy revealed advanced gastric cancer. Abdominal ultrasonography and computed tomography showed liver metastasis and intra-abdominal lymph-node metastasis. Unresectable advanced gastric cancer was diagnosed. Chemotherapy with oral S-1 (120 mg/d), given for four weeks followed by two weeks of rest<sup>[4]</sup>, was started at the outpatient clinic. Computed tomography after seven courses of treatment showed disease progression. The chemotherapy regimen was therefore switched to second-line treatment with paclitaxel (140 mg/m<sup>2</sup>), given once every two weeks<sup>[5]</sup>. After six courses of the second-line treatment, constipation and diarrhea occurred repeatedly. During the seventh course of treatment, melena developed. Gastrointestinal bleeding caused by chemotherapy was suspected, and a gastrointestinal series was performed. An upper gastrointestinal endoscopy showed no bleeding from the primary tumor. Colonoscopic examination disclosed multiple hemorrhagic erosions throughout

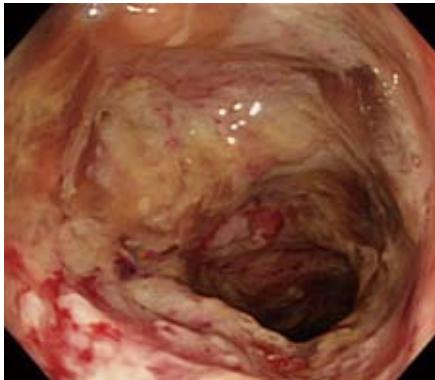
### Abstract

A 52-year-old man had bloody stools during chemotherapy for gastric cancer. A colonoscopy revealed necrotizing ulcer-like changes. A biopsy confirmed the presence of amoebic trophozoites. Subsequently, peritonitis with intestinal perforation developed, and emergency peritoneal lavage and colostomy were performed. After surgery, endotoxin adsorption therapy was performed and metronidazole was given. Symptoms of peritonitis and colitis resolved. However, the patient's general condition worsened with the progression of gastric cancer. The patient died 50 d after surgery. Fulminant amoebic colitis is very rarely associated with chemotherapy. Amoebic colitis should be considered in the differential diagnosis of patients who have bloody stools during chemotherapy.

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**Key words:** Fulminant amoebic colitis; Gastric cancer; Chemotherapy; Amoebiasis

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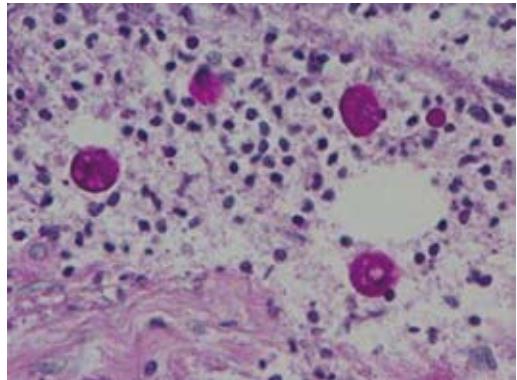
**Figure 1** Colonoscopy on admission, showing reddish, friable mucosa and necrotizing ulcers at the transverse colon.

the colon. A biopsy of the same site revealed only inflammatory-cell infiltration. Subsequently, melena became more frequent, and symptoms worsened. The patient was admitted to the hospital for further evaluation and treatment. On admission, his body temperature was 38.1°C. The palpebral conjunctivae were anemic. There was a palpable mass in the epigastric region, associated with tenderness. Edema of the lower extremities was present. On admission, laboratory examinations showed anemia, hypoalbuminemia, and elevated transaminases. A culture specimen was negative for human immunodeficiency virus.

After admission, the colonoscopy was repeated. Necrotizing ulcer-like changes covered with slough were circumferentially seen from the rectum to the transverse colon (Figure 1). A biopsy of this region showed amoebic trophozoites (Figure 2). Signs of peritoneal irritation developed two days after colonoscopic examination. Intestinal perforation was diagnosed on computed tomography, and emergency surgery was performed. The perforation point was detected in the middle of the transverse colon. Peritoneal lavage and colostomy were performed. After surgery, endotoxin adsorption therapy was performed, and metronidazole was given. The symptoms of peritonitis and colonitis resolved. However, the patient's general condition worsened due to progression of gastric cancer and the patient died 50 d after surgery.

## DISCUSSION

Dysenteric amoebiasis is caused by protozoan parasites, which can be classified into two types: pathogenic *E. histolytica* and non-pathogenic *Entamoeba dispar*<sup>[1]</sup>. The ingestion of food and drink contaminated by pathogenic *E. histolytica* causes amoebiasis. About 90% of infections are asymptomatic, and the remaining 10% of infections produce a spectrum of clinical syndromes, ranging from dysentery to abscesses of the liver or other organs. It is reported that bowel perforation occurs in nearly 1% of the patients hospitalized for amoebiasis<sup>[6]</sup>; delayed diagnosis might have resulted in high mortality even after surgical therapy. Mortality for patients who undergo colectomy with proximal ileostomy and distal colostomy is



**Figure 2** Histological findings of a biopsy specimen, showing *Entamoeba* that have ingested red blood cells, indicating that they are *E. histolytica*.

32% to 76%<sup>[2,3]</sup>. In this case, the perforation was pin hole-like lesion in the middle of transverse colon where friable mucosa and necrotizing ulcers were seen, suggesting that the reason for perforation was due to a discrete ulcer rather than due to the colonoscopic examination. However, the colectomy had not been performed and could not be reviewed pathologically, indicating limitations to diagnostic imaging studies. The possibility of iatrogenic perforation thus cannot be ruled out completely.

It is known that 4%-10% of asymptomatic individuals infected with *E. histolytica* develop disease over a year<sup>[1]</sup>. The onset is often gradual, but several studies have reported that steroids, the presence of diabetes mellitus, chronic alcoholism, and pregnancy can trigger fulminant amoebic colitis<sup>[1,7]</sup>. The classic endoscopic findings of amoebic colitis are described as discrete, round ulcerations that range in size and are covered with white or yellow exudates<sup>[8]</sup>. However, amoebic colitis can be mistaken for other causes of necrotizing colitis, such as idiopathic inflammatory bowel diseases and pseudomembranous colitis of *C. difficile*, because the appearance of amoebic ulcers is variable on endoscopic examination. Oral metronidazole is generally used to treat amoebic colitis. However, complications occur when adequate therapy and appropriate antibiotics are not initiated at an early stage, all the more so in an immunosuppressed state. It is well known that there are two high risk groups in nonendemic areas; one is homosexual men, and the other is travelers from the tropics. As for the route and time of infection, the patient had been to Southeast Asia more than 20 years before disease onset; therefore, the possibility of an acquired oral infection cannot be ruled out. In Japan, dysenteric amoebiasis is most commonly caused by *E. histolytica* infection<sup>[9]</sup>, with a particularly high incidence among homosexuals. However, it is noteworthy that our patient was not homosexual and that colitis developed during chemotherapy. In our patient, cytotoxic drugs are considered a risk factor of invasive amoebiasis, contributing to the immunosuppressed status. Cytotoxic drugs inhibit normal bacterial flora, suppress host defenses, and depress host immunity. In experimental free-living amoebic infections, some drugs suppressed host defenses with an increase in the mortality of infected mice<sup>[10]</sup>.

Amoebic colitis should therefore be included in the differential diagnosis in immunosuppressed patients with gastrointestinal signs and symptoms, such as bloody stools.

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CASE REPORT

## Endoscopic polypectomy: A promising therapeutic choice for esophageal carcinosarcoma

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### Abstract

Esophageal carcinosarcoma is a rare malignant tumor composing of both carcinomatous and sarcomatous elements. Endoscopic therapy is less invasive and may represent an alternative to esophagectomy for superficial esophageal carcinosarcoma. Here, we report a 61-year-old male who was diagnosed as esophageal carcinosarcoma and underwent endoscopic polypectomy with well tolerance and favorable prognosis. We also present a brief review of the literature.

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**Key words:** Carcinosarcoma; Endoscopic polypectomy; Endoscopic ultrasonography

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Ji F, Xu YM, Xu CF. Endoscopic polypectomy: A promising therapeutic choice for esophageal carcinosarcoma. *World J Gastroenterol* 2009; 15(27): 3448-3450 Available from: URL: <http://www.wjgnet.com/1007-9327/15/3448.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.3448>

### INTRODUCTION

Esophageal carcinosarcoma is a rare malignant neoplasm consisting of both carcinomatous and sarcomatous

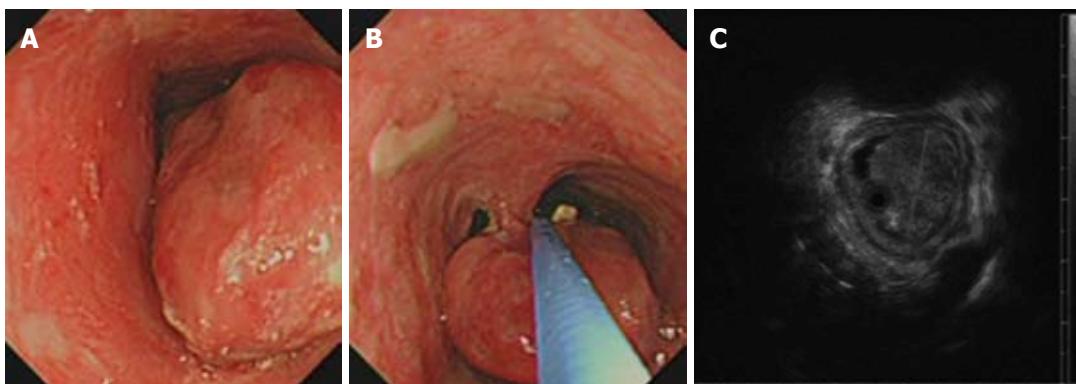
components<sup>[1]</sup>. Esophagectomy has been traditionally considered the first option for esophageal carcinosarcoma patients<sup>[1]</sup>. Endoscopic therapy is less invasive and allows the esophagus to be preserved, and may represent an alternative to esophagectomy for superficial esophageal carcinosarcoma. We here report a case of esophageal carcinosarcoma who underwent endoscopic polypectomy with well tolerance and favorable prognosis.

### CASE REPORT

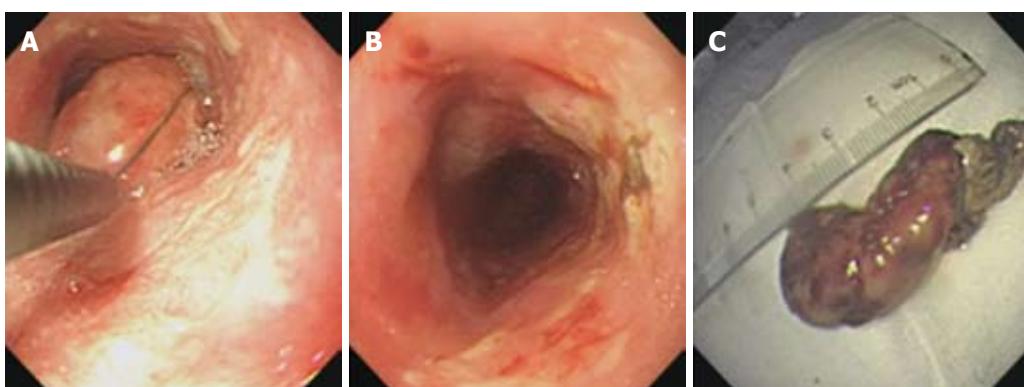
A 61-year-old male was admitted to our hospital with a 20-day history of progressive dysphagia. He was a heavy smoker and alcohol drinker, neither his medical nor family history included any relevant disease. Physical examination revealed no abnormalities. All serum tumor markers were normal. Barium meal showed a regularly-shaped circular stenosis in the upper esophagus. Endoscopy revealed a pedunculated polypoid tumor with ulcer on its surface. The tumor was located at 25-30 cm from the incisor teeth, and packed in the lumen of the esophagus (Figure 1A and B). Biopsy proved the mass to be a carcinosarcoma.

Endoscopic ultrasonography (EUS) and computed tomography (CT) scan were subsequently performed to show the stage of carcinosarcoma. EUS revealed a 5 cm × 3 cm × 3 cm hypoechoic mass with regular margins confined to the mucosal layer with no involvement of other layers or regional lymph nodes (Figure 1C). CT scan displayed an intraluminal mass but no evidence of metastases in brain, chest or abdominal organs. According to the clinical examinations, we performed an endoscopic polypectomy using a polypectomy loop to resect the bulky pedunculated tumor (Figure 2).

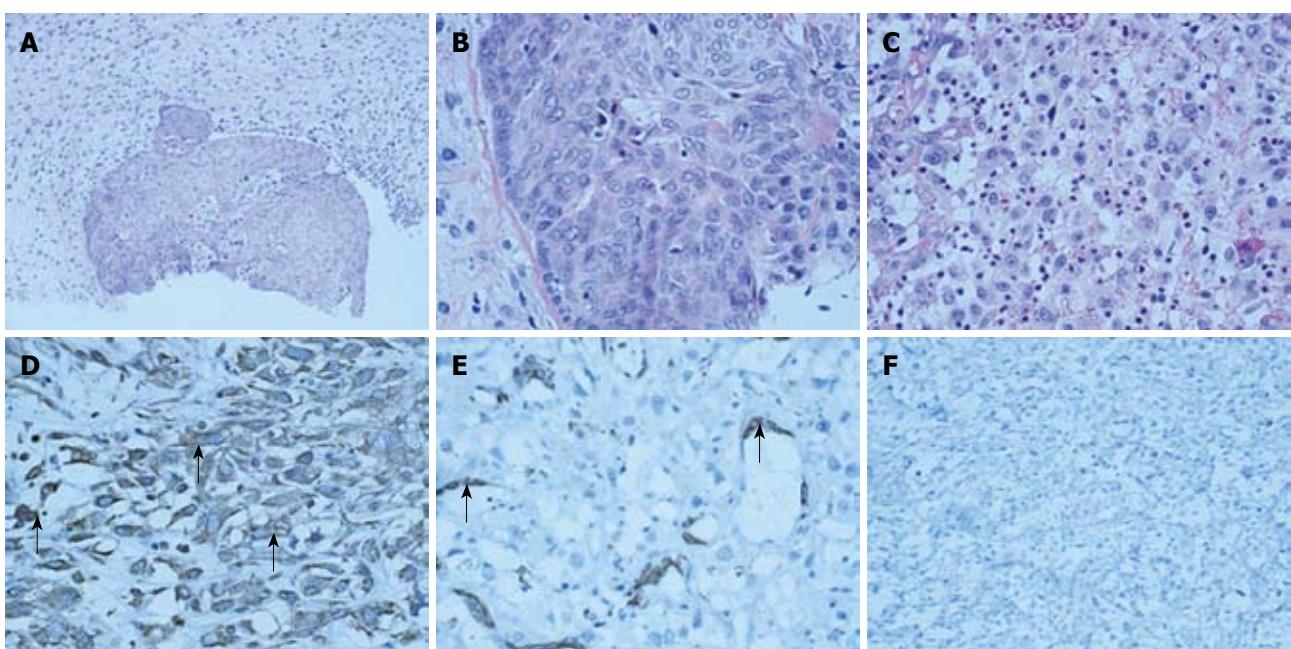
Histology confirmed both carcinomatous and sarcomatous areas in the resected mass (Figure 3A). Large nuclei of polygonal tumor cells with conspicuous nucleoli were observed in the carcinomatous area (Figure 3B) while multiform cells with abundant mitotic figures and bizarre giant cells were observed in the sarcomatous area (Figure 3C). Eosinophilic cytoplasm of tumor cells was found in both carcinomatous and sarcomatous areas with no definite keratinization and intercellular bridges between tumor cells. To further study the histogenesis of sarcoma, we used vimentin, smooth muscle antigen (SMA) and desmin (three well-established immunomarkers for mesenchymal tumor, myofibroblastoma and leiomyoma, respectively) to detect the corresponding antigen expression level in cells of the sarcomatous area. Immunohistology



**Figure 1 Endoscopic examinations.** A and B: Upper gastrointestinal endoscopy showed a pedunculated polypoid tumor of the esophagus; C: EUS found a  $5\text{ cm} \times 3\text{ cm} \times 3\text{ cm}$  hypoechoic mass with regular margins confined to mucosal layer only.



**Figure 2 Endoscopic polypectomy.** A: Pre-polypectomy; B: Post-polypectomy; C: Macroscopy of the esophageal carcinosarcoma.



**Figure 3 Histological findings.** A: Histology showing a transitional area (HE,  $\times 100$ ); B: A carcinomatous area (HE,  $\times 400$ ); C: A sarcomatous area (HE,  $\times 400$ ); D: Immunohistology showing a sarcomatous area positive for vimentin in cytoplasm (arrows, vimentin,  $\times 400$ ); E: Negative for SMA in cytoplasm and positive for internal control (arrows, SMA,  $\times 400$ ); F: Negative for desmin in cytoplasm (desmin,  $\times 400$ ).

revealed positive vimentin (Figure 3D), negative SMA or desmin (Figure 3E and F), suggesting that the sarcoma has a mesenchymal origin.

Polypectomy was performed. The patient recovered well and was discharged three days later with no relapse 17 mo after operation.

## DISCUSSION

Carcinosarcoma is a rare esophageal tumor showing polypoid configurations, with an incidence of 0.1%-1.5% of all esophageal malignancies<sup>[1]</sup>. This type of tumor is usually composed of invasive and/or *in situ* squamous carcinoma cells surrounding the base and surface of exophytic tumor and sarcomatous spindle cells forming the body of polypoid mass<sup>[2]</sup>.

The clinical presentation of esophageal carcinosarcoma is similar to that of squamous cell carcinoma with dysphagia as the most prominent and frequent symptom<sup>[1]</sup>. The mean age of patients at diagnosis in both histological groups is 70 years with a strong male predominance<sup>[1]</sup>. The anatomic distribution of both tumor types follows a similar pattern with the middle third of esophagus being the most common location<sup>[1]</sup>. The bulky nature of esophageal carcinosarcoma is due to its earlier clinical manifestations of dysphagia and obstruction<sup>[3]</sup>, which may also explain why the more favorable prognosis is associated with carcinosarcoma rather than with other esophageal tumors.

The histogenesis of sarcomatous spindle cell component of esophageal carcinosarcoma is still controversial. Some cases of esophageal carcinosarcoma with osteosarcoma, basaloid squamous carcinoma and rhabdomyosarcoma have been reported<sup>[4]</sup>. In our case, the esophageal tumor consisted of both carcinomatous and sarcomatous parts, and immunohistochemistry of sarcomatous parts revealed positive vimentin, a mesenchymal tumor immunomarker.

The treatment modality for esophageal carcinosarcoma include esophagectomy, endoscopic resection, chemo-radiotherapy, etc<sup>[5-7]</sup>. TNM staging is a critical determining factor for treatment decisions. Imaging methods such as EUS, CT and positron emission tomography (PET) play an important role in TNM staging. Compared with CT and PET, EUS is superior for T and N staging of esophageal cancer<sup>[8,9]</sup>. Esophagectomy has been traditionally considered the first option for esophageal carcinosarcoma patients<sup>[1]</sup>. With the advances in micro-invasive techniques, endoscopic procedures, including endoscopic polypectomy, endoscopic mucosal resection and endoscopic submucosal dissection, may represent an alternative to esophagectomy for superficial

esophageal carcinosarcoma<sup>[6]</sup> and are more tolerable, less invasive, less expensive, and most importantly, allow the esophagus to be preserved, compared with traditional esophagectomy.

In our case, endoscopic examination showed a pedunculated polypoid esophageal mass, biopsy indicated the diagnosis of carcinosarcoma, EUS and CT scan showed no evidence of local and distant metastases. Endoscopic polypectomy was subsequently performed. The patient tolerated well to the operation, recovered well, with no relapse during the 17-mo follow-up period, suggesting that endoscopic polypectomy is a promising therapeutic choice for esophageal carcinosarcoma.

In summary, esophageal carcinosarcoma is a rare disease entity. EUS is a useful and accurate method for T and N staging of esophageal carcinosarcoma. Endoscopic therapy is an interesting alternative to surgery for patients with large pedunculated esophageal carcinosarcoma with no involvement of lymph nodes.

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LETTERS TO THE EDITOR

## An exceptional cause of left lower quadrant abdominal pain

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**Figure 1 Abdominal CT scan.** A midline cecum (grey arrow) and dilated appendix with a thickened wall containing fecaliths consistent with acute appendicitis (white arrow).

### Abstract

Acute appendicitis is an exceptional cause of left lower quadrant abdominal pain. Computed tomography scan is the key to its diagnosis and helps to establish its early treatment. We present a case of a 35-year-old male patient who presented acute appendicitis with redundant and loosely attached cecum which was diagnosed based on his left lower quadrant abdominal pain.

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**Key words:** Abdominal pain; Left lower quadrant; Appendicitis; Cecum; Computed tomography scan; Surgery

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### TO THE EDITOR

Acute appendicitis remains an exceptional cause of left lower quadrant abdominal pain. We report a case of an otherwise healthy 35-year-old male who presented with a 6-h increasing abdominal pain located in the left lower quadrant. The patient had a low-grade fever (38.4°C) and physical examination demonstrated that he had rebound

tenderness and guarding in the hypogastric and left paraumbilical areas. Laboratory tests revealed mild left-shifted leucocytosis ( $11.8 \times 10^9/L$ , neutrophils: 86%) and elevated C-reactive protein concentration (86.2 mg/L). According to the subtle presentation, an IV-injected and contrast-ingested computed tomography (CT) abdominal scanning showed a tubular, dilated, blind-ending and fluid-filled structure (Figure 1), strongly suggestive of acute appendicitis next to a midline-located cecum. The patient underwent appendectomy through a midline laparotomy. The appendix was acutely inflamed, while the cecum was redundant and loosely attached. Intestinal malrotation was excluded. The patient's postoperative course was uneventful.

Acute appendicitis remains an uncommon cause of left lower quadrant pain, leading to intestinal malrotation or *situs inversus*<sup>[1]</sup>. Such a clinical presentation is anecdotic in the absence of anatomic anomalies<sup>[2]</sup>. The exceptional mobility of the cecum in this case explains the left-sided symptoms. In the context of atypical acute abdominal pain, CT scan is helpful in reducing mortality and morbidity when its prompt and accurate diagnosis is established<sup>[3,4]</sup>. Actually, it is also useful in adopting the midline approach. Isolated acute appendicitis remains an exceptional cause of left lower quadrant pain which can be most accurately explored with abdominal CT scan.

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## Meetings

### Events Calendar 2009

January 12-15, 2009  
Hyatt Regency San Francisco, San Francisco, CA  
Mouse Models of Cancer

January 21-24, 2009  
Westin San Diego Hotel, San Diego, CA  
Advances in Prostate Cancer Research

February 3-6, 2009  
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)  
Second AACR Conference  
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009  
Hyatt Regency Boston, Boston, MA  
Translation of the Cancer Genome

February 8-11, 2009  
Westin New Orleans Canal Place, New Orleans, LA  
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009  
Hong Kong Convention and Exhibition Centre, Hong Kong, China  
19th Conference of the APASL  
<http://www.apasl2009hongkong.org/en/home.aspx>

February 27-28, 2009  
Orlando, Florida  
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009  
Vienna, Austria  
EASL/ AASLD Monothematic: Nuclear Receptors and Liver Disease  
[www.easl.ch/vienna2009](http://www.easl.ch/vienna2009)

March 13-14, 2009  
Phoenix, Arizona  
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009  
Marriott Wardman Park Hotel  
Washington, DC  
13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009  
Glasgow, Scotland  
British Society of Gastroenterology (BSC) Annual Meeting  
Email: bsg@mailbox.ulcc.ac.uk

April 8-9, 2009  
Silver Spring, Maryland  
2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009  
Colorado Convention Center, Denver, CO  
AACR 100th Annual Meeting 2009

April 22-26, 2009  
Copenhagen, Denmark  
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)  
<http://www.easl.ch/>

May 17-20, 2009  
Denver, Colorado, USA  
Digestive Disease Week 2009

May 29-June 2, 2009  
Orange County Convention Center Orlando, Florida  
45th ASCO Annual Meeting  
[www.asco.org/annualmeeting](http://www.asco.org/annualmeeting)

May 30, 2009  
Chicago, Illinois  
Endpoints Workshop: NASH

May 30-June 4, 2009  
McCormick Place, Chicago, IL  
DDW 2009  
<http://www.ddw.org>

June 17-19, 2009  
North Bethesda, MD  
Accelerating Anticancer Agent Development

June 20-26, 2009  
Flims, Switzerland  
Methods in Clinical Cancer Research (Europe)

June 24-27 2009  
Barcelona, Spain  
ESMO Conference: 11th World Congress on Gastrointestinal Cancer  
[www.worldgicancer.com](http://www.worldgicancer.com)

June 25-28, 2009  
Beijing International Convention Center (BICC), Beijing, China  
World Conference on Interventional Oncology  
<http://www.chinamed.com.cn/wcio2009/>

July 5-12, 2009  
Snowmass, CO, United States  
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009  
Aspen, CO, United States  
Molecular Biology in Clinical Oncology

August 1-7, 2009  
Vail Marriott Mountain Resort, Vail, CO, United States  
Methods in Clinical Cancer Research

August 14-16, 2009  
Bell Harbor Conference Center, Seattle, Washington, United States  
Practical Solutions for Successful Management  
<http://www.asge.org/index.aspx?id=5040>

September 23-26, 2009  
Beijing International Convention Center (BICC), Beijing, China  
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists(IASGO)  
<http://iasgo2009.org/en/index.shtml>

September 27-30, 2009  
Taipei, China  
Asian Pacific Digestive Week  
<http://www.apdwcongress.org/2009/index.shtml>

October 7-11, 2009  
Boston Park Plaza Hotel and Towers, Boston, MA, United States  
Frontiers in Basic Cancer Research

October 13-16, 2009  
Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States  
Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009  
Versailles, France  
Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009  
Boston, MA, United States  
The Liver Meeting

November 15-19, 2009  
John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States  
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009  
London, UK  
Gastro 2009 UEGW/World Congress of Gastroenterology  
[www.gastro2009.org](http://www.gastro2009.org)



### Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.



## Instructions to authors

### GENERAL INFORMATION

*World Journal of Gastroenterology* (*World J Gastroenterol* ISSN 1007-9327 CN 14-1219/R) is a weekly open-access (OA) peer-reviewed journal supported by an editorial board consisting of 1179 experts in gastroenterology and hepatology from 60 countries.

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*Chinese journal article (list all authors and include the PMID where applicable)*

- 2 Lin GZ, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

*In press*

- 3 Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

*Organization as author*

- 4 Diabetes Prevention Program Research Group. Hypertension,

insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

*Both personal authors and an organization as author*

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

*No author given*

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

*Volume with supplement*

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

*Issue with no volume*

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900 DOI:10.1097/00003086-20020800-00026]

*No volume or issue*

- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

## Books

*Personal author(s)*

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

*Chapter in a book (list all authors)*

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

*Author(s) and editor(s)*

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

*Conference proceedings*

- 13 **Hardden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

*Conference paper*

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming-EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

**Electronic journal** (list all authors)

- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

**Patent** (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc, assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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[1]Passed away on October 20, 2007

[2]Passed away on June 11, 2007

[3]Passed away on June 14, 2008



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## Could quantitative liver function tests gain wide acceptance among hepatologists?

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### Abstract

It has been emphasized that the assessment of residual liver function is of paramount importance to determine the following: severity of acute or chronic liver diseases independent of etiology; long-term prognosis; step-by-step disease progression; surgical risk; and efficacy of antiviral treatment. The most frequently used tools are the galactose elimination capacity to assess hepatocyte cytosol activity, plasma clearance of indocyanine green to assess excretory function, and antipyrine clearance to estimate microsomal activity. However, a widely accepted liver test (not necessarily a laboratory one) to assess quantitative functional hepatic reserve still needs to be established, although there have been various proposals. Furthermore, who are the operators that should order these tests? Advances in analytic methods are expected to allow quantitative liver function tests to be used in clinical practice.

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### INTRODUCTION

Liver biopsy is the first-line method for evaluating liver injury. As a result of its limitations and risks, alternative methods have been developed. Many markers and several imaging methods have been evaluated in studies published in peer-reviewed journals. Although the methodological rigor of the design, execution and analysis of the studies proposing new tests has always been ascertained, only their regular use can establish their acceptance among physicians. Probably, nervousness-based medicine plays a role. Fear of litigation is a powerful stimulus to over-investigation, and in an atmosphere of litigation phobia, the only bad test is the test that the physician did not think of ordering<sup>[1]</sup>.

The assessment of liver function has always been considered as important for estimating such things as the severity of any acute or chronic liver disease, and its prognosis and treatment efficacy (Table 1). Generally, methods used to determine cellular damage and related consequences consist of noninvasive and rapid tests, which are easy to perform, e.g. serum enzymes, surrogate serum fibrosis markers, or transient elastography and ultrasound imaging. The first quantitative liver function tests (QLFTs) were suggested almost 50 years ago<sup>[2]</sup>. Their rationale was based on the fact that a drug or a foreign compound is metabolized primarily by the liver cytochrome P450 system, through sequential oxidative processes, and the major metabolite can be detected. The main characteristic of the proposed drug is its relatively high hepatic extraction ratio, but a QLFT depends not only on hepatic metabolic capacity, but also on hepatic blood flow. A more recent study has addressed the clinical utility of measuring galactose elimination capacity (GEC), aminopyrine breath test (ABT) and indocyanine green (ICG) retention test. The results have shown that all QLFTs are predictors of survival in cirrhosis, and that GEC adds new prognostic information to that already available using the Child-Pugh classification<sup>[3]</sup>. In spite of this, relatively few centers perform these tests. Hence, they are currently performed only in very few specialized institutions and usually within the setting of clinical research projects.

More recently, preoperative assessment of liver function and prediction of residual postoperative functional liver parenchymal mass and reserve has been ascertained to be of paramount importance to minimize surgical risk, especially in patients with hepatocellular carcinoma, the

**Table 1** Frequently used QLFTs in patients with liver cirrhosis, chronic viral hepatitis and non-alcoholic liver disease

Type	Methods/drawbacks
GEC	Intravenous administration of galactose; blood samples at 5, 25 and 45 min; urine collected for 5 h
ICG	Measurement of liver plasma flow; injection of ICG must be performed quickly; blood samples at 3, 6 and 49 min
MEGX	Blood samples at 15 and 30 min after i.v. lidocaine administration; allergy to anesthetics
ABT	Resting period of at least 30 min before the breath test that should be repeated three times at 10-min intervals
13C-C breath test	Subjects ingested 2 mg/kg of [3-methyl-13C]-caffeine sitting quietly for 15 min before and throughout the test; breath samples were collected immediately prior to, and 60 min after, caffeine ingestion
13C-M breath test	Measurement of breathed CO <sub>2</sub> by laser-based technology
SCI	Measurement of liver plasma flow. Sorbitol (500 g/L) was administered via a perfusor at 7.5 mL/h. Serum and urinary concentrations of sorbitol were determined at the beginning of the perfusion and after reaching steady-state
TOSCA	Unique sample of saliva to be collected in the morning; rare compliance to drinks containing caffeine by some patients
LURs	Volumetric information as well as functional assessment; expensive
MRI	Expensive; to be validated

QLFTs: Quantitative liver function tests; GEC: Galactose elimination capacity; ICG: Indocyanine green; MEGX: Monoethylglycinexylidide; ABT: Aminopyrine breath test; MRI: Magnetic resonance imaging; LURs: Liver uptake ratios.

majority of whom have liver cirrhosis as a complication. Incorporation of ICG into the decision tree has enabled patients conventionally classified into Child-Pugh class A to be subdivided into several groups in which various hepatectomy procedures are feasible. Applying this choice to 685 patients, during 10 years, only a single fatality was encountered<sup>[4]</sup>. Probably, this is the most important area of application of QLFTs. Indeed, new limits have been established to decrease mortality and morbidity rates after liver resection in cirrhotic and non-cirrhotic patients.

Various laboratory data and imaging techniques have been used to complement the Child-Pugh score to predict liver failure after hepatectomy and to assess functional hepatic reserve. The greatest experience has been made so far with the ABT and GEC, which are decreased among hepatic failure patients after liver resection. However, absence of these changes does not totally exclude hepatic failure. The ICG retention test is the most widely used clearance test. Nevertheless, it remains imperfect because it depends on hepatic blood flow and on the functional capacity of the liver.

Nuclear imaging plays a determinant role in assessing liver function, although it is expensive. Nuclear imaging of the asialoglycoprotein receptors with radiolabeled synthetic asialoglycoproteins provides volumetric information as well functional assessment of the liver<sup>[5]</sup>. Single photon emission computed tomography (SPECT) is superior to the planar method for determining liver uptake ratios (LURs). Evaluation of LURs is a suitable indicator of 99mTc-galactosyl serum albumin clearance from the blood pool and of binding to the asialoglycoprotein receptor, which is a simple and clinically useful indicator for the assessment of hepatic functional reserve in chronic liver diseases<sup>[6]</sup>.

Recent interest toward QLFTs comes from emergency medicine and intensive care physicians. There is no ideal real-time and bedside technique for assessing liver function in critically ill patients. Dynamic tests such as ICG plasma disappearance rate and lidocaine metabolism [monoethylglycinexylidide (MEGX) test], are superior to static tests such as prothrombin time and bilirubin measurement. Recently, the ICG plasma disappearance

rate, which nowadays can be measured reliably by a transcutaneous system in critically ill patients at the bedside and provides results within a few minutes, has been confirmed to correlate well with ICG clearance. In general, the ICG plasma disappearance rate is superior to bilirubin, and comparable or even superior to complex intensive care scoring systems in terms of outcome prediction. Furthermore, ICG plasma disappearance rate is more sensitive than serum enzyme tests for assessing liver dysfunction, and early improvement in the ICG plasma disappearance rate after onset of septic shock is associated with better outcome<sup>[7]</sup>.

## SOME APPLICATIONS OF QLFTs

### Liver cirrhosis

The aim of this report is not to review QLFTs, but to provide a critical appraisal of their extremely selective use. Widespread application of QLFTs as a prognostic tool is controversial. In a recent study, the predictive value of serial evaluations of GEC and MEGX on survival in a cohort of 35 patients was assessed, and secondarily, these tests were compared to Child-Pugh and Model for End Stage Liver Disease (MELD) scores. The end points were patient death or liver transplantation. Statistically significant differences between dead/transplanted patients and survivors were found for basal values of GEC, MEGX, Child-Pugh class and MELD score. Surprisingly, receiver operating characteristic (ROC) curves of Child-Pugh class and MELD score showed a higher prognostic accuracy than GEC and MEGX. On multivariate analysis, neither GEC nor MEGX were independent predictors of survival. Repeated-measures analysis of GEC and MEGX did not increase the prognostic accuracy of these tests, and did not add useful prognostic information on patient outcome during the following 6 mo. These data suggest that neither single nor repeated determinations of GEC and MEGX are superior to Child-Pugh class and MELD score in predicting prognosis of patients with viral cirrhosis<sup>[8]</sup>.

Blood galactose clearance after an intravenous galactose load has been used widely as a QLFT. A novel

QLFT, the galactose single point (GSP) method, has been developed to assess residual liver function in various diseases<sup>[9]</sup>. The goal of that study was to evaluate the influence of non-hepatic factors such as hyperglycemia on GSP and GEC in rats. Four groups of animal studies were carried out, i.e. normal control (NC), streptozotocin-induced diabetes mellitus (DM), CCl<sub>4</sub>-induced hepatotoxicity (CCl<sub>4</sub>), and streptozotocin-induced diabetes with CCl<sub>4</sub>-induced hepatotoxicity (DM + CCl<sub>4</sub>). The serum glucose levels in the diabetic groups (DM and DM + CCl<sub>4</sub>) were significantly increased compared with those in the NC and CCl<sub>4</sub> groups. A significant increase of aspartate aminotransferase and alanine aminotransferase was observed in the CCl<sub>4</sub>-treated groups (CCl<sub>4</sub> and DM + CCl<sub>4</sub>) compared with those in the NC and DM groups. In comparison with the NC group, the values of GSP and GEC in the diabetic groups (DM and DM + CCl<sub>4</sub>) were significantly reduced and increased, respectively. GSP had highly significant correlations with GEC. These results suggest that galactose metabolism tests should be interpreted with caution under conditions of significant hyperglycemia<sup>[9]</sup>.

### **Transplantation**

The unique ability of the liver to regenerate quickly after resection makes living donor liver transplantation (LDLT) possible. However, the quality and course of this regeneration process in humans are still unexplored. In a recent study, GEC, ICG and lidocaine half-life as markers for the quality of liver regeneration in the first 3 mo after LDLT were investigated. Twenty-two consecutive living liver donors and their corresponding recipients were analyzed at baseline and at 10 and 90 d after LDLT. Six recipients lost their grafts during the study period. We compared donors and recipients at the different time points. After LDLT, GEC decreased (-42.6%) and ICG increased (+50.6%) significantly in donors. ICG and GEC remained significantly altered over 3 mo in donors with an improvement between days 10 and 90. ICG and GEC improved significantly in recipients between days 10 and 90. The lidocaine half-life showed no significant changes. The donors had better test results and recovered faster than the recipients. In conclusion, after LDLT, the parameters for liver capacity and flow remain altered in donors and recipients despite rapid volume growth<sup>[10]</sup>.

### **Timing**

The key point of the whole problem is not how to test residual liver function but when. The <sup>13</sup>C-methacetin (13C-M) breath test enables the quantitative evaluation of cytochrome-P450-dependent liver function. 13C-M is metabolized in the liver by O-demethylation to <sup>13</sup>CO<sub>2</sub> and acetaminophen. The aim of a previous study was to evaluate the 13C-M breath test in comparison to the Child-Pugh class and other QLFTs (MEGX and ICG). 13C-M (2 mg/kg) was given orally to 31 patients with histologically proven liver cirrhosis of different etiology and severity (nine Child-Pugh class A, 13 class

B, and nine class C). The increase of exhaled <sup>13</sup>CO<sub>2</sub> was expressed as delta over baseline (DOB; delta/1000). All breath test parameters analyzed provided an excellent discrimination between cirrhotic and non-cirrhotic individuals. The DOB value at 20 min showed a superior correlation with the Child-Pugh class than did MEGX or ICG clearance results. With a cut-off value of  $\leq 25$  delta/1000 at 20 min, sensitivity and specificity to discriminate between cirrhotic and non-cirrhotic individuals was 93.5% and 95%, respectively<sup>[11]</sup>.

### **Pre-cirrhotic stage**

To find out whether this breath test is sensitive in non-cirrhotic patients who also have chronic hepatitis C in the early stages of fibrosis, the following study was carried out. Eighty-one patients with chronic hepatitis C underwent a 13C-M breath test. In all patients, a liver biopsy was performed. The liver histology was classified according to the histology activity index-Knodell score. Patients with early fibrosis did not differ in DOB values from patients at 15 min ( $23.2 \pm 7.9$  per thousand vs  $22.6 \pm 7.2$  per thousand;  $P = 0.61$ ), or cumulative recovery ( $13.6\% \pm 3.7\%$  vs  $13.2\% \pm 3.8\%$ ;  $P = 0.45$ ) from patients with more advanced fibrosis. Conclusively, the noninvasive 13C-M breath test fails to detect early stages of fibrosis in patients with chronic hepatitis C<sup>[12]</sup>.

The 13C-caffeine (13C-C) breath test is a noninvasive, QLFT that is considered to be a valid tool by many authorities. The utility of the 13C-C breath test was measured in 48 patients with chronic hepatitis B and 24 controls, along with its ability to monitor response to lamivudine. In 28 patients on lamivudine, 13C-C breath tests were performed at 1 wk and 1 year after therapy. Patients with Metavir F0-1 fibrosis had a 13C-C breath test similar to the controls. However, patients with F2-3 fibrosis and cirrhosis patients had a decreased 13C-C breath test. Fibrosis correlated best with the 13C-C breath test. The 13C-C breath test independently predicted significant ( $F \geq 2$ ) and advanced ( $F \geq 3$ ) fibrosis and yielded the greatest area under the ROC curve ( $0.91 \pm 0.04$ ) for predicting advanced fibrosis. The 13C-C breath test was unaltered by 1 wk of lamivudine but improved by 61% ( $P < 0.001$ ) in responders to long-term lamivudine, whereas in those with viremia and elevated alanine aminotransferase, values remained stable or deteriorated. The 13C-C breath test distinguishes chronic hepatitis-B-virus-related fibrosis and detects improvement in liver function in response to long-term lamivudine<sup>[13]</sup>.

### **Survival studies**

Caffeine clearance (CCl) has been suggested as a more exact method than those commonly used. The aim of the following study was to assess the usefulness of CCl in survival prediction of patients with liver cirrhosis. Thirty-four patients with cirrhosis of varying etiology were included: 19 were Child-Pugh class A or B and 15 were class C. CCl was determined from saliva samples. The mean length of follow-up was 33.8 mo. A bivariate

survival analysis was carried out following the Kaplan-Meier method, together with a multivariate analysis using the Cox proportional hazards model. Twelve patients died during follow-up. CCl values < 0.24 mL/kg per minutes, age > 60 years, and non-alcoholic cause of cirrhosis were factors predicting lower survival. CCl was the only independent predictive factor in the multivariate analysis. The authors concluded that that CCl enables hepatologists to predict survival in cirrhotic patients and, considering its harmlessness, simplicity and cost, it can be used as a routine procedure in the assessment of these patients<sup>[14]</sup>.

A simplification of this test, the so-called Total Overnight Salivary Caffeine Assessment (TOSCA), comes from another study<sup>[15]</sup> with a further application (patients divided into rapid and slow metabolizers). Furthermore, TOSCA shows near complete safety (patients drink one or two cups of coffee according their habit in the morning). One drawback of QLFTs is the possible occurrence of severe side effects that are sometimes life-threatening (e.g. anaphylaxis).

Magnetic resonance imaging (MRI) offers several advantages. Gadolinium methoxybenzyl diethylenetriamine penta-acetic acid is a newly developed MR contrast agent. Its hepatic extraction fraction is a direct, noninvasive technique for the quantitative evaluation of liver function. It may be a promising alternative, although expensive, for the determination of noninvasive hepatic function in patients with liver disease<sup>[16]</sup>.

### **Antiviral therapy**

Whether and to what extent does antiviral therapy for chronic hepatitis C influence a broad panel of QLFTs? Fifty patients with chronic hepatitis C were treated with interferon ( $n = 8$ ), interferon/ribavirin ( $n = 19$ ) or peg-interferon/ribavirin ( $n = 23$ ). Quantitative testing of liver function, including ABT, GEC, sorbitol clearance (SCI) and ICG clearance was performed before and 3 mo after initiation of antiviral therapy. After 3 mo, 36 patients showed normal transaminases and were negative for hepatitis C virus RNA, and 14 patients did not respond to therapy. ABT and GEC as parameters of microsomal and cytosolic liver function were reduced in all patients before therapy initiation and returned to normal values in the 36 therapy responders after 3 mo. Parameters of liver perfusion (SCI and ICG) were not affected by antiviral therapy. In the 14 non-responders, no changes in QLFT values were observed during the treatment period. ICG and SCI remained unaffected in patients with chronic hepatitis C, while ABT and GEC were significantly compromised. ABT and GEC normalized in responders to antiviral therapy. Early determination of ABT and GEC may differentiate responders from non-responders to antiviral treatment in hepatitis C<sup>[17]</sup>.

### **Assessing liver regeneration**

Improvement of nitrogen balance is desirable in patients with acute or chronic illness. Both growth hormone and insulin-like growth factor-I are promising anabolic agents, and their combined administration has been shown to reverse catabolism more efficiently than each

of the peptides alone. The capacity of urea-nitrogen synthesis [ $\mu\text{mol}/(\text{min} \times 100 \text{ g body weight})$ ] was evaluated in rats, unravelling a neglected QLFT, based on mitochondrial-cytosolic metabolic capacity (M-CMC) for conversion of amino-nitrogen<sup>[18]</sup>. Following this approach, the authors used GEC to assess hepatocyte cytosol activity, plasma clearance of ICG to assess excretory function, antipyrine clearance to estimate microsomal activity, and functional hepatic nitrogen clearance<sup>[19]</sup> to assess M-CMC in females with Turner syndrome<sup>[19]</sup>.

### **Non-alcoholic steatohepatitis (NASH)**

Finally,  $^{13}\text{C}$ -C, a noninvasive tool for the evaluation of the cytochrome P450 system, which is implicated in the development of NASH, has been proposed in patients with metabolic dysfunction. Up-to-date research has demonstrated that  $^{13}\text{C}$ -C can predict reliably severe hepatic fibrosis in patients with the most severe form of non-alcoholic fatty liver disease (NAFLD). Although this test does not quantify the residual functional liver mass, it is safe and easy to perform. Further large-scale studies are needed to verify its reliability<sup>[20]</sup>. A previous study that tested  $^{13}\text{C}$ -M and  $^{13}\text{C}$ -ketoisocaproate for microsomal and mitochondrial liver function has demonstrated its usefulness for better and noninvasive characterization of patients with NAFLD<sup>[21]</sup>. It is worth stressing that every breath test can be affected badly by severe restrictive lung disease, and in elderly patients with chronic heart failure<sup>[22]</sup>.

Unfortunately, MRI findings of liver steatosis and fibrosis in NASH show moderate correlations with histopathological grade of steatosis and stage of fibrosis, but MRI findings of NASH do not demonstrate any significant correlations with MELD score<sup>[23]</sup>. Liver scintigraphy (SPECT) might be a promising noninvasive tool in the follow-up of NASH patients in therapeutic trials<sup>[24]</sup>.

## **CONCLUSION**

While liver function is absolutely complex, a widely accepted test to assess quantitative functional hepatic reserve still needs to be established, although there are various tests currently available. The diagnostic and prognostic gain has been quantified as modest. A new condition in which it may be useful to test residual liver function is acute liver disease<sup>[25]</sup>. Focusing on some aspects of controversial conclusions, or those not supported by very positive results, in the context of the current doctrine is always provocative, although it provides scientists and physicians with responsible and balanced information to support experimental and clinical decisions. Future technical advances may lead to a decrease in time, cost and the number of subjects required to perform QLFTs, therefore, their use in clinical practice is expected to increase.

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TOPIC HIGHLIGHT

Kostas Pantopoulos, Associate Professor, Series Editor

## Alcoholic liver disease and hepatitis C: A frequently underestimated combination

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### Abstract

Alcoholic liver disease (ALD) and hepatitis C virus (HCV) infection represent, either alone or in combination, more than two thirds of all patients with liver disease in the Western world. This review discusses the epidemiology and combined impact of ALD and HCV on the progression of liver disease. ALD and HCV affect the progression of liver disease to liver cirrhosis and hepatocellular carcinoma (HCC) in a synergistic manner. Thus, the risk for HCC increases five times with a daily alcohol consumption of 80 g; in the presence of HCV it is increased 20-fold, and a combination of both risk factors leads to a more than 100-fold risk for HCC development. Alcohol consumption also decreases the response to interferon treatment which is probably due to a lack of compliance than a direct effect on HCV replication. Several molecular mechanisms are discussed that could explain the synergistic interaction of alcohol and HCV on disease progression. They include modulation of the immune response and apoptosis, increased oxidative stress via induction of CYP2E1 and the hepatic accumulation of iron. Thus, both HCV and alcohol independently cause hepatic iron accumulation in > 50% of patients probably due to suppression of the liver-secreted systemic iron hormone hepcidin. A better understanding of hepcidin regulation could help in developing novel therapeutic approaches to treat the chronic disease in the future. For now, it can be generally concluded that HCV-infected patients should abstain from alcohol and alcoholics

should be encouraged to participate in detoxification programs.

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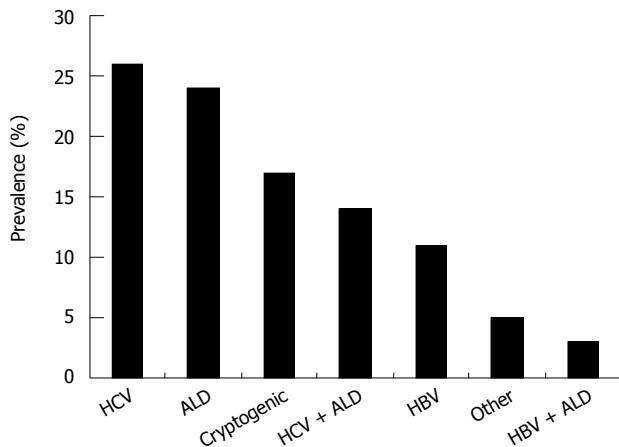
**Key words:** Alcoholic liver disease; Chronic hepatitis C; Steatosis; Steatohepatitis; Fibrosis; Cirrhosis; Reactive oxygen species; Hepatocellular carcinoma; Iron accumulation

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### INTRODUCTION

Together, alcoholic liver disease (ALD) and chronic hepatitis C virus (HCV) infection are the most frequent chronic liver diseases in the Western world. In addition, they frequently coexist in the same individual. While both diseases alone have a similar progression sequence leading to cirrhosis in circa 15% of patients within 10-20 years, their coexistence dramatically enhances disease progression in a so-called synergistic manner. This synergism affects both fibrosis progression and the development of hepatocellular carcinoma (HCC). The basic molecular mechanisms of this synergism are far from being understood but may include increased production of reactive oxygen species (ROS) and deposition of iron. In the present article, we review and discuss the epidemiology of ALD and HCV infection, the synergistic impact of combined alcohol and HCV on the progression of liver disease, viral replication and response to anti-HCV treatment. We finally analyze potentially



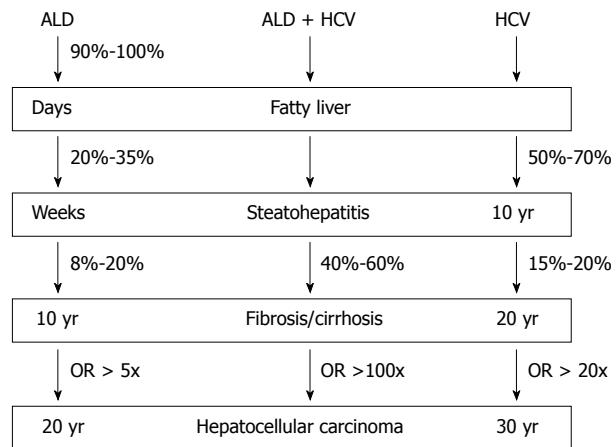
**Figure 1** HCV and ALD, either in combination or alone, represent the majority of liver diseases (data from the US Centers of Disease Control and Prevention 2007). HCV: Hepatitis C virus; ALD: Alcoholic liver disease; HBV: Hepatitis B virus.

underlying mechanisms that may explain the interaction between alcohol and HCV and offer novel molecular strategies for future therapeutic interventions.

## EPIDEMIOLOGY OF ALD AND HCV

Chronic alcohol consumption causes approximately 50% of the chronic liver disease burden in Germany and the death of more than 18000 inhabitants per year<sup>[1]</sup>. In the US alcohol is also responsible for more than 50% of liver related deaths, and ALD is a major health care cost expenditure, accounting for nearly \$3 billion annually<sup>[2]</sup>. At present, the country with the fastest increase in alcohol associated health problems is the Peoples Republic of China with an annual per capita increase in alcohol consumption of 400% and more in some geographic regions<sup>[3]</sup>. The exact number of alcohol related deaths is difficult to obtain due to inaccurate reporting of ethanol use. Since patients with compensated liver cirrhosis may often die by causes not obviously related to liver disease e.g. infectious complications, official mortality tables most likely underestimate the true prevalence of ALD. If the relationship between alcohol intake and prevalence of ALD is examined on a population basis, the risk of developing ALD starts at 20-30 g ethanol per day. Liver cirrhosis develops only in 10%-20% of people consuming more than 80 g of ethanol daily<sup>[2]</sup>. Approximately 5% of the whole population in the US meet diagnostic criteria for alcoholism<sup>[4]</sup>. In Germany, more than 17.8% of the population > 18 years drink more than 20-30 g of alcohol per day<sup>[5]</sup>, and a comparable number of 5% show high risk drinking behavior (> 80 g/d)<sup>[5]</sup>.

In contrast to ALD, the prevalence of HCV is easier to determine based on serological studies. The worldwide seroprevalence of HCV antibodies is estimated to be 3% with marked geographic variations from 1% in North America to 10% in North Africa<sup>[6]</sup>. The prevalence is higher in males than in females (2.5% vs 1.2%) and is highest in the 30-49 years old age group<sup>[7]</sup>. Taken together, there is an estimated prevalence of high risk drinking and HCV of 1%-5% in the Western world. According to re-



**Figure 2** Natural course of ALD and HCV alone or in combination. Estimated risk and time interval for disease states are indicated (for more details see text).

cent data from the Center of Disease Control and Prevention, the prevalence of HCV and ALD is relatively similar at 26% and 24%, respectively. Although there is a selection bias, these prevalence data are somehow reflected by large transplant centers. In our transplant center at the University of Heidelberg, liver cirrhosis due to HCV and ALD are leading causes for liver transplantation accounting for 32% and 24%, respectively, of all liver transplants. In summary, HCV and ALD represent either alone or in combination, more than two thirds of all patients with liver disease in the Western world<sup>[8]</sup> (Figure 1).

## NATURAL COURSE OF ALD AND HCV AND IMPORTANT PROGRESSION FACTORS

ALD is the most important organ manifestation of chronic alcohol consumption. Ninety percent to one hundred percent of heavy drinkers develop alcoholic fatty liver. Ten percent to thirty-five percent of them develop alcoholic steatohepatitis and 8%-20% develop alcoholic liver cirrhosis within 10-20 years<sup>[9]</sup>. The natural course of ALD and HCV is given in Figure 2. Due to better treatment options for complications of liver cirrhosis e.g. variceal bleeding, the prevalence of HCC is increasing with an annual risk of 1%-2%. HCC represents the most common cause of death in patients with ALD. HCV shows a similar progression pattern. In a US study, the mean interval between HCV infection, chronic hepatitis, cirrhosis and HCC was circa 10, 20 and 30 years, respectively<sup>[10]</sup>. A large cohort study with long-term follow up showed that 75% of HCV-infected patients develop persistent infection while severe progressive liver disease occurred in 15%-20%<sup>[11]</sup>.

Factors that contribute to progression of ALD and HCV are summarized in Table 1. For ALD, these include the amount of alcohol consumed over a life time<sup>[12,13]</sup>, drinking patterns, and nutritional status. Both malnutrition and obesity are associated with an increased risk for alcoholic cirrhosis<sup>[14-16]</sup>. This is especially relevant with the endemic occurrence of non-alcoholic fatty liver disease

**Table 1 Comparison of risk factors for ALD and HCV**

HCV	ALD
Male gender	Female gender
	Amount and duration of alcohol consumption
	Continuous drinking ( <i>vs</i> sporadic drinking)
	Overweight/malnutrition
Hepatic iron deposition age > 40	Hepatic iron deposition
Immunosuppression	Vitamin A, co-medication

ALD: Alcoholic liver disease; HCV: Hepatitis C virus.

(NAFLD) in the Western World due to obesity and being overweight associated with diabetes mellitus type II and peripheral insulin resistance. Co-medication of certain drugs together with ethanol may also harm the liver by increased conversion to toxic metabolites due to induced enzyme systems. This is well known for acetaminophen<sup>[17]</sup>, methotrexate<sup>[18]</sup> and the tuberculostatic drug isoniazid<sup>[17]</sup> but also occurs with retinoids such as β-carotin and vitamin A<sup>[19]</sup>. Males and females show different courses of ALD and HCV. While females are more susceptible to alcoholic damage, they progress slower in chronic HCV infection. Other important factors that contribute to disease progression in ALD are co-morbidities such as HBV, hemochromatosis, and Wilson's disease. Factors associated with HCV progression are co-infection with HBV, HIV, schistosomiasis or conditions of immunosuppression. Finally, iron accumulation has been recognized both in ALD and HCV as an independent risk factor for the development of HCC. Pathological hepatocellular iron deposits are found in more than 50% of patients with either HCV or ALD. Underlying mechanisms and potential therapeutic options are still under investigation.

## EFFECT OF ALCOHOL ON THE PREVALENCE OF HCV INFECTION

Chronic alcoholics have an increased prevalence of HCV infection, increasing with the severity of the ALD. Takase *et al*<sup>[20]</sup> showed that HCV prevalence demonstrated by anti-HCV positivity increases with the severity of ALD, having a prevalence of approximately 5% in alcoholic fibrosis, almost 40% in alcoholic cirrhosis and almost in 80% in HCC due to alcohol. This could be due to the lifestyle of chronic alcoholics, since many of them are also intravenous drug abusers, which is a high risk for HCV infection. It could also be due to the immunosuppressive effect of alcohol decreasing the HCV-clearance rate after infection since it has been shown that alcohol suppresses the function of various immune components including natural killer cells, neutrophils, monocytes and others<sup>[21]</sup>.

## ALCOHOL CONSUMPTION, HCV REPLICATION AND RESPONSE TO HCV THERAPY

A great number of studies emphasize the fact that

alcoholics respond poorly to interferon therapy. More than ten years ago, Mochida *et al*<sup>[22]</sup> showed that almost 30% of non-alcoholics responded biochemically and virologically to interferon therapy compared to less than 10% of alcoholics. The question remained open whether this is due to a direct inhibitory effect of alcohol on interferon response or due to poor compliance of these patients. Pessione *et al*<sup>[23]</sup> studied serum HCV RNA in HCV patients with increasing alcohol intake (reported in gram per week). In this study a significant dose-dependent increase in serum HCV RNA was noted starting from 70 g alcohol per week. In line with this observation, a decrease of alcohol consumption prior treatment of hepatitis C significantly reduced viral load. In addition, Cromie *et al*<sup>[24]</sup> showed that viral load decreased highly significantly within 4 mo when patients cut down on alcohol consumption from 39-100 g/d to 0-50 g/d. More recent data, however, clearly suggest that the poor response of alcoholics towards interferon therapy is more likely due to reduced compliance. In this study, the recorded alcohol consumption during the months before HCV treatment was associated with an increased rate of therapy drop out (3% *vs* 26%,  $P = 0.002$ )<sup>[25]</sup> while the response rate was comparable (25% *vs* 23%) after correction for this confounding factor. In conclusion, poor compliance of alcoholics is probably the major cause for poor antiviral response to HCV therapy.

## COMBINED EFFECTS OF ALCOHOL AND HCV ON FIBROSIS PROGRESSION

A huge number of studies have shown that concomitant alcohol consumption in the presence of HCV increases progression of fibrosis<sup>[23,26-54]</sup>. This means that fibrosis occurs at an earlier time point and its development is accelerated. A summary of selected studies on alcohol consumption and fibrosis progression is given in Table 2. Thus, it has been shown in more than 2000 HCV patients that fibrosis progression was significant ( $P < 0.001$ ) if more than 50 g/d alcohol is consumed<sup>[26]</sup>. Similar results were obtained by Roudot-Thoraval *et al*<sup>[27]</sup> with a prevalence of cirrhosis of 35 % *vs* 18 % ( $P < 0.001$ ). Pessione showed in more than 200 HCV patients that weekly alcohol consumption correlated significantly with fibrosis score<sup>[23]</sup>. He also showed that the relative risk for decompensated cirrhosis correlated with alcohol intake. Alcohol-driven fibrogenesis in HCV patients is dose-dependent and starts at less than 30 g/d. Overweight and obese patients as well as type II diabetics are especially sensitive to fibrosis progression<sup>[55]</sup>. HCV patients with excessive alcohol abuse have a 2-3 fold increased risk of severe liver disease compared with HCV patients without a history of drinking<sup>[56]</sup>. So far it is still unclear how long a patient has to abstain from alcohol before the negative effect of alcohol is abolished<sup>[57]</sup>. Alcoholics with HCV infection seem to stop drinking more frequently compared to alcoholics without HCV infection. This is possibly due to a higher awareness in these patients that liver disease can lead to cirrhosis and death without a change in lifestyle<sup>[58]</sup>. Finally, it has been a

**Table 2 Fibrosis progression in HCV and alcohol consumption from selected studies**

No. of patients	Alcohol consumption	Results	Ref.
2235	0 g, < 50 g, > 50 g	> 50 g independent risk factor for fibrosis progression ( $P < 0.001$ )	[26]
6664	> 5/6 drinks (female/male), > 1 year	Higher risk of cirrhosis (35% vs 18%)	[27]
176	> 40/60 g (female/male), > 5 years	Faster cirrhosis progression (58% vs 10%), 2-3 fold increased risk of developing cirrhosis	[29]
168	Low < 30, medium 30-80; high > 80 g/d, > 5 J	Alcohol consumption low/medium/high significantly different between non-cirrhotics (58%/27%/16%) and cirrhotics (76%/15%/9%) ( $P < 0.05$ )	[28]
234	Lifetime alcohol consumption	Cirrhotics have greater alcohol consumption than patients with hepatitis (240 g/wk vs 146 g/wk) ( $P = 0.02$ )	[30]
233	0, 25, 50, 75, 100, > 125 g	Weekly alcohol consumption correlates with serum HCV RNA levels and fibrosis score ( $P < 0.001$ )	[23]
702	0/175 g/d	HCV increases OR for cirrhosis from 1 to 15 (0 g), 9.2 to 147.2 (175 g)	[141]
1667	Subgroup: > 260 g/wk vs < 90 g/wk	Risk for cirrhosis increases by 3.6	[31]
636	> 80	RR for cirrhosis: HCV 7.8, HCV + alcohol 31.1	[32]

continuous debate whether small amounts of alcohol (< 20-30 g/d) alter progression in HCV infection. An answer may come from a Scandinavian study by Westin *et al*<sup>[59]</sup>. These authors investigated 78 patients with hepatitis C infection who underwent two liver biopsies in a mean interval of 6.3 years. Alcohol consumption was less than 40 g/d. The authors found progressive fibrosis with (a) increased total alcohol consumption (15.4 kg vs 3.9 kg;  $P = 0.007$ ), (b) increased daily alcohol consumption (5.7 g vs 2.6 g/d;  $P = 0.03$ ) and (c) increased frequency of drinking occasions (35 vs 8 d per year;  $P = 0.006$ ). These results underscore that even small amounts of alcohol may increase fibrosis progression in HCV. Confirmation comes from another prospective study by Hezode *et al*<sup>[60]</sup> who showed an impact of mild alcohol consumption on histological activity and fibrosis starting as low as 20 g/d.

## ROLE OF ALCOHOL AND HCV ON DEVELOPMENT OF HCC

Various studies have shown that there is an increased risk of HCC in patients with HCV and alcohol abuse compared to either HCV or ALD alone<sup>[61-68]</sup>. Since these studies vary considerably in their definition of alcohol abuse, Table 3 is restricted to comparable studies that tried to identify the independent contribution of HCV and ALD to HCC development. It can be concluded from these data, that a daily uptake of > 80 g alcohol alone increases HCC risk 5-fold while the presence of HCV alone increases HCC 20-fold. A combination of both risk factors increases the risk for HCC development over 100-fold. Thus, HCV and alcohol act truly synergistic on HCC development.

## POSSIBLE MECHANISMS FOR THE SYNERGISM OF ALCOHOL AND HCV

The underlying molecular mechanisms of alcohol and HCV-mediated liver disease are complex and they are still incompletely understood despite intensive efforts over decades.

Both alcohol and HCV can reproduce the four sequential hallmarks of liver disease: steatosis, steato-

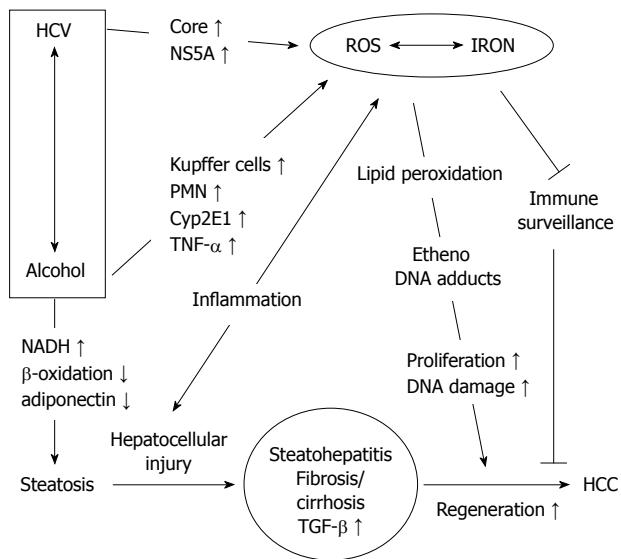
**Table 3 Odds ratio for the development of HCC as a function of HCV seroprevalence and amount of alcohol consumption**

Alcohol consumption (in grams alcohol per day)							
No	Yes	0-40	40-80	> 60	> 80	Ref.	
Without HCV		1	1.5		7.3	[63]	
	1	2.4	1.7		4.5	[68]	
				2		[67]	
With HCV				3		[64]	
			26.1	62.6		126	[63]
	19.1	53.9					[64]

hepatitis, fibrosis and HCC. Molecular key features of ethanol and HCV mediated liver damage include direct biochemical consequences of alcohol metabolism such as the production of acetaldehyde, generation of reactive oxygen species (ROS) and oxidative damage, epigenetic modifications such as hypomethylation of histones and modulation of the signaling machinery. Some of these events lead to similar downstream effects such as fatty liver, ROS and iron accumulation but are based on different mechanisms which could well explain the synergistic effects of alcohol and HCV on the liver. Thus, steatosis in HCV is mainly caused by impairment of mitochondria preventing mitochondrial metabolism of fatty acids, while ethanol primarily stimulates lipogenesis. On the other hand, HCV and ethanol both stimulate ROS generation via distinct mechanisms and they both lead to hepatic iron accumulation, one of the most pro-fibrogenic and protumorigenic factors in liver disease. For this reason, ROS generation and iron accumulation are discussed separately below. It should also be mentioned that alcohol may have direct molecular effects on HCV infection since it exerts stimulatory effects on HCV replication probably via signaling pathways<sup>[69]</sup>. The enhanced quasispecies complexity in the hypervariable region 1 of HCV in alcoholics may be one major cause that sensitizes for faster disease progression<sup>[70]</sup>.

### Alcohol, HCV and liver damage

Ethanol biochemically leads to a shift towards NADH which ultimately stimulates lipogenesis. In addition,



**Figure 3** Potential molecular mechanisms that explain the synergistic effect of alcohol and HCV on the progression of liver disease. Reactive oxygen species (ROS) and iron accumulation seem to be key features of both diseases.

ethanol is metabolized to the mutagenic metabolite acetaldehyde and during that reaction ROS are produced mainly as a byproduct of CYP2E1. Additional mechanisms include the release of cytokines such as TNF- $\alpha$ , which increases free fatty acid release from adipocytes in the periphery of the liver<sup>[71]</sup>, stimulates lipogenesis in hepatocytes<sup>[72]</sup>, and inhibits  $\beta$ -oxidation of fatty acids<sup>[73]</sup>. Chronic ethanol consumption also impairs transport and secretion of triglycerides as VLDL<sup>[74]</sup> which again leads to an increased hepatic fat accumulation. Activation of macrophages by lipopolysaccharides *via* the toll-like receptor 4 (TLR-4) leads to the production of a variety of inflammatory mediators, such as TNF- $\alpha$  and ROS. HCV also leads to steatosis but in contrast to ALD mainly *via* a decreased mitochondrial  $\beta$ -oxidation with ultrastructural alterations of hepatocyte mitochondria in more than half of the patients. This means that HCV and alcohol stimulate fat accumulation in the liver *via* distinct mechanisms. In addition to its role in steatosis, abnormal production of TNF- $\alpha$  is also a critical inflammatory component in the liver induced by chronic ethanol exposure<sup>[75,76]</sup>. Although direct exposure of macrophages in culture can mimic some of the effects of ethanol<sup>[77,78]</sup>, there seem to be multiple hepatic and extra-hepatic consequences of ethanol that finally render Kupffer cells more reactive to LPS, leading to generation of ROS and ROS-modulated signal transduction cascades<sup>[79,80]</sup>. The fat regulating hormone adiponectin also seems to be involved in ethanol mediated steatohepatitis<sup>[81-83]</sup>. Some data indicate that ethanol directly drives fibrosis progression: acetaldehyde is supposed to increase TGF- $\beta$ 1 secretion<sup>[84]</sup> and both ethanol and acetaldehyde induce accumulation of collagen<sup>[85]</sup>. Similar findings have been shown for HCV-replicating hepatoma cells<sup>[86]</sup>.

HCC pathogenesis by ethanol seems to require several factors<sup>[87]</sup> including the presence of cirrhosis, oxidative stress, altered methyl transfer resulting in

DNA hypomethylation, and a decrease in retinoic acid. In addition, co-morbidities such as viral hepatitis, diabetes mellitus and obesity are known to accelerate HCC development in patients with ALD. ROS play an important role in hepatocarcinogenesis<sup>[87,88]</sup>. Chronic ethanol consumption results in the generation of ROS *via* multiple pathways leading to lipid peroxidation (LPO) and LPO-byproducts such as 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA). These DNA-reactive aldehydes in turn form mutagenic exocyclic DNA adducts including 1, N6-ethenodeoxyadenosine (edA) and 3, N4-ethenodeoxycytidine<sup>[89,90]</sup>.

#### Role of ROS in HCV and ALD

The generation of ROS seems to be a hallmark of both ALD and HCV<sup>[91-95]</sup> (Figure 3). While location and mechanisms of their generation differ markedly between ALD and HCV, downstream events of oxidative damage are similar due to the high but rather unspecific reactivity of species such as hydroxyl radicals or lipid peroxidation products. Hepatocyte mitochondria are structurally altered in more than 50% of HCV patients and these conditions are accompanied by a significant depletion of hepatocellular and lymphocyte glutathione (GSH), an increase of oxidized GSH (GSSG) and the lipid peroxidation marker malondialdehyde<sup>[91]</sup>. ROS are either induced directly by the virus or indirectly through activation of inflammatory cells. HCV core<sup>[96]</sup> and NS5A<sup>[96,97]</sup> have been implicated in generating ROS *via* mitochondrial damage and calcium release.

ROS also play an important role in alcohol-induced liver injury and in hepatocarcinogenesis<sup>[87-90]</sup>. Several enzyme systems are capable of producing ROS including the mitochondrial respiratory chain, the cytosolic enzymes xanthine oxidase and aldehyde oxidase, as well as the microsomal cytochrome P450-dependent mono-oxygenases<sup>[88]</sup>. One member of the latter system, cytochrome P450 2E1 (CYP2E1), is involved in the major pathway by which ethanol generates oxidative stress. Expression of CYP2E1 has been shown to correlate well with the generation of hydroxyethyl radicals and with LPO products such as 4-HNE and MDA<sup>[98]</sup>. CYP2E1 is induced by chronic alcohol consumption within a week even at a relatively low ethanol dose (40 g/d), but the degree of CYP2E1 induction shows high variations between individuals<sup>[99]</sup>. Inhibition of CYP2E1 by chlormethiazole, a specific CYP2E1 inhibitor, improved ALD as shown in the Tsukamoto-French rat model<sup>[100]</sup>. An increase of oxidative DNA adducts and of mutagenic apurinic and apyrimidinic DNA sites has been found in chronically ethanol-treated wild-type mice but not in mice that lack functional CYP2E1<sup>[101]</sup> further stressing the importance of CYP2E1 in the generation of DNA damage following ethanol ingestion. Increased levels of Cyp2E1 also potentiate pro-apoptotic effects of TGF- $\beta$  resulting in increased cell death of hepatocytes<sup>[102]</sup>. Recently, we have been able to detect etheno-DNA adducts such as edA in the livers of patients with ALD<sup>[89,103]</sup>.

Kupffer cells are also an important source of ROS

during ethanol exposure<sup>[93]</sup> and in response to LPS<sup>[104]</sup>. NADPH oxidase-dependent production of ROS is implicated in ethanol-induced liver injury since p47phox -/- mice which are deficient in this regulatory subunit of NADPH oxidase are resistant to chronic ethanol-induced injury<sup>[105]</sup>. Chronic ethanol feeding increases the LPS-stimulated production of ROS by Kupffer cells; this increase is primarily due to an increase in NADPH oxidase activation after chronic ethanol feeding<sup>[81]</sup>. Recently, Thakur and colleagues have specifically identified NADPH oxidase-derived ROS as an important contributor to increased TLR-4 mediated signal transduction and TNF- $\alpha$  expression in rat Kupffer cells, particularly after chronic ethanol exposure<sup>[81]</sup>.

### **Ethanol and HCV lead to hepatic iron accumulation**

In contrast to hepatitis B infection, iron deposits are found in more than 50% of patients with HCV infection or chronic ethanol consumption<sup>[96,106-109]</sup>. Even mild to moderate alcohol consumption has recently been shown to increase the prevalence of iron overload<sup>[110]</sup>. Iron localization has been reported in Kupffer cells<sup>[108]</sup> as well as in hepatocytes<sup>[111-113]</sup>. In our experience, iron accumulation is more common in hepatocytes than Kupffer cells in patients with ALD. Increased hepatic iron content is associated with greater mortality from alcoholic cirrhosis, suggesting a pathogenic role of iron in ALD. Genetic hemochromatosis in conjunction with excessive alcohol consumption exacerbates liver injury<sup>[100]</sup>. It should be mentioned that iron *per se* is the most profibrogenic and genotoxic factor and 50% of patients with hereditary hemochromatosis develop fibrosis and have a 200-fold increased risk for HCC<sup>[114]</sup>. On the other hand, immune surveillance can be impaired by iron overload, since it compromises anti-tumor activity of macrophages<sup>[115-117]</sup>.

The underlying mechanisms of iron accumulation observed in ALD and HCV are still poorly understood but seem to involve an inadequate upregulation of the iron hormone hepcidin. Genome wide microarray based screening for candidate genes that could cause iron overload involved several genes not yet linked to iron metabolism<sup>[118]</sup>. Preliminary data from ALD patients and ethanol-treated mice models suggest that hepatic iron uptake pathways are increased in the liver and potential mechanisms involve an increase of the transferrin receptor (TfR)1 and repression of the systemic iron hormone hepcidin that controls duodenal iron absorption and RES-mediated iron release *via* the iron exporter ferroportin<sup>[119,120]</sup>. Using novel *in vitro* and *in vivo* models<sup>[121,122]</sup>, we have recently demonstrated that H<sub>2</sub>O<sub>2</sub> alone increases TfR1 *via* posttranscriptional and translation mechanisms ultimately leading to cellular accumulation of iron<sup>[123,124]</sup>. These data show that chronic exposure of cells to non toxic levels of H<sub>2</sub>O<sub>2</sub> lead to accumulation of iron *via* distinct regulatory mechanisms promoting Fenton chemistry. We suggest that increased oxidative stress in the form of H<sub>2</sub>O<sub>2</sub> is an important regulatory factor that causes continuous iron accumulation and may support ALD progression.

Valuable information on the direct interaction of HCV with host metabolism has been gained from studies with genetically modified animals, though with some controversial results<sup>[125]</sup>. Thus mice transgenic for the total open reading frame of HCV under the murine albumin promoter developed steatosis and liver cancer<sup>[126,127]</sup>, but this association disappeared in later generations of animals, casting doubt on the earlier conclusions that HCV infection alone (in the absence of cirrhosis and iron overload) drives hepatic carcinogenesis<sup>[109,128,129]</sup>. In addition, iron overload induced in mice either through diet<sup>[130,131]</sup> or genetic deletion of the HFE locus<sup>[132]</sup> did not lead to advanced fibrosis or HCC. In HCV transgenic mice, hepcidin was found to be suppressed despite iron loading. This is unexpected, since hepcidin inhibits cellular iron efflux by inducing internalization of ferroportin<sup>[133]</sup>, an iron exporter that is expressed in macrophages, hepatocytes and intestinal cells. The mechanism by which hepcidin was downregulated in the present model remains elusive, since cytokines such as TNF- $\alpha$ , IL-1 $\beta$  and IL-6 which can upregulate hepcidin levels<sup>[134,135]</sup> were not suppressed. Other important players such as iron regulatory proteins (IRP1 and IRP2) which sense iron but also ROS at the cellular level have not been assessed<sup>[136]</sup>.

Finally, it has also been investigated whether iron directly affects HCV replication. In hepatoma cells iron loading promoted reporter expression under the control of regulatory HCV mRNA stem-loop structures by upregulating expression of the translation initiation factor 3eIF3<sup>[137]</sup>. In contrast, iron was shown to suppress HCV replication by inactivating the RNA polymerase NS5B<sup>[138]</sup>. Clinical data indicate that iron status does not significantly influence HCV replication *in vivo*, since the response to therapy of patients with  $\beta$ -thalassemia was not influenced by the degree of iron accumulation<sup>[139]</sup>, and venesection did not reduce hepatitis C viral load<sup>[140]</sup>. Taken together, iron accumulation in patients with HCV and ALD is an important progression factor. The underlying mechanisms are being intensively studied in search for novel therapeutic approaches.

### **CONCLUSION**

ALD and HCV are the most common liver diseases in the Western world either alone or in combination. Coexistence of both diseases has a true synergistic effect on fibrosis progression and HCC development. Thus, a daily consumption of more than 80 g alcohol increases the risk for HCC 5-fold, in the presence of HCV 100-fold while HCV alone increases the risk for HCC 20-fold. Alcohol abusers have an increased prevalence of HCV infection probably due to lifestyle or to immune suppression. Alcoholics also have a decreased response rate to antiviral therapy which is most probably due to poor compliance. There is obviously no safe level of drinking in patients with hepatitis C and it remains unclear how long abstinence is necessary to abolish the negative effect of alcohol on the liver. Potential mechanisms which may explain the synergistic negative effect of alcohol

and HCV infection on liver disease include generation of ROS, iron accumulation, steatosis induction, immune modulation, stimulation of HCV replication and direct DNA damage. Abstaining from drinking in HCV patients who do not respond to antiviral treatment is the sole efficient treatment option to date. A better understanding of the underlying molecular mechanisms could help to develop novel targeted treatment options.

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REVIEW

## Targeting host factors: A novel rationale for the management of hepatitis C virus

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### Abstract

Hepatitis C is recognized as a major threat to global public health. The current treatment of patients with chronic hepatitis C is the addition of ribavirin to interferon-based therapy which has limited efficacy, poor tolerability, and significant expense. New treatment options that are more potent and less toxic are much needed. Moreover, more effective treatment is an urgent priority for those who relapse or do not respond to current regimens. A major obstacle in combating hepatitis C virus (HCV) infection is that the fidelity of the viral replication machinery is notoriously low, thus enabling the virus to quickly develop mutations that resist compounds targeting viral enzymes. Therefore, an approach targeting the host cofactors, which are indispensable for the propagation of viruses, may be an ideal target for the development of antiviral agents because they have a lower rate of mutation than that of the viral genome, as long as they have no side effects to patients. Drugs targeting, for example, receptors of viral entry, host metabolism or nuclear receptors, which are factors required to complete the HCV life cycle, may be more effective in combating the viral infection. Targeting host cofactors of the HCV life cycle is an attractive concept because it imposes a higher genetic barrier for resistance than direct antiviral compounds. However the principle drawback of this strategy is the greater potential for cellular toxicity.

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**Key words:** Host factors; Hepatitis C virus; Novel treatment; Cell entry; Host metabolism; Nuclear receptors; Insulin resistance

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### INTRODUCTION

Chronic hepatitis C virus (CHC) infects approximately 170 million individuals worldwide and is a major cause of mortality and morbidity<sup>[1]</sup>.

Egypt has the highest hepatitis C virus (HCV) prevalence in the world (overall prevalence of HCV is 12% among the general population, reaches 40% in persons above 40 years of age and is more in rural areas)<sup>[2,3]</sup>.

HCV is an RNA virus that belongs to the family *Flaviviridae* with six known genotypes (numbered 1-6) and more than 50 subtypes (e.g. 1a, 1b, 2, etc)<sup>[4]</sup>. In general, the genetic make-up of the HCV genotype varies by about 30%-35% between its different genotypes<sup>[5,6]</sup>, and these differences in genotype are related to response to antiviral treatment.

Current treatment for patients with CHC is interferon-based therapies with ribavirin for 24-48 wk. Unfortunately, a sustained virological response (SVR) is achieved in only 42%-52% of treatment-naïve patients, and the rest of patients either show no response or experience a relapse when therapy is stopped<sup>[7]</sup>, with a wide profile of side effects.

The mechanisms underlying the failure of interferon therapy are not well understood, but evidence indicates that in addition to viral factors, several host factors are also involved<sup>[8]</sup>. So, CHC patients still need a novel approach for treatment of HCV infection.

A major obstacle in combating HCV infection is that the fidelity of the viral replication machinery is notoriously low, thus enabling the virus to quickly develop mutations that resist compounds targeting viral enzymes<sup>[9]</sup>. Therefore, an approach targeting the host factors that are indispensable for the propagation of viruses might be an ideal target for the development of antiviral agents because of a lower rate of mutation compared to that of the viral genome, as long as they have no serious side effects to patients.

A unique aspect of HCV that has not been observed in other viruses is that the entire viral life cycle is associated with cholesterol metabolism in host cells. Thus, drugs that target cholesterol metabolism might be useful for treating HCV infection<sup>[10]</sup>. Also, drugs targeting the host proteins required for HCV infection, nuclear receptor or anti-

receptor antibodies may be more helpful in combating the viral infection<sup>[11]</sup> (Table 1).

## INHIBITION OF VIRAL ENTRY

### Anti-receptor antibodies

HCV circulates in the bloodstream in different forms; either free or in a complex with immunoglobulin or lipoprotein. Implicated lipoproteins are very-low-density lipoprotein (VLDL), intermediate-density lipoprotein, or low-density lipoprotein (LDL)<sup>[12]</sup>. HCV RNA is always found in at least one of these fractions and represents 8% to 95% of the total plasma HCV RNA<sup>[13,14]</sup>. Entry into the host cell is the primary step in the HCV life cycle, which makes it an attractive target for antiviral therapies. Inhibition of viral entry can be accomplished at the level of cell receptor(s) or HCV pseudo-particles (HCVpp) but both approaches require an in-depth knowledge of interactions between host and virus<sup>[15]</sup>.

Attachment and cell entry of HCV is pH dependent and is a clathrin-dependent endocytic pathway<sup>[16,17]</sup>. Although the molecular details regarding how this virus enters a cell are unknown, CD81<sup>[18]</sup> and scavenger receptor class B type 1<sup>[19]</sup> seem to be the key receptor components that mediate viral entry. However, other potential receptors play a role in entry of HCV such as LDL receptor<sup>[20]</sup>, negatively charged glycosaminoglycans, and recently, Evans *et al*<sup>[21]</sup> added another molecule to the list of HCV receptors, namely, the tight junction protein claudin-1 (CLDN1).

The rationale for anti-receptor antibodies as a drug target is based on them not being prone to the problems of viral variability and high density lipoprotein (HDL) attenuation of neutralizing activity<sup>[22]</sup>.

Targeting viral receptors can be accomplished by various methods, including the design of small molecules that bind to proteins and prevent interaction(s) with HCV. The crystal structure of CD81 long extracellular loop enabled the design of small molecules that bind CD81 and prevent its association with HCV E2<sup>[23]</sup>. A recent presentation by Liu *et al*<sup>[24]</sup> identified compounds that displayed a dose-dependent inhibition of HCV infection.

**Scavenger receptor BI (SR-BI):** SR-BI is a lipoprotein receptor with the highest levels found in the liver and adrenal glands, responsible for the selective uptake of cholestryler ester from HDL<sup>[25-27]</sup>. HCV particles have been reported to be complexed with lipoproteins; it is possible that HDL interacts with HCVpp, *via* protein/protein or lipid/protein interactions<sup>[28]</sup>, suggesting an indirect interaction of virus with lipoprotein receptors<sup>[29,30]</sup>. Recent studies have demonstrated the cell culture-derived HCV association with VLDL containing apolipoprotein B (ApoB) and apolipoprotein E, supporting earlier claims of lipo-viral particles in human plasma.

Previous observations implicated SR-B1 as important for infection by different HCV subtypes and support for this hypothesis is the fact that the same SR-B1 protein element is responsible for the recognition of different HCV E2 glycoproteins despite the high level of variability between their amino acid sequences, especially in the HVR1 region previously shown to be involved in

**Table 1 Summary of the potential treatment options of CHC targeting host factors and their mechanism of action**

Drug	Mechanism of action
Nitazoxanide	Induces PKR phosphorylation
HMG-CoA reductase inhibitors	Disruption of HCV replication; depletion of geranylgeranyl lipids
Antisense RNA drugs targeting apoB	Blocks the assembly and secretion of VLDL, inhibits release of HCV particles from infected cells
Microsomal triglyceride protein (MTP) inhibitors	Blocks the assembly and secretion of VLDL, inhibits release of HCV particles from infected cells
Insulin sensitizer	
Metformin	Insulin sensitivity by acting on hepatic AMP-activated protein kinase
Thiazolidindiones (pioglitazone)	Insulin sensitivity by activating peroxisome proliferator-activated receptors (PPARs)
Debio-025	Inhibition of Cyclophilin B
NIM 811	Inhibition of Cyclophilin B
Tamoxifen, other anti-estrogen drugs	Potentially suppresses genome replication
Small molecules (e.g. receptor mimics, soluble intracellular adhesion molecule-1)	Receptor and uptake inhibition
Receptor antibodies (e.g. Anti CD81)	

interaction with SR-B1<sup>[19,31]</sup>.

HCV appears to use SR-BI during cell entry not merely as an additional site for the viral particle entry but also for exploiting its physiological activity, i.e. the capacity to mediate lipid transfer from HDL which is known to facilitate the entry of many different viruses, such as influenza virus, HIV, and HCV<sup>[32,33]</sup>. However, HCVs are many times more sensitive to HDL-mediated infection enhancement than other cholesterol-sensitive viruses. Therefore, enhancement of viral infection might be dependent on the lipid exchange activity of SR-B1<sup>[28]</sup>. Recently, a novel function of SR-Bs for viral antigen uptake and recognition has been suggested; SR-BI may represent a cell-surface receptor for the recognition of viral antigens and be implicated in trafficking exogenous viral antigens toward the MHC class I presentation pathway. The SR-BI-viral antigen interaction may represent a novel target for therapeutic or preventive strategies aiming at the induction of efficient antiviral immune responses<sup>[34]</sup>.

Moreover, HDL with SR-BI is the predominant enhancing factor in infectivity and the presence of HVR1 with HDL protects HCV from neutralizing antibodies as HDL can reduce the neutralizing effect of anti-HCV antibodies<sup>[35,36]</sup>. This phenomenon might be responsible, at least in part, for the limited ability of the humoral immune response to control HCV infection *in vivo*, which raises concerns about the efficacy of anti-HCV antibodies for active or passive immunotherapy<sup>[37]</sup>. Thus, as an alternative to the development of anti-HCV antibodies, one could consider anti-SR-B1 human MAbs or anti-CD-81 capable of interfering with HCV infection as potential therapeutic leads. Agents involved in modulating the normal hepatocellular processes of lipid transport have been reported to have pleiotropic effects on HCV infectivity. Antibodies to ApoB have been shown to have antiviral activity<sup>[13,29-31,38-43]</sup>.

Recent data show that BLT-4 and other inhibitors of SR-BI-mediated lipid transfer not only inhibit HCV entry but also fully restore the potency of neutralizing antibodies in infection assays conducted in the presence of HS/HDL, indicating an intriguing link between neutralization efficiency and stimulation of cell entry<sup>[28,35]</sup>. However, it is too early to know whether the potential for vaccines and passive immunotherapy will be realized. Cholesterol-lowering drugs may be beneficial in patients with chronic hepatitis C by exerting effects on cholesterol metabolism and lipoprotein trafficking via SR-BI (See below).

**CD81:** Recently, Meuleman *et al*<sup>[11]</sup> showed that CD81 is a critical receptor for HCV infection *in vivo*. Prophylactic injection of monoclonal anti-CD81 antibodies prevented infection of human liver-uPA-SCID mice, however once an infection occurred, no significant difference in viremia was observed between anti-CD81-treated and control animals (irrelevant antibody). These results strongly support the use of CD81 as a clinical target for HCV prevention, especially in the context of orthotopic liver transplantation<sup>[11]</sup>.

### **Modes of virus transmission**

Targeting CD81, SR-BI or CLDN1 may be complicated by receptor-independent modes of virus transmission. In general, there are two primary routes of virus transmission: cell-free and cell-to-cell. Cell-free transmission begins when virus is released from an infected cell and enters the extracellular environment. The virion can bind to surface-expressed receptors on naïve or uninfected cells, become internalized, and initiate new rounds of infection. En route from one cell to the next, the virus may encounter neutralising antibodies or other components of the immune response that may limit infection. In the second route of transmission, the virus spreads directly from one cell to another and, in doing so, may bypass receptor-mediated attachment as well as the immune response.

Direct cell-to-cell transmission has been observed with several viruses, including HIV<sup>[44]</sup>, human T-lymphotropic virus type 1<sup>[45]</sup> and vesicular stomatitis virus<sup>[46]</sup> and it was recently proposed that HCV may use this route *in vitro*<sup>[47]</sup>. Whether cell-to-cell HCV transmission occurs *in vivo* remains to be determined. If this mode of transmission exists *in vivo*, targeting cellular receptors alone may not be an effective antiviral therapy<sup>[48]</sup>.

Targeting receptors as antiviral therapy may also be complicated by their ubiquitous expression in human tissue and their essential roles in cell biology.

## **TARGETING HOST METABOLISM**

HCV seems to be not only an infectious hepatotropic virus but also a metabolic disease<sup>[49]</sup> with a wide area of metabolic disarrangement, including lipid metabolism<sup>[50]</sup>, glucose metabolism<sup>[51]</sup> and vitamin D metabolism<sup>[52,53]</sup>. Metabolism refers to all the reactions by which living things break down nutrients to produce energy, along with those reactions by which they rebuild broken-down nutrients into complex molecules (e.g. DNA). Many viruses, including influenza, HIV and hepatitis, dramatically increase cellular metabolism. The fields of metabolomics and fluxomics have emerged to

measure these patterns and to provide insight into diseases with a metabolic component, from diabetes to cancer to infectious diseases such as HCV. Many metabolic processes are essential to the survival of human cells, and so are not candidates for research efforts that would shut them down in an attempt to stop viral replication.

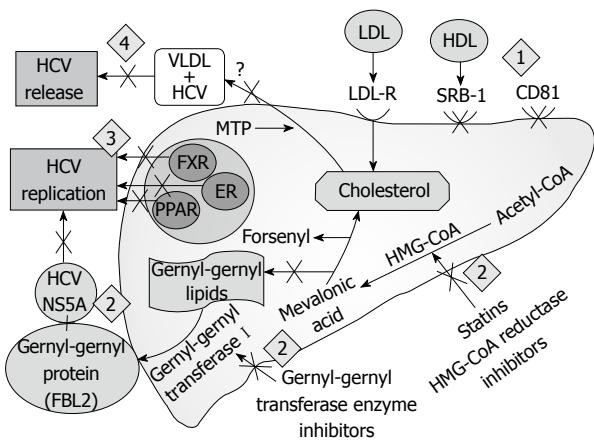
### **Host lipid biosynthesis inhibitors**

Recently, using the new fluxomic techniques, studies revealed that viral infection takes control of cellular metabolism and drives, among other things, marked increases in fatty acid synthesis. Interfering with glucose-to-fatty acid metabolism could stop viral replication, because fatty acid biosynthesis is not essential in adult humans. It does appear, however, to be essential to the ability of HCV to build their envelopes, reproduce and spread. So, targeting of host lipid metabolism by the existing anti-obesity drugs may represent a new way to block these metabolic changes and inhibit viral replication, and may therefore be a potential novel approach that could improve response rates to treatment<sup>[54]</sup>. There are at least two different molecular mechanisms representing a novel target for management of HCV through the modulation of cellular lipid and cholesterol metabolism. *In vitro* data suggest that statins, the widely used cholesterol-lowering drugs, may inhibit HCV RNA replication by depletion of geranylgeranyl lipids<sup>[55,56]</sup>. It was recently demonstrated that dose-dependent strong antiviral effects exist for all the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, except for pravastatin, *in vitro*. Fluvastatin exhibited the strongest antiviral activity, followed by atorvastatin and simvastatin<sup>[57]</sup>.

Recently, Bader *et al*<sup>[58]</sup> reported that fluvastatin inhibits HCV RNA replication in patients with CHC; the study provided evidence that fluvastatin is well tolerated in patients with CHC and at relatively low doses.

These findings, along with other data suggesting synergism with α-interferon, support ‘proof-of-concept’ for trials combining fluvastatin with standard pegylated interferon plus ribavirin. Cautious, prospective and randomized trials are needed before we can call statin therapy an adjuvant treatment panacea<sup>[54]</sup>.

Another class of drugs designed for treating hypercholesterolemia blocks the assembly and secretion of VLDL. These drugs may also be effective in treating HCV infection because they inhibit release of HCV particles from infected cells<sup>[59]</sup>. In this regard, antisense RNA drugs targeting apoB<sup>[60]</sup> and several microsomal triglyceride protein (MTP) inhibitors<sup>[61,62]</sup> have already been tested in clinical trials because of their ability to block VLDL secretion, thereby lowering the plasma levels of VLDL triglycerides and LDL cholesterol. Long-term treatment with MTP inhibitors led to the toxic accumulation of fat in livers<sup>[61,62]</sup>, thus hampering the approval of these drugs for the treatment of hypercholesterolemia on a long-term basis. However, short-term treatment (up to several weeks) reduced the plasma level of VLDL with only minor adverse effects, which disappeared after drug discontinuation<sup>[61]</sup>. It will be interesting to examine whether short-term treatment with MTP inhibitors is beneficial in treating HCV infection (Figure 1).



**Figure 1 Possible sites targeting host factors as a novel antiviral treatment.** (1) Inhibition of HCV entry by anti-receptor antibodies; (2) Interference with the host metabolic factor involved in HCV replication; (3) Modulation of nuclear receptors involved in HCV replication; (4) Inhibition of HCV release. LDL-R: Low density lipoprotein receptor; HDL: High density lipoprotein; VLDL: Very low density lipoprotein; SRB-1: Scavenger receptor B1; FXR: Farnesoid X receptor; ER: Estrogen receptors; MTP: Microsomal triglyceride protein; HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A.

### Cyclophilin B inhibitors

Another host cell factor involved in HCV RNA replication is the human protein cyclophilin B protein which interacts with the C-terminal region of NS5B and appears to stimulate its RNA binding activity<sup>[63]</sup>. The cyclophilin B inhibitor Debio-025 potently suppresses genotype 1 HCV replication *in vivo*<sup>[64]</sup>.

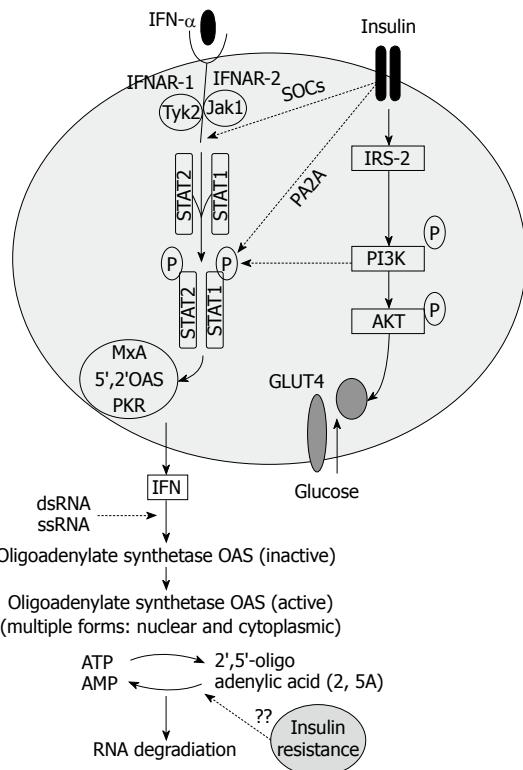
### Insulin resistance

Insulin resistance emerges as a very important host factor in patients with CHC, mainly because it has been related to steatosis development, fibrosis progression and non-response to peg-interferon plus ribavirin<sup>[65]</sup>. Insulin resistance is the main pathogenic factor in the development of steatosis in chronic hepatitis C; both viral-induced insulin resistance and metabolic insulin resistance could be implicated in the development of steatosis<sup>[66]</sup>.

Insulin resistance, calculated by the homeostasis model assessment (HOMA), has been found to be one of the most important host factors related to the impermanence of virological response to combined therapy in chronic hepatitis C patients<sup>[67]</sup>.

Recently, obesity has been identified as a modifiable host factor associated with a lower SVR. An elevated BMI is associated with reduced insulin sensitivity and HCV treatment outcome. This observation has led experts to suggest that managing insulin resistance might improve hepatitis treatment outcome and that insulin resistance seems to be a new target in the management of hepatitis C.

The rationale of increasing insulin sensitivity in patients with chronic hepatitis C is based on the premise that insulin resistant state directly or indirectly inhibits the antiviral action of interferon (IFN)- $\alpha$ - $\beta$ , or increases the viral fitness making it more resistant to therapy, or both<sup>[8,68]</sup>. Since HCV appears to directly interfere with the glucose homeostasis, several studies have tried to analyze in detail the potential interactions between viral products and insulin signaling. Experimental data suggest a direct interference of HCV



**Figure 2 Interaction between insulin and interferon-alpha signaling pathway.** SOCs: Suppressor of cytokine signaling; PA2A: Protein phosphatase 2A; PI3K: Phosphatidyl-Inositol 3-kinase; JAK: Janus kinase; STAT: Signal transduction and activator of transcription; TYK2: Tyrosine kinase 2; Dotted lines: Represent inhibition; Continuous lines: Represent activation.

with the insulin signaling cascade *via* proteasome degradation of the insulin receptor substrates-1 and -2<sup>[69,70]</sup>. HCV may also impair insulin signaling transduction indirectly, that is, through increased levels of proinflammatory cytokines such as tumor necrosis factor (TNF)- $\alpha$ <sup>[71,72]</sup>. The interference with insulin signaling seems to proceed *via* HCV genotype-specific mechanisms and insulin resistance levels vary according to the infecting HCV genotype, although all genotypes induce insulin resistance. Interestingly, intracellular factors dysregulated by HCV and responsible for the insulin resistant phenotype may play promiscuous effects as they are also involved in regulating IFN- $\alpha$  signaling (Figure 2). These factors include some members of the suppressor of cytokine signalling (SOCS) family<sup>[69,70,73]</sup> and the protein phosphatase 2A<sup>[74]</sup>. Thus, modulating the levels and/or the activity of these factors may not only reverse hepatic insulin resistance but also help in establishing the IFN- $\alpha$ -induced antiviral state at the site of HCV replication. This is one of the reasons for trying to restore insulin sensitivity in chronic hepatitis C patients, especially those who failed to respond to therapy. Although increasing insulin sensitivity may be a rational option in chronic hepatitis C patients, especially in those with metabolic syndrome, the modalities of this intervention, however, have not been established. In addition, it is unclear whether one should start the antiviral treatment together with the insulin sensitizer or only once the HOMA-IR score has been decreased to a level predicting sufficient SVR rate<sup>[67]</sup>.

However, specific inhibitors of SOCS family members and of the protein phosphatase 2A are either not suitable

for *in vivo* administration or are toxic. Alternatively, increasing insulin sensitivity may be achieved by modulating serum levels of specific cytokines, such as TNF- $\alpha$ , associated with insulin resistance<sup>[71,72]</sup>, but the administration of anti-TNF- $\alpha$  antibodies to chronic hepatitis C patients may be risky<sup>[73]</sup>. Insulin sensitizers not only increase insulin sensitivity but may also inhibit HCV replication by decreasing serum free fatty acid flow to hepatocytes; saturated and monounsaturated free fatty acids have indeed been shown to stimulate HCV replication in an *in vitro* model<sup>[57]</sup>. It is not clear whether the best approach would be using a thiazolidindione, activating peroxisome proliferator-activated receptors (PPARs) (see below), or a biguanide such as metformin, whose mechanism of action is specifically directed to the hepatic AMP-activated protein kinase.

Recently, metformin-based triple therapy has been shown to be safe, improving insulin sensitivity and increasing SVR rate by 10% in patients with hepatitis C genotype 1 and insulin resistance (HOMA > 2). This therapy was especially effective in females in whom metformin significantly raised the SVR rate<sup>[76]</sup>.

## NUCLEAR RECEPTORS

### PPAR receptor

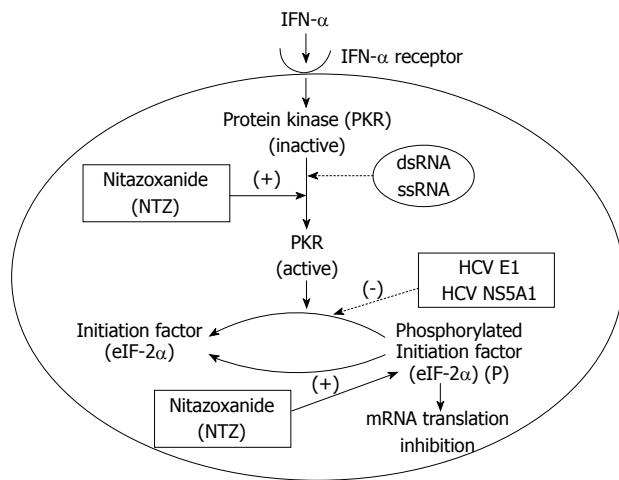
The PPARs are nuclear factors (amongst others) involved in the regulation of glucose homeostasis. In addition to the direct effects on factors involved in lipid and glucose homeostasis<sup>[77-81]</sup>, PPARs may have insulin sensitizing effects via their anti-inflammatory activity<sup>[82,83]</sup>. Thus, treatment with PPAR agonists results in improved insulin sensitivity via diverse mechanisms, both direct and indirect, and both at the level of the liver and at the level of extrahepatic tissues<sup>[77]</sup>. The relationship between HCV replication, protein expression and PPARs has been the focus of some recent studies. However, the data available so far are quite scanty and concern only the HCV genotype 3a<sup>[77]</sup>.

In a recent randomized, double-blind, placebo-controlled study, adding concurrent (PPAR- $\gamma$  agonist) pioglitazone 30 mg QD to the standard of care (i.e. without a preceding administration as monotherapy) markedly increased the on-treatment virological response, but failed to increase the SVR after the end of treatment<sup>[84]</sup>. In a related but smaller and shorter study, another research team reported that pioglitazone given as an adjuvant to pegylated interferon/ribavirin in HCV genotype one patients improved viral kinetic response during the first 4 wk of therapy<sup>[85]</sup>.

Also, in a recent study, the level of PPAR $\alpha$  mRNA was found to be profoundly suppressed in the liver of chronic hepatitis C patients (about 85% compared to control livers)<sup>[86]</sup>. The suppression of PPAR- $\alpha$  leads to the upregulation of nuclear factor (NF)- $\kappa$ B. NF- $\kappa$ B has been shown to accelerate virus replication<sup>[87]</sup>, and it has been speculated that activation of PPAR- $\alpha$  with subsequent NF- $\kappa$ B suppression leads to decreased HCV replication in hepatocytes<sup>[88]</sup>. Given the availability of potent agonists, PPARs may represent a novel pharmacological target in the treatment of liver lesions observed in chronic hepatitis C.

### Farnesoid X receptor (FXR)

The bile acid receptors were found to play a role in



**Figure 3** Antiviral mechanism of Nitazoxanide. Dotted lines: Represent inhibition; Continuous lines: Represent activation.

bile acid-mediated promotion of HCV replication<sup>[89]</sup>. Furthermore, it was discovered that bile acids compromised the anti-HCV effect of IFN in the cells. These findings suggest a mechanism for persistent infections of HCV in hepatocytes and for the failure of IFN-based treatment for certain HCV patients<sup>[89,90]</sup>. These data suggest a novel mechanism for bile acid-mediated gene regulation at virus and host levels. Importantly, these data may contribute to the finding of better regimens for the treatment of chronic HCV infections by including agents altering the bile acid-mediated FXR pathway<sup>[89]</sup>.

### Estrogen receptor (ESR)

ESR belongs to the steroid hormone receptor family of the nuclear receptor super family. There are two different forms of the estrogen receptor, usually referred to as  $\alpha$  and  $\beta$ , each encoded by a separate gene<sup>[91]</sup>. The novel role of ESR  $\alpha$  in regulation of HCV replication has been recently reported<sup>[92]</sup>. Tamoxifen and other anti-estrogens suppress genome replication, as part of ESR resides on the endoplasmic reticulum and interacts with HCV RNA polymerase NS5B, so ESR is suggested to serve as a potential novel target for anti-HCV therapies<sup>[92]</sup>.

## OTHER PRINCIPLES

### Nitazoxanide

Nitazoxanide is an oral prodrug of a thiazolidine (tizoxanide), and was approved for the treatment of protozoal infections<sup>[93]</sup>. In addition to having antiprotozoal and antibacterial activity, nitazoxanide coincidentally was discovered to inhibit HCV replication<sup>[94]</sup> through a recently identified host-mediated mechanism of action. The antiviral mechanism of action of nitazoxanide appears to be different from the mechanism of action of nitazoxanide in protozoa and anaerobic bacteria. Recent studies suggest that nitazoxanide and other thiazolidides selectively induce PKR phosphorylation, which leads to increased cell concentration of phosphorylated eIF2, a naturally occurring antiviral intracellular protein (Figure 3)<sup>[95]</sup>. This mechanism of action is only triggered when a cell is infected with HCV

while nitazoxanide has no effect in uninfected cells, which provides a possible explanation for its very low rate of toxicity.

Furthermore, nitazoxanide does not appear to induce antiviral resistance, based on an attempt to produce a resistance to nitazoxanide and tizoxanide in HCV replicon-containing cell lines<sup>[96]</sup>. With serial exposure to nitazoxanide or tizoxanide, direct HCV viral resistance did not emerge, suggesting that the genetic barrier to the development of resistance to nitazoxanide is high. The drug has been recently studied in combination with the standard of care in 96 treatment-naïve patients in Egypt infected with genotype 4 HCV infection. The combination of nitazoxanide, peginterferon α-2a, and ribavirin increased the percentage of patients with rapid and sustained virologic responses, compared with patients given peginterferon plus ribavirin, without an increase in adverse events<sup>[97]</sup>.

Nitazoxanide, a novel protein kinase inducer, has the potential not only to increase the SVR rate but also potentially to shorten the duration of therapy.

In summary, the suboptimal response to the currently available standard therapy has led to an extensive search for novel therapies with new therapeutic approaches. Targeting host cofactors of the HCV life cycle by different strategies (inhibition of viral entry, targeting host metabolism, nuclear receptors and other principles) may be a novel rational option, especially because they impose higher genetic barriers for resistance than direct antiviral compounds. However, the principle drawback of these strategies is the greater potential for cellular toxicity.

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ORIGINAL ARTICLES

## Characteristic pathological findings and effects of ecabet sodium in rat reflux esophagitis

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greater than that in the controls ( $26.0 \pm 5.5$  and  $21.0 \pm 6.5 \mu\text{m}$ , respectively) ( $P < 0.05$ ). The mean number of ulcers per animal in the ES group in the entire esophagus was  $5.4 \pm 2.5$ , which was significantly less than that in the esophagitis group ( $9.0 \pm 3.5$ ) ( $P < 0.05$ ). The epithelial thickness in the ES group was  $97.5 \pm 32.2 \mu\text{m}$  in the lower esophagus, which was decreased compared with that in the esophagitis group ( $210.8 \pm 47.7 \mu\text{m}$ ) ( $P < 0.05$ ).

**CONCLUSION:** Mucosal inflammation extended to the upper esophagus close to the hypopharynx. Our study suggested that ES may have a useful defensive role in reflux esophagitis.

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**Key words:** Gastroesophageal reflux disease; Upper esophagus; Laryngopharyngeal reflux disease; Extraesophageal syndrome

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### Abstract

**AIM:** To explore the pathological findings in the entire esophagus in rats with reflux esophagitis, and the effects of ecabet sodium (ES).

**METHODS:** A rat model of chronic acid reflux esophagitis was used. In the treatment group, ES was administered after surgery ( $n = 16$ ). No drug was administered postoperatively to the esophagitis group ( $n = 9$ ). Sham-operated rats were used as a control group ( $n = 5$ ). Rats were sacrificed on day 7 after the operation. The epithelial thickness and leukocyte infiltration were examined in the upper, middle and lower areas of the esophagus. The survival rate, incidence of esophageal ulcer, and mean surface area and number of esophageal ulcers were determined in the esophagitis and ES groups. Esophageal histology was assessed in all three groups.

**RESULTS:** Leukocyte infiltration in the esophagitis group was  $26.3 \pm 22.0$  in the middle esophagus and  $8.2 \pm 4.9$  in the upper esophagus, which was significantly greater than that in the controls ( $1.3 \pm 1.1$  and  $1.4 \pm 1.0$ , respectively) ( $P < 0.05$ ). The thickness of the epithelium in the esophagitis group was  $210.8 \pm 47.7 \mu\text{m}$  in the lower esophagus and  $204.2 \pm 60.1 \mu\text{m}$  in the middle esophagus, which was significantly

### INTRODUCTION

Against the background of an aging society and changing dietary habits that now include western-style food, and despite a low rate of infection with *Helicobacter pylori* (*H pylori*), the number of patients with gastroesophageal reflux disease (GERD) has increased recently in Japan<sup>[1,2]</sup>. The symptoms of GERD decrease quality of life<sup>[3]</sup>, and long-term reflux of gastric acid is known to increase the risk of Barrett's esophagus and Barrett's adenocarcinoma. As a result of the global ground swell

of GERD, a worldwide consensus definition of GERD has been developed recently<sup>[4]</sup>. Laryngopharyngeal reflux disease (LPRD) is a common condition in the primary care setting and is one of the extraesophageal syndromes regarded as secondary to GERD<sup>[5,6]</sup>. Exactly how reflux of gastric juice influences the esophagus and/or extraesophageal structures is unknown, and the causal association between gastric-juice reflux and pathogenesis of reflux esophagitis is a controversial subject.

There are limitations associated with the investigation of the pathophysiology of GERD in humans, thus, experiments using animal models are fundamental to this investigation. Proton pump inhibitors (PPIs) and histamine-2 receptor antagonists are likely to be used for reflux esophagitis, but there have been few reports about using mucosal-protective drugs. We previously induced chronic acid-reflux esophagitis in a rat model and investigated the underlying mechanism of reflux esophagitis, with a focus on the mechanism of esophageal mucosal resistance<sup>[7,8]</sup>.

In the current study, we used a rat model of chronic acid-reflux esophagitis to explore the esophageal mucosal damage macroscopically and microscopically throughout the entire esophagus, including the upper esophagus close to the hypopharynx, and to investigate the protective effects of ecabet sodium (ES) on the esophageal mucosa.

## MATERIALS AND METHODS

### Chronic acid-reflux esophagitis model

Specific-pathogen-free male Wistar rats aged 9 wk were purchased from SLC (Tokyo, Japan). They were used after acclimatization for 1 wk in an animal room with a controlled temperature ( $23 \pm 2^\circ\text{C}$ ). The rats were fed a standard diet but fasted for 12 h prior to the surgical induction of chronic acid-reflux esophagitis, which was induced by modifying the method of Omura *et al*<sup>[9]</sup>. Anesthesia was induced by inhalation of isoflurane. After laparotomy, duodenal stenosis was accomplished by wrapping the duodenum near the pylorus with a piece of 18 Fr Nelaton catheter (length: 3.0 mm, diameter: 4.0 mm; Terumo Inc., Tokyo, Japan). To prevent dislodgement, we sutured the edge of the catheter to the serosa of the pylorus using 4-0 nylon thread. The transitional zone between the fore-stomach and the glandular portion (i.e. the limiting ridge) was ligated with 2-0 silk thread. The animals were fasted for 48 h after the operation but were allowed free access to drinking water. In those allocated to the ES treatment group ( $n = 16$ ), ES (65 mg/kg) was intragastrically administered only once immediately after surgery, and drinking water including ES ( $21.39 \pm 2.74$  mg/kg per day) was given from the day after surgery until day 7. ES was obtained from Tanabe Seiyaku Co. Ltd. (Osaka, Japan). No drug was administered after surgery to the animals in the esophagitis group ( $n = 9$ ). Sham-operated rats were used as a control group ( $n = 5$ ). The animals were sacrificed on day 7 after the operation. All

the procedures performed on laboratory animals were approved by the Institutional Animal Care and Use Committee of Juntendo University School of Medicine (Tokyo, Japan), and all the animal experiments were carried out in compliance with the guidelines for animal experimentation of Juntendo University School of Medicine.

### Measurement of gross esophageal lesions

In the chronic acid-reflux esophagitis and ES groups, the survival rate and incidence of esophageal ulcers were determined. The esophagus was resected up to the upper segment close to the hypopharynx. After taking photographs of esophageal lesions, the numbers of ulcers were measured in the upper, middle and lower segments of the esophagus. The surface areas of ulcers in these three segments of the esophagus were measured using a high-resolution computerized image analyzer (KS 400; Carl Zeiss Imaging Solutions GmbH, Hallbergmoos, Germany).

### Histological assessment

After being fixed in 10% buffered formalin, the tissues were embedded in paraffin, and 3- $\mu\text{m}$  sections were prepared and stained with HE. The epithelial thickness was assessed in the upper (approximately 5 mm below the cricopharyngeus), middle [midpoint between the cricopharyngeus and the esophagogastric (EG) junction] and lower (approximately 0.5 mm above the EG junction) segments of the esophagus, using light microscopy (high-power fields). The numbers of leukocytes that infiltrated each high-power field were counted in these three segments of the esophagus.

### Statistical analysis

All data are presented as mean  $\pm$  SD. Student's *t* test and  $\chi^2$  test were used, and  $P < 0.05$  was regarded as statistically significant.

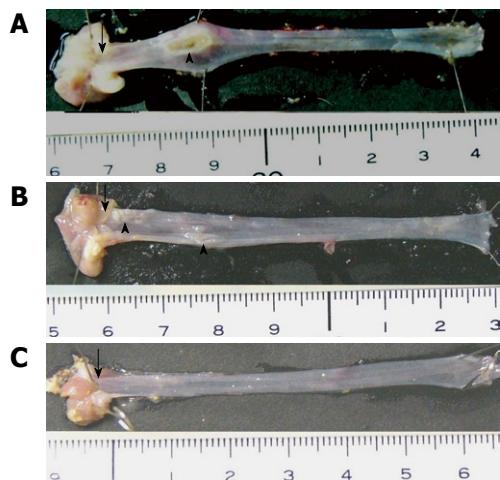
## RESULTS

### Modified chronic acid-reflux esophagitis model vs ES model

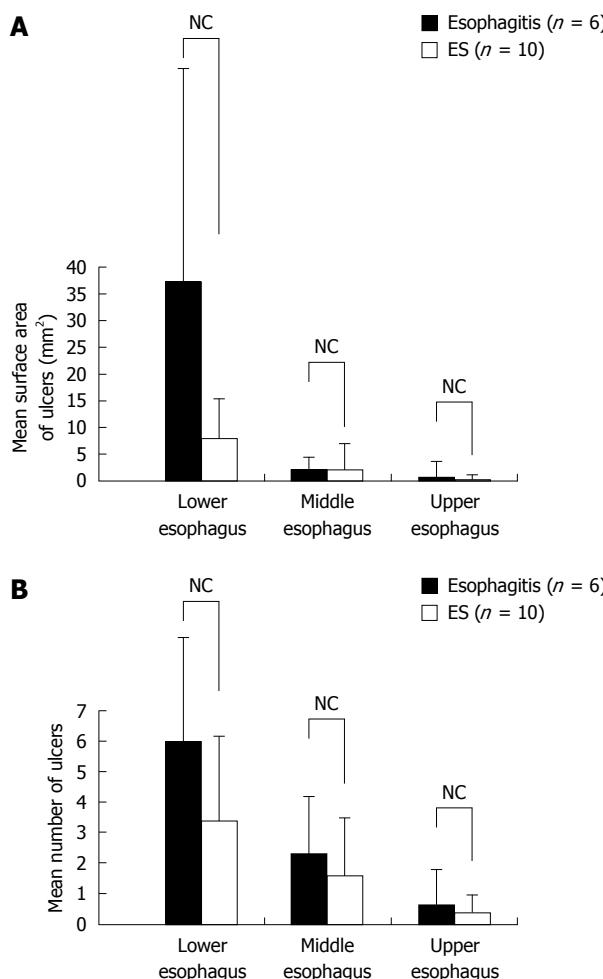
In the esophagitis group, the 7-d survival rate after the operation was 66.7% (6/9 rats), and the incidence of ulcers in the survivors was 100%. In the ES group, the 7-d survival rate after the operation was 62.5% (10/16), and the incidence of ulcers was 90.0% (9/10 rats). There were no statistically significant between-group differences in the survival rate and incidence of ulcers.

### Macroscopic assessment

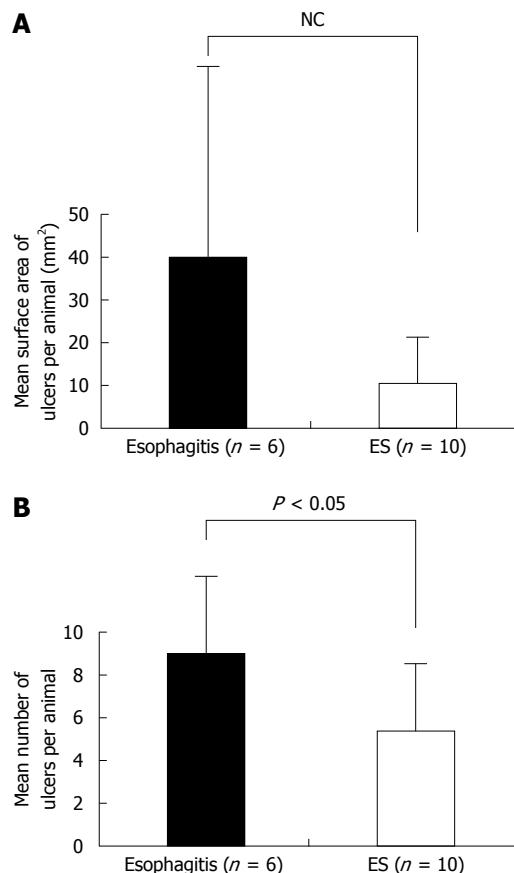
In both the esophagitis and ES groups, ulcers were noted in the mucosa, but none occurred in the control group. Also in the esophagitis and ES groups, macroscopic esophageal ulcers were found in the lower and middle parts of the esophagus in most cases (Figure 1). The mean number of ulcers per animal in the ES group in the



**Figure 1 Macroscopic findings in the three groups.** A: Esophagitis group on day 7 after operation; B: ES group on day 7 after operation; C: Control group on day 7 after operation. In the esophagitis and ES groups, ulcers were noted in the mucosa, but none occurred in the control group. The arrows indicate the EG junction. The arrowheads indicate ulcers.



**Figure 3 Mean surface area and number of ulcers in each of the three individual segments of the esophagus.** A: Mean surface area of ulcers in each part of the esophagus in the esophagitis and ES groups; B: Mean number of ulcers in each part of the esophagus in the esophagitis and ES groups. There were no significant between-group differences in terms of the number and mean area of ulcers in each of the three individual segments of the esophagus.

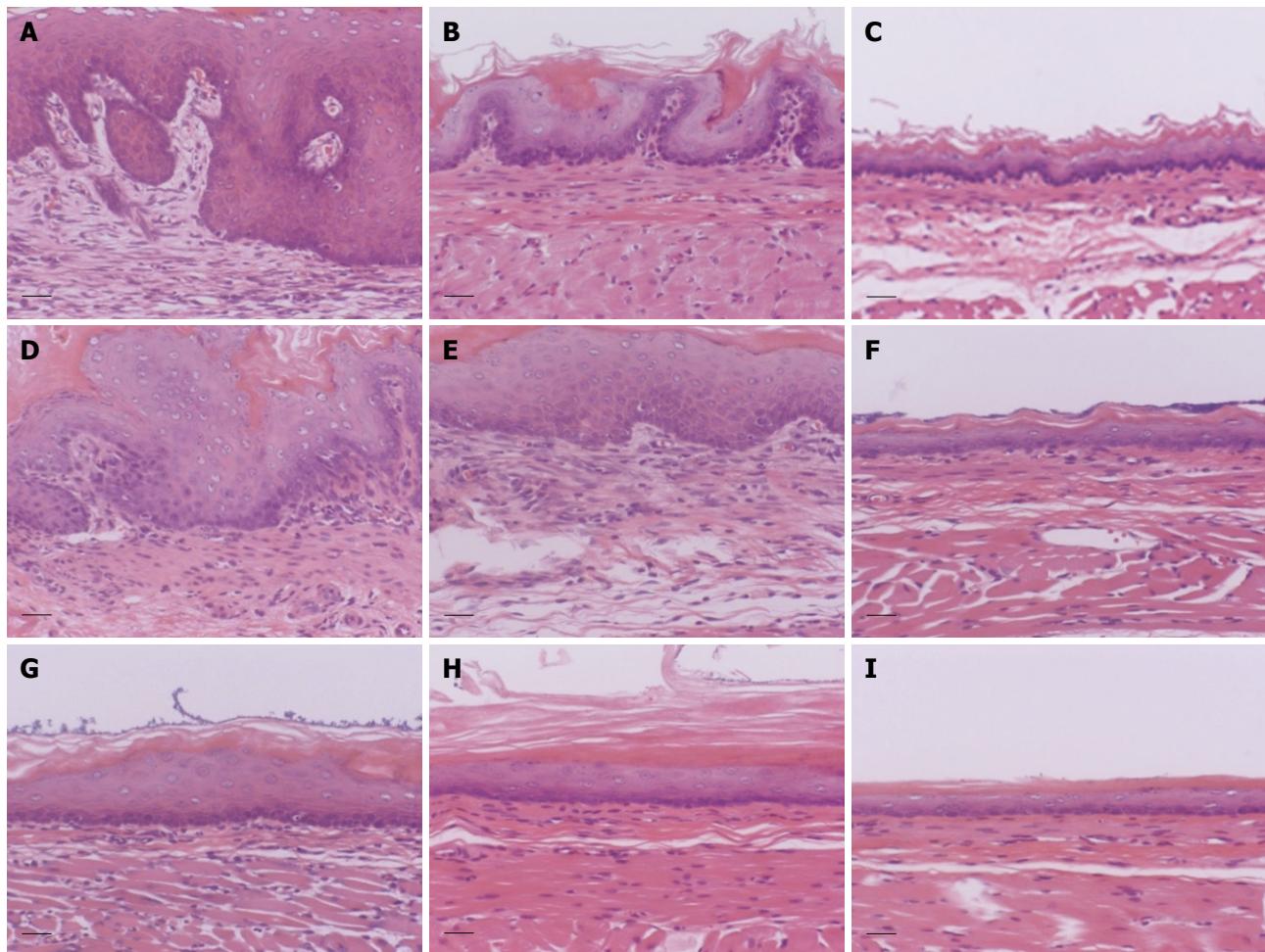


**Figure 2 Mean surface area and number of ulcers per animal.** A: Mean surface area of ulcers per animal in the esophagitis and ES groups. There were no significant differences in the mean area of ulcers between the two groups; B: Mean number of ulcers per animal in the esophagitis and ES groups. In the ES group, the number of ulcers was significantly less than in the esophagitis group ( $5.4 \pm 2.5$  vs  $9.0 \pm 3.5$ ,  $P < 0.05$ ).

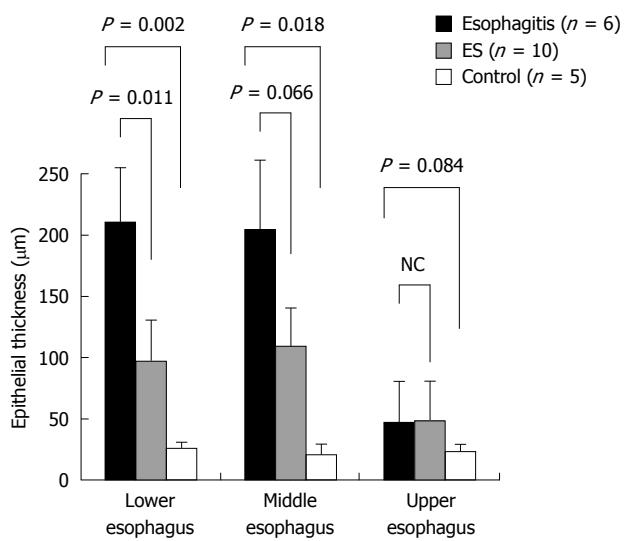
entire esophagus was  $5.4 \pm 2.5$ , which was significantly less than the number in the esophagitis group ( $9.0 \pm 3.5$ ) ( $P < 0.05$ ) (Figure 2). The mean surface area of ulcers per animal was  $40.0 \pm 56.8$  and  $10.5 \pm 8.6 \text{ mm}^2$  in the esophagitis and ES groups, respectively ( $P = 0.120$ ) (Figure 2). No significant between-group differences were found in terms of the number and mean area of ulcers in each of the three individual segments of the esophagus (Figure 3).

#### Histological assessment

Histologically, the esophagus showed a thin epithelial layer with squamous cells in the control group. In the esophagitis group, the epithelium was thickened markedly, and elongation of the lamina propria papillae into the epithelium was noted. Basal cell hyperplasia and inflammatory cell infiltration were marked in the lamina propria (Figure 4). The thickness of the epithelium in the lower esophagus was the most severely affected of the three areas. The thickness of the epithelium in the esophagitis group was  $210.8 \pm 47.7 \mu\text{m}$  in the lower esophagus and  $204.2 \pm 60.1 \mu\text{m}$  in the middle esophagus, which was significantly greater than that in the controls ( $26.0 \pm 5.5$  and  $21.0 \pm 6.5 \mu\text{m}$ , respectively) ( $P < 0.05$ )



**Figure 4** Histological findings in each part of the esophagus in the three groups (HE staining). Lower esophagus: esophagitis (A), ES (B) and control (C) groups. Middle esophagus: esophagitis (D), ES (E) and control (F) groups. Upper esophagus: esophagitis (G), ES (H) and control (I) groups. In the ES group, the thickness of the epithelium in the lower esophagus was significantly decreased compared with that in the esophagitis group. Scale bar, 30  $\mu\text{m}$ .



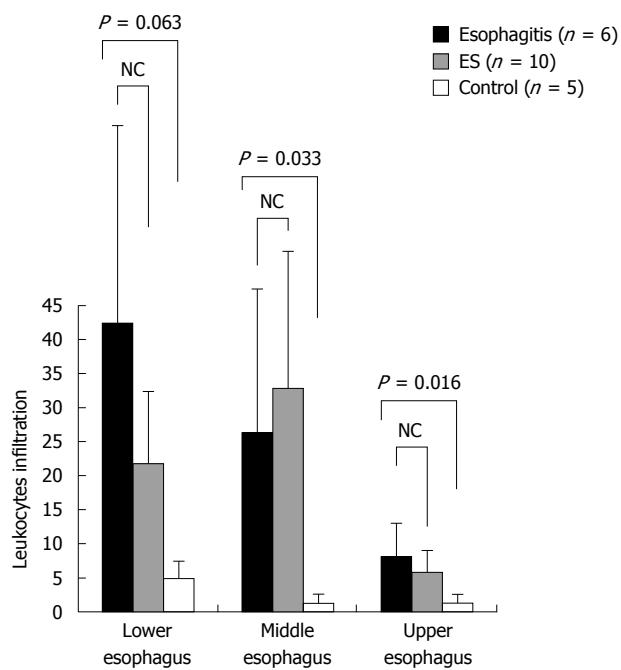
**Figure 5** Thickness of the esophageal epithelium in the three groups. In the esophagitis group, the thickness of the epithelium in the lower and middle esophagus was significantly greater than that in the controls ( $P < 0.05$ ). In the ES group, the thickness of the epithelium in the lower esophagus was significantly less than that in the esophagitis group ( $P < 0.05$ ), and the thickness of the epithelium in the middle esophagus tended to be less than that in the esophagitis group ( $P = 0.066$ ).

(Figure 5). Also, the epithelial thickness in the ES group was  $97.5 \pm 32.2 \mu\text{m}$  in the lower esophagus, which was decreased compared with that in the esophagitis group ( $210.8 \pm 47.7 \mu\text{m}$ ) ( $P < 0.05$ ), and the epithelial thickness in the ES group in the middle esophagus also tended to be less than that in the esophagitis group.

Leukocyte infiltration in the esophagitis group was  $26.3 \pm 22.0$  in the middle esophagus and  $8.2 \pm 4.9$  in the upper esophagus, which was significantly greater than that in the controls ( $1.3 \pm 1.1$  and  $1.4 \pm 1.0$ , respectively) ( $P < 0.05$ ) (Figure 6).

## DISCUSSION

We revealed that epithelial thickening occurs at the same time as inflammatory cell infiltration in the middle to lower esophagus in chronic acid-reflux esophagitis. An imbalance between offensive factors (e.g. gastric acid and pepsin) and defensive factors (e.g. mucin, saliva and the esophageal epithelial lining) results in the development of reflux esophagitis<sup>[7,10,11]</sup>. However, there have been few reports about the pathological findings in the esophageal squamous epithelium, and there are differing opinions among pathologists about the findings considered



**Figure 6 Leukocyte infiltration in the esophageal epithelium on day 7 after operation in the three groups.** In the esophagitis group, leukocyte infiltration in the middle and upper esophagus was significantly greater than that in the controls ( $P < 0.05$ ).

characteristic of chronic reflux esophagitis<sup>[12-14]</sup>. Some authors have reported the relationship between chronic inflammation and epithelial changes in other parts of the gastrointestinal tract. Yasunaga *et al*<sup>[15]</sup> have suggested that increased interleukin 1 $\beta$  and hepatocyte growth factor production caused by *H pylori* infection may contribute to fold thickening of the stomach by stimulating epithelial cell proliferation and foveolar hyperplasia in patients with enlarged fold gastritis<sup>[15]</sup>. In patients with erosive reflux disease, the thickness of the basal cell layer and length of the papillae were associated with the severity of esophagitis. After esomeprazole treatment, the basal layer thickness and length of papillae were reduced in both non-erosive and erosive reflux disease<sup>[16]</sup>. However, these studies were performed in human subjects, so detailed pathological investigation in chronic reflux esophagitis was not performed.

Recently, the mechanism by which acid may induce inflammation has been proposed by Tobey *et al*<sup>[17]</sup>. They clarified that the permeability of the esophageal epithelium to acid is increased by dilatation of the intracellular space, and PPIs may decrease inflammation of the esophageal epithelium<sup>[17,18]</sup>. These results suggest that acid may diffuse through the esophageal epithelium and induce infiltration of inflammatory cells. This increases the release of proinflammatory cytokines, which may induce thickening of the esophageal epithelium. Furthermore, we demonstrated that inflammatory cells infiltrated the epithelium of the upper esophagus close to the hypopharynx, where there was no evidence of ulcers. It has been reported previously that inflammation mainly occurs in the lower esophagus near the EG junction in cases of reflux esophagitis<sup>[8]</sup>, but how the reflux of

gastric juice influences esophageal and/or extraesophageal symptoms is unknown. Recently, GERD-related extraesophageal syndromes have attracted attention and it has been suggested that gastric-juice reflux can extend to the upper esophagus close to the hypopharynx. In a study of the pathogenesis of LPRD, which is one of the extraesophageal syndromes, Tokashiki *et al*<sup>[19]</sup> have found that patients with LPRD show a significantly longer acid reflux time in the upper esophagus than healthy volunteers do.

In our chronic acid-reflux esophagitis model, gastric juice passed through the EG junction and diffused directly into the esophagus. This model resembles the reflux esophagitis that is seen in the clinical setting. The direct acid injury to the hypopharynx appeared to be reflected in the microscopic pathological changes in the upper esophagus.

Secondarily, we demonstrated that ES inhibited the epithelial thickening of the lower esophagus, which was the most severely inflamed segment of the esophagus, and ES also tended to inhibit the epithelial thickening of the middle esophagus. ES, a dehydroabietic acid derivative from pine resin, has been used clinically in the treatment of gastritis and gastric ulcer, and is believed to exert its effects through various mechanisms<sup>[20-24]</sup>. ES binds directly to the gastric mucosa, thereby protecting the mucosa against ethanol binding, and it has been shown to inhibit pepsin activity in rat and human gastric juices.

In the present study, ES may decrease the number of ulcers in the entire esophagus by binding directly to the esophageal mucosa and inhibiting pepsin activity. ES was shown to be useful in preventing inflammation from the lower to the middle esophagus. ES may inhibit the increase in cytokines which are released as part of the inflammatory process and induce epithelial thickening. However, the relationship between the occurrence of ulcers and epithelial thickening is unknown, and further study about this relationship is necessary.

In conclusion, this study revealed that mucosal inflammation extended to the upper esophagus close to the hypopharynx, even where there was no evidence of ulcers. This finding of inflammation suggested that direct injury to the hypopharynx may occur as a result of the reflux of gastric juice. Our study also suggested that ES may play a useful defensive role in the prevention of reflux esophagitis. Further studies are necessary to explore the factors that are responsible for the protective effects of ES in reflux esophagitis.

## COMMENTS

### Background

Recently, the number of patients with gastroesophageal reflux disease (GERD) has increased in Japan. Although GERD-related extraesophageal syndromes have attracted attention, how gastric-juice reflux influences the esophagus and/or extraesophageal structures is unknown. In the present study, the authors explored the pathological findings in the entire esophagus and the effects of esacet sodium (ES).

### Innovations and breakthroughs

The authors revealed that epithelial thickening occurred at the same time as

inflammatory cell infiltration in the middle to lower esophagus in chronic acid-reflux esophagitis. Furthermore, they demonstrated that inflammatory cells infiltrated the epithelium of the upper esophagus close to the hypopharynx, where there was no evidence of ulcers. These findings suggested that the reflux of gastric juice can extend to the upper esophagus close to the hypopharynx.

### Terminology

Laryngopharyngeal reflux disease is a common condition in the primary care setting and is one of the extraesophageal syndromes regarded as secondary to GERD.

### Peer review

The authors evaluated the pathological findings in rat chronic acid-reflux esophagitis and the defensive effects of ES on esophageal mucosal injury. This an interesting, well-executed study.

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BRIEF ARTICLES

## A survey of ampullectomy practices

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**CONCLUSION:** Among biliary experts, there is less variation in ampullectomy practices than is reflected in the literature.

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**Key words:** Endoscopic retrograde cholangiopancreatography; Ampullectomy; Papillectomy; Ampulla of Vater; Common bile duct neoplasms; Adenoma

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### Abstract

**AIM:** To investigate the endoscopic ampullectomy practices of expert biliary endoscopists.

**METHODS:** An anonymous survey was mailed to 79 expert biliary endoscopists to assess ampullectomy practices.

**RESULTS:** Forty six (58%) biliary endoscopists returned the questionnaire. Of these, 63% were in academia and in practice for an average of 16.4 years ( $\pm$  8.6). Endoscopists performed an average of 1.1 ( $\pm$  0.8) ampullectomies per month. Prior to ampullectomy, endoscopic ultrasound was "always" utilized by 67% of respondents vs "sometimes" in 31% of respondents. Empiric biliary sphincterotomy was not utilized uniformly, only 26% "always" and 37% "sometimes" performed it prior to resection. Fifty three percent reported "never" performing empiric pancreatic sphincterotomy prior to ampullectomy. Practitioners with high endoscopic retrograde cholangiopancreatography volumes were the most likely to perform a pancreatic sphincterotomy (OR = 10.9;  $P$  = 0.09). Participants overwhelmingly favored "always" placing a prophylactic pancreatic stent, with 86% placing it after ampullectomy rather than prior to resection (23%). Argon plasma coagulation was the favored adjunct modality (83%) for removal of residual adenomatous tissue. Practitioners uniformly (100%) preferred follow-up examination to be within 6 mo post-ampullectomy.

### INTRODUCTION

Ampullary tumors account for approximately 5% of all gastrointestinal neoplasms<sup>[1]</sup>. In autopsy series, these tumors are seen in 0.04%-0.64% of the general population<sup>[2,3]</sup>. The most commonly affected patients are those with familial adenomatous polyposis with a 50%-100% lifetime incidence of peri-ampullary adenomas<sup>[4-6]</sup>. Given that the adenoma-carcinoma sequence for ampullary adenomas follows a similar progression to that of colorectal cancer, there is a need for prophylactic removal<sup>[7]</sup>. However, the associated morbidity and mortality of surgical resection for ampullary adenomas have led clinicians to seek less invasive techniques. Endoscopic ampullectomy was first described in the 1980s<sup>[8-10]</sup>. Since then, numerous case and cohort series of ampullectomies, both retrospective and prospective, have been reported<sup>[11-19]</sup>. The first prospective, randomized, controlled trial of the use of prophylactic pancreatic duct stenting for endoscopic ampullectomy was published in 2005<sup>[20]</sup>. The trial was prematurely terminated because of an elevated incidence of pancreatitis in the unsplinted group (33% vs 0%) and suggested that pancreatic stent placement confers a protective effect.

Endoscopic ampullectomy guidelines have not been established. Desilets *et al*<sup>[14]</sup> performed endoscopic ampullectomy only in tumors less than 4.0 cm in size without induration/ulceration, and with the ability to be lifted by saline solution/epinephrine injection in the absence of

extension or strictureting into the pancreatic or biliary ducts. Similarly, Cheng *et al*<sup>[18]</sup> performed endoscopic ampullectomy in lesions less than 4.5 cm without endoscopic or pathologic evidence of malignancy and a soft consistency on palpation with any device. A recent editorial by Baillie *et al*<sup>[21]</sup> suggested a tumor size greater than 3 cm requires endoscopic ultrasound (EUS) assessment prior to ampullectomy. However, a recent literature review continues to reveal diverse endoscopic practices regarding the use of biliary/pancreatic sphincterotomy, use and timing of pancreatic stenting, thermal ablation therapy and follow-up in ampullectomy<sup>[22]</sup>. The majority of the literature guiding ampullectomy practice is comprised of case reports, retrospective and prospective clinical series, except for the aforementioned randomized, controlled trial by Harewood *et al*<sup>[20]</sup>. Subsequently, a consensus for endoscopic ampullectomy practices has not been established. In this respect, it is helpful to assess opinions on endoscopic ampullectomy practices in that it may set priorities for future research. Also, this type of data could be helpful for guideline development. Therefore, we surveyed expert biliary endoscopists on their endoscopic ampullectomy techniques to determine if a consensus exists in ampullectomy practice.

## MATERIALS AND METHODS

### Sample population

Seventy-nine expert biliary endoscopists were identified by the investigators, representing 55 medical centers in 33 States and Canada. Expert biliary endoscopists were identified by selecting the primary biliary endoscopists at tertiary care centers with a medium or large gastroenterology fellowship (2 or more fellows per year) ( $n = 52$ ). Additional expert biliary endoscopists ( $n = 27$ ) from private practice were selected based on the senior investigator's (G.E.) knowledge. No surveys were distributed at the sponsoring institution (University of Michigan and the Ann Arbor Veterans Affairs hospital). By utilizing biliary endoscopists from gastroenterology fellowship training institutions our study was likely to reflect routine gastroenterology practice since trainees tend to have similar practice patterns as their teachers.

### Survey methods

An anonymous survey was sent to 79 potential respondents from May 2006 through October 2006. A self-addressed envelope was included with the survey to facilitate survey return. After 6 wk, a second survey was sent out to obtain results from endoscopists who did not respond initially with instructions to ignore the second mailing if they had already submitted the survey.

The survey instrument (Table 1) was composed of 16 questions based on an extensive literature review which identified diverse endoscopic ampullectomy practices. We performed an extensive literature search to identify endoscopic ampullectomy practices using the following search terms: ampullectomy, papillectomy, endoscopic ampullectomy/papillectomy, ampulla of Vater, major duodenal papilla and endoscopic retrograde

**Table 1 Ampullectomy survey**

1 Please list your age.
2 Gender:
Male
Female
3 Please specify your type of practice?
Private practice
Multi-specialty group
Academic practice
Health maintenance organization (HMO)
Other
4 How many years have you been in practice?
5 On average, how many ERCPs do you perform in a month?
6 On average, how many ampullectomies for ampullary adenomas do you perform in a month?
7 How often do you perform an EUS or IDUS of the ampulla prior to ampullectomy?
Always
Sometimes-if there are concerning features known ahead of time
Never
8 How often do you perform an empiric biliary sphincterotomy prior to ampullectomy?
Always
Sometimes
Never
9 How often do you perform an empiric pancreatic sphincterotomy prior to ampullectomy?
Always
Sometimes
Never
10 How often do you place a prophylactic pancreatic stent prior to ampullectomy?
Always
Sometimes
Never
11 How often do you place a prophylactic stent after ampullectomy?
Always
Never
Only if there is delayed pancreatic duct drainage or a remnant lesion close to the pancreatic orifice that needs additional treatment
12 How often do you perform a submucosal injection of the ampullary adenoma prior to resection?
Always
Sometimes
Never
13 For endoscopic ampullectomy, what type of electrosurgical currents do you use most often?
Pure coagulation current
Blended current
Pure cutting current
ERBE-adjustable current
14 What is the largest ampullary adenoma that you have removed endoscopically?
15 What adjunct modality do you use most commonly to remove residual tissue after ampullectomy?
Cold forceps biopsy
Argon plasma coagulation
Monopolar/multipolar electrocoagulation probe
Nd: YAG laser photoablation
16 In general, after ampullectomy, at what interval do you recommend a follow-up endoscopic examination?
1 mo
3 mo
6 mo
12 mo

cholangiopancreatography (ERCP). Based upon the results of this literature search, we determined diverse approaches to the following practices: use of EUS, timing of pancreatic stent placement, pre-ampullectomy biliary sphincterotomy and pancreatic sphincterotomy, type of electrosurgical currents used, type of adjunct modality for residual tumor removal and interval for follow-up. In order

**Table 2** Respondent characteristics (mean  $\pm$  SD)

Characteristic	
Male, n (%)	46 (100)
Practice type, n (%)	
Academic	29 (63)
Private	14 (30)
Multi-specialty group	3 (7)
Years in practice	16.4 $\pm$ 8.6
ERCPs per month	36.78 $\pm$ 26.2
Ampullectomies per month	1.1 $\pm$ 0.8

ERCP: Endoscopic retrograde cholangiopancreatography.

to establish content validity, the results of this literature search were used to develop a draft questionnaire which was then reviewed by expert biliary endoscopists at the University of Michigan, followed by revision of the survey instrument. Since an adequate sample of expert biliary endoscopists were not available, test-retest reliability of the survey instrument could not be performed.

The study was approved by the Institutional Review Board (IRB) at the University of Michigan. In accordance to standard IRB guidelines, the need for documentation of informed consent was waived because of survey's anonymity.

### Statistical analysis

All returned surveys were included in the analysis, regardless of the completeness of the survey. Percentage calculations were performed to determine if there were variations among expert biliary endoscopists in ampullectomy practices, including the use of biliary sphincterotomy, pancreatic sphincterotomy, timing of placement of pancreatic stents, use of submucosal injection, adjunctive ablative therapies and EUS. Multivariate models were used to determine if ampullectomy practices varied by academic or private practice and by volume of ERCPs. Since our ampullectomy practice data were collected as 3-level categories of "always", "sometimes" or "never", we first used multinomial logistic regression models for the 3-level outcomes and followed with logistic regression models for 2-level outcomes after collapsing the 3 levels into appropriate 2 levels. Given that the volume of ERCPs was highly skewed to the right, we considered this in various ways: as the number of ERCPs performed per month, as the number categorized into intervals ( $\leq$  20, 20-40, 40-60,  $>$  60 per month), and as the number dichotomized to high ( $>$  10 per month) versus low volume<sup>[23]</sup>. All statistical analyses were done using STATA 9.2 (StataCorp LP, College Station).

## RESULTS

### Demographic data

Forty-six respondents completed and returned the survey (58% response rate). Two-thirds of participants were from an academic medical practice. All respondents were male. Respondents had been in practice for a mean of 16.4 years ( $\pm$  8.6 SD) at the time of the survey. There was

**Table 3** Pre-ampullectomy practices

Practice	n (%)
EUS	
Always	30 (67)
Sometimes	14 (31)
Never	1 (2)
Biliary sphincterotomy	
Always	11 (26)
Sometimes	16 (37)
Never	16 (37)
Pancreatic sphincterotomy	
Always	10 (23)
Sometimes	10 (23)
Never	23 (53)
Pancreatic stent	
Always	10 (23)
Sometimes	15 (35)
Never	18 (42)
Submucosal injection	
Always	5 (12)
Sometimes	21 (49)
Never	17 (39)

EUS: Endoscopic ultrasound.

a wide range in ERCP volume among respondents (ranging from 5 to 135 ERCPs per month) with an average of 36.7 ( $\pm$  26.2, median = 30) ERCPs per month. One respondent stated that he no longer performed ERCPs. Thirty-nine (85%) reported a high volume ( $>$  10) of ERCPs per month. Participants reported an average of 1.1 ( $\pm$  0.79) ampullectomies per month (Table 2).

### Practices performed prior to ampullectomy

Prior to ampullectomy, 67% of participants "always" used EUS to correctly assess tumor infiltration, 31% "sometimes" used EUS prior to ampullectomy, while only 2% "never" used EUS regularly. To maximally expose the affected ampullary epithelium, 26% of the respondents "always" performed biliary sphincterotomy and 23% "always" performed pancreatic sphincterotomy prior to resection. However, 53% of participants "never" performed pancreatic sphincterotomy and 37% "never" performed biliary sphincterotomy prior to ampullectomy. Only 12% of respondents "always" utilized submucosal injection of the ampullary adenoma to decrease the depth of thermal injury to the duodenal wall, while 49% and 39% of participants "sometimes" and "never" utilized this technique, respectively. For endoscopic ampullectomy, the most common type of electrosurgical current utilized was ERBE (67%) and blended current (17%) (Table 3).

### Pancreatic stenting

For both pre- and post-ampullectomy, 98% of respondents reported placing a prophylactic pancreatic stent. A majority of participants (86%) favored "always" placing a pancreatic stent after resection. Some overlap in practice was identified with our 2 separate questions assessing the specific timing of pancreatic stent placement. Twenty-three percent of respondents always placed a pancreatic stent prior to resection, 35% "sometimes"

**Table 4** Post-ampullectomy practices

Practice	n (%)
Pancreatic stent	
Always	37 (86)
Sometimes	4 (9)
Never	2 (4)
Adjunct therapy <sup>1</sup>	
APC	35 (83)
Multi/multipolar	3 (7)
Nd-Yag	1 (2)
Cold biopsy	3 (7)
Follow-up <sup>2</sup>	
One month	6 (16)
Three months	21 (55)
Six months	11 (29)
One year	0 (0)

<sup>1</sup>One respondent who checked both argon plasma coagulation (APC) and multipolar/monopolar electrocoagulation probe is not included here; <sup>2</sup>Four respondents who checked 2 follow-up intervals are not included here. They each checked 1 and 6 mo, 3 mo and 1 year, 1 and 3 mo, 3 and 6 mo.

placed a pancreatic stent prior to ampullectomy whereas 42% “never” placed a pancreatic stent prior to resection.

#### Practices performed after ampullectomy

The most frequently used adjunct modality to remove residual tissue post-ampullectomy was Argon Plasma Coagulation (83%). Follow-up examination at 3 mo was the most common time frame chosen (55%) by expert biliary endoscopists. Repeat examination at 6 mo (29%) and 1 mo (16%) were less frequently used. The largest reported adenoma removed by experts was 8.0 cm (Table 4).

#### Predictors of ampullectomy practices

**Academic vs non-academic:** Multinomial logistic regression showed that for empiric biliary sphincterotomy, the relationships between factors associated with a response of “sometimes” were similar to those associated with “never”. On the other hand, for pancreatic sphincterotomy, the relationships between factors associated with “always” were similar to “sometimes”. Thus for biliary sphincterotomy, we dichotomized the practice responses to “always” vs “sometimes or never”, while for pancreatic sphincterotomy, we dichotomized the responses to “always or sometimes” vs “never”. Logistic regression analysis showed that after controlling for years in practice and high ERCP volume, an odds of “always” doing empiric biliary sphincterotomy was 0.22 (95% CI = 0.05, 1.04;  $P = 0.06$ ) for academic relative to private physicians, and an odds of “always or sometimes” doing empiric pancreatic sphincterotomy was 0.23 (95% CI = 0.05, 1.04;  $P = 0.06$ ) for academic relative to private physicians. These indicated that academic practitioners tended to be less likely to do sphincterotomy than non-academic practitioners. Regarding prophylactic stenting, academic practitioners tended to be less likely to “always” do pre-ampullectomy prophylactic pancreatic stenting ( $OR = 0.42$ ;  $P = 0.28$ ), while they tended to be more likely to “always” do post-ampullectomy prophylactic

pancreatic stenting ( $OR = 2.1$ ;  $P = 0.45$ ); however, these differences between academic *vs* private physicians were not statistically significant. Of the 18 practitioners who “never” placed a prophylactic pancreatic stent prior to ampullectomy, 17 “always” placed a prophylactic pancreatic stent after ampullectomy and only 1 “never” placed a prophylactic pancreatic stent after ampullectomy. These findings emphasize that regardless of the timing, almost all respondents utilized pancreatic stenting.

#### Volume of ERCPs

ERCP volumes were not associated with practice variation in empiric biliary sphincterotomy, but practitioners with high volumes of ERCPs (> 8 per month) tended to be more likely to “always or sometimes” do empiric pancreatic sphincterotomy ( $OR = 10.9$ ;  $P = 0.09$ ), controlling for academic status and years in practice. Practitioners with high volumes of ERCPs were also significantly less likely to “always” place prophylactic pancreatic stents prior to ampullectomy ( $OR = 0.08$ ;  $P = 0.04$ ), and more likely to “always” place prophylactic pancreatic stents after ampullectomy ( $OR = 12.8$ ;  $P = 0.06$ ).

## DISCUSSION

This research describes the most commonly used endoscopic ampullectomy techniques by expert biliary endoscopists. This survey raises some interesting findings about current practices, showing some uniformity by expert endoscopists, which is important for future guideline development. For other practices, there is more variability. Therefore, these practices should be studied in prospective trials to help refine the best practice for our patients. In this regard, our survey has helped to identify key questions for future studies.

Universal agreement among participants regarding the use of prophylactic pancreatic stenting for ampullectomy was seen. This corresponds to findings by Brackbill *et al*<sup>[24]</sup> where 100% of respondents utilized prophylactic pancreatic stenting when performing ampullectomy. Previously, in some retrospective case series, prophylactic pancreatic stenting was performed only in the setting of delayed pancreatic duct drainage<sup>[11,15,16,19]</sup>. However, recent findings by Harewood *et al*<sup>[20]</sup> showing a markedly reduced rate of pancreatitis in those receiving prophylactic pancreatic stenting. Prophylactic pancreatic stenting was most commonly performed after ampullectomy by our expert biliary endoscopists. An argument against pre-ampullectomy pancreatic stenting is that it precludes *en bloc* removal of the adenomatous tissue by practitioners who favor complete transection of the polyp with a snare, rather than piecemeal resection. Only a minority of our respondents placed pancreatic stents prior to ampullectomy. For some practitioners, the possibility of not being able to find the pancreatic duct post-resection, and the increased risk of post-ampullectomy pancreatitis without a pancreatic stent may dictate their practice of pre-ampullectomy stent placement. To alleviate this concern, endoscopists may also consider wire placement in the pancreatic duct before ampullectomy, with snare

resection over the wire as an option.

EUS is frequently utilized by biliary experts prior to resection. EUS has the benefits of assessing the depth of tumoral infiltration with 70%-90% accuracy since endoscopic biopsies are not always reliable because of sampling error<sup>[25-31]</sup>. Size and characteristics of the ampullary tumor (evidence of ulceration, friability or spontaneous bleeding) should determine the need for EUS. Baillie suggested that EUS should be performed in large lesions to determine the need for surgery<sup>[32]</sup>. If concerning findings are noted, it obviates the need for endoscopic therapy. In the literature, there has been concern about overstaging the tumor with EUS. Desilets *et al*<sup>[14]</sup> felt that the suspicion for invasive disease is more accurately predicted by the behavior of the lesion with submucosal injection and careful evaluation of the cholangiogram and pancreatogram. Adding fine needle aspiration at the time of EUS is also a consideration. Defrain *et al* found adenocarcinoma in lesions ranging in size of 1.3-3.0 cm with sensitivity, specificity, positive and negative predictive values of 82.4%, 100%, 100% and 76.9%, respectively.

Endoscopic biliary and pancreatic sphincterotomy is utilized to assess ductal involvement prior to ampullectomy. In the literature, the use of biliary sphincterotomy prior to ampullectomy is not well defined. Ideally, biliary sphincterotomy maximally exposes the affected ampullary epithelium, aiding in future surveillance and preventing biliary stenosis. In the 2 largest series reporting ampullectomy outcomes, both reported using biliary sphincterotomy at the discretion of the endoscopists, although this was not well defined<sup>[18,33]</sup>. In other series, authors always performed biliary sphincterotomy in patients undergoing ampullectomy<sup>[13-16,19,20]</sup>. However, in a recent "Expert's Corner" on endoscopic ampullectomy, biliary sphincterotomy was not mentioned<sup>[32]</sup>. Auiria *et al*<sup>[34]</sup> argued against biliary sphincterotomy since it carries the risk of bleeding, may interfere with *en bloc* resection and has the theoretical risk of seeding malignant cells present within the tumor. Unlike biliary sphincterotomy, the routine use of pancreatic sphincterotomy prior to ampullectomy has been advocated in the literature<sup>[14,19,32]</sup>. Our respondents utilized pancreatic sphincterotomy less frequently than biliary sphincterotomy prior to ampullectomy. However, Desilets *et al*<sup>[14]</sup>, Kahaleh *et al*<sup>[19]</sup>, and Baillie<sup>[32]</sup> all preferred pre-ampullectomy pancreatic sphincterotomy. The pancreatic sphincterotomy techniques that Desilets *et al*<sup>[14]</sup> described were wire-guided, involving sphincterotomies extending into normal duodenal tissue within the limits of safety. This was performed to further isolate the lesion, to remove the pancreatic orifice from the resection site and to ensure adequate drainage post-resection. Kahaleh and Baillie go on to further specify performing the pancreatic sphincterotomy solely with pure cutting current<sup>[19,32]</sup>. Our respondents with a high volume of ERCP were more likely to perform pre-resection sphincterotomy. However, Lee *et al*<sup>[35]</sup> questioned the use of pre-resection pancreatic sphincterotomy because of the higher risk of bleeding

with papillary tumors, and the distortion of the resected specimen resulting from mechanical and thermal injury, making histopathologic interpretation of the lesion difficult.

Submucosal injection prior to ampullectomy has been recommended by some authors<sup>[14,19,36]</sup>. The technique is performed to separate the tumor from the muscularis propria. As a submucosal cushion, the fluid prevents deeper coagulation into the duodenal wall and theoretically reduces the risk of perforation and pancreatitis<sup>[37,38]</sup>. Epinephrine is added to prevent bleeding. Another benefit of submucosal injection is that it can serve as an indicator of malignancy. Lack of elevation with injection suggests invasive tumor growth. However, submucosal injection may actually impede optimal snare placement. This can be seen particularly in smaller tumors since the center of these lesions are tethered by the ducts and may not lift well. The surrounding normal tissue will lift and mushroom around the adenoma, thus partially burying it<sup>[36]</sup>. However, few respondents "always" performed this technique prior to ampullectomy. Factors determining when 48% of practitioners "sometimes" utilized submucosal injection were not defined.

Our study has several potential limitations. First, because of our study population, our findings may not apply to other practice settings. Unfortunately, a strict definition for "expert biliary endoscopists" does not exist. We therefore relied on the personal knowledge of leaders in the field to choose our sample population. This was the same method utilized by Brackbill *et al*<sup>[24]</sup>; however, we expanded the field to include more community gastroenterologists. Recall bias may also be present in these data since we are relying on self-reported data. There is also the possibility that our results may reflect what the respondents think they should do versus what they actually do in everyday practice.

In endoscopic ampullectomy, experts agreed (> 50%) on the use of EUS for large lesions, prophylactic pancreatic stenting, follow-up examination and adjunct modality for residual tissue removal. Few respondents used empiric pancreatic sphincterotomy. Practitioners with high volumes were more likely to "always" perform biliary and pancreatic sphincterotomy and place pancreatic stents after ampullectomy. Among biliary experts, there was less variation in ampullectomy practices than is reflected in the literature.

## COMMENTS

### Background

Ampullary tumors account for 5% of gastrointestinal neoplasms. Because of the morbidity and mortality associated with surgery, endoscopic retrograde cholangiopancreatography (ERCP) has been utilized as a less invasive procedure to perform an ampullectomy for removal of the ampullary tumor. Specific endoscopic guidelines for ampullectomy have not been established.

### Research frontiers

Endoscopic ampullectomy has been reported widely in case reports and case series. Further study needs to be dedicated to the techniques involved in ampullectomy, in particular the use of biliary and pancreatic sphincterotomy, timing of pancreatic stenting and possible routine use of submucosal injection and endoscopic ultrasound (EUS).

## Applications

This survey demonstrated the ampullectomy practices of expert endoscopists which is important for future guideline development. Experts agree on the use of EUS for large lesions, prophylactic pancreatic stenting, follow-up examination and adjunct modality for residual tissue removal. Variability existed among experts regarding the use of biliary and pancreatic sphincterotomy prior to ampullectomy. Therefore, these practices should be studied in prospective trials to help refine the best practice for our patients.

## Terminology

The ampulla is an orifice in the second portion of the duodenum where the biliary tree and pancreas drain. Ampullectomy describes a technique to remove a tumor at the ampulla. This can be performed by surgery or using a less invasive procedure named ERCP. ERCP is an endoscopic procedure used to access the biliary tree and pancreatic duct from the second portion of the duodenum. EUS is an endoscopic procedure where an endoscope with an ultrasound probe is used to obtain images of the internal organs.

## Peer review

This article is very interesting and helpful to assess guidelines in endoscopic ampullectomy techniques for expert biliary endoscopists in North America.

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## Cyclooxygenase-2 polymorphisms and the risk of esophageal adeno- or squamous cell carcinoma

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*GG* genotype resulted in a higher risk of developing esophageal adenocarcinoma (OR = 3.85, 95% CI: 1.45-10.3) compared with the *-1195 AA* genotype as a reference. The *-765 G→C* genotype distribution was not different between the two groups. The *GG/GG* haplotype was present more often in esophageal adenocarcinoma patients than in controls (OR = 3.45, 95% CI: 1.24-9.58; with *AG/AG* as a reference). The same trends were observed in patients with squamous cell carcinomas, however, the results did not reach statistical significance.

**CONCLUSION:** Presence of the *COX-2 -1195 GG* genotype and of the *GG/GG* haplotype may result in a higher risk of developing esophageal carcinoma.

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**Key words:** Adenocarcinoma; Cyclooxygenase-2; Esophagus; Genetic polymorphism; Squamous cell carcinoma

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### Abstract

**AIM:** To determine whether *-1195 A→G* and/or *-765 G→C* polymorphisms in Cyclooxygenase-2 (*COX-2*) may have a risk modifying effect on the development of esophageal carcinoma in a Dutch Caucasian population.

**METHODS:** Two study groups were recruited, 252 patients with esophageal carcinoma and 240 healthy controls, matched for race, age, gender and recruiting area. DNA was isolated from whole blood and used for genotyping. PCR products were digested with restriction enzymes and products were analyzed by agarose gel electrophoresis. Odds ratios (OR) and 95% confidence intervals (CI) were estimated.

**RESULTS:** The distribution of the *-1195 A→G* polymorphism was significantly different in esophageal cancer patients compared to controls. The *-1195*

### INTRODUCTION

During the last few decades, the incidence of esophageal carcinoma has sharply increased in Western-lifestyle countries. Two main types of esophageal carcinoma exist, adenocarcinoma and squamous cell carcinoma. The main difference between adenocarcinoma and squamous cell carcinoma is the cell type from which the tumor originates; glandular or squamous epithelial cells, respectively.

Adenocarcinoma of the esophagus predominantly occurs in Western societies. There is a strong and probably

causal relation between gastro-esophageal reflux and the development of esophageal adenocarcinoma<sup>[1]</sup>. Gastro-esophageal reflux may cause damage to the esophageal tissue due to the high concentrations of acid and bile salts, which may induce metaplasia and cell proliferation, thereby increasing the risk of mutations. This can lead to Barrett's esophagus with high grade dysplasia and ultimately to adenocarcinoma of the esophagus<sup>[1,2]</sup>.

In contrast to adenocarcinoma, squamous cell carcinoma of the esophagus is thought to be caused predominantly by specific lifestyle or environmental factors such as heavy smoking in combination with alcohol use, chewing of tobacco or consumption of spicy foods and hot beverages<sup>[3]</sup>. In certain developing countries such as China, India or Iran, squamous cell carcinoma of the esophagus is very common, probably due to particular lifestyle habits<sup>[3]</sup>. As a result, damage to esophageal tissue may occur and tissue renewal may increase. This increased cell proliferation can lead to mutations, dysplasia and carcinoma.

Cell proliferation may play a key role in tumor genesis and cyclooxygenases (COXs) are important regulatory enzymes in this process. COXs are enzymes that catalyze the conversion of free arachidonic acid into prostaglandin H<sub>2</sub>, which is the precursor of prostaglandins, prostacyclin and thromboxanes. These regulatory compounds play a role in many biological processes such as cell proliferation, angiogenesis, immune function and inflammation, which are all crucial in the development and progression of neoplasms<sup>[4]</sup>. The human COX family consists of three members, COX-1-3<sup>[4,5]</sup>. COX-1 is found in most tissues and plays a role in homeostasis of many physiologic processes. COX-3 is an alternative splice product of COX-1 and is believed to be involved in the regulation of pain and fever. COX-2 is probably very important in the development and progression of neoplasms. COX-2 is an inducible enzyme whose expression can be induced by pro-inflammatory and mitogenic stimuli like cytokines and growth factors. COX-2 plays an important role in the development of otherwise healthy tissue into metaplastic and dysplastic tissue, as well as in the development and progression of a tumor, by taking part in the regulation of cell proliferation, cell transformation, tumor growth, metastasis and invasion. COX-2 is often found over-expressed in gastrointestinal tumors, including those of the esophagus<sup>[6-10]</sup>. Tumors which exhibit a high level of COX-2 seem to be more aggressive<sup>[6]</sup> and patients bearing those tumors showed a significantly reduced survival<sup>[10]</sup>. In addition, when COX-2 expression in laboratory animals was suppressed with medication, fewer animals developed esophageal adenocarcinoma<sup>[11]</sup>. Therefore, the role of COX-2 in the development of normal or metaplastic tissue into neoplasms seems evident.

Recently, several functional Single Nucleotide Polymorphisms in the COX-2 gene have been discovered which may contribute to the variance in inter-individual COX-2 expression. The -1195 A→G substitution in the COX-2 promotor was found to be associated with a lower expression of COX-2 in a Chinese population<sup>[12]</sup>.

Another SNP, -765 G→C was first described in a UK

population<sup>[13]</sup>. This polymorphism was shown to result in a lower promoter activity, which could subsequently lead to a lower expression of COX-2.

The purpose of this study is to determine the possible modulating effect of the COX-2 polymorphisms -1195 A→G and -765 G→C on the risk for developing esophageal cancer in a Dutch Caucasian population.

## MATERIALS AND METHODS

### **Patients and controls**

A group of 252 patients with esophageal carcinoma was recruited during the period October 2002 to January 2008, in four hospitals all localized in the South-East area of The Netherlands. These hospitals were: (1) Radboud University Nijmegen Medical Center, (2) Canisius Wilhelmina Hospital, Nijmegen, (3) Hospital Gelderse Vallei, Ede and (4) Rijnstate Hospital, Arnhem. Only patients with a diagnosis of esophageal carcinoma as confirmed by a pathologist were included in the study.

Following an advertisement in local papers, a group of 240 healthy controls was recruited from the same geographical area of The Netherlands. Controls were matched with the esophageal carcinoma patients for age, ethnicity and gender.

The study was approved in 2002 by the Medical Ethical Review Committee, region Arnhem-Nijmegen (CMO 2002/114). EDTA blood was collected from patients and controls. The whole blood samples were stored at -22°C until use. DNA was extracted from whole blood by using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, USA) according to the manufacturer's instructions. The extracted DNA was stored at 4°C until use.

The extracted DNA was used for determination of the -1195 A→G and -765 G→C polymorphisms in the COX-2 promoter by polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP), exactly as described by Zhang *et al*<sup>[12]</sup>.

### **Statistical analysis**

The differences between characteristics of patients with esophageal carcinoma and controls were analysed with the Student's *t*-test. All genotypes of controls and patients were tested to determine whether they were distributed according to the Hardy-Weinberg equilibrium. The chi-square test was used to test for differences in distribution of genotypes between the two groups, or to estimate differences in allele frequencies. Odds ratios (OR) with 95% confidence interval (95% CI) were calculated for genotypes associated with predicted normal versus predicted altered enzyme activities (variant genotypes). COX-2 haplotypes were studied using the PL-EM software as described by Qin *et al*<sup>[14]</sup>. *P* < 0.05 was considered to be statistically significant. All data were processed using SPSS software for Windows version 16.0 (SPSS Inc, Chicago Illinois, USA).

## RESULTS

Patients with esophageal carcinoma and controls were

**Table 1** Characteristics of patients with oesophageal carcinoma and controls *n* (%)

Characteristics	Patients with oesophageal carcinoma			Controls	
	Total	Adeno carcinoma	Squamous cell carcinoma	Mixed	
<i>n</i>	252	174 (69.0)	70 (27.8)	8 (3.2)	240
Age (yr; mean ± SD)	64.3 ± 10.8	64.7 ± 11.0	62.7 ± 10.2	69.9 ± 8.0	64.6 ± 10.9
Gender					
Female	51 (20.2)	24 (13.8)	26 (37.1)	1 (12.5)	51 (21.2)
Male	201 (79.8)	150 (86.2)	44 (62.9)	7 (87.5)	189 (78.8)

**Table 2** Distribution of the COX-2 -1195A→G and -765 G→C genotypes and corresponding ORs in patients with oesophageal adenocarcinoma or squamous cell carcinoma versus controls

Genotype COX-2	Adenocarcinoma		Squamous cell carcinoma		Controls <i>n</i> (%)
	<i>n</i> (%)	OR (95% CI)	<i>n</i> (%)	OR (95% CI)	
-1195A→G					
-1195A/-1195A	100 (58)	Reference	39 (56)	Reference	154 (64)
-1195G/-1195A	59 (34)	1.13 (0.75-1.73)	26 (37)	1.28 (0.73-2.26)	80 (33)
-1195G/-1195G	15 (9)	3.85 (1.45-10.3)	5 (7)	3.29 (0.95-11.4)	6 (3)
Total	174		70		240
-765 G→C <sup>1</sup>					
-765G/-765G	112 (69)	Reference	41 (69)	Reference	157 (66)
-765G/-765C	46 (28)	0.88 (0.57-1.37)	16 (27)	0.84 (0.44-1.60)	73 (31)
-765C/-765C	5 (3)	1.17 (0.35-3.92)	2 (3)	1.28 (0.25-6.56)	6 (3)
Total	163		59		236

<sup>1</sup>In both the cases and control group, there are some missing data because of unsuccessful PCR; OR: Odds ratio; CI: Confidence interval.**Table 3** COX-2 haplotype distribution and corresponding ORs in patients with oesophageal adenocarcinoma or squamous cell carcinoma carcinoma versus controls

Haplotype COX-2	Adenocarcinoma		Squamous cell carcinoma		Controls <i>n</i> = 236 (%)
	<i>n</i> = 163 (%)	OR (95% CI)	<i>n</i> = 59 (%)	OR (95% CI)	
AG/AG	59 (36.2)	Reference	18 (30.5)	Reference	94 (39.8)
AG/AC	29 (17.8)	0.87 (0.50-1.52)	12 (20.3)	1.18 (0.53-2.64)	53 (22.4)
AC/AC	5 (3.1)	1.33 (0.39-4.55)	2 (3.4)	1.74 (0.33-9.32)	6 (2.5)
GC/AC	0 (0)	-	0 (0)	-	0 (0)
GG/AC	17 (10.4)	1.29 (0.63-2.64)	4 (6.8)	0.99 (0.31-3.24)	21 (8.9)
GG/AG	40 (24.5)	1.14 (0.68-1.91)	19 (32.2)	1.77 (0.86-3.66)	56 (23.7)
GG/GG	13 (8.0)	3.45 (1.24-9.58)	4 (6.8)	3.48 (0.89-13.6)	6 (2.5)

matched for race, age, gender and recruiting area. Table 1 shows the characteristics of the patients and controls. The COX-2 genotype distributions in patients and controls are summarized in Table 2. The polymorphisms tested here were distributed according to the Hardy-Weinberg criteria, *P*-values in patients and controls were 0.98 and 0.47 for the -765 G→C polymorphism and 0.21 and 0.24 for the -1195 A→G polymorphism, respectively.

No significant differences in the distribution of the -765 G→C polymorphism between patients with esophageal carcinoma and controls were observed (*P* = 0.80;  $\chi^2$  test). However, a significant difference in the distribution of the -1195 A→G polymorphism between patients and controls was observed (*P* = 0.02; chi square test). The -1195 G/-1195 G genotype was present more often in patients with esophageal carcinoma (whole group) as compared to the -1195 A/-1195 A genotype in controls (OR 3.57, 95% CI 1.39-9.13, *P* = 0.005). When analyzed according to the type of tumor, ORs were 3.85 (95% CI 1.45-10.3) for patients with adenocarcinoma and

3.29 (0.95-11.4) for patients with squamous cell carcinoma (Table 2). Allele frequencies in all patients with esophageal cancer (-1195 A vs -1195 G) also differed significantly from those in controls (*P* = 0.02).

When comparing the squamous cell carcinoma group (*n* = 70) with the adenocarcinoma group (*n* = 174), there were no significant differences with respect to the -1195 genotype distribution: -1195 AA, 55.7% vs 57.5%; -1195 AG, 37.1% vs 33.9% and -1195 GG, 7.2% vs 8.6% (*P* = 0.97). For the -765 genotypes no differences in distribution between the squamous cell carcinoma and adenocarcinoma groups were found: -765 GG, 69.5% vs 68.7%; -765 GC, 27.1% vs 28.2% and -765 CC, 3.4% vs 3.1% (*P* = 0.95).

Table 3 shows the results of a comparison of the distribution of the COX-2 -765 and -1195 haplotypes, according to the type of tumor. Only one significant difference was found, the GG/GG haplotype was present more often in the esophageal adenocarcinoma group than in the control group (OR = 3.45, 95% CI = 1.24-9.58). However, the number of individuals

present in these subgroups was very small ( $n = 13$  vs  $n = 6$ , respectively). The same trend was observed in the squamous cell carcinoma group, however, statistical significance was not reached.

## DISCUSSION

The -1195 GG genotype was present more often in patients with esophageal carcinoma than in controls. This is in contrast to the findings of Zhang *et al*<sup>[12]</sup> who identified the -1195 AA genotype as a risk factor for esophageal carcinoma. It is commonly reported that COX-2 expression is higher in cancerous tissue, because high COX-2 expression contributes to and sustains inflammatory and pre-cancerous processes<sup>[4,6]</sup>. Zhang *et al*<sup>[12]</sup> also concluded that COX-2 mRNA expression in -1195 AA genotypes was much higher than the mRNA expression in tissues of patients with the -1195 GG genotype. Our findings now suggest that the COX-2 -1195 polymorphism has the opposite effect on esophageal carcinoma risk in Caucasians, as compared to Chinese patients. However, two limitations must be noted: firstly, we did not measure whether the COX-2 mRNA expression in -1195 AA genotypes was highest in our group of Caucasian patients, similar to the findings of Zhang *et al*<sup>[12]</sup> in Chinese patients. Secondly, there is a difference between our study population and that of Zhang *et al*<sup>[12]</sup>; the majority of our patients had adenocarcinoma (69%) and the minority suffered from squamous cell carcinoma (28%), whereas the Chinese patients in the study by Zhang *et al*<sup>[12]</sup> all had squamous cell carcinoma. In China, esophageal squamous cell carcinoma is significantly more common than adenocarcinoma, as it is mainly caused by lifestyle factors such as drinking hot beverages and eating spicy foods, whereas adenocarcinoma is associated with acid reflux as a result of the Western lifestyle<sup>[11]</sup>. In our patient group, we found no differences in the distribution of both COX-2 polymorphisms between patients with adenocarcinoma and squamous cell carcinoma, which suggests that the differences found when compared to the results of Zhang *et al*<sup>[12]</sup> could be assigned merely to racial differences rather than to differences in the type of tumor.

Another indication that racial differences in the study populations may explain the apparent contradictory results is obtained by comparing the distribution of the COX-2 polymorphisms in the Chinese and Dutch control populations. The genotype frequencies found in our Dutch controls for the -765 G→C and -1195 A→G polymorphisms were: 66.5% GG, 30.9% GC, 2.9% CC and 64.2% AA, 33.3% GA, 2.5% GG, respectively. Zhang *et al*<sup>[12]</sup> in a Chinese population reported genotype frequencies of 95.7% GG, 4.3% GC, 0% CC and 24.1% AA, 53.4% GA and 22.5% GG, respectively. Tan *et al* in Chinese controls more recently reported approximately the same genotype frequencies as Zhang *et al*: 95.2% GG, 4.8% GC, 0% CC and 23.7% AA, 53.2% GA and 23.1% GG, respectively<sup>[15]</sup>.

On the other hand, our control group data on the COX-2 -765 genotype were in good agreement with other

European control data recently reported from Denmark, being 73.2%, 24.8% and 2.0% for -765 GG, GC and CC genotypes, respectively<sup>[16]</sup>. In addition, the COX-2 polymorphism data in our patients are very similar to the recently reported COX-2 -765 and -1195 genotype distributions in Dutch esophageal adenocarcinoma patients by Moons *et al*<sup>[17]</sup>, except for the -1195 GG genotype, which was present in 8.0% of our patients vs only 2.0% in the patients in the study by Moons *et al*<sup>[17]</sup>.

The distribution of the -765 genotypes in the control group was not found to be significantly different when compared to the esophageal carcinoma group, whereas Moons *et al*<sup>[17]</sup> reported a significantly different -765 CC genotype distribution between a Dutch esophageal carcinoma group ( $n = 140$ ) and a Barrett's esophagus ( $n = 255$ ) or reflux esophagitis ( $n = 240$ ) patient group. It should be noted, however, that the number of -765 CC genotype individuals in these patient groups was very low, being seven, four and zero individuals, respectively<sup>[17]</sup>. Two main reasons for the difference in results between the two Dutch studies are as follows: firstly, our study was performed on a larger patient population than the study by Moons *et al*<sup>[17]</sup> (252 vs 140 patients), and secondly in our study, similar to the study by Zhang *et al*<sup>[12]</sup>, a comparison between patients with esophageal cancer and healthy controls was made, in contrast to the study by Moons *et al*<sup>[17]</sup> where patients with Barrett's esophagus or reflux esophagitis, both of which are at risk for esophagus carcinoma, were used for comparison.

Analyzing the COX-2 haplotypes showed that the GG/GG haplotype was present more often in the esophageal carcinoma group, which again is not in accordance with the results of Zhang *et al*<sup>[12]</sup> and Moons *et al*<sup>[17]</sup>, who both found that the CA containing haplotypes carried the highest risk. Since the results of Zhang *et al*<sup>[12]</sup> and Moons *et al*<sup>[17]</sup> on different types of tumors (squamous cell carcinoma vs adenocarcinoma, respectively) are very similar, and more or less contradict our results, it was of interest to compare the haplotype distribution between our patients with squamous cell carcinoma vs adenocarcinoma. However, no significant differences were found.

In conclusion, the presence of the COX-2 -1195 GG genotype and of the GG/GG haplotype may result in a higher risk of developing esophageal adenocarcinoma and possibly also squamous cell carcinoma.

## COMMENTS

### Background

Cyclooxygenase-2 (COX-2) is claimed to be a key enzyme in the development and progression of neoplasms. COX-2 is often found over-expressed in gastrointestinal tumors, including those of the esophagus. The corresponding COX-2 gene is polymorphic and two single nucleotide polymorphisms; -1195 A→G and -765 G→C were demonstrated to influence the expression of COX-2. Therefore, these polymorphisms might modulate the risk for gastrointestinal cancers, including cancer of the esophagus.

### Research frontiers

In this study, the COX-2 -1195 GG genotype was found to be present more often in Caucasian patients with esophageal carcinoma than in controls. This is in contrast to earlier findings in a Chinese population, where the -1195 AA genotype was revealed as a risk factor for esophageal carcinoma.

### Innovations and breakthroughs

Presence of the COX-2 -1195 GG genotype and of the GG/GG haplotype may result in a higher risk of developing esophageal carcinoma.

### Applications

Screening for the COX-2 -1195 GG genotype in a population at risk for esophageal cancer may be valuable in the future in order to select high risk patients. Information and prevention programs can then be focused on these patients.

### Terminology

COX-2 is an enzyme that catalyzes the conversion of arachidonic acid in prostaglandin H<sub>2</sub>, the precursor of other prostaglandins, prostacyclin and thromboxanes. These regulatory compounds play a role in many biological processes such as cell proliferation, angiogenesis, immune function and inflammation, which are all crucial in the development and progression of neoplasms.

### Peer review

This study offered a controversial view of COX-2 polymorphisms in the esophageal carcinomas, compared with existing studies in Europe and China. The authors thoroughly discussed various possibilities that may lead to the different findings among studies. This manuscript is well written. Although the finding is controversial, the authors discussed this issue very well.

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BRIEF ARTICLES

## Gallbladder emptying in patients with primary sclerosing cholangitis

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### Abstract

**AIM:** To assess gallbladder emptying and its association with cholecystitis and abdominal pain in patients with primary sclerosing cholangitis (PSC).

**METHODS:** Twenty patients with PSC and ten healthy subjects were investigated. Gallbladder fasting volume, ejection fraction and residual volume after ingestion of a test meal were compared in patients with PSC and healthy controls using magnetic resonance imaging. Symptoms, thickness and contrast enhancement of the gallbladder wall and the presence of cystic duct strictures were also assessed.

**RESULTS:** Median fasting gallbladder volume in patients with PSC [67 (19-348) mL] was twice that in healthy controls [32 (16-55) mL] ( $P < 0.05$ ). The median postprandial gallbladder volume in patients with PSC was significantly larger than that in healthy controls ( $P < 0.05$ ). There was no difference in ejection fraction, gallbladder emptying volume or mean thickness of the gallbladder wall between PSC patients and controls. Contrast enhancement of the gallbladder wall in PSC patients was higher than that in controls; (69%  $\pm$  32%) and (42%  $\pm$  21%) ( $P < 0.05$ ). No significant association was found between the gallbladder volumes and occurrence of abdominal pain in patients and controls.

**CONCLUSION:** Patients with PSC have increased fasting gallbladder volume. Gallbladder mucosal dysfunction secondary to chronic cholecystitis, may be a possible mechanism for increased gallbladder.

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**Key words:** Cholecystitis; Ejection fraction; Gallbladder volume; Magnetic resonance imaging

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Said K, Edsborg N, Albiin N, Bergquist A. Gallbladder emptying in patients with primary sclerosing cholangitis. *World J Gastroenterol* 2009; 15(28): 3498-3503 Available from: URL: <http://www.wjgnet.com/1007-9327/15/3498.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.3498>

### INTRODUCTION

Primary sclerosing cholangitis (PSC) is an idiopathic chronic cholestatic inflammatory liver disease characterized by diffuse fibrosing inflammation of intra- and/or extrahepatic bile ducts, resulting in bile duct obliteration, biliary cirrhosis, and eventually hepatic failure<sup>[1,2]</sup>. Gallbladder abnormalities, including gallstones, cholecystitis and gallbladder masses are common in patients with PSC<sup>[3,4]</sup>, and seems to be part of the spectrum of the disease. Inflammation of the bile ducts in PSC is similar to that found in the gallbladder epithelium in these patients.

Functional impairment of the gallbladder in PSC is rarely studied. Van de Meeberg *et al*<sup>[5]</sup> showed enlarged fasting gallbladder volumes and increased postprandial volumes in patients with PSC compared with patients with primary biliary cirrhosis and healthy controls, although the ejection fraction of bile was normal. Other studies in patients with PSC have not found gallbladder enlargement<sup>[6,7]</sup>. Increased gallbladder volume or gallbladder retention are known to occur in conditions other than PSC such as truncal vagotomy<sup>[8,9]</sup>, chronic

pancreatitis<sup>[10]</sup>, octreotide therapy<sup>[11]</sup>, obesity<sup>[12]</sup>, diabetes mellitus<sup>[13]</sup>, pregnancy<sup>[14]</sup> and distal biliary obstruction<sup>[15]</sup>. PSC associated inflammation of the gallbladder epithelium and cholangiographic abnormalities of the cystic duct have been reported in patients with PSC<sup>[3,7,16]</sup>.

One of the most common symptoms at the time of presentation of PSC is mild to severe abdominal pain localized in the right upper quadrant<sup>[17-20]</sup>. The cause of abdominal pain is unclear, it seems, however, unrelated to the grade of bile duct strictures. In addition, cholecystectomy seldom improves abdominal pain in these patients. A possible association between enlarged fasting gallbladder volume, ejection fraction and abdominal pain has never been investigated in patients with PSC.

The primary aim of the present study, using magnetic resonance imaging (MRI), was to evaluate the fasting and postprandial gallbladder volumes and to assess whether or not gallbladder emptying is associated with abdominal pain in patients with PSC. Secondly, we studied if the presence of imaging signs of chronic cholecystitis is correlated to gallbladder volume, the emptying process or abdominal pain.

## MATERIALS AND METHODS

### Ethics

The study was approved by the Ethics committee at Karolinska University Hospital, Huddinge and written informed consent was received from all patients and controls.

### Subjects

Twenty patients, (14 men and 6 women) who ranged in age from 24 to 59 years (mean age  $39 \pm 10$  years), with well-defined PSC<sup>[21]</sup> treated at the Liver Unit, Karolinska University Hospital, Huddinge were included in the study between January 2005 and July 2006. Clinical data were obtained by review of the complete medical history collected from patient files. Patients with hepatobiliary malignancy, diabetes mellitus, chronic pancreatitis or distal biliary obstruction (dominant extrahepatic strictures) were excluded. Ten healthy subjects (5 men and 5 women), who ranged in age from 31-79 years (mean age  $47 \pm 13.5$  years), without any history of gastrointestinal disease or previous abdominal surgery served as controls. Informed consent for study participation was received from all patients and controls.

### Procedure

After overnight fasting, two intravenous cannulas were inserted into the antecubital veins: one for blood sampling and one for intravenous injection of a contrast agent for MRI. The fasting gallbladder volume was analysed by MRI, prior to injection of the contrast agent, time = 0 min. One hour later (time = 1 h) a test meal consisting of 200 g "Swedish hash" (fried diced meat, onions and potatoes served with beetroot), 250 mL milk (3% fat) and an apple, totalling 2064 kJ including 21 g fat was ingested. Postprandial gallbladder volume and

ejection fraction were obtained at 2.5 h (time = 2.5 h), that is an hour and a half after ingestion of the fat-meal at which point gallbladder contraction is supposed to be maximal<sup>[5,22-24]</sup>.

### Laboratory data

Biochemical variables including alkaline phosphatase, serum transaminases, total bilirubin, International normalized ratio (INR), serum albumin and CRP were obtained at the beginning of the procedure and analysed using standard procedures at the Karolinska University Hospital.

### Questionnaire

Every subject filled in a questionnaire for the assessment of abdominal pain localized in the right upper quadrant, abdominal discomfort and nausea, before the first MRI, just before, and one and three hours after meal ingestion. The questionnaire consisted of visual analogue scales (VAS) where the patient marked the degree of symptoms including abdominal pain, nausea and abdominal discomfort.

### Magnetic resonance imaging (MRI)

Examinations including the gallbladder and the hepatobiliary system were performed (after overnight fasting) using a 1.5 T magnetic resonance system [Magnetom Symphony ( $n = 1$  PSC), Vision ( $n = 7$  PSC) or Avanto ( $n = 12$  PSC and 10 controls]; Siemens, Erlangen, Germany]. Each patient was examined using the same unit before and after the meal combining the spine and the flexible body array coil. The use of different units was therefore not considered to influence the results. Gd-BOPTA (MultiHance® 0.5 mmol/mL, Bracco, Milan, Italy) at a dosage of 0.1 mmol/kg of was injected. Axial breath-hold 3D-T1-weighted scans (VIBE, slice thickness 1.7-2.5 mm) were performed natively and dynamically in arterial, portal-venous and delayed 5 min phase for clinical diagnosis. Postprandially, in the hepatobiliary phase, the hepatobiliary system was rescanned (VIBE).

### Gallbladder wall thickness, cystic duct and gall stones

The thickness of the gallbladder wall was measured on the axial T2 Haste slices at three different areas of the gallbladder. The mean values of the measurements were calculated for each patient. The presence of biliary stones and perivesical fluid was noted and the cystic duct was evaluated for the presence of strictures. Cystic duct abnormalities were defined as mural irregularities of the cystic duct on magnetic resonance cholangiography (MRC).

### Gallbladder wall contrast enhancement

Contrast enhancement of the gallbladder wall was analyzed in % using the formula: Contrast enhancement =  $[SI(\text{portalvenousphase}) - SI(\text{native})]/SI(\text{native}) \times 100$ .

In each patient the signal intensity (SI) of the wall was measured, in a single voxel, in three different areas, trying to avoid vessels and adjacent intestinal loops or the liver parenchyma. The same areas were measured natively

and in the portal venous phase and the enhancement was calculated for each part. The mean of the measurements was calculated for each patient.

### Gallbladder volume measurements

The 3D-T1-weighted scans were analysed using a Voxar® 3D workstation (Barco NV, Kortrijk, Belgium) using 3D segmentation and volume measurements. The volume of the gallbladder was measured fasting (delayed 5 min phase) and in the postprandial phase. In the latter hepatobiliary phase, contrast filling of the gallbladder was also noted. The analyses were made in consensus by two radiologists (NE and NA).

The ejection volume was measured in microliter using the formula: Ejection volume = volume (fasting) - volume (postprandial).

The ejection fraction or gallbladder emptying was measured in % using the formula: Ejection fraction = [volume (fasting) - volume (postprandial)]/volume (fasting) × 100.

Gallbladder fasting volume, ejection fraction and postprandial gallbladder volume of patients with PSC were compared with healthy controls. Postprandial gallbladder refilling of bile (that is, of contrast excreted to the common bile duct) was noted.

### Statistical analysis

Data were analyzed using statistical software (v 7.0, Stat Soft Inc.). Values are expressed as mean and standard deviation or as median (range). For comparison of categorical data, the Chi-square test was used, or Fisher's exact test when appropriate. Spearman's correlation test was used to determine the association between contrast enhancement and gallbladder wall thickness.  $P < 0.05$  was considered significant.

## RESULTS

The clinical characteristics of the 20 PSC patients and the healthy controls are shown in Table 1. There was no significant difference between the two groups regarding age and body mass index (BMI). The mean levels of plasma alkaline phosphatase (ALP) and plasma bilirubin were significantly higher in patients with PSC than in the healthy controls.

The median fasting gallbladder volume in patients with PSC was twice that of the healthy controls; 67 (range 19-348) mL and 32 (range 16-55) mL, respectively ( $P < 0.05$ ) (Figure 1A). The mean fasting gallbladder volume (mean ± SD) in patients with PSC was 91 ± 78 mL compared with 35 ± 11 mL in healthy controls. The median postprandial gallbladder volume in patients with PSC was significantly higher than that in healthy controls; 40 (9-345) mL and 16 (9-26) mL, respectively ( $P < 0.05$ ) (Figure 1B). Median ejection volume was 16 (1-100) mL in PSC patients and 16 (8-38) mL in healthy controls (n.s.) (Figure 1C).

There was no significant difference in ejection fraction between PSC patients and controls as shown in Figure 1D. Ninety percent (18/20) of patients had

**Table 1 Clinical characteristics of 20 patients with PSC and 10 healthy controls**

Clinical characteristic	PSC patients (n = 20)	Healthy controls (n = 10)	P value
Gender			
Male	70% (14/20)	50% (5/10)	NS
Female	30% (6/20)	50% (5/10)	NS
Age in years (± SD)	39 ± 10	47 ± 13	NS
Associated IBD	90% (18/20)	0	
UC	85% (17/20)		
CD	5% (1/20)		
Liver cirrhosis	10% (2/20)	0	
Treatment with UDCA	85% (17/20)	0	
Cholangiographic distribution of PSC		-	
Extra-and intrahepatic involvement	75% (15/20)		
Intrahepatic changes	25% (5/20)		
Duration of PSC in years	9.7 ± 5	-	
Mean P-ALP (< 1.9 µkat/L)	7 ± 6	1 ± 0.3	P < 0.05
Mean P-ALT (< 0.76 µkat/L)	1.4 ± 1.2	0.4 ± 0.1	P < 0.05
Mean P-Bilirubin (< 26 µmol/L)	20 ± 14	10 ± 5	NS
Mean P-Albumin (36-45 g/L)	40 ± 6	39 ± 5	NS
Mean P-Cholesterol (3.9-7.8 mmol/L)	5 ± 0.1	5 ± 0.1	NS
Mean BMI			
Male	25 ± 4	25 ± 2	NS
Female	25.5 ± 2.5	25 ± 5	NS

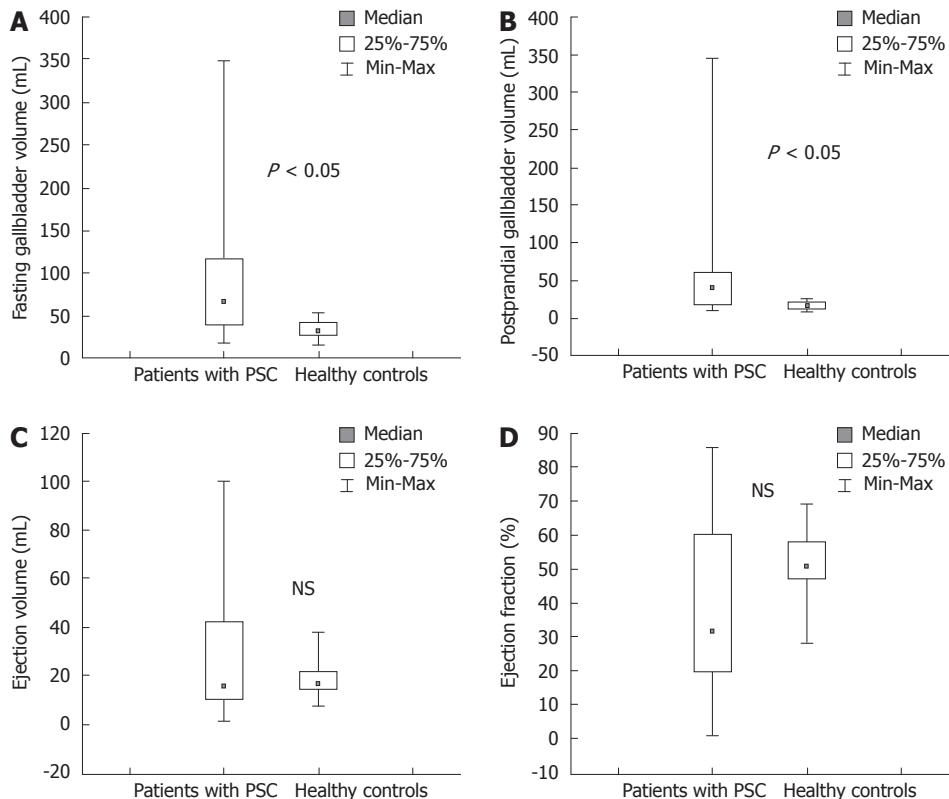
PSC: Primary sclerosing cholangitis; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; IBD: Inflammatory bowel diseases; UDCA: Ursodeoxycholic acid; UC: Ulcerative colitis; CD: Crohn's disease; NS: Not significant; BMI: Body mass index.

inflammatory bowel disease (IBD) and two of these patients had active disease. We found no significant difference in gallbladder volumes between the PSC patients with active IBD and patients who were in remission. None of the women in the PSC group were pregnant at the time of the study.

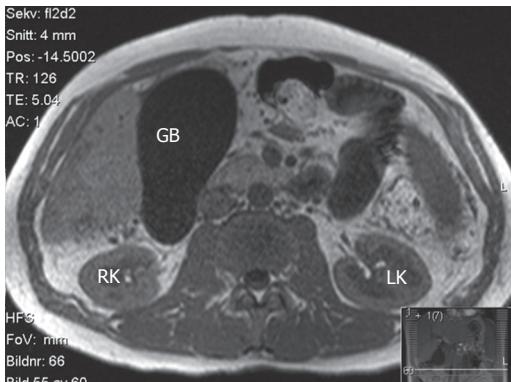
Two of the patients with PSC had liver cirrhosis. One of them, a 42-year-old man had the largest fasting gallbladder volume (348 mL) with an ejection fraction of only 1% (Figure 2). The other cirrhotic patient was a 37-year-old man with a fasting gallbladder volume of 20 mL and an ejection fraction of 55%.

Gallstones were found in the gallbladder in 20% (4/20) of the PSC patients but not in the controls. The median gallstone size was 2.5 mm (2-6 mm), these gallstones had no impact in the cystic duct in preprandial images. None of the patients had visible stones in the common bile duct. Perivesical fluid was seen in small amounts in 20% (4/20) of the PSC patients. None of the patients had a previous history of pancreatitis and we found no significant difference in gallbladder size between patients with and without abdominal pain.

The thickness of the gallbladder wall (mean ± SD) did not differ significantly between PSC patients and controls; 2.2 ± 0.5 mm and 1.9 ± 0.3 mm, respectively. However, there was a significant increase in contrast enhancement of the gallbladder wall (mean ± SD) in PSC patients compared to controls; 69% ± 32% and



**Figure 1** Fasting gallbladder volume (A), postprandial gallbladder volume (B), ejection fraction (C, ejection volume as percentage of fasting gallbladdervolume) and Ejection volume (D) measured with magnetic resonance imaging (MRI) in patients with primary sclerosing cholangitis (PSC) ( $n = 20$ ) and in healthy controls ( $n = 10$ ).



**Figure 2** MRI showing a large gallbladder volume of 348 mL in a patient with PSC.

$43\% \pm 21\%$ , respectively ( $P < 0.05$ ). There were no other signs of acute cholecystitis.

In all subjects there was a significant correlation between high contrast enhancement of the gallbladder wall and large gallbladder volume at fasting ( $P < 0.05$ ) ( $r = 0.39$ ). Postprandial gallbladder volume and ejection fraction were not significantly correlated to contrast enhancement of the gallbladder wall.

We found no correlation between patients with increased gallbladder volume, increased postprandial gallbladder volume and decreased ejection fraction and levels of P-Bilirubin, P-Cholesterol, P-Albumin, P-ALP, duration and distribution of PSC, treatment with ursodeoxycholic acid (UDCA), presence of IBD or age. In all subjects there was no significant correlation between BMI and fasting gallbladder volume or ejection fraction of the gallbladder. Seventeen of 20 patients with PSC were taking 10–15 mg/kg per day of UDCA.

The fasting gallbladder volume and ejection fraction was similar in patients who were on UDCA treatment ( $96 \pm 83$  mL and  $38\% \pm 27\%$ ) and patients who were not treated with UDCA ( $57 \pm 19$  mL and  $36\% \pm 15\%$ ) (n.s.). Seventy five percent (15/20) of the PSC patients and 100% (10/10) of the healthy controls showed contrast in the gallbladder in the hepatobiliary phase (n.s.). Abnormalities of the cystic ducts were visualized in 13 (60%) of the PSC patients and in none in the control group. No significant correlation was found between increased gallbladder fasting volume, decreased ejection fraction or lack of postprandial gallbladder refilling and presence of abnormalities in the cystic ducts.

### Symptoms

No significant association was found between gallbladder volumes or contrast enhancement and occurrence of abdominal pain, abdominal discomfort and nausea in PSC patients and controls. Before the fatty meal, 25% of PSC patients experienced abdominal pain, the visual analogue scale (VAS) ranging from 1 to 4. Twenty five percent of PSC patients experienced nausea with the VAS ranging from 1 to 2. Twenty percent of PSC patients experienced abdominal discomfort with the VAS ranging from 1 to 4. There was no significant increase in symptoms in the PSC group at one or three hours after meal ingestion. None of the healthy controls experienced symptoms pre- and postprandially.

### DISCUSSION

In the present study, we showed that patients with PSC have a significant increase in gallbladder volume both pre- and postprandially compared with healthy control

subjects. This is in agreement with a previous sonographic study by van de Meeberg *et al*<sup>[5]</sup>, who reported a fasting gallbladder volume of  $73 \pm 13.7$  mL compared with  $91 \pm 77.9$  mL in our study. The reason for the increased fasting gallbladder volume in patients with PSC is unclear. Several mechanisms may cause gallbladder enlargement, for example obstruction of the cystic duct or the common bile duct distal to the cystic duct, gallbladder dysmotility or gallbladder mucosal dysfunction may all contribute to increased fasting gallbladder volumes. In our study we excluded patients with significant extrahepatic strictures. Seventy-five percent of the PSC patients showed contrast in the gallbladder indicating the absence of a dominant stricture in the cystic duct. We also found that the gallbladder ejection fraction in PSC patients was similar to that of the healthy controls. Taken together, these findings do not suggest mechanical obstruction and/or gallbladder dysmotility as reasons for the enlarged gallbladder fasting volume in these patients.

Gallbladder abnormalities are common in PSC, and include cholecystitis which is found in 25% of all PSC patients<sup>[4]</sup>. In experimental cholecystitis the process of fluid absorption in the gallbladder epithelium changes to fluid secretion<sup>[25,26]</sup>. The secretory function of the gallbladder in PSC has been described previously in a case report of one PSC patient with concomitant cholecystitis. This patient produced between 39 mL and 52 mL of fluid daily from the gallbladder epithelium<sup>[27]</sup>. We found a similar gallbladder wall thickness in cases and controls. This finding may represent an underestimation of the wall thickness in PSC patients since the gallbladder is larger and more distended. The increased enhancement of the gallbladder wall may indicate the presence of inflammation of the gallbladder epithelium and wall in PSC patients. This sign of cholecystitis, in combination with normal ejection fraction of the gallbladder in PSC patients, support the notion that gallbladder mucosal dysfunction is a possible cause of the increased fasting gallbladder volume and residual volume in patients with PSC. The presence of inflammatory changes in the gallbladder epithelium and its effect on absorption/secretory functions is difficult to evaluate in a clinical setting. There are obvious problems in obtaining daily gallbladder volume measurements in patients, and biopsies are needed for the proper evaluation of inflammation. Measurement of bile concentration in the gallbladder could be a surrogate marker for absorption/secretory dysfunction and such measurements may be possible in the future using MR spectroscopy techniques.

The effect of UDCA treatment on gallbladder motility is unclear. Several studies have shown that UDCA treatment results in increased fasting and postprandial gallbladder volume, whereas gallbladder emptying has not been shown to be reduced or modified<sup>[28-31]</sup>. PSC patients in the study conducted by Van de Meeberg *et al*<sup>[5]</sup>, which showed similar results to ours, discontinued their UDCA medication for four weeks before commencement of the study. We decided not to discontinue therapy with UDCA based on the above studies in order to study the patients' symptoms in a true clinical setting. Eighty-five percent of

our PSC patients were treated with UDCA. We did not ascertain any significant difference in fasting gallbladder volume and gallbladder emptying between UDCA treated and untreated patients. However, this should be interpreted with caution due to the small number of patients involved.

Up to a third of all PSC patients experience pain in the upper right quadrant of the abdomen<sup>[19,20]</sup>. This abdominal pain is most often intermittent, but may occasionally be of a more continuous nature<sup>[32]</sup>. Abdominal pain has been hypothesized to result from constriction of the bile ducts. One third of patients with small duct PSC, which is characterized by the absence of strictures in the large ducts also suffer from abdominal pain<sup>[33]</sup> indicating that biliary strictures do not play a role in the development of abdominal pain. In our study, we did not find any significant difference among PSC patients regarding gallbladder dysfunction and the occurrence of abdominal pain, abdominal discomfort and nausea pre- or postprandially. Gallbladder motility dysfunction as a pathophysiological factor in the development of abdominal pain in patients with PSC is therefore unlikely.

In conclusion, patients with PSC have increased fasting and residual gallbladder volumes, whereas gallbladder emptying is normal. The reason for the increased fasting gallbladder volume is unclear. However, gallbladder mucosal dysfunction secondary to chronic inflammation of the gallbladder is a possible mechanism. Gallbladder size or emptying does not seem to be involved in the development of abdominal pain in patients with PSC.

## COMMENTS

### Background

The mechanisms responsible for the abdominal pain in primary sclerosing cholangitis (PSC) are not fully understood. The aim of the present study was to assess gallbladder emptying and its association with cholecystitis and abdominal pain in patients with PSC.

### Research frontiers

The authors compared gallbladder volumes at fasting and after ingestion of a test meal in patients with PSC and healthy controls using magnetic resonance imaging (MRI). Symptoms, thickness and contrast enhancement of the gallbladder wall and the presence of cystic duct strictures were also assessed.

### Innovations and breakthroughs

The increased enhancement of the gallbladder wall on MRI may indicate the presence of inflammation of the gallbladder epithelium and wall in PSC patients. Gallbladder size or emptying does not seem to be involved in the development of abdominal pain in patients with PSC.

### Applications

The present study provides information on the possible mechanisms of increased fasting gallbladder volume in patients with PSC.

### Peer review

This is an interesting report addressing a clinically important question: whether gallbladder emptying is associated with cholecystitis and the abdominal pain in patients with PSC. The authors analyzed 20 patients with PSC and compared with ten healthy subjects. Results indicate that patients with PSC have increased fasting and residual gallbladder volumes, probably resulting from gallbladder mucosal dysfunction secondary to chronic cholecystitis. However, gallbladder size or the emptying process does not seem to cause abdominal pain in patients with PSC.

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BRIEF ARTICLES

## Perianal disease, small bowel disease, smoking, prior steroid or early azathioprine/biological therapy are predictors of disease behavior change in patients with Crohn's disease

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duration of  $9.0 \pm 7.2$  years. In a logistic regression analysis corrected for disease duration, perianal disease, smoking, steroid use, early AZA or AZA/biological therapy use were independent predictors of disease behavior change. In a subsequent Kaplan-Meier survival analysis and a proportional Cox regression analysis, disease location ( $P = 0.001$ ), presence of perianal disease ( $P < 0.001$ ), prior steroid use ( $P = 0.006$ ), early AZA ( $P = 0.005$ ) or AZA/biological therapy ( $P = 0.002$ ), or smoking ( $P = 0.032$ ) were independent predictors of disease behavior change.

**CONCLUSION:** Our data suggest that perianal disease, small bowel disease, smoking, prior steroid use, early AZA or AZA/biological therapy are all predictors of disease behavior change in CD patients.

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**Key words:** Crohn's disease; Smoking; Azathioprine; Infliximab; Monoclonal antibodies; Colectomy; Risk; Reoperation

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Lakatos PL, Czegledi Z, Szamosi T, Banai J, David G, Zsigmond F, Pandur T, Erdelyi Z, Gemela O, Papp J, Lakatos L. Perianal disease, small bowel disease, smoking, prior steroid or early azathioprine/biological therapy are predictors of disease behavior change in patients with Crohn's disease. *World J Gastroenterol* 2009; 15(28): 3504-3510 Available from: URL: <http://www.wjgnet.com/1007-9327/15/3504.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.3504>

### Abstract

**AIM:** To assess the combined effect of disease phenotype, smoking and medical therapy [steroid, azathioprine (AZA), AZA/biological therapy] on the probability of disease behavior change in a Caucasian cohort of patients with Crohn's disease (CD).

**METHODS:** Three hundred and forty well-characterized, unrelated, consecutive CD patients were analyzed (M/F: 155/185, duration:  $9.4 \pm 7.5$  years) with a complete clinical follow-up. Medical records including disease phenotype according to the Montreal classification, extraintestinal manifestations, use of medications and surgical events were analyzed retrospectively. Patients were interviewed on their smoking habits at the time of diagnosis and during the regular follow-up visits.

**RESULTS:** A change in disease behavior was observed in 30.8% of patients with an initially non-stricturing, non-penetrating disease behavior after a mean disease

### INTRODUCTION

Inflammatory bowel disease (IBD) is a multifactorial disease with probable genetic heterogeneity<sup>[1]</sup>. In addition, several environmental risk factors (e.g. diet, smoking, measles or appendectomy) may contribute to its pathogenesis. During the past decades, the incidence pattern of both forms has significantly changed<sup>[2]</sup>, showing some common but also quite distinct characteristics for the two disorders.

Phenotypic classification of Crohn's disease (CD) plays an important role in determining treatment and may assist in predicting the likely clinical course of disease<sup>[3]</sup>. In 2005, the Montreal revision of the Vienna classification system was introduced<sup>[4]</sup>. Although the broad categories for CD classification remained the same, changes were made within each category. Upper gastrointestinal disease can now exist independently of, or together with, disease present at more distal locations. Finally, perianal disease, which occurs independently of small bowel fistulae, is no longer classified as penetrating disease. Instead, a perianal modifier has been introduced, which may coexist with any disease behavior.

Using the Vienna classification system, it has been shown in clinic-based cohorts that there can be a significant change in disease behavior over time, whereas disease location remains relatively stable<sup>[3,5]</sup>. An association between the presence of perianal disease and internal fistulation (OR: 2.6-4.6) was reported earlier by Sachar *et al*<sup>[6]</sup> in patients with colonic disease. Most recently, Australian authors<sup>[3]</sup> have shown that although > 70% of CD patients had inflammatory disease at diagnosis, the proportion of patients with complicated disease increased over time. Progression to complicated disease was more rapid in those with small bowel than colonic disease location ( $P < 0.001$ ), with perianal disease being also a significant predictor of change in CD behavior (HR: 1.62,  $P < 0.001$ ). Similarly, small bowel location and stricturing disease were predictors for surgery in a long-term follow-up study<sup>[7]</sup>. Finally, perianal lesions, the need for steroids to treat the first flare-up and ileocolonic location, but not an age below 40 years were confirmed as predictive markers for developing disabling disease (according to the predefined criteria) at 5 years<sup>[8]</sup>. In the same study, stricturing behavior (HR: 2.11, 95% CI: 1.39-3.20) and weight loss ( $> 5$  kg) (HR: 1.67, 95% CI: 1.14-2.45) at diagnosis were independently associated with the time to development of severe disease.

A further environmental factor which may be of importance in determining change in disease behavior is smoking. In CD, smoking was reported to be associated with disease location: most, but not all, studies report a higher prevalence of ileal disease and a lower prevalence of colonic involvement in smokers<sup>[9,10]</sup>. A recent review<sup>[10]</sup> and previous data have demonstrated that smoking, when measured up to the time-point of disease behavior classification, was more frequently associated with complicated disease and penetrating intestinal complications<sup>[9,11,12]</sup>, a greater likelihood of progression to complicated disease, as defined by the development of strictures or fistulae<sup>[10]</sup>, and a higher relapse rate<sup>[13]</sup>. In addition, the risk of surgery as well as the risk for further resections during disease course were also noted to be higher in smokers in some studies<sup>[9,14]</sup> and a recent meta-analysis<sup>[15]</sup>. The need for steroids and immunosuppressants was found to be higher in smokers compared to non-smokers<sup>[16]</sup>. Noteworthy, in one study by Cosnes *et al*<sup>[17]</sup>, immunosuppressive therapy was found to neutralize the effect of smoking on the need for surgery. In a recent paper by Aldhous *et al*<sup>[18]</sup>, using the Montreal classification, the harmful effect of smoking was only partially confirmed. Although

current smoking was associated with a lower rate of colonic disease, the smoking habits at diagnosis were not associated with time to development of stricturing disease, internal penetrating disease, perianal penetrating disease, or time until first surgery.

Finally, early postoperative use of azathioprine (AZA, at a dose of 2-2.5 mg/kg per day) appeared to delay postoperative recurrence in comparison to a historical series or placebo groups in randomized, controlled trials<sup>[19]</sup>. Furthermore, in a recent withdrawal study by the GETAID group<sup>[20]</sup>, the authors provide evidence for the benefit of long-term AZA therapy beyond 5 years in patients with prolonged clinical remission. In contrast, initial requirement for steroid use [OR: 3.1 (95% CI: 2.2-4.4)], an age below 40 years (OR: 2.1, 95% CI: 1.3-3.6), and the presence of perianal disease (OR: 1.8, 95% CI: 1.2-2.8) were associated with the development of disabling disease in the study by Beaugerie *et al*<sup>[21]</sup>. The positive predictive value of disabling disease in patients with two and three predictive factors for disabling disease was 0.91 and 0.93, respectively.

In this study, the authors aimed to assess the combined effect of disease phenotype, smoking, and medical therapy (steroid, AZA, AZA/biological) on the probability of disease behavior change in a cohort of Hungarian CD patients.

## MATERIALS AND METHODS

### Patients

Three hundred and forty well-characterized, unrelated, consecutive CD patients (age:  $38.1 \pm 13.2$  years, M/F: 155/185, duration:  $9.4 \pm 7.5$  years) with a complete clinical follow-up were included. A detailed clinical phenotypic description of these patients is presented in Table 1.

The diagnosis was based on the Lennard-Jones Criteria<sup>[22]</sup>; age, age at onset, presence of familial IBD, presence of extraintestinal manifestations; arthritis: peripheral and axial; ocular manifestations: conjunctivitis, uveitis, iridocyclitis; skin lesions: erythema nodosum, pyoderma gangrenosum; and hepatic manifestations: primary sclerosing cholangitis, frequency of flare-ups (frequent flare-up:  $> 1$  per year). The disease phenotype (age at onset, duration, location, and behavior) was determined according to the Montreal Classification<sup>[4]</sup> (non-inflammatory behavior: either stricturing or penetrating disease). Perianal disease and behavior change during follow-up were also registered. Medical therapy was registered in detail [e.g. steroid and/or immunosuppressive/biological therapy use, AZA intolerance as defined by the ECCO (European Crohn's and Colitis Organisation) Consensus Report<sup>[23]</sup>], need for surgery/reoperation (resections) in CD, and time-point of surgery/reoperation and smoking habits, were investigated by reviewing the medical charts and completing a questionnaire. Only patients with a confirmed diagnosis for more than 1 year were enrolled.

Definitions of AZA/biological therapy use and smoking: patients were regarded as AZA users if they took a dose of  $\geq 1.5$  mg/kg body weight for at least 6 mo. Early use was considered if the use of immuno-

**Table 1 Clinical characteristics of patients with Crohn's disease n (%)**

	<b>CD (n = 340)</b>
Male/female	155/185
Age (yr)	38.1 ± 13.2
Age at presentation (yr)	28.7 ± 12.4
Duration (yr)	9.4 ± 7.5
Familial IBD	39 (11.4)
Location	
L1	75
L2	99
L3	161
All L4	22
L4 only	5
Behavior at diagnosis	
B1	198
B2	65
B3	77
Behavior change from B1 to B2/B3	61 (30.8)
Perianal disease	117 (34.4)
Frequent relapse	126 (37.1)
Arthritis	127 (37.5)
PSC	10 (2.9)
Ocular	17 (5.0)
Cutaneous	43 (12.6)
Steroid use	264 (77.6)
Azathioprine use	216 (63.5)
Azathioprine intolerance	36 (14.3)
Biological use	71 (20.9)
Surgery/reoperation	158 (46.5)/52 (32.8)
Smoking habits	
No	151
Ex	36
Yes	153

L1: Ileal; L2: Colonic; L3: Ileocolonic; L4: Upper gastrointestinal disease; B1: Inflammatory; B2: Stenosing; B3: Penetrating disease behavior. IBD: Inflammatory bowel disease.

modulatory therapy preceded the behavior change by at least 6 mo. According to the Center's policy, if AZA was started, its use was not halted even in patients with long-term clinical remission. The rate of AZA intolerance was 14.3% (including 36 additional patients), and these patients were classified as AZA-non-users. Common intolerance reactions were leukopenia, abdominal pain, and in three patients, pancreatitis. Biological therapy use was considered if the patient received at least a full, anti-tumor necrosis factor induction therapy at an appropriate dose. The definition of smoking consisted of smoking ≥ 7 cigarettes/wk for at least 6 mo<sup>[15-18]</sup> at the time of diagnosis and/or during follow-up, within 1 year of diagnosis or behavior change. Patients were interviewed on their smoking habits at the time of diagnosis and during the regular follow-up visits. Moreover, due to Hungarian health authority regulations, a follow-up visit is obligatory for IBD patients at a specialized gastroenterology centre every 6 mo. Otherwise, the conditions of the health insurance policy change and they forfeit their ongoing subsidized therapy. Consequently, the relationship between the IBD patients and their specialists is a close one. The referred patients were retrospectively interviewed about their smoking habits at the time of referral and thereafter. Smoking cessation was defined as complete abstinence of at least 1 year's duration. Only 16 (4.7%) CD patients

stopped smoking during the course of the disease, while 2 additional CD patients started smoking after the diagnosis. In patients with change in disease behavior, all CD patients stopped smoking following the change. Since macroscopic lesions on the ileal side of the anastomosis observed 1 year following surgery were not different between smokers and non-smokers and there was no significant difference reported between ex-smokers and non-smokers in reoperation rates in a recent meta-analysis<sup>[15,24]</sup>, ex-smokers at the time of diagnosis were included in the non-smoker group.

Detailed clinical phenotypes were determined by thoroughly reviewing the patients' medical charts, which had been collected in a uniform format. The central coordination of sample and database management was completed at the 1st Department of Medicine, Semmelweis University (by PLL, TS). Data capture was prospective except for referred patients in whom disease course until referral was registered at the date of the referral and prospectively thereafter. Data analysis was done retrospectively. The study was approved by the Semmelweis University Regional and Institutional Committee of Science and Research Ethics.

### Statistical analysis

Variables were tested for normality using Shapiro Wilk's W test. The *t*-test with separate variance estimates, ANOVA with *post hoc* Scheffe test,  $\chi^2$ -test, and  $\chi^2$ -test with Yates correction were used to evaluate differences within subgroups of IBD patients. Kaplan-Meier survival curves were plotted for analysis with the LogRank and Breslow tests. Additionally, forward stepwise Cox regression analysis was used to assess the association between categorical clinical variables and surgical requirements.  $P < 0.05$  was considered as significant. For the statistical analysis, SPSS 15.0 (SPSS Inc, Chicago, IL) was used with the assistance of a statistician (Dr. Peter Vargha).

## RESULTS

### Association between clinical markers and disease behavior change in CD patients

In a univariate analysis, behavior change from B1 to B2/B3 during follow-up was associated with disease duration, location, presence of perianal disease, smoking at diagnosis, frequency of relapses, steroid use, early AZA use, AZA/biological therapy use and need for resective surgery (Table 2). Although ocular manifestations were also associated with behavior change (3.6% vs 11.5%,  $P = 0.033$ ), this became non-significant after Bonferroni correction. Patients with a change in disease behavior had significantly longer disease duration ( $12.3 \pm 7.6$  years vs  $7.4 \pm 6.5$  years,  $P < 0.001$ ).

In a logistic regression model, disease duration, presence of perianal disease, smoking, steroid use, and early AZA use prior to behavior change were independent predictors for change in disease behavior (Table 3). If early AZA use was changed to early AZA and/or biological therapy use (Coefficient: -1.221,  $P = 0.002$ , OR: 0.29, 95% CI: 1.34-0.64) in the same logistic regression model, the associations remained unchanged.

**Table 2** Clinical factors associated with behavior change from inflammatory to complicated disease behavior *n* (%)

Factor	Prevalence without behavior change	Prevalence with behavior change	P-value	OR	95% CI
Disease location					
L1	19 (13.9)	17 (27.9)			
L2	55 (40.1)	18 (29.5)	0.04	-	-
L3	63 (46)	25 (41)			
L4	0	1 (1.6)			
Perianal disease	30 (22.2)	30 (49.2)	< 0.001	3.4	1.78-6.46
Frequent relapses	18 (13.1)	19 (31.1)	0.003	3.0	1.43-6.23
Disease duration (> 10 yr)	28 (20.4)	35 (57.4)	< 0.001	5.3	2.7-10.1
Smoking	51 (37.2)	32 (52.5)	0.04	1.9	1.02-3.45
Steroid use	106 (77.4)	59 (96.7)	0.001	8.6	2.00-37.3
Early azathioprine use	79 (57.7)	24 (39.3)	0.017	0.48	0.26-0.88
Early azathioprine/biological use	83 (60.6)	25 (41)	0.01	0.45	0.24-0.84
Need for operation	19 (13.9)	37 (60.7)	< 0.001	9.6	4.72-19.4

**Table 3** Logistic regression: predictive factors for behavior change from non-stricturing, non-penetrating to complicated disease behavior in Crohn's disease

Factor	Coefficient	P-value	OR	95% CI
Disease location	-0.385	0.13	-	-
Longer disease duration ( $\leq 10$ yr vs > 10 yr)	1.476	< 0.001	4.37	2.04-9.38
Perianal disease	1.351	0.001	3.86	1.72-8.67
Frequent relapses	0.388	0.404	-	-
Smoking	1.015	0.009	2.76	1.29-5.89
Steroid use	2.089	0.01	8.07	1.64-39.7
Early azathioprine use	-1.055	0.006	0.35	0.16-0.74

The coefficient is equivalent to the natural log of the OR.

### Association between clinical markers and time to disease behavior change in CD patients

Disease location, perianal disease, early AZA or AZA/biological therapy, steroid use (LogRank  $P = 0.004$  and Breslow  $P = 0.005$ ) and smoking were significant determinants for time to behavior change surgery in a Kaplan-Meier analysis using LogRank and Breslow tests (Figure 1).

To further evaluate the effect of the above variables on the probability of behavior change, we performed a forward stepwise proportional Cox regression analysis. Each of the above variables was independently associated with the probability of disease behavior change (Table 4). The result was the same if early AZA/biological therapy use ( $P = 0.002$ , HR: 0.43, 95% CI: 0.25-0.73) was incorporated in the same analysis.

## DISCUSSION

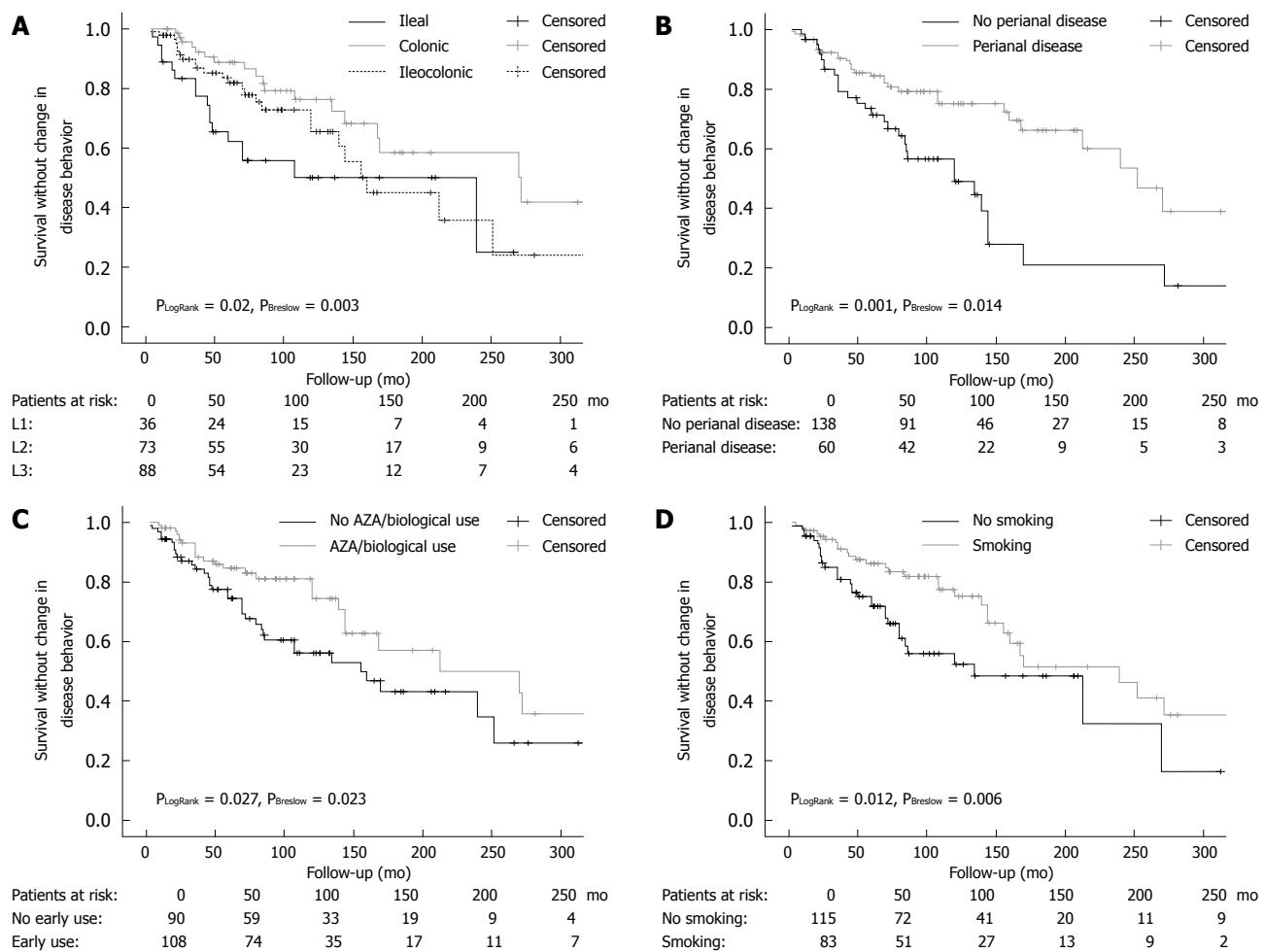
In the present study, we assessed, for the first time, the combined effect of disease phenotype and medical therapy on the probability of disease behavior change in a well-characterized CD cohort with a strict clinical follow-up. This study has shown that perianal disease, disease location, prior steroid, early AZA or AZA/biological therapy were significant independent predictors of disease behavior change during follow-up in patients with CD, in a stepwise proportional Cox regression and Kaplan-Meier analysis.

**Table 4** Summary of Cox regression model: factors affecting time to disease behavior change

	P-value	Hazard ratio	95% CI
Disease location	0.001		
L1	0.023	2.13	1.11-4.08
L2	0.05	0.54	0.27-1.001
L3		Reference	
Perianal disease			
Yes	< 0.001	3.26	1.90-5.59
No		Reference	
Steroid use			
Yes	0.006	7.48	1.79-31.2
No		Reference	
Early azathioprine use			
Yes	0.005	0.46	0.27-0.79
No		Reference	
Smoking status			
Yes	0.032	1.79	1.05-3.05
No		Reference	

In accordance with the findings by Tarrant *et al*<sup>[3]</sup>, the presence of perianal disease was associated with a 3-4-fold increased risk of developing complicated disease behavior in patients with non-stricturing, non-penetrating disease at the time of diagnosis. Interestingly, in both studies, approximately 50%-55% of patients with perianal disease and only 15%-20% of patients without perianal disease developed a complicated disease behavior after 10 years of follow-up. In the present study, however, only 60% of the patients presented with B1 behavior, representing the more severe disease population in referral centers. In contrast, the Australian study reports the results of a population-based cohort follow-up. Therefore, our results are not only confirmatory, but enable us to extrapolate on the finding that perianal disease is a poor prognostic factor in referral center IBD populations.

In addition, similar to the Australian study, the present study found that progression to complicated disease was more rapid in those with small bowel than colonic disease location. The HR was significantly lower (0.54,  $P = 0.05$ ) for colonic disease and significantly higher for ileal disease (2.13,  $P = 0.023$ ) compared to an ileocolonic location in the Cox regression analysis. The same conclusions were reached when data were analyzed



**Figure 1 Association between disease location (A), perianal disease (B), early azathioprine/biological therapy use (C) or smoking (D) and disease behavior change from non-stricturing, non-penetrating to complicated disease behavior in Crohn's disease.**

by the Kaplan-Meier method using the LogRank and Breslow tests for comparison. Of note, while the L1 and L3 curves ran parallel after 150 mo, the relatively small number of patients in the L1 group may have introduced a bias in the analysis. These results were only partially confirmed by Aldhous *et al*<sup>[18]</sup>. In 275 CD patients, colonic disease was negatively associated with the time required for development of stricturing complications ( $P < 0.001$ ), while any upper gastrointestinal disease (and tendentially ileocolonic disease,  $P = 0.066$ ) was the only factor significantly increasing the risk of development of fistulizing complications. Finally, small bowel involvement, stricturing disease, and a young age at diagnosis were associated with disease recurrence in another Dutch population-based study<sup>[7]</sup>.

In the present study, we could not confirm frequency of relapses as an independent prognostic factor for disease behavior change. This is somewhat in contrast with the findings by Munkholm *et al*<sup>[25]</sup>, where the relapse rate within a year of diagnosis and in the following 2 years, was positively correlated ( $P = 0.00001$ ) with the relapse rate in the following 5 years in patients with CD.

The findings were slightly different if the authors assessed the clinical factors associated with the development of irreversible structural damage. After a multivariate analysis, only stricturing behaviour at diagnosis (HR:

2.11,  $P = 0.0004$ ) and weight loss ( $> 5$  kg) at diagnosis (HR: 1.67,  $P = 0.0089$ ) were independently associated with time to the development of severe disease in the study by Loly *et al*<sup>[8]</sup>. The definition of severe, non-reversible damage was, however, much more rigorous. It was defined by the presence of at least one of the following criteria: the development of complex perianal disease, any colonic resection, two or more small-bowel resections (or a single small-bowel resection measuring more than 50 cm in length) or the construction of a definite stoma. Nonetheless, medical therapy was not included in either of the previous studies.

Similarly, although the effect of smoking was extensively investigated in IBD, most studies failed to investigate the complex associations between smoking, disease phenotype, and medical therapy. A recent review<sup>[10]</sup> and previous data have demonstrated that current smoking was more frequently associated with complicated disease, penetrating intestinal complications<sup>[9,10]</sup>, and greater likelihood to progress to complicated disease, as defined by the development of strictures or fistulae<sup>[11]</sup>. In accordance, in the present study, smoking at the time of diagnosis was independently associated with time to behavior change from a non-stricturing, non-penetrating phenotype to complicated disease behavior in CD in a Cox regression model.

However, this was not a universal finding. In a recent paper by Aldhous *et al*<sup>[18]</sup>, using the Montreal classification, the harmful effect of smoking was only partially confirmed. Although current smoking was associated with less colonic disease, the smoking habits at diagnosis were not associated with time to development of strictureting disease, internal penetrating disease, perianal penetrating disease, or time to first surgery. In contrast, disease location was associated with the need for surgery.

A more solid end-point was also deleteriously affected by smoking in the present study; the need for intestinal resection but not reoperation was also increased in smokers (HR: 3.19,  $P < 0.001$ ) not treated with immunosuppressive therapy, especially in females, in accordance with data from Cosnes *et al*<sup>[26]</sup>. Much emphasis was also placed, by some authors, on investigating the association between the amount of smoking and the above variables in both CD and ulcerative colitis. In a recent publication, the authors<sup>[18]</sup> did not find a significant association between pack-years of smoking and disease behavior or need for surgery. In addition, in a recent French publication<sup>[27]</sup>, light smokers had higher resection rates compared to non-smokers in CD, suggesting that complete smoking cessation should be advised for all smokers with CD.

The key to explaining these conflicting results lies partly in the study by Cosnes *et al*<sup>[17]</sup>, where the authors have demonstrated that immunosuppressive therapy neutralizes the effect of smoking on the need for surgery. Therefore, we aimed to analyze the effect of smoking in a more complex setting. After obtaining the results of the univariate and Kaplan-Meier analyses, we performed both a logistic regression analysis adjusted to disease duration as an independent variable and a step-wise proportional Cox regression analysis to investigate the relative weight of the risk factors. In this analysis, perianal disease, smoking, steroid use, and AZA or AZA/biological therapy use before the behavior change were independently associated with time to disease behavior change. However, a partial recall bias, especially in the referral patients, where smoking habits were analyzed in a partially retrospective manner, cannot be excluded.

Finally, the initial requirement for steroid use (OR: 3.1, 95% CI: 2.2-4.4), an age below 40 years at diagnosis (OR: 2.1, 95% CI: 1.3-3.6), and the presence of perianal disease (OR: 1.8, 95% CI: 1.2-2.8) were associated with the development of disabling disease in the study by Beaugerie *et al*<sup>[21]</sup>. The positive predictive values of disabling disease in patients with two and three predictive factors for disabling disease were 0.91 and 0.93, respectively. Nonetheless, the prevalence of disabling disease was approximately 80.5% at 5 years in the entire patient group, which makes these criteria less valuable in clinical practice. Moreover, the authors classified the need for immunosuppressive therapy as one potential disabling factor, which in light of the present study, is rather controversial. In the present study, prior steroid use was an independent predictor of time to change in disease behavior. However, again, because of the high prevalence of overall steroid use, the confidence interval is wide and, consequently, the clinical usefulness of this marker is relatively low.

In adults, early postoperative use of AZA at a dose

of 2-2.5 mg/kg per day seemed to delay postoperative recurrence in comparison to a historical series or placebo groups in randomized, controlled trials<sup>[19]</sup>. Furthermore, in a recent withdrawal study by the GETAID group<sup>[20]</sup>, the authors provided evidence for the benefit behind long-term AZA therapy beyond 5 years in patients with prolonged clinical remission. The most convincing data to support the benefit from early use of AZA, however, comes from the pediatric literature<sup>[28]</sup>, where in a randomized, controlled trial in 55 children, early 6-mercaptopurine use was associated with a significantly lower relapse rate (only 9%) compared with 47% of controls ( $P = 0.007$ ). Moreover, the duration of steroid use was shorter ( $P < 0.001$ ) and the cumulative steroid dose lower at 6, 12 and 18 mo ( $P < 0.01$ ). More recently, also in a pediatric setting, this strategy was found to be associated with a lower hospitalization rate<sup>[29]</sup>. Similarly, in the present study, early AZA or AZA/biological therapy was an independent preventive factor associated with decreased probability of developing complicated disease behavior during the course of the disease (HR: 0.46 and 0.43).

In conclusion, in the present study, we have shown that the complex analysis of disease phenotype, medication history, and smoking habits is needed, in order to study the factors associated with change in disease behavior in patients with IBD. Our data suggest that perianal disease, current smoking, prior steroid use, early AZA or AZA/biological therapy are predictors of disease behavior change in patients with CD.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Using the Vienna classification system, it has been shown in clinic-based cohorts that there can be a significant change in inflammatory bowel disease (IBD) disease behavior over time, whereas disease location remains relatively stable. Early age at diagnosis, disease location, perianal disease and, in some studies, smoking were associated with the presence of complicated disease and surgery in previous studies.

### Research frontiers

The combined effect of markers of disease phenotype (e.g. age, gender, location, perianal disease) and medical therapy (steroid use, early immunosuppression) on the probability of disease behavior change have not, however, been studied in detail thus far in the published literature.

### Innovations and breakthroughs

In the present study, the authors have shown in a well-characterized Crohn's disease (CD) cohort with strict clinical follow-up, that the complex analysis of disease phenotype, medication history, and smoking habits is needed in order to study the factors associated with change in disease behavior in patients with IBD. Their data suggest that perianal disease, current smoking, prior steroid use, early azathioprine (AZA) or AZA/biological therapy are predictors of disease behavior change in patients with CD.

### Applications

New data with easily applicable clinical information may assist clinicians in everyday, practical decision-making, when choosing a treatment strategy for their CD patients.

## Terminology

Vienna-Montreal classification: classification systems of CD disease phenotypes. The Vienna classification assesses the age at presentation, disease location and disease behavior. In the Montreal classification, the broad categories for CD classification remain the same; however, changes were made within each category. First, a new category was introduced for those aged 16 years or younger at the time of diagnosis, to separate pediatric from adult-onset IBD. Second, upper gastrointestinal disease and perianal disease became disease modifiers, which may coexist with any disease behavior or location.

## Peer review

The present article provides valuable information regarding clinical prognostic factors of phenotype changes of CD over time. The study was performed in a large cohort of patients.

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# Barrett's esophagus: Prevalence and risk factors in patients with chronic GERD in Upper Egypt

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was detected in eight cases (0.8%), six of them in BE patients. There was no significant difference between patients with BE and GERD regarding sex, smoking, alcohol consumption or symptoms of GERD. Patients with BE had significantly longer esophageal acid exposure time in the supine position, measured by pH monitoring.

**CONCLUSION:** The prevalence of BE in patients with GERD who were referred for endoscopy was 7.3%. BE seems to be associated with older age and more in patients with nocturnal gastroesophageal reflux.

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**Key words:** Barrett's esophagus; Gastrointestinal; Endoscopy; Gastroesophageal reflux; Risk factors

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## Abstract

**AIM:** To determine the prevalence and possible risk factors of Barrett's esophagus (BE) in patients with chronic gastroesophageal reflux disease (GERD) in El Minya and Assuit, Upper Egypt.

**METHODS:** One thousand consecutive patients with chronic GERD symptoms were included in the study over 2 years. They were subjected to history taking including a questionnaire for GERD symptoms, clinical examination and upper digestive tract endoscopy. Endoscopic signs suggestive of columnar-lined esophagus (CLE) were defined as mucosal tongues or an upward shift of the squamocolumnar junction. BE was diagnosed by pathological examination when specialized intestinal metaplasia was detected histologically in suspected CLE. pH was monitored in 40 patients.

**RESULTS:** BE was present in 7.3% of patients with chronic GERD symptoms, with a mean age of  $48.3 \pm 8.2$  years, which was significantly higher than patients with GERD without BE ( $37.4 \pm 13.6$  years). Adenocarcinoma

## INTRODUCTION

Barrett's esophagus (BE) was first identified by N.R. Barrett in 1950, who described the replacement of the normal squamous mucosa of the distal esophagus by a columnar epithelium of both gastric and intestinal types<sup>[1,2]</sup>. The definition of BE has been modified over subsequent years to include only intestinal metaplasia within the tubular esophagus.

The exact cause of BE remains unclear. A popular explanation for the occurrence of BE is that it results from mucosal damage caused by gastroesophageal reflux<sup>[3,4]</sup>. When visible upon upper gastrointestinal endoscopy, this mucosal damage is termed erosive esophagitis. However, not all patients with gastroesophageal reflux and erosive

esophagitis go on to develop BE, and not all patients with BE have a history of gastroesophageal reflux<sup>[5]</sup>. In most patients with reflux esophagitis, the epithelium heals through regeneration of the normal squamous lining<sup>[6]</sup>. Other patients, however, will develop BE with the risk of ultimately progressing to esophageal adenocarcinoma (EAC)<sup>[7]</sup>.

The prevalence of BE varies in different geographic areas worldwide. Multiple risk factors for the development of BE besides reflux have been studied, including being Caucasian and/or male, a history of smoking, and hiatus hernia<sup>[8]</sup>.

The aim of this study was to determine the prevalence and possible risk factors of BE in patients with chronic gastroesophageal reflux disease (GERD) symptoms in Upper Egypt.

## MATERIALS AND METHODS

### Ethics

This study was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. All patients provided informed written consent.

### Location

The study was performed at two clinical centers in Upper Egypt (Southern part of Egypt): the endoscopy units of the Department of Tropical Medicine at El Minya University, and the Department of Internal Medicine at Assuit University, from January 2006 to January 2008. Both centers are referral centers for a large number of patients in Upper Egypt.

### Patients

In a prospective manner, 1000 consecutive patients with chronic GERD symptoms were recruited. All patients had a history of longstanding heartburn and or regurgitation for at least three times weekly for the last year. A questionnaire was completed by every patient, including age, sex, occupation, smoking and alcohol consumption. The symptom questionnaire also included the following criteria: primary referral symptom; frequency of GERD symptoms such as heartburn, regurgitation, and acid taste; extra esophageal symptoms; and history of systemic diseases such as scleroderma and diabetes.

### Endoscopic examination

Endoscopic examination was performed using an Olympus Evis CLV-U 200 Videoscope (Olympus, Japan). All patients were examined in our units by a well-trained endoscopist. BE was diagnosed by the presence of columnar-lined esophagus at endoscopy and the confirmed presence of intestinal metaplasia upon biopsy. In addition, information on the presence of intestinal metaplasia, evidence of dysplasia (a premalignant condition characterized by increased cell growth, cellular atypia, and altered cell differentiation) and its severity,

and the presence of coexistent EAC was obtained from histopathology records. We defined short-segment BE by the presence of less than 3 cm of columnar-lined esophagus at endoscopy. The distinction between long- and short-segment BE was made. We also recorded the presence of esophagitis or any esophageal lesions. Repeat endoscopy was done in patients with erosive esophagitis after complete healing, to confirm the presence of BE. Then the patients were classified into two groups according to presence or absence of BE.

### Histopathological examination

Fresh endoscopic biopsy samples were obtained from the operating theatre and fixed in 10% formalin within 13 h at room temperature. Tissues were subjected to a series of processing steps, which included fixation, dehydration with ethanol, clearing with xylene, and wax impregnation with paraffin, and then stained with hematoxylin and eosin (HE).

Ambulatory pH monitoring was done for 40 patients (20 patients with BE and 20 without). All patients were studied with ambulatory pH monitoring using an antimony pH electrode placed 5 cm above the proximal border of the manometrically located lower esophageal sphincter, and another electrode placed 10 cm above this point in the proximal esophagus, connected to a portable Digitrapper (Synectics, San Antonio, TX, USA) data storage unit. Intraesophageal pH was recorded continuously, with sampling obtained every 4 s. All pH tracings were analyzed for the percentage time that the distal and proximal esophageal pH was < 4, determined for the upright and recumbent time periods in each study. Average esophageal acid clearance (EAC) time was calculated for each patient by dividing the total time (in minutes) that distal esophageal pH remained < 4 by the total number of GERD episodes. This was calculated for both the upright and supine periods.

### Statistical analysis

Statistical analyses were performed using Stats Direct version 2.2.5 statistical software (Stats Direct Ltd., Sale, Cheshire, UK) and SPSS for Windows version 11.5 (SPSS, Inc., Chicago, IL, USA). All data are presented as means  $\pm$  SD.  $\chi^2$  and Fisher's tests were used to compare BE and GERD groups according to sex, smoking and alcohol consumption. Student's *t* test was used for some factors. ANOVA was used for pH measurement analysis. Significance was accepted at  $P \leq 0.05$ .

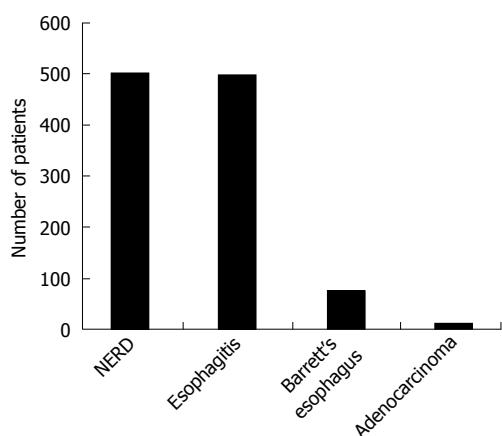
## RESULTS

This study included 1000 patients with chronic GERD symptoms (764 male and 236 female) with a mean age of  $38.81 \pm 14.52$  years. Seventy-three of these patients (7.3%) had BE suspected endoscopically and detected histopathologically. The remaining 927 patients (92.7%) were negative for BE. Accordingly, we classified the patients into two groups: group A included patients with

**Table 1** Age, sex, symptoms and endoscopic findings *n* (%)

	<b>Group A</b>	<b>Group B</b>	<b>P value</b>
Age (yr, mean $\pm$ SD)	48.3 $\pm$ 8.2	37.6 $\pm$ 13.4	< 0.05
Sex			NS
Male	68 (93)	785 (85)	
Female	5 (7)	142 (15)	
Total	73	927	
Smoking (508 patients)			
Smokers	45 (61.6)	463 (49.9)	NS
Non-smokers	28 (38.4)	464 (50.1)	
Alcohol consumption (60 patients)			
Alcoholic	6 (8)	54 (6)	NS
Non-alcoholic	67 (92)	871 (94)	
Main symptoms			
Heartburn	71 (97)	908 (98.1)	NS
Regurgitation	61 (83.6)	769 (82.9)	NS
Dyspepsia	58 (80)	742 (80.1)	NS
Epigastric pain	58 (80)	797 (86.2)	NS
Dysphagia	23 (31.5)	222 (24)	NS
Endoscopic findings			
Hiatus hernia	29 (39.7)	389 (42.1)	0.8 (NS)
Gastritis	53 (72.7)	667 (71.6)	0.8 (NS)
Duodenitis	21 (28.7)	297 (31.8)	0.7 (NS)

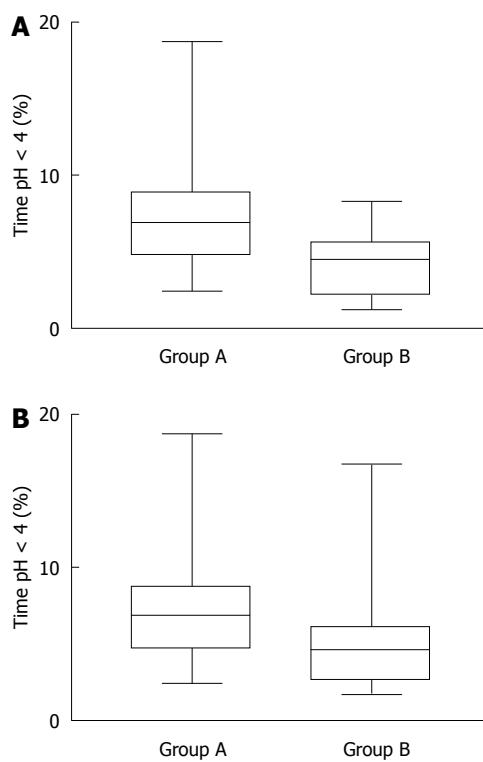
NS: Not significant;  $\chi^2$  and Student's *t* test used for statistical analysis. The patients with BE (group A) were significantly older than those with GERD (group B) ( $P < 0.05$ ).



**Figure 1** Endoscopic findings. Esophagitis was detected in 498 patients (49.8%) and NERD was seen in 502 patients. BE was present in 73 patients. EAC was detected in eight patients.

BE and group B included those with chronic GERD without BE. The mean length of BE was  $5.3 \pm 2.6$  cm. Short-segment BE was present in 61 patients (84%), while long-segment BE was present in 12 (16%). Four cases with BE were detected in endoscopy-negative patients, while the remainder was detected in patients with esophagitis. EAC was detected in eight patients (six in group A and two in group B).

Regarding age and sex, the mean age of patients in group A ( $48.3 \pm 8.2$  years) was significantly older than that in group B ( $37.6 \pm 13.4$  years) ( $P < 0.05$ ), and there was no significant difference regarding sex between the groups (Table 1). Although smoking and alcohol consumption were more frequent in the BE group, there was no significant difference from those without BE.



**Figure 2** Distal esophageal acid exposure in both groups. A: In the supine position. The median time at pH < 4 in the distal esophagus in the supine position was significantly longer in patients with BE (group A) than in those with GERD without BE (group B) ( $P < 0.03$ ); B: In the upright position. The median time at pH < 4 in the distal esophagus in the upright position did not differ significantly between patients with BE (group A) and those with GERD without BE (group B).

Also, there was no significant difference detected between the groups regarding GERD symptoms (Table 1).

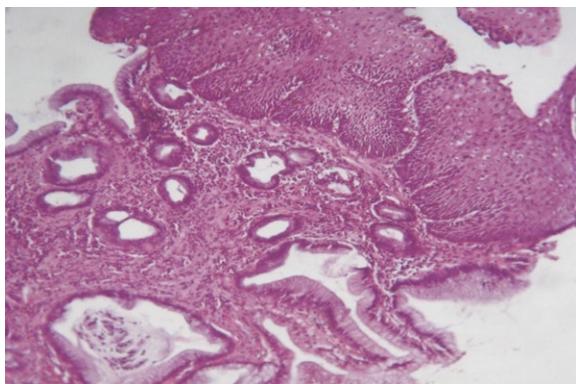
Endoscopic examination detected esophagitis in 498 patients (49.8%) and non-erosive reflux disease (NERD) was seen in 502 patients (Figure 1). Only four cases with BE were seen among patients with NERD.

The percentage of patients with abnormal esophageal acid exposure was higher in the supine position in group A (16 patients, 80%) than in group B (nine patients, 45%). The median time at pH < 4 in the distal esophagus in the supine position was significantly longer in group A than group B, while no significant difference was detected in the upright position (daytime) between the groups (Figure 2A and B).

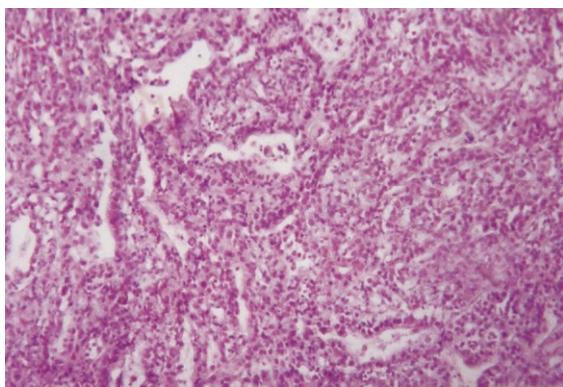
The histopathology of BE and EAC is shown in Figures 3 and 4.

## DISCUSSION

To the best of our knowledge, this is the first study reporting the prevalence of BE in patients with chronic GERD symptoms in Upper Egypt. We recruited patients with GERD symptoms. We could not perform the study on the general population as it is difficult to convince asymptomatic people to undergo endoscopic procedures. One thousand consecutive patients were referred to endoscopy units and evaluated for chronic GERD



**Figure 3** BE without dysplasia, showing true barrel-shaped goblet cells and intervening columnar cells, with incomplete brush-border, intestinal type metaplasia. The squamous epithelium of the normal esophagus was transformed into columnar epithelium (HE,  $\times 200$ ).



**Figure 4** Well-differentiated adenocarcinoma showing gland formation, and low levels of nuclear pleomorphism and atypia (HE,  $\times 200$ ).

symptoms by a well-trained endoscopist in a prospective manner.

The prevalence of BE was 7.3% in patients with GERD symptoms. Taking into considerations the type of subjects included, the prevalence could have been much lower in the general population because of the known association of BE and GERD. In Northern Egypt, Hak *et al*<sup>[9]</sup> have found a prevalence rate of 9.9% of BE in patients with GERD, which agrees with the results of our study in the Southern part of Egypt (Upper Egypt). Hak *et al*<sup>[9]</sup> recruited symptomatic patients with GERD, but with an emphasis on the effect of acid and bile reflux on the esophageal mucosa.

The prevalence of BE varies around the world and it seems to be higher in western than eastern countries. Focusing on patients who presented for their initial endoscopy in the setting of suspected GERD, Westhoff *et al*<sup>[8]</sup> studied 378 consecutive patients who had biopsies taken from areas suspicious for BE. The overall prevalence of BE was found to be 13.2%. The majority of patients diagnosed had short-segment BE, which agreed with previous data that showed the prevalence of endoscopically recognizable short-segment BE at 5%-7% vs 1%-3.4% for long-segment BE<sup>[9,10]</sup>.

Ronkainen and colleagues have used a population-based study to estimate the prevalence of BE in Sweden. Of

19 000 subjects within a target age range of 20-80 years, a random sample of 3000 was surveyed by questionnaire. A random sub-sample of 1000 subjects then underwent upper digestive system endoscopy, in which an overall BE prevalence of 1.6% was observed. However, when reflux symptoms were present, the prevalence rose to 2.3%<sup>[11]</sup>. In another study in Korea, Kim *et al*<sup>[12]</sup> have found that, in the general population, the prevalence of BE was < 1%, and remained less common in Korea than in western countries.

In our study, the prevalence of BE did not differ significantly between men and women, although the number of men recruited was much higher than women. Probably, men have more reflux symptoms or seek medical advice and endoscopic evaluation more than women do. Lin *et al*<sup>[13]</sup> have studied 543 patients with GERD symptoms, and have shown that while male and female patients demonstrated an equal severity of erosive esophagitis, only 14% of female patients had BE, compared to 23% of male patients ( $P < 0.05$ ). However, Banki *et al*<sup>[14]</sup> have shown that there was an equal prevalence of BE in men and women diagnosed with severe reflux by 24-h pH monitoring. A chart review of almost 22 000 first endoscopies identified 492 patients with BE, and suggested that there was a 20-year age shift between men and women in prevalence patterns, which resulted in a male to female OR of 4.15 (95% CI: 2.99-5.77)<sup>[15]</sup>.

In our study, the mean age in patients with BE was significantly older than in those without BE. Other studies have demonstrated that increased age is a risk factor for developing BE, as well EAC<sup>[16,17]</sup>.

We found no significant difference between the groups regarding smoking and alcohol consumption, but it seemed that the number of smokers was high in both groups. While Ronkainen *et al*<sup>[11]</sup> and Kim *et al*<sup>[12]</sup> have found that alcohol consumption and smoking are significant risk factors, others have shown no significant importance of alcohol consumption and smoking in patients with BE<sup>[18-20]</sup>.

Trying to explore the pattern of acid reflux in patients with BE, we found a significant difference between patients with BE and those with GERD for night-time acid reflux, which was more evident in patients with BE. The nocturnal gastroesophageal reflux that occurs in the recumbent position causes more injury to esophageal mucosa and may contribute to more severe chronic esophageal mucosal changes. Hak *et al*<sup>[9]</sup> have found more prolonged reflux periods in patients with BE than in those with GERD or NERD without BE. Gutschow *et al*<sup>[21]</sup> have reported that patients with BE have significantly more acid reflux events and a higher percentage of reflux time during the supine and upright phase than patients with NERD and GERD without BE. Also, Koek *et al*<sup>[22]</sup> in a multivariate analysis have found that BE is associated with male sex and exposure to both acid and duodenogastroesophageal reflux.

We conclude that BE is present in about 7.3% of patients with chronic GERD symptoms in our area.

It may be associated with older age and nocturnal gastroesophageal reflux.

## COMMENTS

### Background

Barrett's esophagus (BE) is gaining importance because of its association with esophageal adenocarcinoma (EAC). Its prevalence varies around the world. However, there are no data about its prevalence in upper Egypt.

### Innovations and breakthroughs

This is the first study to report prevalence of BE in Upper Egypt.

### Applications

This study may represent a future strategy for screening for BE and AEC in patients with chronic gastroesophageal reflux disease.

### Peer review

This was a well-conducted and interesting study that investigated the prevalence of BE in Upper Egypt.

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BRIEF ARTICLES

## Bone mineral density and disorders of mineral metabolism in chronic liver disease

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### Abstract

**AIM:** To estimate the prevalence and identify the risk factors for metabolic bone disease in patients with cirrhosis.

**METHODS:** The study was performed on 72 Indian patients with cirrhosis (63 male, 9 female; aged < 50 years). Etiology of cirrhosis was alcoholism ( $n = 37$ ), hepatitis B ( $n = 25$ ) and hepatitis C ( $n = 10$ ). Twenty-three patients belonged to Child class A, while 39 were in class B and 10 in class C. Secondary causes for metabolic bone disease and osteoporosis were ruled out. Sunlight exposure, physical activity and dietary constituents were calculated. Complete metabolic profiles were derived, and bone mineral density (BMD) was measured using dual energy X ray absorptiometry. Low BMD was defined as a Z score below -2.

**RESULTS:** Low BMD was found in 68% of patients. Lumbar spine was the most frequently and severely

affected site. Risk factors for low BMD included low physical activity, decreased sunlight exposure, and low lean body mass. Calcium intake was adequate, with unfavorable calcium: protein ratio and calcium: phosphorus ratio. Vitamin D deficiency was highly prevalent (92%). There was a high incidence of hypogonadism (41%). Serum estradiol level was elevated significantly in patients with normal BMD. Insulin-like growth factor (IGF) 1 and IGF binding protein 3 levels were below the age-related normal range in both groups. IGF-1 was significantly lower in patients with low BMD. Serum osteocalcin level was low (68%) and urinary deoxypyridinoline to creatinine ratio was high (79%), which demonstrated low bone formation with high resorption.

**CONCLUSION:** Patients with cirrhosis have low BMD. Contributory factors are reduced physical activity, low lean body mass, vitamin D deficiency and hypogonadism and low IGF-1 level.

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**Key words:** Bone mineral density; Liver disease; Chronic disease; Cirrhosis; Bone mineral metabolism; Hepatic osteodystrophy

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### INTRODUCTION

Metabolic bone disease is a common complication of long-standing liver disease, ranging from cholestatic disorders to alcoholic, autoimmune and post-viral cirrhosis<sup>[1]</sup>. Often known as hepatic osteodystrophy (HO),

it is well-recognized among individuals with chronic liver disease (CLD). Its etiology is poorly understood and is thought to vary according to the type, severity and progression of the liver disease, along with a multitude of other contributing factors including the ethnicity of the population studied. It can result in spontaneous low-trauma fractures that significantly impact on the morbidity, quality of life, and even survival, through pain, deformity and immobility. With liver transplantation steadily taking the center stage in treatment of end-stage cirrhosis of varying etiology and offering long-term survival, bone disease has snowballed into one of the major determinants of survival and quality of life in this cohort<sup>[1]</sup>.

Keeping in view the numerous therapeutic options for bone disease<sup>[2]</sup> already available and those under development, it is prudent to characterize this condition in order to give these patients a better chance of survival. The medical fraternity around the world has recognized this and has started characterizing the disorder. In various international studies, the overall incidence has varied from 11% to 48%<sup>[3]</sup>, with a fracture rate of 3%-44%<sup>[3]</sup>. This has not been studied extensively in the Indian population<sup>[4]</sup>.

## MATERIALS AND METHODS

### **Patients**

The study was performed on 72 Indian patients with cirrhosis. The group consisted of 63 men and nine women with a median age of 45 years ( $43.1 \pm 7.4$ , range 22-50 years). Twenty-five patients had hepatitis B (22 men and 3 women), 10 had hepatitis C (5 men and 5 women), and 37 had alcoholic cirrhosis (36 men and 1 woman). A diagnosis of cirrhosis was confirmed histologically or clinically if biopsy was not available. A clinical diagnosis was established in patients who demonstrated a Child-Pugh index  $> 6$  or ultrasound findings suggestive of cirrhosis (the presence of at least two of the findings of nodular irregular surface, distorted vascular pattern, or ascites). Signs of portal hypertension (endoscopically proven esophageal varices or dilated portal venous system with ultrasonography) were taken as additional corroborative evidence. The etiology of post-viral cirrhosis was proven if any of the serological markers were positive [hepatitis B surface antigen by ELISA, anti-hepatitis C virus (HCV) by third generation ELISA, or HCV RNA]. Diagnosis of alcoholic cirrhosis was made with a positive answer to more than one question in the CAGE questionnaire and a previous history of alcohol intake of  $> 80$  g/d in men and  $> 40$  g/d in women for  $> 10$  years. An aspartate aminotransferase to alanine aminotransferase (AST/ALT) ratio of  $> 1.5$  was taken as corroborative evidence. We selected only patients who had abstained from alcohol for  $> 3$  mo prior to the study.

All patients with acute exacerbation or flair of disease (suggested by a bilirubin concentration  $> 5$  mg/dL, AST  $> 2.5$  times the upper limit of normal, leukocytosis

$> 10\,000/\text{mm}^3$ , or diagnostic lesions of hepatitis on biopsy) and those with recent gastrointestinal bleeding were excluded. Patients with serum creatinine levels  $> 1.4$  mg/dL were excluded, as were those with any form of acute illness. None of the patients had a previous history of chronic disorders associated with changes in mineral metabolism (thyroid disorders, parathyroid disorders, Cushing's syndrome, diabetes, immobilization in the past, or renal failure). None had a family history of osteoporosis, nor did they receive calcium, vitamin D or any medication which may have influenced bone metabolism (corticosteroids, hormone replacement therapy, calcitonin, bisphosphonates, cytotoxics, antimetabolites, anticoagulants, anticonvulsants, thyroxine, interferon or lamivudine). Nineteen patients were receiving spironolactone and 14 were receiving spironolactone and furosemide. Patients with major sclerosis of the aorta, osteophytes, or scoliosis on X-ray, which precluded accurate measurements of lumbar bone mineral density (BMD) by dual energy X ray absorptiometry (DXA), and those who had the criteria for more than one etiology of chronic liver disease were also excluded. All patients signed informed consent and the protocol was approved by the institutional ethics committee.

### **Methods**

Demographic and disease-related data including anthropometry at the time of enrollment were captured. Each subject was interviewed to characterize sunlight exposure, physical activity and dietary intake.

Sunlight exposure was calculated in terms of length of usual weekly outdoor activity, sunscreen use, and usual outdoor attire. The "rule of nine" was adapted to estimate the fraction of body surface area (BSA) exposed to sunlight by each subject's usual outdoor attire<sup>[5]</sup>. With this, sun index was calculated as the product of hours of sun exposure per week and fraction of BSA exposed to sunlight. Mumbai is at latitude  $18^\circ 56'$  North and all of the study population were from areas below  $37^\circ$  latitude. Only sunlight exposure between 8 am to 5 pm in summer and 9 am to 3 pm in winter was measured. All our patients belonged to the same ethnicity and were of skin type 5. Physical activity was assessed using the Global Physical Activity Questionnaire (GPAQ) developed by WHO ([www.who.int/chp/steps](http://www.who.int/chp/steps)). Nutritional intake was calculated using a questionnaire with specific reference to calorie, protein, calcium (dairy and non-dairy), phosphorus and salt intake. These parameters were calculated in two different periods of life; prior to illness (5 years prior to patient perceived onset) and present state of illness.

### **Biochemical and hormonal determinations**

Blood samples were drawn in the morning after an overnight fast. In addition to standard liver function tests, serum levels of calcium, phosphate, magnesium, alkaline phosphatase, and creatinine were measured on the same day with an auto analyzer (Biosystems S.A.,

**Table 1 Demographic data of patients with normal and low BMD**

Parameter	Low BMD	Normal BMD	P value
Age (yr)	44.4 ± 6.1 (47)	42 ± 8.7 (45.5)	0.220
BMI ( $\text{kg}/\text{m}^2$ )	21.14 ± 3.55 (20.3)	23.16 ± 5.46 (22.4)	0.275
Child score	7.4 ± 1.8 (7)	7.85 ± 1.9 (8)	0.350
Lean body mass (kg)	43.3 ± 7.3 (43)	46.7 ± 8.75 (44.7)	0.290
Fat mass (kg)	11.6 ± 6.5 (11.2)	14.4 ± 7.1 (12.43)	0.150

BMD: Bone mineral density. Parameters are expressed as mean ± SD. Median value is given in parentheses.

Barcelona, Spain). The rest of the sample was centrifuged immediately and stored at -70°C for measurement of hormonal parameters, which were analyzed in a single batch. Serum was assayed using commercially available kits for 25 hydroxy vitamin D [25 (OH)D; radioimmunoassay (RIA); DiaSorin Inc., Stillwater, MN, USA], 1,25, dihydroxy vitamin D [1,25 (OH)<sub>2</sub>D; enzyme immunoassay; Immunodiagnostic Systems Inc, Fountain Hills, AZ, USA], parathyroid hormone (PTH), osteocalcin, follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), testosterone (RIA; Diagnostic Products Corp., Los Angeles, CA, USA), sex hormone binding globulin (SHBG), free T4 (FT4), thyroid stimulating hormone (TSH), insulin like growth factor 1 (IGF-1) and IGF binding protein 3 (IGFBP3). Free testosterone was calculated from total testosterone, SHBG and albumin concentration ([www.issam.ch](http://www.issam.ch)). All hormonal investigations except testosterone, 25 (OH)D and 1,25 (OH)<sub>2</sub>D were done by chemiluminescent immunometric assay with Immulite 1000 systems (Diagnostic Products Corporation). Serum testosterone, estradiol, FSH and LH were estimated from pooled sera collected three times at 20-min intervals. The morning second void urinary sample was used for urinary parameters. Urine was analyzed for calcium, creatinine and free deoxypyridinoline (UDPD). UDPD was expressed as the ratio to creatinine.

#### BMD and X-ray measurements

BMD of the lumbar spine (L1-L4) and proximal femur (femoral neck and trochanter) was measured by DXA (Delphi W 70460; Hologic Inc., USA). All scans were carried out on the same machine by the same operator and were analyzed with the same software. BMD was expressed as  $\text{g}/\text{cm}^2$  as well as Z score, compared to reference data for Caucasian populations. As there are no normative data available for the Indian population, no such comparison could be made. Low BMD was considered to be a Z score of -2 or less obtained at any site. X-ray analysis of lumbosacral spine (lateral view) and pelvis (antero-posterior view) was done to rule out any fracture. Lean body mass was also assessed by DXA.

#### Statistical analysis

Statistical analysis was done using SPSS version 14

software (Chicago, IL, USA). All results are expressed as means ± SD and median. The statistical significance between means was calculated by Student's *t* test, analysis of variance (ANOVA), or Mann-Whitney *U* test when appropriate. Differences between proportions were assessed by the  $\chi^2$  test. *P* < 0.05 was considered significant.

## RESULTS

### BMD

Among the 72 patients, 49 (68%) had low BMD. There were no significant differences in demography between the patients with normal and low BMD (Table 1). When patients were classified according to etiology of liver disease, the incidence was 56.7% alcoholic, 72% hepatitis B, and 100% hepatitis C. Incidence of low BMD was the same across all Child classes. Lumbar spine was the most frequently and severely affected site. It was involved in all patients with low BMD. Mean BMD at each site was: spine, -2.28 ± 1.1; hip, -1.27 ± 0.74; trochanter, -1.3 ± 0.8; and femoral neck, 0.75 ± 0.86. Bone mass loss in trabecular bone (lumbar spine) was more severe than that in cortical bone (femoral neck). The percentage of patients with low BMD of the hip was 14%, trochanter was 18%, and femoral neck was 7%.

### Risk factors for low BMD

Patients were evaluated further for the possible predisposing factors for low BMD. The following data pertain to the 63 men in the study. As there were only nine women, they were analyzed separately. Patients were subdivided into low BMD (Z score ≤ -2, 43 patients) and normal BMD (Z score > -2, 20 patients) groups and further analyzed.

### Relationship of BMD with physical activity, sunlight exposure and diet

Both groups showed considerable reduction in sunlight exposure and physical activity after the onset of illness (Table 2). Past and present sunlight exposure was lower in the group with low BMD, although it reached significance (*P* < 0.05) only with present exposure. Low physical activity (defined as < 600 MET·min/wk) was seen in 15 patients (23%) prior to disease onset but in 76% after developing chronic liver disease. It can be seen that the median activity level and sunlight exposure in the affected population was zero, compared to some amount of activity (120 MET·min/wk) and sunlight exposure (sun index of 0.15) in patients who were able to maintain their bone strength.

Dietary intake was comparable between the two groups. Calorie and protein intake were adequate. Calcium intake was also adequate according to the Indian Council of Medical Research guidelines (ICMR)<sup>[6]</sup>. The calcium:protein ratio (8.5-11.5) was much below the advocated range of 16-20<sup>[6]</sup>. The calcium:phosphorus ratio (0.45) was also not in the recommended range of 1:1<sup>[6]</sup>.

**Table 2** Sunlight exposure, physical activity and dietary parameters in men with normal and low BMD

Parameter		Low BMD	Normal BMD	P value
Sun index	Past	2.49 ± 3.3 (1.3)	3.54 ± 5.3 (2.5)	0.13
	Present	0.21 ± 0.41 (0)	0.83 ± 1.4 (0.15)	0.035 <sup>a</sup>
Physical activity (MET.min/wk)	Past	2188 ± 2340 (1920)	2378 ± 1855 (2450)	0.7
	Present	468 ± 1260 (0)	351 ± 659.7 (120)	0.47
Total Calorie (kcal/d)	Past	2111 ± 768 (2078)	1867 ± 524 (1878.0)	0.18
	Present	2020 ± 662 (2071)	1997 ± 422 (2078)	0.65
Total Proteins (g/d)	Past	59.9 ± 22.6 (59.7)	54.9 ± 20 (56)	0.3
	Present	59.3 ± 20.7 (50.6)	60.2 ± 18.6 (61.2)	0.56
Calcium (mg/d)	Past	550 ± 160 (426.5)	582.6 ± 197 (557.8)	0.45
	Present	528 ± 180.7 (391.3)	620.3 ± 259.7 (639.8)	0.3
Phosphorus (mg/d)		1436 ± 652 (1478)	1214.8 ± 535.7 (1202)	0.1
Salt intake (g/d)		6.9 ± 2.9 (7)	7.2 ± 2.8 (7.4)	0.53

<sup>a</sup>Statistically significant, P < 0.05.

**Table 3** Biochemical parameters and markers of bone turnover in men with normal and low BMD

Parameter	Low BMD	Normal BMD	P value
Serum calcium (mg/dL <sup>1</sup> )	9.17 ± 0.52 (9.22)	9.29 ± 0.47 (9.33)	0.29
Serum phosphorus (mg/dL)	3.63 ± .9 (3.59)	3.74 ± 1 (3.8)	0.69
Serum alkaline phosphatase (IU/L)	138 ± 61 (132.7)	140.2 ± 65.6 (128.5)	0.96
Serum magnesium (mg/dL)	2.0 ± 1.9 (1.58)	1.42 ± 0.3 (1.48)	0.08
Urine calcium/creatinine ratio	0.06 ± 0.1 (.03)	0.053 ± 0.053 (0.035)	0.72
25 (OH) vit D (ng/mL)	11.2 ± 7.6 (9.4)	10.5 ± 5.7 (8)	0.90
1,25 (OH) <sub>2</sub> vitD (pg/mL)	46.54 ± 26.4 (41)	36.6 ± 21.6 (30)	0.19
Serum PTH (pg/mL)	45.5 ± 28.1 (43.9)	42.2 ± 18.9 (42.1)	0.95
Serum osteocalcin (ng/mL)	2.99 ± 2.5 (1.8)	2.14 ± 1.8 (1.14)	0.37
UDPD/creatinine ratio <sup>2</sup>	11.5 ± 4.5 (12.1)	12.5 ± 6.2 (11.6)	0.84

<sup>1</sup>Corrected calcium is used; <sup>2</sup>Unit - nM DPD/mmol/L creatinine.

**Table 4** Hormone parameters in men with normal and low BMD

Parameter	Low BMD	Normal BMD	P value
FSH (IU/L)	8.2 ± 9.4 (4.62)	19.8 ± 40.2 (7.53)	0.34
LH (IU/L)	6.8 ± 6.2 (4.78)	11.1 ± 13.9 (6.47)	0.31
Estradiol (pg/mL)	76.1 ± 61.5 (63.8)	100.6 ± 61.8 (79.2)	0.02 <sup>1</sup>
SHBG (nmol/L)	76.7 ± 31.1 (75.4)	72.3 ± 25.4 (72.6)	0.60
Free testosterone <sup>2</sup> (ng/dL)	7.6 ± 4.7 (7.27)	7.2 ± 4.0 (7.06)	0.85
IGF-1 (pmol/L)	44.8 ± 24.7 (35.2)	73.7 ± 58.54 (38.4)	0.049 <sup>1</sup>
IGFBP3 (μg/mL)	1.21 ± 0.6 (1.03)	1.8 ± 1.2 (1.51)	0.071

<sup>1</sup>Statistically significant, P < 0.05; <sup>2</sup>Calculated from total testosterone.

#### Relationship of BMD with biochemical parameters and markers of bone turnover

Biochemical parameters were comparable between both groups (Table 3). Serum calcium, magnesium, phosphorus and alkaline phosphatase levels were normal. Prevalence of vitamin D deficiency was high among patients with CLD. Among the 63 patients, vitamin D values were < 10 ng/mL in 38 patients (60%), 10-20 ng/mL in 20 patients (32%), and > 20 ng/mL in 5 patients (8%). Despite having a low vitamin D level in 92%, PTH was within the physiological range in 87% of patients.

Markers of bone turnover indicated high resorption with low formation. Serum osteocalcin was low in 43 patients (68%) and UDPD: creatinine ratio was high

in 50 patients (79%). This suggests uncoupling of bone remodeling as the cause for low BMD in CLD. The levels of bone turnover markers were comparable between the two groups.

#### Relationship of BMD to hormone parameters

There was a high incidence of hypogonadism in patients with cirrhosis. Twenty-six patients (41%) had low calculated free testosterone. This was distributed equally between the low and normal BMD groups. Among the hypogonadal patients, 18 (69%) had central hypogonadism and eight (31%) had primary testicular failure. An FSH value of > 10 IU/L with normal free testosterone was seen in an additional nine (14%) patients. Serum estradiol level was significantly elevated ( $P < 0.05$ ) in patients with normal BMD as compared to those with low BMD (Table 4). Forty-six patients (73%) had a high estradiol level, which was distributed unequally within the groups, with 90% of patients with normal BMD and 65% in the group with low BMD having values above the physiological upper range of 50 pg/mL.

IGF-1 levels were below the age-related normal range in both groups, but significantly lower ( $P < 0.05$ ) in patients with low BMD (Table 4). Forty-one patients (95%) of patients in the low BMD group and 15 (75%) in the normal BMD group had IGF-1 level below normal, which accounted for 89% of patients with CLD.

Low IGFBP3 was almost a universal finding in patients with CLD (61 of 63 patients; 97%), although it did not differ significantly between the groups ( $P = 0.071$ ).

## DISCUSSION

The purpose of the current study was to determine the prevalence of low BMD, to estimate the bone turnover and hormonal status, and to identify the factors associated with bone disease in patients with CLD. The only available Indian data on this subject are those of Sachdev *et al*<sup>[4]</sup> from 1976. The current study shows that patients with CLD have a high prevalence of decreased BMD, with the lumbar spine being the most frequently and intensely affected site. Furthermore, there was no relation between severity of hepatic dysfunction (Child class) and incidence of low BMD. These factors point to the need for early evaluation for HO in patients with CLD.

In the present cohort, low BMD was found in 68% of patients. This is comparable to the only available Indian data of Sachdev *et al*<sup>[4]</sup>, in which 64% of cirrhotic patients had low BMD. The method of evaluation and diagnosis differed greatly in that era. In the earlier study of 25 patients with cirrhosis (all aged < 40 years), diagnosis of cirrhosis was made from liver biopsy and osteoporosis from iliac crest biopsy. Scanning through western studies has indicated marked heterogeneity in BMD findings in CLD, ranging from no effect to a large BMD deficit. Leslie *et al*<sup>[7]</sup> have pooled the results from uncontrolled and controlled studies of bone mineral content in CLD. They have shown a Z score less than -2 in 21% of patients. Studies on patients with end-stage liver disease of varying etiology confirm a high but variable incidence of osteoporosis (11%-48%) and osteopenia (18%-35%)<sup>[5]</sup>. The incidence of 68% in the present study is much higher than that in any previous study. This may be because Indians have a lower BMD as compared to Caucasians<sup>[8,9]</sup>. Thus, the use of Z scores based on a Caucasian database might have resulted in overestimation of osteoporosis. However there are no published data for BMD in healthy Indian populations.

The major influences on bone metabolism are genetic, but also essential are mechanical stress (exercise and muscle activity), good nutrition, adequate calcium and vitamin D, and a normal hormonal environment. The patient with CLD could have any of these factors acting alone or in concert, which potentially predispose him/her to thin bones. Each of the above factors were assessed and compared between patients with low and normal BMD. It was found that patients with CLD had all the above and known risk factors: low sunlight exposure, reduced physical activity, low lean body mass, vitamin D deficiency and hypogonadism. The presence of risk factors in the low and normal BMD groups was probably the reason for the absence of a statistically significant difference in risk factors between the normal and low BMD groups. This indicates that all patients with cirrhosis, unless prevented, will develop

the disease. In addition, although the calcium intake was adequate by ICMR guidelines, it was well below the internationally accepted daily allowance. This added to an unfavorable calcium:protein ratio of 8.5-11.5 mg/g, and calcium:phosphorus ratio of 0.45 may have resulted in inadequate recommended daily allowance of calcium in these patients.

Vertebrae consist of 50% trabecular bone, while other bones (hip, neck and trochanter) consist mainly of cortical bone. Sites with a high proportion of trabecular bone are affected earliest in diseases that produce increased bone turnover<sup>[10]</sup>. In the present study, serum osteocalcin was low in 68% and UDPD: creatinine ratio was high in 79% of patients, which indicated a high resorptive state added to low formation. This suggests uncoupling of bone remodeling as the cause of low BMD in CLD. This can explain the predominant involvement of the spine in HO. This is also compatible with other similar studies<sup>[11,12]</sup>.

In the present study, 41% of patients were hypogonadal, although this was not correlated with the severity of bone loss. Diamond *et al*<sup>[13]</sup> and Monegal *et al*<sup>[11]</sup> have shown that hypogonadism is common in men with cirrhosis but it is not correlated with osteoporosis. A particularly interesting finding in the present study was the significantly elevated estradiol level ( $P < 0.05$ ) in patients with normal compared with low BMD. Estrogen is known to have a positive influence on the male skeleton<sup>[14]</sup>. It is also known to be increased in men with cirrhosis. Probably the anabolic and antiresorptive qualities of estrogens in bone act as protective factors in preventing bone loss in these patients with cirrhosis.

More than 90% of circulating IGF-1 is synthesized in the liver. It is a proven early marker of hepatocellular functional capacity<sup>[15,16]</sup>, and shows a marked decline in the early stages of cirrhosis (Child-Pugh class A). It starts decreasing before other liver-function parameters such as albumin, bilirubin and prothrombin become involved<sup>[15]</sup>. GH levels are increased correspondingly, which creates a state of IGF resistance<sup>[17]</sup>. IGF-1 is also a major determinant of BMD in healthy men<sup>[18]</sup>. In the present study, IGF-1 levels were below the age-related normal range in both groups, and were significantly lower ( $P < 0.05$ ) in patients with low BMD. IGF-1 values were low in 89% of patients with CLD. Previous studies have shown IGF-1 levels to correlate directly with BMD and inversely with disease severity<sup>[12,19,20]</sup>. Studies have described a role for decreased serum IGF-1, even in idiopathic osteoporosis<sup>[21]</sup>. IGF-1 expression is increased during early osteoblast recruitment, but declines as these cells undergo differentiation. It is known to stimulate osteoblast proliferation<sup>[22]</sup> and play a key role in bone remodeling and maintenance of bone mass. Simonet *et al*<sup>[23]</sup> have shown that low levels of IGF-1 may lead to increased bone resorption. Thus, the link between cirrhosis and bone loss also involves low levels of IGF-1. A significant difference in IGF-1 level between normal and low BMD patients may be a pointer to why some patients deteriorate faster, despite sharing equally all the risk factors.

IGFBP3 play a very important role in bioavailability of circulating IGF-1. It forms a stable ternary complex with an acid-labile subunit and IGF-1, and binds > 95% of circulating IGF-1. In the present cohort, low IGFBP3 was seen in 97% of patients with CLD, although this did not differ significantly between the normal and low BMD groups ( $P = 0.071$ ). This is plausible because hepatocytes are the major site of IGFBP3 synthesis. This may have further decreased the tissue bioavailability of IGF-1<sup>[24,25]</sup>.

In conclusion, the present study confirms the high incidence of low BMD in patients with CLD. Disease onset is early in the course of cirrhosis. Decreased bone formation with increased bone resorption imply that uncoupling of bone remodeling is the mechanism involved. Contributing factors are inadequate sunlight exposure, reduced physical activity, low lean body mass, vitamin D deficiency and hypogonadism. The presence of most risk factors in low and normal BMD groups indicates that all patients with cirrhosis are vulnerable, and unless prevented, will develop the disease. Our results provide evidence of the key roles played by IGF-1 and estrogen in this condition. Although risk factors are prevalent in all patients, the severity of bone loss may be accelerated in patients with low IGF-1 level. The present study also suggests a possible protective role for the high estrogen level seen in cirrhosis.

## COMMENTS

### Background

Long-standing liver disease has long been recognized to result in fragile bones with increased risk of fractures. In various international studies, the overall incidence has varied from 11% to 48%, with a fracture rate of 3%-44%. The reason for this is poorly understood. With liver transplantation becoming a viable option in liver disease and offering complete cure and long-term survival, bone disease is becoming the major determinant of survival and quality of life in these patients. The present study tried to characterize the problem and identify contributing risk factors.

### Research frontiers

Much work has been done and is still going on in the field of hepatic osteodystrophy (HO). It is a hot topic of research, as liver transplantation is improving survival of patients with end-stage liver disease, and bone disease and fracture are limiting the survival and quality of life of these patients. The medical fraternity has recognized that bone health has to be taken care of to fully translate the benefits of modern treatment into patient survival.

### Innovations and breakthroughs

Most of the data obtained in this study conform to those in the literature. Two significant findings of the study (that low levels of IGF-1 is a risk factor for decreased BMD, and increased estrogen is protective) are relatively new.

### Applications

This article provides an entirely new frontier in research, namely, to look forward to the therapeutic benefit of IGF-1 therapy in these patients. Synthetic IGF-1 is available under the name mecasermin and is used currently for the long-term treatment of growth failure in children with severe primary IGF-1 deficiency.

### Peer review

This work represents an original contribution regarding HO in patients with advanced liver disease in India. The study was well-conducted. The authors identified in cirrhotic patients that low levels of IGF-1 are a risk factor for decreased BMD, while increased estrogens protect against osteopenia.

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# Clinical outcomes of self-expandable metal stents in palliation of malignant anastomotic strictures caused by recurrent gastric cancer

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migration occurred with a covered stent in 3 patients who underwent a subtotal gastrectomy with Billroth-II reconstruction. Two cases of partial stent migration were easily treated with a second stent or stent repositioning. The median stent patency was 56 d (range, 5-439 d). The median survival was 83 d (range, 12-439 d).

**CONCLUSION:** Endoscopic insertion of a SEMS provides safe and effective palliation of a recurrent anastomotic stricture caused by gastric cancer. A meticulous evaluation of the presence of other strictures before inserting the stent is essential for symptom improvement.

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**Key words:** Stents; Surgical anastomosis; Stricture; Endoscopic gastrointestinal surgery; Stomach neoplasms

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## Abstract

**AIM:** To examine the technical feasibility and clinical outcomes of the endoscopic insertion of a self-expandable metal stent (SEMS) for the palliation of a malignant anastomotic stricture caused by recurrent gastric cancer.

**METHODS:** The medical records of patients, who had obstructive symptoms caused by a malignant anastomotic stricture after gastric surgery and underwent endoscopic insertion of a SEMS from January 2001 to December 2007 at Kangnam St Mary's Hospital, were reviewed retrospectively.

**RESULTS:** Twenty patients (15 male, mean age 63 years) were included. The operations were a total gastrectomy with esophagojejunostomy ( $n = 12$ ), subtotal gastrectomy with Billroth-I reconstruction ( $n = 2$ ) and subtotal gastrectomy with Billroth-II reconstruction ( $n = 8$ ). The technical and clinical success rates were 100% and 70%, respectively. A small bowel or colon stricture was the reason for a lack of improvement in symptoms in 4 patients. Two of these patients showed improvement in symptoms after another stent was placed. Stent reobstruction caused by tumor ingrowth or overgrowth occurred in 3 patients (15%) within 1 mo after stenting. Stent

## INTRODUCTION

Local recurrence causing dysphagia occurs in approximately 20% of stomach cancer patients treated with a gastrectomy<sup>[1]</sup>. These patients are usually poor surgical candidates because of advanced malignancy, poor performance status or malnutrition. Palliative surgery carries a high risk of mortality and morbidity.

A self-expandable metal stent (SEMS) is currently the main palliative nonsurgical treatment for malignant gastric outlet obstructions<sup>[2]</sup>. Metal stents are also used to treat malignant anastomotic obstructions after esophagojejunostomy, gastrojejunostomy and gastroduodenostomy. However, there are only a few

reports on the clinical outcome of SEMS for the palliation of a recurrent anastomotic obstruction after gastric surgery<sup>[3-11]</sup>. In particular, there is one study on endoscopic insertion of a SEMS in a recurrent anastomotic stricture<sup>[11]</sup> instead of a fluoroscopically-guided method. The clinical outcomes and complications might differ according to the surgical technique because of the different anastomotic angle or different anatomical alterations during surgery.

This study evaluated the technical feasibility and clinical effectiveness of endoscopic SEMS placement in the palliation of patients with a recurrent anastomotic obstruction after gastric surgery.

## MATERIALS AND METHODS

### **Patients**

Twenty consecutive patients (M:F = 15:5, mean age 63.1 ± 10.3 years), who had a documented postoperative anastomotic stricture caused by recurrent gastric cancer and had undergone endoscopic SEMS insertion from January 2001 to December 2007, were enrolled in this study. All patients had a symptomatic obstruction characterized by nausea, vomiting, reduced oral intake and weight loss. The recurrent gastric cancer, which was the underlying cause of the obstruction, was confirmed by pathological diagnosis in all patients. None of the patients were surgical candidates based on the presence of advanced, metastatic disease or medical comorbidity.

The exclusion criteria were patients who were mildly symptomatic or patients in whom an adult endoscope could be passed through the malignant anastomotic stricture or patients showing evidence of peritonitis. An abdominal computed tomography (CT) scan or contrast media radiographic study to document multiple strictures was not performed routinely.

The surgical technique was a total gastrectomy with esophagojejunostomy in 10 patients, subtotal gastrectomy with Billroth-I reconstruction in 2 patients and subtotal gastrectomy with Billroth-II reconstruction in 8 patients. The type of reconstruction after total gastrectomy was loop esophagojejunostomy except for 2 patients with a Roux-en-Y esophagojejunostomy. Gastrojejunostomy without jeunojejunostomy was used for Billroth-II reconstruction after subtotal gastrectomy. Strictures occurring in the efferent loop were included in this study. One patient had a stricture in both afferent and efferent loops. Therefore 2 stents were inserted in both sites. Patients with recurrent cancer only in the afferent loop were excluded. Table 1 lists the patients' characteristics.

### **Methods**

NITI-S® stents (Taewoong, Seoul, Korea, n = 10), Choo stent (M.I. Tech, Seoul, Korea, n = 10) were used. These stents are commonly used commercial pyloric stents. The degree, length and site of the stenosis were evaluated using an endoscopic procedure or barium meal prior to stent insertion. Thirteen covered stents and 7 uncovered stents were inserted. The covered stent was coated with polyurethane around the body and contained the proximal flare portion. The diameter of the body and flare portions

**Table 1 Patients' characteristics**

Age ± SD (yr)	63.1 ± 10.3
Male:Female	15:5
Prior surgery (n)	
Total gastrectomy with esophagojejunostomy (10)	Covered stent (6) Uncovered stent (4)
Subtotal gastrectomy with Billroth-I reconstruction (2)	Covered stent (1) Uncovered stent (1)
Subtotal gastrectomy with Billroth-II reconstruction (8)	Covered stent (6) Uncovered stent (2)
Chemotherapy after stent insertion (number of patients)	10
Follow-up loss (n, %)	3 (15%)
30-d mortality	3/17 (18%)
Survival [median (range)]	83 (12-439) d
Stent patency duration [median (range)]	56 (5-439) d

were 18 and 26 mm, respectively. The length of these SEMS ranged from 8 to 22 cm. The outer diameter of the delivery system was 10F to 11F with an overall length of 180 cm. The stent delivery system was advanced over the guidewire. Under direct guidance of endoscopic and fluoroscopic vision, a guidewire was passed through the malignant stricture. The stent was then released and the position and location of the stent were assessed by both endoscopy and fluoroscopy. Compensatory hydrostatic dilatation of the stent was not required in any of the patients. The patients usually resumed a water or a liquid diet 24 h after stent placement. The patients started a soft or solid diet after the follow up X-ray showed full extension. There was one patient whose stent was not sufficiently expanded. He could not restart a soft diet.

After inserting the stent, a combination of 5-fluorouracil, cisplatin, and epirubicin or paclitaxel-based or docetaxel-based chemotherapy was administered when the oral intake improved and the Eastern Cooperative Oncology Group performance status was ≤ 2 (graded as follows: 0 = normal activity, 1 = symptoms but ambulatory, 2 = in bed less than 50% of time, 3 = in bed more than 50% of time, and 4 = totally bedridden). Palliative chemotherapy after stent insertion was performed in 10 patients (50%).

### **Definitions**

The outcome of the stent was evaluated using the following parameters: (1) technical success and clinical success; (2) complications; (3) stent patency.

Technical success was defined as the successful insertion of a stent in the proper position and the confirmation of patency using a combination of endoscopy and fluoroscopy with oral contrast opacification.

Clinical success was defined as an improvement in the obstructive symptoms and oral intake 1 to 3 d after placing the stent. The degree of oral intake was assessed using the Gastric Outlet Obstruction Scoring System as follows: 0 = no oral intake; 1 = exclusively liquid diet; 2 = exclusively soft solids diet; 3 = full diet possible. The improvement in oral intake was evaluated as the best degree at least 3 d after stent insertion. A primary stent dysfunction was defined as a failure to resume an oral intake after stent insertion.

**Table 2** Improvement in the oral intake status compared to before stent insertion ( $n = 20$ )

Oral intake status (by GOOSS)	Number of cases	
	Pre-stenting	Post-stenting
No oral intake (0)	15	3
Liquids only (1)	3	6
Soft solids (2)	2	8
Low-residual or full diet (3)	0	3
Mean score <sup>b</sup>	0.35 ± 0.61	1.55 ± 0.94

<sup>b</sup> $P < 0.01$  by Wilcoxon signed rank test; GOOSS: Gastric Outlet Obstruction Scoring System.

The stent patency time was defined as the duration between the initial stent placement and the recurrence of obstructive symptoms caused by a stent occlusion. It was considered to be equal to the survival time if there were no obstruction symptoms or stent occlusion.

### Follow-up

The patients were followed up to determine their clinical outcomes until they died or the stent malfunctioned, such as by migration or occlusion by tumor ingrowth or overgrowth. The data were obtained from the hospital records, radiology or endoscopic records, the patients themselves during a clinical visit and their relatives by a telephone survey. The status of oral food intake was monitored at 1 mo intervals on an outpatient basis. A follow-up barium study or endoscopy was performed only if obstructive symptoms recurred in order to evaluate stent occlusion or migration.

### Statistical analysis

The values for the patients' characteristics are expressed as the median (range). The categorical data were examined using Fisher's exact test. The degree of oral intake before and after stent insertion was compared by a Wilcoxon signed rank test. The overall survival and stent patency were estimated by Kaplan-Meier life table analysis. A  $P$ -value  $< 0.05$  was considered significant. All analyses were carried out using SPSS version 10.0 (SPSS Inc, USA).

## RESULTS

### Technical and clinical success

Endoscopic stent placement was technically successful in all patients. Clinical success was achieved in 14 out of the 20 cases (70%). The reasons for the lack of improvement in obstructive symptoms were small bowel or colon stricture ( $n = 4$ ), ileus induced by peritoneal dissemination ( $n = 1$ ) and primary stent dysfunction caused by stent expansion failure ( $n = 1$ ). The symptoms in 2 of the 4 patients with single small bowel or colon stricture improved after placing a second stent. Table 2 summarizes the improvement in the dietary status.

### Complications

There was no procedure-related mortality. In one patient who underwent a distal gastrectomy with Billroth-I

reconstruction, the uncovered stent did not expand fully and was compressed by the tumor mass until 5 d after stent placement. The symptoms were not improved. However, he refused further treatment and was lost to follow-up 7 d after stent placement.

Recurrent symptoms of an obstruction were observed in 3 patients (15%) as a result of tumor overgrowth ( $n = 2$ ) or tumor ingrowth ( $n = 1$ ) within 1 mo after stenting. The reobstruction rate (1/13 vs 2/7,  $P = 0.55$ ) of a covered stent and uncovered stent, and stent patency duration [56 d (range, 7-439) for the covered stent vs 37 d (range, 15-141) for the uncovered stent,  $P = 0.7$ ] were similar. Tumor overgrowth occurred in patients who underwent a total gastrectomy with esophagojejunostomy. Tumor ingrowth occurred in a patient who underwent a subtotal gastrectomy with Billroth-II reconstruction, in whom an uncovered stent was inserted. Two patients were treated successfully with an overlapping second covered stent. Stent migration occurred in 3 patients (15%) who underwent a subtotal gastrectomy with Billroth-II reconstruction, in whom covered stents were inserted. Complete stent migration occurred at 64 d in one patient who received palliative chemotherapy. The migrated stent was not detected until the endoscopy or radiologic study revealed no stent remaining at the previous stricture site. Therefore, the stent was believed to have migrated downward and pass out of the anus without the patient's awareness. She was asymptomatic even though there was stent migration. The reobstructive symptoms appeared 319 d after stent migration. She was treated with the placement of 3 stents at the efferent loop, the afferent loop and distal colon stricture.

Partial stent migration to the more distal side of the efferent loop occurred in a patient 2 d after stent placement. The patient was treated by overlapping a second stent into the first stent. The proximal half of one stent slipped upward to the body of the stomach in one patient, which was repositioned by grasping with the forceps. The symptoms improved. Table 3 gives a summary of the complications.

### Survival

Three patients were lost during the follow-up period and the remaining 17 patients died. The median survival period was 83 d (range, 12-439 d) and the median stent patency was 56 d (range, 5-439 d, Figure 1). There were no differences in median survival or stent patency between the patients who received palliative chemotherapy and those who did not ( $P = 0.66$ ).

## DISCUSSION

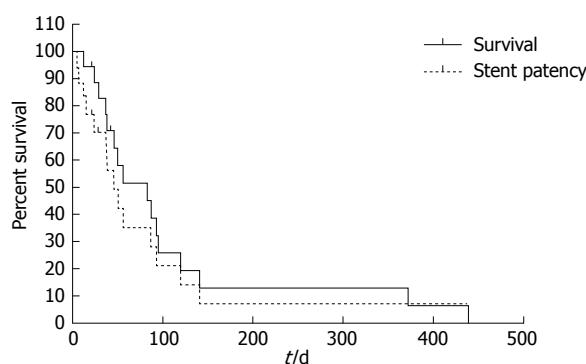
A SEMS is a simple, safe and effective palliation treatment for patients with a malignant obstruction of the gastrointestinal tract<sup>[12,13]</sup>. A SEMS has clinical advantages, compared with surgical gastrojejunostomy, such as rapid resumption of oral intake, shorter hospital stay and rapid improvement in the quality of life in malignant gastric obstruction<sup>[14,15]</sup>.

Patients with an anastomotic stricture caused

**Table 3 Complications associated with stent placement**

Patient	Complication	Type of operation	Type of stent	Days after stenting	Treatment
1	Expansion failure	Billroth- I subtotal gastrectomy	Uncovered	5	Refusal of treatment
2	Tumor overgrowth	Total gastrectomy	Covered	7	Second stent
3	Tumor overgrowth	Total gastrectomy	Uncovered	28	TPN
4	Tumor ingrowth	Billroth- II subtotal gastrectomy	Uncovered	15	Second stent
5	Stent migration (complete)	Billroth- II subtotal gastrectomy	Covered	64	Not needed
6	Stent migration (partial)	Billroth- II subtotal gastrectomy	Covered	20	Reposition
7	Stent migration (partial)	Billroth- II subtotal gastrectomy	Covered	2	Second stent

TPN: Total parenteral nutrition.



**Figure 1 Cumulative survival and stent patency of 20 patients obtained using the Kaplan-Meier method.**

by recurrent gastric cancer are likely to be severely debilitated. These patients generally have a relatively short life expectancy. Bypass or resective operations are usually impossible because of the extensive tumor invasion and metastasis<sup>[5]</sup>. Therefore, a less invasive procedure is preferred. This study evaluated the clinical effectiveness and the technical feasibility of SEMS insertion in the palliation of patients with a recurrent anastomotic stricture after various gastric surgical procedures.

The surgical techniques used were total gastrectomy with esophagojejunostomy ( $n = 10$ ), subtotal gastrectomy with Billroth- I reconstruction ( $n = 2$ ), subtotal gastrectomy with Billroth- II reconstruction ( $n = 8$ ). All procedures were performed using endoscopic guidance. The technical success rate was 100%, which is comparable to those with a primary malignant gastric outlet obstruction (83%-100%)<sup>[15]</sup>. There is one report on endoscopically-guided stent insertion in a recurrent anastomotic stricture<sup>[11]</sup>. The advantages of endoscopically-guided stent insertion are the ease of accessing the stricture site and the avoidance of looping the delivery system through the dilated gastric lumen because endoscopy offers sufficient stiffness, so that the delivery system can easily pass through the dilated gastric lumen. There was no erroneous stent placement in the incorrect loop. The efferent loop was differentiated by identifying the ampulla of Vater in the afferent loop by endoscopy, which was confirmed by fluoroscopy during stent insertion. Before stenting, knowledge of the anatomy is important because it can be altered by the surgical procedures or recurrent tumor mass occluding the efferent loop<sup>[7]</sup>.

The dietary intake improved in 14 out of the 20

patients (70%) after stent placement, which is comparable to the clinical success rate of SEMS insertion in a malignant gastric outlet obstruction (75%-85%)<sup>[13]</sup>. The improvement in symptoms after SEMS insertion in the anastomotic stricture caused by recurrent gastric cancer was reported to be 80%-90%<sup>[3-8]</sup>. The average score of the dietary status improved from  $0.35 \pm 0.61$  to  $1.55 \pm 0.94$  ( $P < 0.01$ ). Five patients whose symptoms did not improve had another single stricture at the small intestine or colon, or ileus by peritoneal dissemination. The dietary state in 2 of them improved after inserting an additional stent. This suggests that a precise study of the distal bowel loop using a CT scan or barium study before stent insertion is essential in order to exclude a concealed obstruction. A single stent may not be helpful if there are multiple strictures. Moreover, the insertion of 2 stents at one time may be necessary if the patients have another single stricture.

Stent reobstruction caused by tumor ingrowth or overgrowth occurred in 3 patients (15%) within 1 mo after stent placement. A recent study reported that early restenosis within 1 mo tended to occur more frequently in postoperative anastomosis than a gastric outlet obstruction caused by primary cancer (4/6 vs 2/6,  $P < 0.01$ )<sup>[16]</sup>. The covered stents had the merit of less frequent reobstruction by tumor ingrowth<sup>[17]</sup>. However, in this study, the reobstruction rate and stent patency duration of covered stents and uncovered stents were similar. The incidence of stent reobstruction in recurrent anastomotic stricture after gastric surgery was reported to be 0%-17%<sup>[3-8]</sup>. Most studies used covered stents. In 2 studies using uncovered stents, Lee *et al*<sup>[6]</sup> reported that one out of 4 patients had tumor ingrowths, and Song *et al*<sup>[7]</sup> reported a 50% stent reocclusion rate within 2 wk of stent placement. A recent retrospective study suggested that a double coaxial stent had a longer patency and lower migration rate than an uncovered stent in postoperative anastomotic obstructions<sup>[11]</sup>. A prospective, randomized, comparative study to determine which stent is favorable in this situation will be needed.

Three cases of stent migration (15%, 3/20) were encountered in patients who underwent a subtotal gastrectomy with Billroth-II reconstruction and had a covered stent inserted. Complete stent migration occurred in one patient who received palliative chemotherapy after approximately 64 d. Because chemotherapy might stabilize or reduce the tumor burden, it could influence stent migration. Two cases of partial stent migration were

easily treated by repositioning the stent and overlapping a second stent. The incidence of stent migration was reported to be 0%-16% in studies using a covered stent in an anastomotic stricture in various types of gastric cancer surgery<sup>[3-8]</sup>. The surgical technique can influence the rate of migration. The relatively acute angle between anastomosis and the efferent loop in gastrojejunostomy compared with the relatively obtuse angle in esophagojejunostomy or gastroduodenostomy, the radial force of the stent in the angulated loop, or the use of a covered stent may influence stent migration.

In this study, the 30-d mortality was 18%. The median survival was 83 d (range, 12-432 d). The median stent patency was 56 d (range, 5-439 d). Because the median survival in an anastomotic obstruction is comparable to that in a malignant gastric outlet obstruction, strategies to prolong stent patency and avoid the need for additional intervention are important in patients with recurrent cancer, particularly those with a relatively good performance status or who are expected to have a longer survival.

In summary, endoscopic insertion of a SEMS is a safe, technically feasible, and effective treatment for the palliation of anastomotic strictures caused by recurrent gastric cancer. A meticulous evaluation of the presence of another stricture before inserting the stent is essential for symptom improvement.

## COMMENTS

### Background

A self-expandable metal stent (SEMS) was used to treat malignant anastomotic obstruction during esophagojejunostomy, gastrojejunostomy and gastroduodenostomy. There are only a few reports on the clinical outcome of SEMS insertion for the palliation of a recurrent anastomotic obstruction after gastric surgery.

### Research frontiers

The authors aimed to evaluate the technical feasibility and clinical effectiveness of an endoscopic SEMS placement in the palliation of patients with a recurrent anastomotic obstruction after gastric surgery.

### Innovations and breakthroughs

This retrospective study has shown that the technical and clinical success of SEMS insertion for anastomotic strictures caused by recurrent gastric cancer were 100% and 70%, respectively. The main reasons for the clinical failure were small bowel or colon stricture in addition to anastomotic stricture. Stent migration (15%) was encountered in patients who underwent subtotal gastrectomy with Billroth-II reconstruction and had a covered stent inserted.

### Applications

A meticulous evaluation of the presence of other strictures is essential before inserting the stent for anastomotic strictures from recurrent gastric cancer. The possibility of stent migration is a consideration in anastomotic strictures after gastrojejunostomy.

### Peer review

This paper is a good retrospective report on the usage of a SEMS for palliation of malignant anastomotic stricture caused by recurrent gastric cancer, showing that it is a safe and effective palliation treatment.

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BRIEF ARTICLES

## Osteoporosis in adult Sri Lankan inflammatory bowel disease patients

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### Abstract

**AIM:** To determine if inflammatory bowel disease (IBD) is a risk factor for osteoporosis in adult Sri Lankans.

**METHODS:** We identified eligible subjects from among consecutive patients diagnosed with IBD who attended our outpatient clinic. We included only patients aged between 20 and 70 years. Patients who were pregnant, had significant comorbidity, or were on calcium supplements or treatment for osteoporosis within the past 6 mo, were excluded. Healthy, age- and sex-matched controls were also recruited, in

a control to patient ratio of 3:1. Both groups were screened for osteoporosis using peripheral dual energy X-ray absorptiometry scanning.

**RESULTS:** The study population consisted of 111 IBD patients (male:female = 43:68; mean age 42.5 years) and 333 controls (male:female = 129:204; mean age 43.8 years). The occurrence of osteoporosis among IBD patients (13.5%) was significantly higher than among controls (4.5%) ( $P = 0.001$ ). The frequency of osteoporosis was not significantly different between ulcerative colitis (14.45%) and Crohn's disease (10.7%). However, on multivariate analysis, only age ( $P = 0.001$ ), menopause ( $P = 0.024$ ) and use of systemic steroids ( $P < 0.001$ ) were found to be associated independently with the occurrence of osteoporosis, while IBD, severity of disease, number of relapses, duration of illness or treatment other than systemic steroids were not.

**CONCLUSION:** IBD does not appear to be an independent risk factor for the occurrence of osteoporosis in this population. However, the use of systemic steroids was a risk factor.

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**Key words:** Osteoporosis; Inflammatory bowel disease; Asians; Crohn's disease; Ulcerative colitis

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### INTRODUCTION

The incidence of inflammatory bowel disease (IBD)

is rising in Asian populations<sup>[1]</sup>. IBD, both ulcerative colitis (UC) and Crohn's disease (CD), is a recognized risk factor for development of osteoporosis among Caucasians<sup>[2-4]</sup> but this association does not seem to have been investigated adequately in Asian populations<sup>[5]</sup>.

Osteoporosis is usually diagnosed by dual energy X-ray absorptiometry (DEXA) scanning<sup>[6]</sup>. However, peripheral DEXA (pDEXA), quantitative computed tomography (QCT), radiographic absorptiometry, and ultrasound have become useful in community screening<sup>[7-9]</sup>. In the literature, the reported prevalence of osteoporosis/osteopenia in IBD varies from 7% to 56%<sup>[10,11]</sup>. A retrospective study of a Caucasian population showed a 40% increase in the risk of fracture compared to healthy controls<sup>[12]</sup>. CD seems to be associated with a slightly higher risk than UC does for osteoporosis and subsequent fractures, although this has been disputed in some studies<sup>[13,14]</sup>. The mechanism for development of osteoporosis in IBD patients seems to be multifactorial<sup>[15]</sup>. The slightly higher incidence of osteoporosis in CD could be attributed to the presence of ileal disease or small intestinal resection causing vitamin D malabsorption, malnutrition or estrogen deficiency<sup>[16]</sup>. Some studies have shown a genetic predisposition to osteoporosis in IBD patients<sup>[17]</sup>. The identified genes involve the pro-inflammatory cytokine interleukin-6<sup>[18,19]</sup>. It is important to identify IBD patients with osteoporosis, as treatment with bisphosphonates has been found to be effective<sup>[20,21]</sup>.

There have been no large published studies regarding an association between osteoporosis and IBD in Asian populations<sup>[22]</sup>. It is important to investigate such an association because IBD among Asians seems to be genetically and phenotypically different to that in the West<sup>[23]</sup>.

## MATERIALS AND METHODS

### **Patients**

Consecutive patients with previously diagnosed IBD from a single tertiary care center in Sri Lanka were eligible for inclusion in the study. IBD was diagnosed using standard criteria<sup>[24]</sup>. Inclusion criteria were age > 20 and < 70 years and the presence of IBD. Exclusion criteria were pregnancy; uncontrolled diabetes; renal, hepatic, cardiovascular or psychiatric disease; rheumatoid arthritis; ankylosing spondylitis; primary sclerosing cholangitis; or treatment with teriparatide, calcitonin, bisphosphonates, fluoride, androgens, anabolic steroids or active metabolites of vitamin D within the past 6 mo.

### **Controls**

For each case, three age- ( $\pm$  5 years) and sex-matched healthy controls were selected from among individuals who were selected randomly from the community for a large population study that screened for non-communicable diseases. The controls were screened for diabetes and were not taking active metabolites of vitamin D or calcium supplements.

### **Steroid use**

Steroid use was defined as the continuous use of systemic steroids for > 3 mo. Others were considered steroid naïve.

### **Ethics**

The study protocol was approved by the Ethics Committee of the Faculty of Medicine, University of Kelaniya. Written informed consent was obtained from all participants.

### **Study design**

A comparative study involving IBD cases and age- and sex-matched community controls at ratio of 1:3.

### **DEXA scanning**

Both cases and controls underwent pDEXA with the accuDEXA (ADX-finger) (Schick, New York, NY, USA) using the right index finger. The bone mineral density (BMD) and *T* scores were recorded.

### **Diagnosis of osteoporosis and osteopenia**

Osteoporosis and osteopenia were diagnosed using WHO criteria<sup>[25]</sup>. Osteoporosis was defined as a *T* score of -2.5 or below, while osteopenia was diagnosed with a *T* score between -1 to -2.49.

### **Statistical analysis**

Previous studies have reported a 56% prevalence of osteoporosis among Caucasian IBD patients, and we assumed a 40% prevalence of osteoporosis among controls. We calculated that a sample size of 111 IBD patients and 333 controls was required to have 80% power to detect this difference at a significance level of 0.05. Quantitative data were compared using the *t* test, and categorical data were compared using a  $\chi^2$  test. Multiple logistic regression was used to identify independent risk factors for osteoporosis. The analysis was carried out using the statistical program SPSS version 16 (Chicago, IL, USA).

## RESULTS

One hundred and eleven IBD patients [male:female = 43:68; mean age 42.5 years; 83 (74.8%) with UC, 28 (25.2%) with CD, and 333 age- and sex-matched healthy controls (male:female = 129:204; mean age 43.8 years) were recruited (Table 1). The site of disease was mainly proctitis for UC and colonic for CD (Table 2). Osteopenia was significantly more common among IBD patients (13.51%) than the controls (4.5%) ( $P = 0.001$ ). Osteopenia ( $T < -1$ ) was also significantly more common in IBD patients than in controls (35.1% vs 22.5%,  $P = 0.008$ ). The prevalence of osteoporosis was not significantly different between patients with UC (14.45%) and CD (10.71%) ( $P = 0.616$ ). On bivariate analysis, age, female sex, menopause, presence of IBD, severity of disease and use of systemic steroids were found to be associated independently with the

**Table 1 Characteristics of inflammatory bowel disease (IBD) patients and controls *n* (%), mean  $\pm$  SD**

	<b>IBD patients (n = 111)</b>	<b>Controls (n = 333)</b>	<b>P value</b>
Male:Female	43:68	129:204	1.000
Age (yr)	42.5 $\pm$ 14.19	43.8 $\pm$ 11.2	0.368
Postmenopausal women	29 (42.6)	83 (40.7)	0.778
Fractures	12 (10.8)	20 (6)	0.073
BMI (kg/m <sup>2</sup> )	21.3 $\pm$ 4.45	23.8 $\pm$ 4.47	< 0.001
Disease duration (yr)	5.66 $\pm$ 5.72		
Corticosteroid use	74 (66.7)	3 (0.9)	< 0.001
Current smokers	4 (3.6)	29 (8.7)	0.094

**Table 2 Disease location in IBD patients**

	<b>Frequency</b>	<b>Percent</b>
Ulcerative		
Distal	46	56.8
Left sided	23	28.4
Total	12	14.8
Total	81	100.0
Crohn's disease		
Upper GI	2	6.7
Small bowel	4	13.3
Colon	21	70.0
Small bowel & colon	3	10.0
Total	30	100.0

**Table 3 Summary of multiple logistic regression analyses**

<b>Variable</b>	<b>Regression coefficient</b>	<b>P value</b>	<b>OR (95% CI)</b>
Constant	-7.478		
Age	0.78	0.001	1.081 (1.032-1.133)
Sex <sup>1</sup>			
Female (pre-menopausal)	0.898	0.213	2.456 (0.597-10.108)
Female (menopausal)	1.271	0.024	3.563 (1.179-10.763)
Using steroids <sup>2</sup>	2.082	< 0.001	8.021 (2.693-23.891)

<sup>1</sup>Comparison group is males; <sup>2</sup>Comparison group is not using steroids.

occurrence of osteoporosis. In the multivariate logistic regression model, age, sex, menopausal status and use of steroids were significant predictors of osteoporosis (Table 3). IBD was not a significant predictor of osteoporosis. With each advancing year of age, there was a 1.081 times increase in the likelihood of the development of osteoporosis. Premenopausal women were 2.5 times more likely to have osteoporosis than men, and menopausal women were 3.6 times more likely to have osteoporosis than men. Steroid use increased the risk of osteoporosis by eightfold.

## DISCUSSION

The prevalence of osteoporosis and osteopenia in our IBD patients was significantly higher than in community controls. However, on multiple logistic regression analysis, only use of systemic steroids, age and menopause were found to be significant independent risk factors for osteoporosis. The presence of IBD and its severity were not, nor were the number of relapses, duration of illness, or treatment other than systemic steroids. The increased frequency of osteoporosis in our IBD patients was likely to have been caused by use of systemic steroids rather than by IBD itself. This is different to western studies, and we cannot explain this difference.

We did not find a significant difference in prevalence of osteoporosis between patients with UC and CD, although we admit that the number of CD patients in our sample was small, with mainly colonic involvement. This is in agreement with some but not all western data<sup>[26,27]</sup>. Our finding that there was no association between the occurrence of osteoporosis and severity

of IBD, number of relapses, duration of illness, and treatment other than systemic steroids, agrees with the findings of Western studies<sup>[28,29]</sup>.

We also noted a difference in the fracture risk between the two groups: 10.8% in the IBD group and 6% in the control group. However, this did not reach statistical significance, as our study was probably not adequately powered to investigate this complication. This finding is not surprising and could be attributed to steroid use as in western studies<sup>[30]</sup>.

There are several methodological weaknesses in our study. We designed this as a comparative study rather than a case-control study, as that would have been difficult to perform in an Asian country where the prevalence of IBD is much lower than in the West. We also used pDEXA scanning instead of central DEXA to diagnose osteoporosis. However, although central DEXA scanning is accepted widely as the gold standard for diagnosis of osteoporosis, there have been many studies showing that pDEXA is a good alternative, especially in the community setting<sup>[7,8]</sup>.

In conclusion, IBD does not appear to be an independent risk factor for the occurrence of osteoporosis in this population. The increased frequency of osteoporosis in our IBD patients is likely to be related to the use of systemic steroids. However, our finding that osteoporosis is more common in IBD patients, even though it may only be related to steroid use, has a direct bearing on patient management, as new guidelines advise the routine use of bisphosphonates in IBD patients with a BMD of < -1.5<sup>[31]</sup>.

## COMMENTS

### Background

Inflammatory bowel disease (IBD) is a well-recognized risk factor for osteoporosis in Caucasian patients. However, there have been very few studies on Asian patients that have investigated this problem. To the best of our knowledge, there have been no studies on this topic in Southern Asians. However, since there are obvious genetic differences between the two populations it an important area of study that has been neglected.

### Research frontiers

The genetics of IBD is a rapidly expanding field. To support this type of work, good phenotypic data from different cohorts of patients across continents are important. In studying osteoporosis, it is important to have similar data that will help in subsequent genetic studies.

### Innovations and breakthroughs

In the present study, the authors showed that IBD was not an independent risk

factor for osteoporosis, but rather the use of systemic steroids was a risk factor for the development of osteoporosis.

### Applications

It is important to know that not all Asian patients with IBD need routine bisphosphonates, as these are expensive drugs. This study will help to target whom to treat. Also, in future genetic studies, phenotypic racial differences will be important in the search for specific genes.

### Terminology

Osteoporosis is a metabolic bone disease that is characterized by reduced bone mineral density. It is usually asymptomatic until it results in fractures. It is diagnosed using dual energy X-ray absorptiometry. IBD is a chronic disease of unknown etiology that comprises Crohn's disease and ulcerative colitis.

### Peer review

This study dealt with the prevalence and risk factors of osteoporosis in adult Sri Lankan IBD patients. It is a well conceived and analyzed study.

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BRIEF ARTICLES

## Assessment of the hepatic microvascular changes in liver cirrhosis by perfusion computed tomography

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**Author contributions:** Chen ML designed the study, performed the majority of experiments and wrote the manuscript; Zeng QY and Huo JW provided reagents and analytical tools and were also involved in editing the manuscript; Yin XM and Li BP coordinated and provided the collection of all the human material; Liu JX provided some materials.

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**CONCLUSION:** The hepatic microvascular changes in patients with liver cirrhosis can be quantitatively assessed by perfusion CT. Hepatic microvascular parameters (MTT and PS), as measured by perfusion CT, were significantly altered in decompensated cirrhosis.

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**Key words:** Tomography; X-ray computed; Hepatic microcirculation; Cirrhosis

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### Abstract

**AIM:** To assess the hepatic microvascular parameters in patients with liver cirrhosis by perfusion computed tomography (CT).

**METHODS:** Perfusion CT was performed in 29 patients without liver disease (control subjects) and 39 patients with liver cirrhosis, including 22 patients with compensated cirrhosis and 17 patients with decompensated cirrhosis, proved by clinical and laboratory parameters. CT cine-scans were obtained over 50 s beginning with the injection of 50 mL of contrast agent. Hepatic microvascular parameters, mean transit time (MTT) and permeability surface area product (PS) were obtained with the Perfusion 3 software (General Electric, ADW 4.2).

**RESULTS:** The overall differences of MTT and PS between control subjects, patients with compensated cirrhosis and those with decompensated cirrhosis were statistically significant ( $P = 0.010$  and  $P = 0.002$ , respectively). MTT values were  $15.613 \pm 4.1746$  s,  $12.592 \pm 4.7518$  s, and  $11.721 \pm 4.5681$  s for the three groups, respectively, while PS were  $18.945 \pm 7.2347$  mL/min per 100 mL,  $22.767 \pm 8.3936$  mL/min per 100 mL, and  $28.735 \pm 13.0654$  mL/min per 100 mL. MTT in decompensated cirrhotic patients were significantly decreased compared to controls ( $P = 0.017$ ), whereas PS values were remarkably increased ( $P = 0.001$ ).

### INTRODUCTION

Mean transit time (MTT) and permeability surface area product (PS) of contrast material are two important parameters of perfusion computed tomography (CT). MTT is defined as the time that a contrast agent takes to go through the liver, from entry to exit and averaged over all possible paths. The PS is considered as the speed of transfer of a contrast agent from the capillary endothelium to the intercellular space (one-way). These parameters are, to a certain extent, two descriptors of parenchymal microvascular changes.

In chronic liver diseases, a capillarization of the sinusoids is observed, which is characterized by endothelial fenestration, collagen deposition in the extravascular Disse's space, formation of basal laminae<sup>[1-3]</sup>, and increased intrahepatic vascular resistance<sup>[4,5]</sup>. All these microvascular changes in cirrhosis have great influence on the development of the hepatic function<sup>[6-8]</sup>. In order to evaluate the quantitative and qualitative microvascular alterations in liver cirrhosis, these hepatic perfusion parameters (both MTT and PS) were measured in patients with liver cirrhosis by perfusion CT and the results compared to those of healthy volunteers.

## MATERIALS AND METHODS

### **Patients**

Sixty-eight adult patients were studied, including 29 controls and 39 patients with cirrhosis (22 with compensated cirrhosis and 17 with decompensated cirrhosis, divided by two experienced physicians specialized in hepatology). Four additional patients with cirrhosis were excluded because of surgically confirmed hepatocellular carcinoma. Three cirrhotic patients were excluded due to a disagreement on the assignment to the compensated or decompensated cirrhosis group. The 29 controls without liver disease (8 women, 21 men; age range: 28–78 years; average age: 49.5 years) performed perfusion CT examination of the abdomen for unrelated causes. In these 29 controls, lack of liver disease was documented by history, physical examination, laboratory screening, and Doppler sonography of the liver. Twenty-two patients (4 women, 18 men; age range: 31–78 years; average age: 58.6 years) had compensated cirrhosis. Among them, one had alcoholic cirrhosis, two had primary biliary cirrhosis, three had cryptogenic cirrhosis and 16 had posthepatitic cirrhosis. Seventeen patients (5 women, 12 men; age range: 31–78 years; average age: 55.9 years) had decompensated cirrhosis. The origin of cirrhosis in these cases was alcohol in one, hepatitis C virus in one, cryptogenic in one, primary biliary cirrhosis in one, and hepatitis B in the remaining 13 patients. All diagnoses were confirmed by appropriate clinical and laboratory examinations. No patient had portal thrombosis at ultrasonography. The study was approved by the ethics committee at our institution and was in conformity with the ethical guidelines of the 1975 Declaration of Helsinki<sup>[9]</sup>. The patients gave informed consent to participate in the study.

### **Imaging**

After an overnight fast, multiple-slices dynamic sequences were carried out. A fixed 5 mm thick slice, which was selected to include the right hepatic lobe, spleen and portal trunk, was repeatedly scanned with cine mode. Scanning was carried out using a low radiation dose (120 kV, 60 mA), cine-scan mode, standard reconstruction algorithm, 35 cm display field of view (LightSpeed RT, GE Medical Systems, Milwaukee, WI) with a 50 s of continuous scanning time set at 8 s after the injection of contrast material. A bolus infusion of 50 mL of contrast material [Omnipaque (iohexol); 350 mg I/mL; Beijing, China] was given at 5.5 mL/s via a 20 gauge intravenous catheter in the antecubital vein. Patients were advised to hold their breath as long as possible, and oxygen inhalation at 2 L/min was provided to facilitate long breathholding during scanning.

### **Data analysis**

These images were transferred to a workstation (GE Advantage workstation 4.2) for data analysis by using Perfusion 3 software. Three regions of interest (ROI) were set on the abdominal aorta, the portal vein trunk, and the right liver lobe respectively, by two radiologists

with 10 years of experience in gastrointestinal and hepatobiliary imaging. The former two ROIs were set as input function. The latter ROI of the right liver lobe was drawn on the whole visible right lobe carefully, avoiding blood vessels and margin of liver parenchyma. The value of the latter ROI was measured and averaged for the hepatic perfusion parameters.

### **Statistical analysis**

The results of the two perfusion parameters (MTT, PS) were expressed as the mean  $\pm$  SD. All data were analyzed by SPSS 11.5 software. The independent samples *t* test was used to determine differences between cirrhotics and controls. Data of the three groups (normal control subjects, patients with compensated cirrhosis, and patients with decompensated cirrhosis) were compared using one-way analysis of variance. Bonferroni's correction was applied. Statistical significance was defined as a *P* < 0.05.

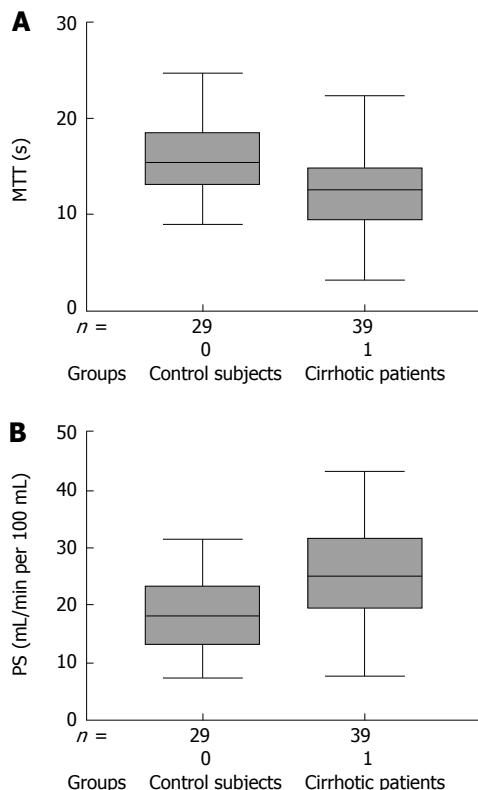
## RESULTS

### **Differences between the controls and the cirrhotics**

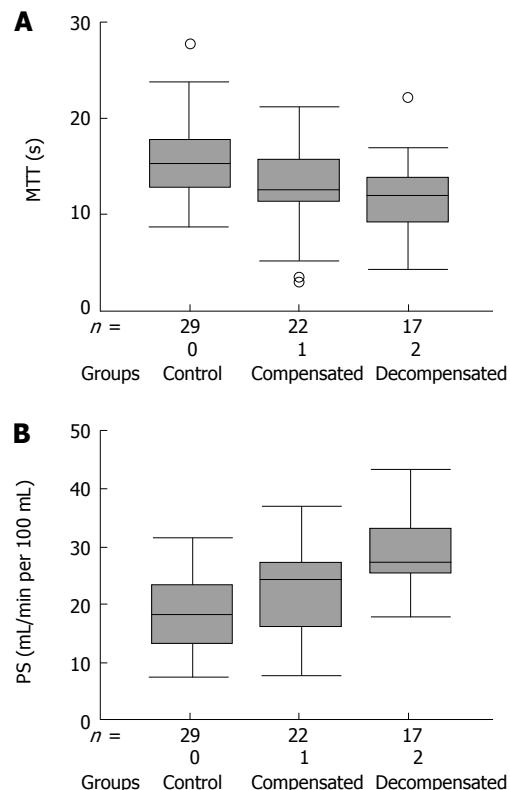
The results summarizing the MTT and PS of the controls and the cirrhotics are shown in Figure 1 and Table 1. The MTT in cirrhotic livers ( $12.212 \pm 4.632$  s) was significantly shorter than in controls subjects ( $15.613 \pm 3.942$  s), the difference being statistically significant (*P* = 0.002). PS was increased in cirrhosis compared with controls ( $24.964 \pm 8.298$  against  $18.945 \pm 7.235$  mL/min per 100 mL), and it was also significant (*P* = 0.003).

### **Differences among the three groups**

The hepatic perfusion parameters for the control subjects group, compensated cirrhosis group, and decompensated cirrhosis group are shown in Figure 2 and Table 2. Compared with the control subjects, the MTT of patients with compensated cirrhosis were decreased ( $12.592 \pm 4.7518$  s vs  $15.613 \pm 3.942$  s), and it diminished further in decompensated cirrhosis patients ( $11.721 \pm 4.568$  s) compared to compensated patients. The difference between controls and compensated cirrhotics was not significant (*P* = 0.059), nor was the difference between compensated and decompensated cirrhotics (*P* = 1.000). However, there was a markedly significantly difference between the control subjects and the decompensated cirrhotic patients (*P* = 0.017). One of the subjects, a 75 years old individual, had a MTT of 27.7 s, the highest value observed in the whole study. Another patient, with posthepatitic decompensated cirrhosis, had a rather high MTT value of 22.3 s. Other two patients in the compensated cirrhosis group showed much lower values (3.32 s and 3.79 s). The results of the PS were  $18.945 \pm 7.235$  mL/min per 100 mL,  $22.767 \pm 8.3936$  mL/min per 100 mL and  $27.806 \pm 7.4730$  mL/min per 100 mL in the three groups (controls, compensated cirrhotics and decompensated cirrhotics, respectively). The PS in compensated cirrhotic patients did not show statistically significant difference compared to control subjects (*P* = 0.25), nor was significant the



**Figure 1 Distribution of hepatic microvascular parameters in controls and cirrhotics.** Box-whisker plots are shown, in which the lower box boundary indicates the 25th percentile, the line within the box marks the median and the top box boundary indicates the 75th percentile. Error bars below and above the boxes indicate 10th and 90th percentiles. Other data are represented as individual dots. The graphs show box-whisker plots of mean transit time (MTT) (A) and permeability surface area product (PS) (B). n: Number of patients.



**Figure 2 The box-whisker plots show the distribution of hepatic microvascular parameters in controls as well as in compensated and decompensated cirrhosis patients. The values of MTT (A) and PS (B) are shown.**

**Table 2 Comparison among control subjects and compensated and decompensated cirrhosis patients on perfusion parameters (mean  $\pm$  SD)**

Group	n	MTT (s)	PS (mL/min per 100 mL)
Control	29	15.613 $\pm$ 4.175	18.945 $\pm$ 7.235
Compensated	22	12.592 $\pm$ 4.752	22.767 $\pm$ 8.394
Decompensated	17	11.721 $\pm$ 4.568	27.806 $\pm$ 7.473

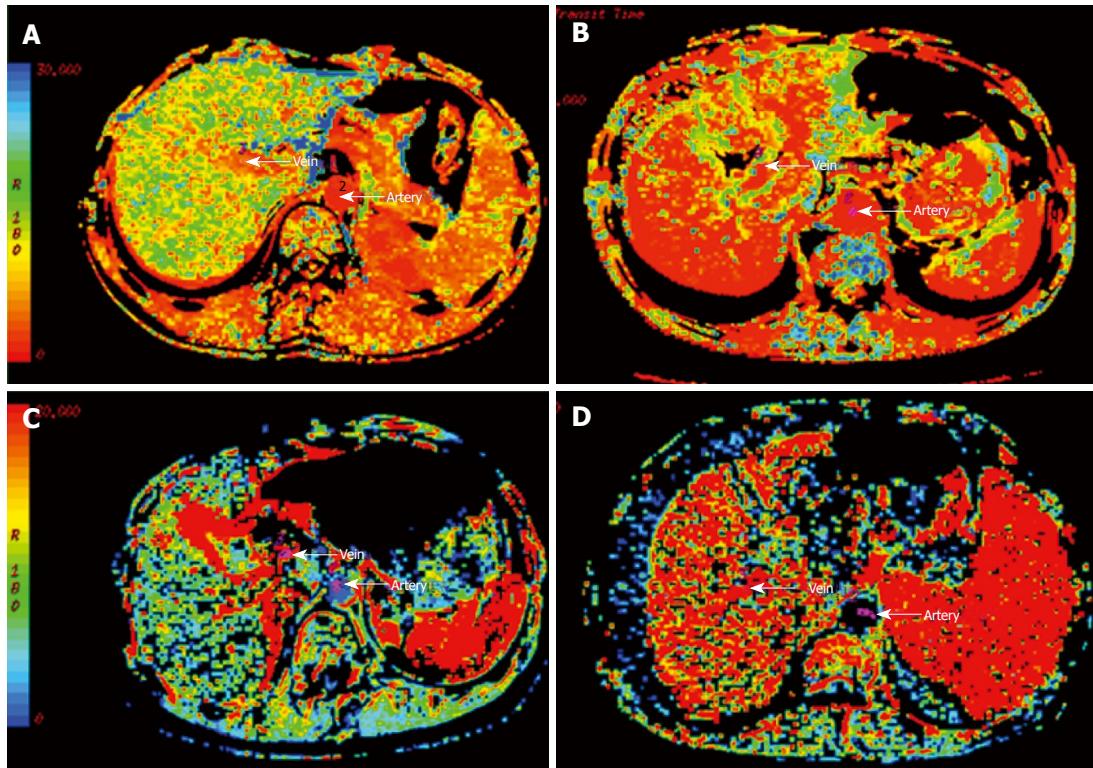
Mean transit time (MTT) in cirrhotics was shorter than in the controls ( $P = 0.002$ ). Permeability surface area product (PS) was higher in cirrhotics than in controls ( $P = 0.003$ ).

difference between compensated and decompensated cirrhotics ( $P = 0.139$ ). However, compared with controls, the PS of decompensated cirrhotic patients increased remarkably, the difference reaching statistical significance ( $P = 0.001$ ).

## DISCUSSION

A simple, noninvasive technique to separately quantify the changes of arterial and portal venous components in cirrhosis has always been a long-standing ambition of both pathologists and physicians. Most authors have reached an agreement on the notion that the increase of intrahepatic vascular resistance leads to a decrease of the portal fraction of liver perfusion<sup>[4,5]</sup>. This decreased portal perfusion is partially compensated by an increase of arterial flow, with the total liver perfusion being

reduced in cirrhotic patients<sup>[4,10,11]</sup>. More importantly, since the changes of the hepatic microcirculation in cirrhosis influence the progression of the disease, such a technique would be of greater value in hepatology. Various methods exist for the determination of hepatic microcirculation in clinical practice<sup>[12-15]</sup>. Most of them, however, are invasive or of controversial significance<sup>[12,16]</sup>. It's possible to measure both arterial and portal venous flow at the level of the main vessels using Doppler sonography, but this gives an indirect measurement of the circulation at parenchymal level. It is much harder to assess alterations in capillary blood, i.e. at the level of the microcirculation. Nuclear medicine techniques have been hindered by limited spatial and temporal resolution, and used only to estimate the arterial relative to the portal venous blood flow<sup>[17-19]</sup>, since they can hardly differentiate the overlapping fractions of hepatic artery and portal vein perfusion within the liver tissue.



**Figure 3 MTT and PS maps between controls and cirrhotic patients.** MTT in a control subject (A) was higher than in a cirrhotic patient (B). PS in a control subject (C) was lower than in a cirrhotic patient (D).

Reports have shown that the hepatic microcirculation can be estimated by the hepatic clearance of sorbitol<sup>[8,13]</sup> due to its high extraction fraction in normal subjects. The clearance of sorbitol is flow-limited and reflects hepatic microcirculation through functional sinusoids. However, sorbitol has been used as a marker of functional perfusion, which raises the issue of the decrease of the hepatocyte extraction of sorbitol in cirrhosis<sup>[20]</sup>. Moreover, the sorbitol clearance method cannot separate the measurements of arterial and portal flows. In addition, it needs to collect urine to determine the renal clearance of sorbitol after several hours, which may cause logistic problems in a busy clinical department<sup>[20]</sup>.

CT is suited for the measurement of perfusion. Because of the high spatial and temporal resolution, modern CT scanners have been used widely in clinical studies, diagnosis and treatment. The methods for quantifying tissue perfusion by dynamic CT were described previously<sup>[14,21]</sup>. Many studies have been conducted using this similar method with the use of iodinated contrast agents<sup>[5,22,23]</sup>. This technique measures the slope of the time-attenuation curve, and only the peak time-points of the aortic and portal time-attenuation curves have been applied. The procedure can quantify the arterial and portal venous blood flow at tissue level, but the use of single-slice CT hampers its limited temporal resolution and Z-axis spatial resolution<sup>[22,24,25]</sup>. Some respiratory motion artifacts are unavoidable, and they considerably influence the quantification. We used here a 16 slices spiral-CT with super high Z-axis spatial resolution and temporal

resolution, which avoided the motion artifacts in an efficient way. The perfusion parameters were measured by the deconvolution method, which uses a well-established compartmental modeling technique<sup>[26]</sup>. Several points of the time-attenuation curves were used. Arterial and portal venous perfusion was detected quantitatively. In particular, the MTT and the PS could be quantified. These parameters were important and critical to assess the function of liver, as shown by many studies in humans and animals.

The MTT mainly reflects the blood capillary's time. Some early studies almost exclusively depended on *ex vivo* evaluations using the same multiple indicator dilution technique described previously<sup>[8,13]</sup>. A study<sup>[25]</sup> on MTT had already relied upon CT, and its results were in agreement with the findings of the indicator dilution techniques. These investigators observed that the small-molecular-weight contrast agent used in their study had an increased transit time in cirrhotic patients. Some other studies<sup>[27,28]</sup> reported no difference in MTT between controls and cirrhotic patients. In the present study, however, the MTT values observed in control subjects (Figure 3A) were higher than in cirrhotic patients (Figure 3B). The MTT of contrast agent measured in the cirrhotic livers was significantly shorter than among controls ( $P = 0.002$ ) (Figure 1A). He *et al*<sup>[29]</sup> had already reported similar results, but the difference was not statistically significant. We hypothesize that there may be some differences as to etiopathogenesis, disease severity and gender distribution among the cirrhotic patients in previous studies<sup>[5,30,31]</sup>. The patients enrolled in our study (women/men: 9/30) had a cirrhosis mostly

due to hepatitis B virus (29/39) and only few had alcoholic cirrhosis (2/39), whereas no patients with hepatitis B virus infection and nine alcoholics of 18 cirrhotic patients (women/men: 6/12) were included in a previous study<sup>[25]</sup>. Histopathological examinations have shown that in the cirrhotic liver several sinusoids become capillarized and many terminal hepatic venules are distended<sup>[32,33]</sup>: the combination of these changes may lead to low-resistivity, high-speed vascular flow, with increased inflow and outflow. In addition, intrahepatic portal veins and hepatic veins cross cirrhotic areas, leading directly or indirectly into the central venous compartment. All of the above causes may have brought about the shorter MTT in cirrhotic patients.

The PS shows the permeability of blood capillaries. As shown in this study, PS may be quantified noninvasively using perfusion CT scans acquired from control subjects and patients with cirrhosis. We observed that the PS of controls (Figure 3C) was lower than in cirrhotic patients (Figure 3D): PS was in fact significantly increased in cirrhosis ( $P = 0.003$ ), (Figure 1B). This change may be explained by the results obtained with multiple indicator dilution techniques in human cirrhosis or rat cirrhotic liver. The increased microvascular resistance in the cirrhotic liver reduces capillary blood flow; the reduction in capillary blood flow is compensated by liver arterialization, increasing the capillary perfusion<sup>[4,10,11]</sup>.

In addition, we observed that MTT and PS measured by the perfusion CT tended to change significantly in patients with decompensated cirrhosis (Table 2). Compared to controls, the MTT of patients with compensated cirrhosis was decreased, while the PS was increased: however, these differences failed to reach a statistically significant level in our study. We cannot exclude that some microvascular changes might indeed occur also in cases with compensated cirrhosis (Figure 2), and that the differences between compensated cirrhotic patients and the other groups may become significant simply by increasing the size of the study population. In contrast, the microvascular perfusion parameters (both MTT and PS) were significantly altered in decompensated cirrhosis.

However, because it was difficult to control all the aspects of the study, there are several limitations associated with this study. First, the radiation dose may be one of the most important issues when using the perfusion CT, especially with cine-scan mode. Secondly, during CT imaging, patients are requested to hold their breath for long periods of time. Besides, a further limitation of our study is the small overall sample size. Perhaps, the differences between compensated cirrhosis group and the other groups would turn significant if the overall sample size increased.

Despite these limitations, our study with perfusion CT has proven that the perfusion parameters (MTT and PS) are significantly changed in patients with cirrhosis. The decreased MTT and increased PS of an iodinated contrast agent correlate with the severity of liver cirrhosis. They may be two acceptable indicators of the degree of hepatic microcirculation alteration in

patients with cirrhosis. In addition, the quantification by perfusion CT may also improve our understanding of the effects on the hepatic microcirculation of vasoactive drugs and interventional procedures for the treatment of cirrhosis<sup>[34-36]</sup>.

These findings underscore the possibility of using perfusion CT as a marker of hepatic microcirculation. Perfusion CT may be used as a noninvasive tool to quantify hepatic microvascular parameters in cirrhotic liver.

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## COMMENTS

### Background

Hepatic microcirculation is of great importance in hepatology. Its changes in cirrhosis may heavily influence the progression of the disease. Various methods exist for the determination of hepatic microcirculation in clinical practice. However, most of them are invasive or controversial.

### Research frontiers

Mean transit time (MTT) and permeability surface (PS) area product of contrast material for quantifying hepatic vascular changes by dynamic computed tomography (CT) were rarely measured in previous studies. The research hotspot is whether hepatic microvascular changes can be quantified by perfusion CT and what kind of modifications can be measured.

### Innovations and breakthroughs

Previous applications of perfusion CT to the cirrhotic liver focused on hepatic haemodynamics. Most studies have reached an agreement on the fact that the increase of intrahepatic vascular resistance leads to a decrease of the portal fraction of liver perfusion. This decreased portal perfusion is partially compensated by an increase of arterial inflow, while the total liver perfusion is reduced in cirrhotic patients. However, hepatic microvascular changes were ignored. Few studies on MTT and PS have already relied upon CT. In order to explore hepatic microvascular parameters in patients with liver cirrhosis by using the perfusion CT, the authors compared hepatic microvascular parameters in patients with cirrhosis with those found in healthy volunteers. MTT and PS were significantly altered in liver cirrhosis. In order to obtain some meaningful correlations of the MTT and PS values with the severity of cirrhosis, they measured these parameters in three groups: control subjects, and patients with compensated or decompensated cirrhosis. They found that these parameters were significantly altered in patients with decompensated cirrhosis.

### Applications

Hepatic microvascular parameters may be used to quantitatively assess hepatic microcirculation: the findings correlate with the severity of liver cirrhosis. These data may improve our understanding of the hepatic microcirculation effects of vasoactive drugs and interventional procedures for the treatment of cirrhosis.

### Terminology

MTT: Mean transit time, a parameter defined as the time that a contrast agent takes to cross the liver, from entry to exit, averaged across all possible paths.

PS: Permeability surface area product, which is considered as the speed of a contrast agent from the capillary endothelium to the intercellular space (one-way).

### Peer review

The authors examined the differences in MTT and PS between normal individuals and patients with cirrhosis; they propose that perfusion CT may be utilized to quantify changes in microcirculation among stages of cirrhosis. Overall, an interesting study.

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BRIEF ARTICLES

## Urokinase perfusion prevents intrahepatic ischemic-type biliary lesion in donor livers

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reduce the incidence of IITBLs.

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**Key words:** Cadaver donor; Liver transplantation; Ischemic-type biliary lesion; Urokinase

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### Abstract

**AIM:** To evaluate whether urokinase perfusion of non-heart-beating cadaveric donor livers reduces the incidence of intrahepatic ischemic-type biliary lesions (IITBLs).

**METHODS:** A prospective study was conducted to investigate potential microthrombosis in biliary microcirculation when non-heart-beating cadaveric livers were under warm or cold ischemic conditions. The experimental group included 140 patients who underwent liver transplantation during the period of January 2006 to December 2007, and survived for more than 1 year. The control group included 220 patients who received liver transplantation between July 1999 and December 2005 and survived for more than 1 year. In the experimental group, the arterial system of the donor liver was perfused twice with urokinase during cold perfusion and after trimming of the donor liver. The incidence of IITBLs was compared between the two groups.

**RESULTS:** In the control group, the incidence of IITBLs was 5.9% (13/220 cases) after 3-11 mo of transplantation. In the experimental group, two recipients (1.4%) developed IITBLs at 3 and 6 mo after transplantation, respectively. The difference in the incidence between the two groups was statistically significant ( $P < 0.05$ ).

**CONCLUSION:** Double perfusion of cadaveric livers from non-heart-beating donors with urokinase may

### INTRODUCTION

Intrahepatic ischemic-type biliary lesions (IITBLs) after orthotopic liver transplantation are the most frequent cause of non-anastomotic biliary strictures in liver grafts<sup>[1-4]</sup>. They affect 2%-19% of patients after liver transplantation and have become a leading cause of liver re-transplantation in China<sup>[5,6]</sup>. Several risk factors for IITBLs have been identified, including ischemia-related injury, immunologically induced injury, and cytotoxic injury<sup>[7-9]</sup>. Although IITBLs have a multifactorial origin, ischemia-reperfusion injury and hepatic arterial thrombosis are considered to be the major causes<sup>[10-12]</sup>. In recent years, however, impaired biliary microcirculation has led to an increasing concern. Improving microcirculation can increase oxygen supply and might prevent biliary injuries. Urokinase is a proteolytic enzyme produced by the kidney, which is found in the urine. Urokinase acts on the endogenous fibrinolytic system by converting plasminogen to plasmin, which, in return, degrades fibrin clots. Since January 2006, our center has used a procedure based on the therapeutic principle of urokinase to prevent potential microthrombosis in biliary microcirculation. We have used non-heart-beating cadaveric donor grafts in which warm or cold ischemic insult was induced and which were perfused with urokinase; this procedure has produced good results.

In the present study, we evaluated prospectively 140 liver transplantation patients who received grafts with urokinase perfusion.

## MATERIALS AND METHODS

### Ethics

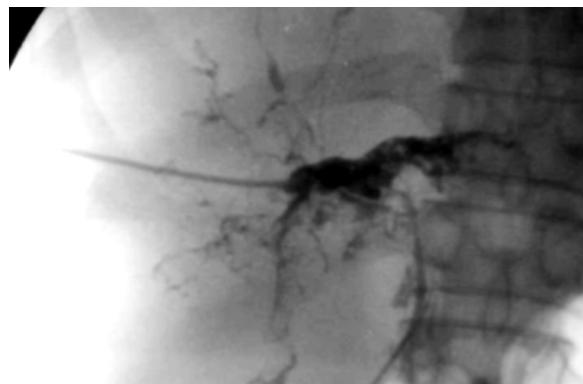
This study was approved ethically by Beijing Chaoyang Hospital. All patients provided written consent. All donors were volunteers and signed consent documents before donation.

### Clinical data

Between July 1999 and December 2005, 220 patients (176 male, 44 female; age, 14–71 years) received orthotopic liver transplantation in the Department of Hepatobiliary Surgery, Beijing Chaoyang Hospital, which is affiliated to Capital Medical University (Beijing, China). All patients survived for more than 1 year. They received ABO-compatible non-heart-beating cadaveric donor livers without urokinase perfusion. The arterial system of the donor livers was perfused with 2000 mL cold histidine-tryptophan-ketoglutarate (HTK) solution (4°C). The portal system was perfused with 2000 mL HTK solution, followed by 2000 mL University of Wisconsin (UW) solution for preservation (4°C). The biliary system was decompressed using the trocar technique. The common bile duct was perfused with normal saline at low pressure. The warm ischemia time for the donor livers was 2–8 min, and the cold preservation time was 2–13.5 h. The secondary warm ischemia for the biliary tract lasted 25–90 min. Primary diseases of the patients included posthepatitis cirrhosis in 148, primary hepatic carcinoma in 56, alcoholic cirrhosis in seven, fulminant hepatitis in four, hepatolenticular degeneration in three, and primary sclerosing cholangitis in two.

Since January 2006, all patients received donor livers with urokinase perfusion. Until December 2007, 140 patients (108 male, 32 female; age, 16–69 years) underwent liver transplantation and survived for more than 1 year. We used 2000 mL HTK solution that contained 2 MU urokinase for perfusion through the arterial system. After trimming of the donor liver, the arterial system was perfused again with 1 MU urokinase. The residual urokinase was washed out using 500 mL HTK solution before implantation. The warm ischemia time for donor livers was 2–10 min, and the cold preservation time was 2.5–15 h. The secondary warm ischemia for the biliary tract lasted 20–115 min. Primary diseases of the recipients included posthepatitis cirrhosis in 96, primary hepatic carcinoma in 34, alcoholic cirrhosis in three, fulminant hepatitis in two, hepatolenticular degeneration in two, primary sclerosing cholangitis in two, and autoimmune liver disease in one.

The immunosuppressive regimen for all the patients included cyclosporin A (CsA) (Novartis, Switzerland) or Prograf (FK506) (Astellas, Ireland), mycophenolate mofetil (CellCept; Roche, United States), and steroids. The trough CsA level was maintained at 200–300 µg/L and that of FK506 was maintained at 8–12 µg/L at 3 mo after operation. We compared the incidence of IITBLs in the patients who received grafts with or without donor urokinase perfusion.



**Figure 1** Percutaneous transhepatic cholangiography shows intrahepatic ITBL. Variant degrees of diffuse stricture and segmental dilatation of the intrahepatic bile duct in the biliary tree.

### Statistical analysis

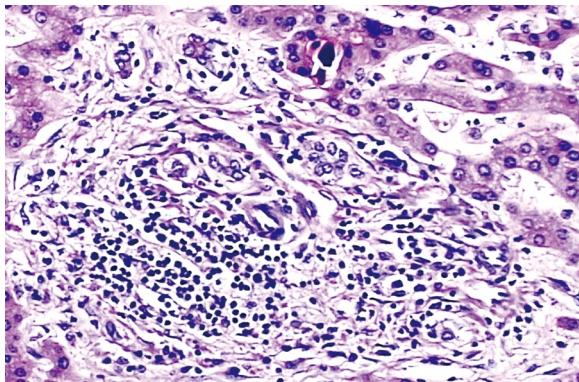
The difference between the two groups of patients was analyzed by a  $\chi^2$  test.  $P < 0.05$  was considered statistically significant. Analysis was performed using SPSS version 11.5 (Chicago, IL, USA).

## RESULTS

The incidence of IITBLs was 5.9% in patients who received grafts without urokinase perfusion (13/220 patients) 3–11 mo (mean, 5.1 mo) after transplantation, and 1.4% in the patients who received grafts with urokinase perfusion (2/140 patients) at 3 and 6 mo after transplantation. The difference in the incidence between the two groups was statistically significant ( $P < 0.05$ ). The main symptom noted in the 15 IITBL patients in both groups was progressive hyperbilirubinemia. Cholangitis was observed as a complication in three of the 15 patients. The results of a biochemical assay showed that the level of total bilirubin increased progressively. The level of direct-reacting bilirubin increased the most, followed by the level of biliary enzymes such as alkaline phosphatase and  $\gamma$ -glutamyltransferase. A slight increase in the levels of aspartate aminotransferase and alanine aminotransferase levels was detected.

Of the 13 IITBL patients who received grafts without urokinase perfusion, four were diagnosed with IITBLs by T-tube cholangiography; two by percutaneous transhepatic cholangiography; four by endoscopic retrograde cholangiography; and three by magnetic resonance cholangiography (MRCP). In the two IITBL patients who received grafts with urokinase perfusion, IITBLs were diagnosed by MRCP. The imaging of the 15 IITBL patients showed varying degrees of diffuse stricture and segmental dilatation of the intrahepatic bile duct, with withered-branch-like changes in the biliary tree (Figure 1). The stricture or embolic lesion of the hepatic artery and its major branches was excluded by Doppler ultrasonography, and acute or chronic rejections were excluded by liver biopsy.

Eight of 13 IITBL patients who received grafts without urokinase perfusion required liver re-trans-



**Figure 2 Cholestasis of hepatocytes, infiltration of inflammatory cells and proliferation of fibrous tissue in the portal area, fibrosis and luminal stenosis of bile ducts.** (HE stain,  $\times 200$ ).

plantation, and the other five patients died from liver failure while still on the waiting list. The two IITBL patients who received grafts with urokinase perfusion underwent liver re-transplantation. The injured grafts were removed from the 10 re-transplantation patients and observed pathologically. Gross findings showed patchy necrosis of the biliary endomembrane, thickened and sclerosed duct wall, and biliary sludge in the narrowed lumen. Microscopic findings showed severe cholestasis of hepatocytes, spotty or patchy necrosis, infiltration of inflammatory cells and mild to moderate proliferation of fibrous tissue in the portal area, necrosis and exfoliation of a great number of endothelial cells in small biliary lumens, formation of bile thrombus, and fibrosis and luminal stenosis of some bile ducts (Figure 2).

## DISCUSSION

IITBLs have become a leading cause of liver re-transplantation in China<sup>[13,14]</sup>. This is closely associated with the fact that non-heart-beating cadaveric livers constitute a major source of donor livers. Several studies conducted in China and other countries have suggested that cadaveric donor grafts with warm ischemic injury leads to a higher incidence of IITBLs than grafts from brain-dead donors. Abt *et al*<sup>[15]</sup> have reported that the ratio of IITBLs in biliary complications was 66.6% with non-heart-beating cadaveric livers and 19.2% with livers from brain-dead donors. Nakamura *et al*<sup>[16]</sup> have reported that the incidence of IITBLs was only 1.4% in recipients grafted with brain-dead donor livers. Zhang *et al*<sup>[17]</sup> have reported that in 235 patients transplanted with cadaveric livers and 36 transplanted with living-donor livers, the incidence of biliary complications was 19.1% and 5.6%, respectively. The incidence of IITBLs was 7.29% and 0%, respectively, and the difference was statistically significant. Researchers in China and other countries have agreed that the difference was mainly caused by warm ischemia.

What kinds of pathological or physiological changes associated with IITBLs may occur in a donor liver during warm ischemia? Generally, the initiation of intrinsic coagulation requires stoppage or slowing of blood flow, high blood viscosity, and injury of vascular

endothelial cells. After these three requirements are met, most non-heart-beating cadaveric donors are usually in a hypercoagulable state during warm ischemia. Thus, blood coagulation or microthrombi may develop rapidly in the arterioles and peribiliary capillary plexus of the donor liver within a short period. The blood clots or microthrombi are not washed out easily by subsequent cold perfusion under normal pressure, which results in inadequate perfusion of the peribiliary capillary plexus. Even after blood circulation in the donor liver returns to normal, the arterioles and peribiliary capillary plexus still lack perfusion with arterial blood. This may lead to ischemia and hypoxia of the bile duct wall; formation of biliary sludge caused by degeneration, necrosis or even exfoliation of a great number of endothelial cells; and luminal stenosis caused by gradual fibrosis of the muscular layers. By periodically sampling livers from rats dying suddenly from cardiac arrest for light microscopy, we observed blood coagulation in most arterioles in the portal area in hepatic tissue samples collected after 25 min of cardiac arrest. For the reasons described above, we adopted the strategy of double perfusion with urokinase, which aimed to achieve maximum dissolution of microthrombi, ensure better effects of cold perfusion and reperfusion, and prevent the development of IITBLs. In the present study, we found that the difference between the two groups was significant, which indicated that urokinase perfusion can effectively reduce the incidence of IITBLs.

The *in vitro* use of urokinase may not have any effect on the coagulation function of patients and is not dose-limited. Thus, urokinase usually can be administered *in vitro* at a higher concentration than when injected intravenously. For better blood circulation in arterioles and the peribiliary capillary plexus, a higher concentration of urokinase could be used to reperfuse the liver after trimming. A urokinase-free preservation solution should be used to perfuse the artery to wash out excess urokinase before completing the whole perfusion process. The activity and function of urokinase at 0–4°C have not been reported yet. In separate tests, a significant thrombolytic effect was observed when fresh blood clots in tubes at 4°C were immersed in urokinase at the above-mentioned concentrations.

The results of the present study confirm that: (1) liver transplantation from non-heart-beating cadaveric donors may lead to a higher incidence of IITBLs and a higher rate of re-transplantation; and (2) double perfusion of cadaveric livers from non-heart-beating donors with urokinase may reduce the incidence of IITBLs.

## COMMENTS

### Background

Biliary complications are a major cause of morbidity and graft failure in patients after orthotopic liver transplantation (OLT). The most troublesome is the so-called intrahepatic ischemic-type biliary lesion (IITBL), which is one of the most important reasons for liver re-transplantation. Therefore, it is clinical significance to reduce the incidence of IITBLs in order to decrease the re-transplantation rate and improve long-term life quality.

## Research frontiers

The incidence of IITBL varies between 2% and 19% after OLT. Although the exact pathophysiological mechanism of IITBL is still unknown, several risk factors of this often cumbersome complication have been identified, strongly suggesting a multi-factorial origin. Therefore, the etiology, development and prophylaxis of IITBL have been hot topics of research.

## Innovations and breakthroughs

Improving biliary microcirculation might prevent biliary injury. In this study, urokinase was perfused through the arterial system during harvesting and after trimming of donor liver in non-heart-beating cadaveric donors. This procedure has produced good results and provides the possibility of reducing IITBLs after OLT.

## Applications

This study may provide a method for clinical research into the prophylaxis of IITBLs after liver transplantation. This technique for prevention of IITBLs is easy to establish, and the results of this study confirm that urokinase perfusion may reduce the incidence of IITBLs from non-heart-beating cadaveric donors.

## Terminology

ITBL is defined as non-anastomotic destruction of the graft's biliary tree after OLT, and is characterized by bile duct destruction, subsequent stricture formation, and sequestration.

## Peer review

This is a good descriptive study in which the authors analyze the preventive effect of urokinase perfusion on IITBLs in non-heart-beating donors after OLT. The results are interesting and the conclusion is encouraging.

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BRIEF ARTICLES

## Clinical application of subjective global assessment in Chinese patients with gastrointestinal cancer

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**Author contributions:** Cao WX designed the study and contributed to the study coordination; Wu BW and Yan M performed all the clinical investigation; Gu ZD took charge of the clinical laboratory data; Wang XJ conducted the data analysis; Wu BW wrote the manuscript; Cao WX and Yin T contributed to the critical review; Liu BY aided in the study coordination.

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### Abstract

**AIM:** To investigate the role of subjective global assessment (SGA) in nutritional assessment and outcome prediction of Chinese patients with gastrointestinal cancer.

**METHODS:** A total of 751 patients diagnosed with gastrointestinal cancer between August 2004 and August 2006 were enrolled in this study. Within 72 h after admission, SGA, anthropometric parameters, and laboratory tests were used to assess the nutritional status of each patient. The outcome variables including hospital stay, complications, and in-hospital medical expenditure were also obtained.

**RESULTS:** Based on the results of SGA, 389 (51.8%), 332 (44.2%), and 30 (4.0%) patients were classified into well nourished group (SGA-A), mildly to moderately malnourished group (SGA-B), and severely malnourished group (SGA-C), respectively. The prevalence of malnutrition classified by SGA, triceps skinfold thickness (TSF), mid-upper arm muscle circumference (MAMC), albumin (ALB), prealbumin (PA), and body mass index (BMI) was 48.2%, 39.4%, 37.7%, 31.3%, 21.7%, and 9.6%, respectively. In addition, ANOVA tests revealed significant differences in body mass index (BMI), TSF, PA, and ALB of patients in different SGA groups. The more severely malnourished the patient was, the lower the levels of BMI, TSF, PA, and ALB were ( $P < 0.05$ ).  $\chi^2$  tests showed a significant difference in SGA classification between patients receiving different types of treatment (surgery vs chemotherapy/radiotherapy). As the nutritional status classified by SGA deteriorated, the patients stayed longer in hospital and their medical expenditures increased significantly. Furthermore, multiple regression analysis showed that SGA and serum ALB could help predict the medical expenditures and hospital stay of patients undergoing surgery. The occurrence of complications increased in parallel with the increasing grade of SGA, and was the highest in the SGA-C group (23.3%) and the lowest in the SGA-A group (16.8%).

**CONCLUSION:** SGA is a reliable assessment tool and helps to predict the hospital stay and medical expenditures of Chinese surgical gastrointestinal cancer patients.

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**Key words:** Gastrointestinal cancer; Subjective global assessment; Surgery; Nutritional assessment; Hospital stay; Medical expenditures; Complication

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## INTRODUCTION

Cancer, one of the serious global health problems today, is considered by the public as a frightening, painful, and untreatable disease that implies death. Approximately 10 million people get cancer and 5 million people face death every year throughout the world. It is estimated that the number of new cancer patients will reach 15 million in 2020<sup>[1,2]</sup>. It was reported about 20% cancer patients die of malnutrition or its relative complications rather than the malignant disease itself<sup>[3]</sup>. Many researchers have suggested that the nutritional status of cancer patients after diagnosis is associated with cancer recurrence and survival rate<sup>[4-6]</sup>, and is generally accepted as an important prognostic factor that determines patients' outcomes including treatment response, survival, and hospital stay<sup>[7-13]</sup>. Furthermore, some studies showed that good nutrition in patients with cancer can improve their quality of life<sup>[14-16]</sup>. The objective of nutritional assessment is to accurately define the nutritional status of patients, diagnose clinically relevant malnutrition, and monitor changes in nutritional status. Comprehensive and accurate information on nutritional status of patients with gastrointestinal cancer helps decide whether surgery or chemotherapy can be delayed. A number of tools have been developed for the assessment of nutritional status<sup>[17]</sup>.

Subjective global assessment (SGA) is an easy, noninvasive, and cost-effective method for the assessment of nutritional status by identifying whether the patients are malnourished or at a risk of becoming malnourished<sup>[18]</sup>. Although SGA has been originally developed to identify poor nutritional status in patients undergoing gastrointestinal surgery<sup>[19]</sup>, it can be used to quantify the prevalence of malnutrition in patients with chronic and end-stage renal failure during hemodialysis or peritoneal dialysis<sup>[20-22]</sup>. In addition, SGA is a powerful predictor of postoperative complications in general surgery<sup>[23]</sup>, liver transplantation<sup>[24]</sup>, and in patients on dialysis<sup>[25]</sup>. Although SGA has been used widely for more than two decades all over the world, few studies are available on its clinical value in Chinese cancer patients. This study was to investigate whether SGA can reliably identify malnourished patients and predict the clinical outcomes of Chinese gastrointestinal cancer patients.

## MATERIALS AND METHODS

### Ethics

This study was approved by the relevant research board and the ethics committee in Shanghai, China. All patients gave their informed consent to participate in this study.

### Patients

Adult patients diagnosed with gastrointestinal cancer (including stomach, colon, or rectal cancer) from August 2004 to August 2006 were enrolled in this study. Eligibility criteria included (a) patients diagnosed by pathology or cytology, (b) patients scheduled to undergo treatment modalities including radiotherapy

or chemotherapy or surgery, (c) patients older than 18 years, (d) patients able to read and comprehend Chinese, and (e) patients giving their informed consent. Patients with cognitive impairment, mental disorder, or communication problems were excluded from this study. The final number of recruited subjects was 751 (including 591 newly diagnosed and 160 previously diagnosed cancer patients). Of them, 384 (51.1%) were gastric cancer patients, 367 (48.9%) were colorectal cancer patients. The male/female ratio was 455/296 with a median age of 69 years (range 23-92 years). Of the 591 newly diagnosed cancer patients, 505 underwent surgery and 86 underwent chemotherapy or radiotherapy due to tumor metastasis, while the 160 previously diagnosed cancer patients received radiotherapy or chemotherapy during their hospital stay.

### Nutritional assessment

An initial assessment of nutritional status in all recruited patients was made within 72 h after admission. To avoid possible variance among observers, SGA was performed by trained researchers. Anthropometric data including body weight, height, triceps skinfold thickness (TSF), mid-upper arm circumference (MUAC), and laboratory data including albumin (ALB) and prealbumin (PA) were collected.

### Subjective global assessment

SGA of nutritional status in patients was performed based on their medical history and physical examination. Changes in weight, dietary intake, functional capacity, gastrointestinal symptoms, metabolic stress, loss of subcutaneous fat, muscle wasting, and ankle/sacral edema of the patients were recorded. After careful assessment, the changes in medical history and physical examination were classified as grade A, B, or C (Table 1). Finally, the assessment results were accumulated. If the total number of grade C was more than 5, the nutritional status of patients was classified as severely malnourished. If the total number of grade B was more than 5, the nutritional status of patients was classified as mildly to moderately malnourished. If the total number of grade C and B was less than 5, the nutritional status of patients was classified as well nourished<sup>[26]</sup>. Therefore, based on the results of SGA, patients were assigned to one of the three categories: A (well nourished), B (mildly to moderately malnourished), or C (severely malnourished).

### Anthropometric measurement

Body height and weight, and other anthropometric parameters were measured by SGA. Body mass index (BMI) was calculated based on body height and weight. BMI less than 18.5 was regarded as malnourished. MUAC and TSF were measured with intertape and adipometer. MAMC was calculated following the formula: MAMC = MUAC (mm) - 3.14 × TSF (mm). TSF ≤ 10.17 mm in males and ≤ 13.41 mm in females, or MAMC ≤ 20.52 cm in males and ≤ 18.81 cm in females was the diagnostic criterion for malnutrition. These standards of anthropometric parameters

**Table 1 Parameters and diagnostic criteria for subjective global assessment (SGA)**

Parameters	Grade A	Grade B	Grade C
Food intake	No deficiency	Definite decrease in intake or liquid diet	Severe deficiency in intake or starvation
Weight loss (during the past 6 mo)	No weight loss or weight loss > 10% during the past 6 mo but weight gain over the past month	Continuous weight loss of 5%-10%	Continuous weight loss > 10%
Gastrointestinal symptoms (nausea, vomit, diarrhea)	None	Mild or moderate GI symptoms for less than 2 wk	Continuous severe GI symptoms for more than 2 wk
Activities and function	No limitation	Not normal, but able to do fairly normal activities or do not know most things, but in bed or chair for less than half a day	Able to do little activity and spend most of the day in bed or chair; or much bedridden, rarely out of bed
Metabolic stress	No fever	Temperature > 37°C and < 39°C during the past 72 h	Continuous temperature ≥ 39°C during the past 72 h
Subcutaneous fat loss	No	Mild to moderate	Severe
Muscle wasting	No	Mild to moderate	Severe
Ankle edema/Ascites	No	Mild to moderate	Severe

Nutritional status: SGA-A (Normal); SGA-B (Mildly to moderately malnourished); SGA-C (Severely malnourished).

**Table 2 Classification standards for nutritional parameters in assessing malnutrition**

Nutritional parameter	Normal nutrition	Mildly malnourished	Moderately malnourished	Severely malnourished
TSF (mm)				
Male	> 10.17	9.04-10.17	6.78-9.03	< 6.78
Female	> 13.41	11.92-13.41	8.94-11.91	< 8.94
MAMC (cm)				
Male	> 20.52	18.24-20.52	13.68-18.23	< 13.68
Female	> 18.81	16.72-18.81	12.54-16.71	< 12.54
PA (mg/L)	≥ 200	160-199	120-159	< 120
ALB (g/L)	≥ 35	31-34	26-30	≤ 25

TSF: Triceps skinfold thickness; MAMC: Mid-upper arm muscle circumference; PA: Prealbumin; ALB: Albumin.

for classifying nutritional status were formulated in accordance with the Chinese Anthropometric Reference Data (Table 2)<sup>[27]</sup>.

### Blood measurement

Blood samples were collected at anthropometric assessment, before initiation of IV fluids. ALB and PA were measured with a standard clinical analyzer. The cut-off value for PA and ALB was set at 200mg/L (measured by immune turbidimetry) and 35 g/L (measured by biuret method), respectively. The standards for classifying nutritional status in serum proteins were also formulated in accordance with the Chinese Anthropometric Reference Data (Table 2)<sup>[27]</sup>.

### Outcome variables related to health care

Outcome variables related to health care, such as hospital stay, medical expenditures, occurrence of complications, and pathological stage of cancer were also detected. Patients were discharged according to the hospital policy. Hospital stay (d) was recorded. All patients were followed up until discharge or death. Complications, including infectious complications (septicemia, incisional, respiratory, abdominal, pelvic, and urinary tract infection) and non-infectious complications (rupture of incision, intestinal obstruction, ascites, cerebrovascular accident, bleeding, and organ failure, etc,

were monitored and recorded daily. Pathological stage of cancer was described by TNM staging according to Union International Contere Cancer (UICC) version 5.0.

### Statistical analysis

Data analyses were carried out using StatView 6.12 (SAS Institute, Cary, NC, USA). Data were expressed as mean ± SD. Differences in mean values were tested with one-way analysis of variance and Student's *t*-test.  $\chi^2$  test was used to compare differences in categorical data. Bivariate correlation analysis (Pearson's R) was performed to show the correlation between SGA grades and other nutritional parameters. Multiple regression analyses were carried out to assess the relation between SGA, other nutritional parameters, and health care outcome variables. *P* < 0.05 was considered statistically significant.

## RESULTS

### Nutritional status and cancer stage of gastrointestinal cancer patients

Based on different nutritional parameters, the number of patients with malnutrition was 362 (48.2%), 296 (39.4%), 283 (37.7%), 230 (31.3%), 145 (21.7%), and 72 (9.6%) for SGA, TSF, MAMC, ALB, PA, and BMI, respectively.

In our study, 71.1% patients were at advanced cancer stage. The number of cancer patients was 142 (18.9%), 179 (23.8%), 205 (27.3%), 225 (30.0%) at stage I, stage II, stage III, and stage IV, respectively.

### Comparison of nutritional status classified by SGA and other nutritional parameters

Based on the results of SGA, 389 (51.8%), 332 (44.2%), and 30 (4.0%) patients were classified into well nourished group (SGA-A), mildly to moderately malnourished group (SGA-B), and severely malnourished group (SGA-C), respectively. One-way analysis of variance revealed that SGA grade was closely related with other nutritional parameters (Table 3). Further analyses of Post Hoc least significant difference comparisons (LSD tests) identified that there were differences in percentage of weight loss, BMI, PA, and ALB between each two

**Table 3 Comparison of nutritional parameters in different SGA grades**

Nutritional parameters	The grade of SGA			F	P	Correlation coefficient ( <i>r</i> )	P
	SGA-A	SGA-B	SGA-C				
Weight loss (%)	2.2 ± 2.9	9.7 ± 7.0	23.2 ± 12.6	296.0	0.000	0.65	0.00
BMI	23.4 ± 3.0	21.2 ± 2.8	19.0 ± 3.3	70.8	0.000	0.40	0.00
TSF (mm)							
Male	16.0 ± 8.5	10.5 ± 6.2	9.2 ± 6.5	31.9	0.000	0.34	0.00
Female	25.0 ± 10.2	17.4 ± 8.3	14.4 ± 10.0	26.6	0.000	0.38	0.00
MAMC (cm)							
Male	21.8 ± 2.3	21.9 ± 2.1	20.6 ± 2.5	2.4	0.095	0.03	0.50
Female	18.2 ± 2.5	18.6 ± 2.4	16.8 ± 2.5	4.1	0.018	0.02	0.71
ALB (g/L)	37.7 ± 4.2	35.7 ± 5.7	30.5 ± 6.6	36.9	0.000	0.29	0.00
PA (mg/L)	246.7 ± 41.5	221.7 ± 49.2	159.6 ± 52.9	59.5	0.000	0.37	0.00

**Table 4 Comparison of SGA grades between patients before surgery and chemotherapy/radiotherapy**

Treatment	n	Grade of SGA (%)			$\chi^2$	P
		SGA-A	SGA-B	SGA-C		
Surgery	505	275 (70.7)	214 (64.5)	16 (53.3)	5.91	0.05
Chemotherapy and radiotherapy	246	114 (29.3)	118 (35.5)	14 (46.7)		
Total	751	389	332	30		

of the three SGA groups ( $P < 0.05$ ). Therefore, in general, when the patients were classified by the SGA grade as more severely malnourished, the value of the other nutritional parameters, such as levels of BMI, ALB, and PA was lower. Bivariate correlation analysis showed that SGA grade was significantly correlated with the percentage of weight loss, BMI, TSF, ALB, and PA (Table 3), even though the correlation coefficient was less than 0.3 between SGA grade and ALB level.

$\chi^2$  tests showed that SGA grade was significantly different between patients receiving surgery and chemotherapy/radiotherapy (Table 4). In addition, the percentage of weight loss ( $5.4\% \pm 6.7\% vs 8.4\% \pm 8.8\%$ ,  $P = 0.000$ ) and the serum of PA ( $235.3 \pm 46.5 vs 223.8 \pm 55.6$ ,  $P = 0.013$ ) existed obviously differences between the patients receiving surgery and chemotherapy/radiotherapy.

#### Could SGA and other nutritional parameters predict hospital stay?

One-way analysis of variance revealed that the hospital stay of 751 gastrointestinal cancer patients was not statistically different in different SGA groups ( $F = 2.46$ ,  $P = 0.086$ ). Preliminary multiple regression analysis using hospital stay as an outcome variable showed that the type of treatment was the biggest predictor for hospital stay in our study (Table 5). In general, patients receiving surgery stayed in the hospital much longer than those receiving chemotherapy/radiotherapy. Further ANOVA analysis revealed that the hospital stay was significantly longer in accordance with the increasing grade of SGA, both in patients receiving surgery and in patients receiving chemotherapy/radiotherapy (Table 6). Subgroup multiple regression analysis using hospital stay as an outcome variable, showed that SGA and serum

ALB could help explain the length of hospital stay only in surgical gastrointestinal (GI) cancer patients (Table 7), but not in patients receiving chemotherapy/radiotherapy ( $F = 1.22$ ,  $P = 0.27$ ).

#### Could SGA and other nutritional parameters predict in-hospital medical expenditures?

One-way analysis of variance revealed that the in-hospital medical expenditures of different SGA groups of patients were significantly different ( $P < 0.01$ ) (Table 6). SGA-C group had the highest expenditures, SGA-A group the lowest expenditures, and SGA-B group the medium expenditures. Multiple regression analysis using medical expenditures as an outcome variable showed that the type of treatment was the biggest predictor of medical expenditures for GI cancer patients in our study (Table 5). The multiple regression analysis revealed that SGA, serum ALB, and cancer stages (TNM) could independently influence the medical expenditures of surgical GI cancer patients (Table 7). On the contrary, no significant predictors could be found for those not undergone surgery.

#### Could SGA and other nutritional parameters predict occurrence of complications?

The occurrence of complications increased with the increasing SGA grade. SGA-C group had the highest occurrence of complications (23.3%), SGA-A group the lowest occurrence of complications (16.8%), and SGA-B group the medium occurrence of complications (19.1%) ( $\chi^2 = 1.21$ ,  $P = 0.546$ ). In addition, hospital stay of patients with complications was significantly longer than that of those without complications ( $26.1 \pm 12.1 vs 15.5 \pm 7.8$ ,  $t = -9.67$ ,  $P = 0.00$ ).

During hospital stay, 8 patients died of various

**Table 5 Factors influencing hospital stay and in-hospital costs of GI cancer patients (multiple regression analysis)**

Factors	Factors influencing hospital stay			Factors influencing in-hospital costs		
	Standardized coefficients $\beta$	t	P	Standardized coefficients $\beta$	t	P
Age	0.02	0.64	0.52	-0.06	-1.37	0.17
Sex	-0.07	-1.64	0.10	-0.04	-0.90	0.37
Education background	-0.04	-1.04	0.30	-0.01	-0.25	0.80
Weight loss (%)	0.03	0.67	0.50	0.07	1.26	0.21
BMI	-0.03	-0.51	0.61	0.05	0.73	0.47
MAMC	-0.04	-0.69	0.49	-0.06	-0.82	0.41
TSF	0.03	0.42	0.68	0.01	0.18	0.86
ALB	-0.04	-1.04	0.30	-0.06	-1.43	0.15
SGA-A/SGA-C	0.18	1.68	0.09	0.18	1.45	0.15
SGA-B/SGA-C	0.17	1.80	0.07	0.09	0.86	0.39
TNM	-0.05	-1.54	0.12	0.06	1.49	0.14
Tumor site	-0.02	0.59	0.56	-0.07	-1.76	0.08
Type of treatment	-0.49	-13.99	0.00	-0.25	-6.30	0.00

Factors influencing hospital stay model F = 19.20, P < 0.05; Factors influencing in-hospital costs model F = 5.62, P < 0.01.

**Table 6 Comparison of hospital stay and medical expenditures of patients with different SGA grades**

	Grade of SGA (%)			F	P
	SGA-A	SGA-B	SGA-C		
Hospital stay (d)	17.1 ± 9.7	17.3 ± 9.0	21.1 ± 14.6	2.46	0.086
Surgery	20.8 ± 8.6	21.2 ± 7.8	29.1 ± 15.1	7.07	0.001
Chemotherapy and radiotherapy	8.2 ± 5.1	10.3 ± 6.4	12.1 ± 6.8	5.02	0.007
Medical expenditure (RMB)	6522.4 ± 6670.9	8353.7 ± 9575.9	12550.0 ± 10579.7	9.85	0.000
Surgery	7987.9 ± 6963.9	10025.8 ± 10009.6	17654.2 ± 11678.5	11.51	0.000
Chemotherapy and radiotherapy	3033.5 ± 3430.5	5358.0 ± 7945.0	6268.0 ± 3632.5	4.58	0.011

**Table 7 Factors influencing hospital stay and in-hospital costs of surgical GI cancer patients (multiple regression analysis)**

Factors	Factors influencing hospital stay			Factors influencing in-hospital costs		
	Standardized coefficient $\beta$	t	P	Standardized coefficient $\beta$	t	P
Age	0.05	1.01	0.32	-0.06	-1.22	0.22
Sex	-0.11	-1.80	0.07	-0.07	-1.17	0.24
Education background	-0.04	-0.89	0.37	-0.04	-0.88	0.38
weight loss (%)	0.02	0.26	0.80	-0.01	-0.05	0.96
BMI	-0.05	-0.62	0.54	0.06	0.81	0.42
MAMC	-0.05	-0.66	0.51	-0.07	-0.86	0.39
TSF	0.12	1.35	0.18	0.06	0.66	0.51
ALB	-0.10	-2.11	0.04	-0.16	-3.17	0.002
SGA-A/SGA-C	0.41	2.36	0.02	0.43	2.51	0.01
SGA-B/SGA-C	0.39	2.52	0.01	0.31	2.06	0.04
TNM	-0.01	-0.26	0.08	0.10	2.11	0.04
Tumor site	-0.06	1.21	0.23	-0.09	-1.90	0.06

Factors influencing hospital stay model F = 2.35, P < 0.01; Factors influencing in-hospital costs model F = 3.92, P < 0.01.

complications (5 in SGA-B group, 2 in SGA-A group, and 1 in SGA-C group). SGA grade was not related with the number of deaths in our study.

## DISCUSSION

Severe malnutrition is associated with increased

morbidity and mortality of gastrointestinal cancer, decreased treatment efficacy, and increased hospital stay<sup>[28]</sup>. Nutritional status is conventionally assessed by anthropometric measurement and laboratory assessment<sup>[29]</sup>. In this study, the prevalence of malnutrition for the same group of subjects ranged 9.6%-48.2%. The highest prevalence of malnutrition

was detected by SGA, the lowest by BMI. The purpose of nutritional assessment in cancer patients is to discover mild or moderate malnutrition before the patients become overtly wasted in order to prevent further deterioration and improve their quality of care. In clinical settings, some of the anthropometric measurements and laboratory assessments are not ideal because they are neither accurate nor convenient.

Although the British Association for Parenteral and Enteral Nutrition (BAPEN) has recommended that the measurements used for screening malnutrition should be based upon the changes in BMI and the percentage of weight loss, our study demonstrated that only a small number of patients were diagnosed with malnutrition by BMI, suggesting that BMI cannot precisely assess malnutrition in Chinese cancer patients. The established cut-off point of malnutrition for BMI largely depends on studies in younger patients<sup>[30]</sup>, and therefore, cannot be directly applied to the elderly population, which may explain why only a small number of patients were diagnosed with malnutrition by BMI in this study. It has been shown that a BMI value of 20 should alert clinicians to suspect malnutrition in the elderly<sup>[29]</sup>. It was reported that the optimal range of BMI in elderly people should be increased from 20 kg/m<sup>2</sup> to 25 kg/m<sup>2</sup> in order to identify the elderly at a risk of malnutrition<sup>[31]</sup>. On the other hand, some elderly patients spend most of their day time in bed or totally bedridden, so it is not always easy or sometimes even impossible to measure their weight or height changes.

In addition, SGA was not significantly correlated with ALB level compared to other anthropometric parameters. The ALB level alone is not a good representative marker of nutritional status of cancer patients as shown in our study. It has been shown that ALB level may be considered as an indicator of illness or as a prognostic factor for complications and mortality, but not as a major indicator of nutritional status<sup>[32]</sup>. Our study showed that ALB level was an important factor for prolong hospital stay and medical expenditures of surgical cancer patients. In patients with malignancy diseases, the ALB level can be affected by nutritional status and the malignant disease itself, or by inflammatory reactions due to any causes, such as severe liver disease, dehydration, and edema<sup>[29]</sup>. In fact, serum ALB, a negative acute phase protein<sup>[33]</sup>, is decreased in response to acute or chronic inflammation by altering the normal hepatic protein metabolism and inducing capillary leak<sup>[34-36]</sup>. Irrespective of the value of biochemical indicators, ALB level measurement is more time consuming and expensive than SGA.

SGA, one of the better available tools, can assess nutritional status, not only because it is patient centered by combining clinical history and physical examination, but also because it is associated with patient outcomes<sup>[37-39]</sup>. This is why SGA has been used widely in Western countries yielding trustworthy results. In the present study, the values of BMI, TSF, PA, and ALB were lower in more severely malnourished patients, which is consistent with previous findings<sup>[23,29]</sup>.

It has been shown that SGA grade is closely correlated with TSF, MAMC, and ALB<sup>[29]</sup>. SGA can be used as a benchmark to validate new assessment methods, such as bioelectrical impedance analysis<sup>[40]</sup> and mid-upper arm anthropometry<sup>[41]</sup>.

Although SGA is now considered a clinical method for assessing nutritional status, it was originally developed to identify patients with poorer outcomes following surgery. Baker *et al*<sup>[23]</sup> showed that patients classified as 'malnutrition' suffer more infections, use more antibiotics, and have a longer hospital stay. We hypothesized that SGA grade of patients at admission could help to predict the occurrence of complications, hospital stay, and in-hospital medical expenditures of Chinese gastrointestinal cancer patients, and found that the more severely malnourished patients had a longer hospital stay, a higher occurrence of complications, and higher in-hospitalization costs. Multiple regression analysis displayed that SGA grade could only predict hospital stay and medical expenditures of surgical cancer patients, but not those of chemotherapy/radiotherapy patients, indicating that type of treatment may influence the predictive value of SGA. Wakahara *et al*<sup>[42]</sup> reported that although SGA can be used to predict the hospital stay of patients with digestive diseases, cancer staging is a better prognostic index of cancer patients. However, the results of our study do not support the fact that advanced cancer would lead to worse nutritional status, longer hospital stay, and higher incidence of postoperative complications. In addition, patients with complications had a longer hospital stay than those with no complications. Since cancer patients are more prone to develop complications when their nutritional status deteriorates, more treatment modalities are needed to help them recover.

Although SGA could provide useful information for predicting certain outcome variables in our study, SGA was not related with death of patients. Eight patients (5 in SGA-B group, 2 in SGA-A group and 1 in SGA-C group) died of complications during hospital stay. The reason why only one patient died in the most severely malnourished SGA-C group was due to the small subgroup sample size. Whether SGA can predict the risk of in-hospital death remains unclear.

This study had some limitations. For example, the small sample size in SGA-C group resulted in a quite unbalanced distribution of nutritional status in different SGA classification groups, which may limit the power of data analysis. As an assessment tool, SGA consists of both history taking and physical examination of the patients<sup>[40,43]</sup>. Thus, reliable SGA grading depends on collection of correct history and physical examination. During our study, since some patients could not remember their exact body weight and detail dietary intake when information was collected to assess the nutritional status, the relevant information was obtained from the recall of patients and their relatives. Recently, quantification of SGA has been advocated as a way to improve the sensitivity and specificity of SGA in diagnosing malnutrition<sup>[44,45]</sup>.

In conclusion, SGA is a safe, inexpensive and reliable method for assessing nutritional status of Chinese gastrointestinal cancer patients and only can predict their hospital stay and medical expenditures in surgical GI patients. Further study is needed on the role of SGA in predicting the occurrence of in-hospital deaths.

## COMMENTS

### Background

Cancer is one of the serious health problems worldwide. A large number of cancer patients die of malnutrition or its relative complications rather than the disease itself. Malnutrition has a negative impact on the well-being of patients and evolution of the disease. A timely efficient nutritional assessment would provide a better basis for deciding whether nutritional support is given. Many nutritional assessment methods are now available. Each method has its own advantages and disadvantages. An accurate, convenient, and inexpensive method should be available for clinicians.

### Research frontiers

Subjective global assessment (SGA) has been originally developed to identify poor nutritional status in subjects undergoing gastrointestinal surgery, it can be used to quantify the prevalence of malnutrition in patients at the end-stage of renal failure, and is a powerful predictor of postoperative complications in general surgery, liver transplantation, and in patients on dialysis. In addition, a recent study revealed that although SGA can predict hospital stay of patients with benign digestive disease, its predictive power is limited in patients with malignant diseases.

### Innovations and breakthroughs

Although SGA has been used widely for more than two decades all over the world, few studies are available on SGA in Chinese gastrointestinal cancer patients. In this study, SGA was used to assess the nutritional status of Chinese patients with gastrointestinal cancer. The results show that SGA helps predict certain outcomes such as hospital stay and medical expenditures of surgical gastrointestinal (GI) cancer patients.

### Applications

As a convenient and reliable method, SGA can be used to assess the nutritional status of cancer patients since it helps predict certain outcomes of surgical GI cancer patients.

### Peer review

This manuscript is valuable and offers important data for the clinical management of GI cancer patients. The results of this study demonstrate that SGA is superior over other nutritional parameters in the assessment of nutritional status of GI cancer patients.

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BRIEF ARTICLES

## Sonographic evaluation of vessel grafts in living donor liver transplantation recipients of the right lobe

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### Abstract

**AIM:** To evaluate the vessel grafts (VG) used to reconstruct the middle hepatic vein (MHV) tributaries with ultrasonography.

**METHODS:** Twenty-four patients undergone living donor liver transplantation were enrolled in our study. MHV tributaries larger than 5 mm in diameter were reconstructed with interposition VG. Blood flow of the graft and interposition VG was checked by Doppler ultrasonography daily in the first 2 postoperative weeks and monthly followed up after discharge. The sensitivity of VG detected by ultrasonography was assessed using surgical records as references. Student's *t* test was used to compare the velocity of VG and occluded VG in chronic patients (> 3 mo).

**RESULTS:** Thirty-one VG were used to reconstruct the MHV tributaries. Ultrasonography identified 96.7% (30/31) of large MHV tributaries and 90.3% (28/31) of VG. The diameter of VG was  $5.6 \pm 0.8$  mm and the velocity of VG was  $19.7 \pm 8.1$  cm/s. Two VG (2/31, 6.5%) were occluded on the first postoperative day in one patient who suffered from persistent ascites and had a prolonged recovery of liver function. Twenty-six VG (26/31, 83.9%) were patent 2 wk after operation. Six (6/31, 19.4%) VG were patent over 3 mo after operation. Intrahepatic venous collaterals were detected in 29.2% (7/24) patients. The velocity of VG and

occluded VG was  $30.1 \pm 5.6$  cm/s,  $16.5 \pm 5.8$  cm/s, respectively, in chronic patients. The difference between two groups was statistically significant ( $P < 0.001$ ).

**CONCLUSION:** Our results indicate that most VG are patent in the first postoperative week while only a small portion with a higher velocity remains patent after 3 mo. Intrahepatic venous collaterals can be observed in some patients after occlusion of VG.

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**Key words:** Living donor liver transplantation; Ultrasonography; Vessel graft; Venous collateral; Middle hepatic vein

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Lu Q, Wu H, Fan YT, Luo Y, Zhang ZW. Sonographic evaluation of vessel grafts in living donor liver transplantation recipients of the right lobe. *World J Gastroenterol* 2009; 15(28): 3550-3554 Available from: URL: <http://www.wjgnet.com/1007-9327/15/3550.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.3550>

### INTRODUCTION

Adult-to-adult living donor liver transplantation (AALDLT) is emerging as an alternative to cadaveric liver transplantation due to shortage of graft organs<sup>[1,2]</sup>. A right lobe liver graft is now commonly used in this procedure, and the middle hepatic vein (MHV) trunk is usually preserved for the donor for his or her safety<sup>[3,4]</sup>. Because hepatic venous blood of the anterior segment usually drains to the inferior vena cava through the V5 and V8 MHV tributaries, this type of grafts is inherently prone to cause congestion of the right paramedian sector (segments V and VIII), leading to tissue atrophy or necrosis<sup>[5,6]</sup>. Because establishment of optimal hepatic venous outflow is essential to a successful outcome of AALDLT, there has been emerging interest in hepatic venous congestion in the paramedian sector of the right lobe graft after AALDLT. Recently, surgical reconstruction of the major MHV tributaries using an interposition VG has been advocated to achieve optimal hepatic venous outflow

of the paramedian sector in AALDLT recipients using a modified right lobe graft. Although the indication for reconstruction of MHV is still controversial<sup>[3,7,8]</sup>, MHV tributaries in the anterior segment of the modified right lobe graft are routinely reconstructed in our institution if they are larger than 5 mm in diameter. However, compared with the recent surgical interest and investigations designed to prevent hepatic venous congestion in the right paramedian sector of the right lobe grafts after AALDLT, little attention has been given to postoperative radiological surveillance for thick MHV tributaries, VG and intrahepatic venous collaterals.

Therefore, this study was to assess the value of ultrasound in the evaluation of interposition VG and intrahepatic venous collateral formation after occlusion of the VG in AALDLT.

## MATERIALS AND METHODS

### Patients

From January 2006 to August 2007, 51 consecutive adult patients underwent AALDLT using the right lobe grafts at West China Hospital. The indications for liver transplantation included hepatitis-B virus cirrhosis in 26 patients, acute hepatic failure in 8 patients, hepatitis-B cirrhosis associated with hepatocellular carcinoma in 16 patients, retransplant for diffused ischemic intrahepatic biliary stenosis of cadaveric graft with liver failure in 1 patient. Twenty-four (47.1%) patients (21 males and 3 females) who underwent MHV reconstruction were enrolled in our study. Their median age was 38 years (range, 22–54 years). The donors (14 males and 10 females) with a median age of 36 years (range, 22–53 years) included 6 wives, 7 brothers, 2 sisters, 4 sons, 2 daughters, 2 fathers and 1 husband. All cases were approved by the ethics committee of local authority and informed consent was obtained from each patient.

### Preoperative assessment

Right liver volume was estimated by multi-slice computed tomography (CT). Candidates in whom the right liver represented more than 70% of the whole liver were excluded from prospective donors. An estimated graft volume to recipient standard liver volume ratio of 40% was the lower limit for right lobe liver transplantation. The number and diameter of thick MHV tributaries draining the right paramedian sector were evaluated by CT.

### Intraoperative evaluation

Donor hepatectomy was performed through a J-shaped incision. Intraoperative ultrasound was performed to confirm the hepatic vein anatomy and verify the transection plane. Major MHV tributaries were isolated and preserved if present and greater than 5 mm in diameter. Hepatic venous congestion in the right paramedian sector was investigated intraoperatively after parenchyma transaction by clamping test<sup>[9]</sup>. Liver surface discoloration in the right paramedian sector was observed 5 min after simultaneous clamping of MHV tributaries and the right hepatic artery. If the congested

area was dominant as determined by the clamping test or the diameter of MHV tributaries was larger than 5 mm, reconstruction of MHV tributaries was preceded. The autogenous great saphenous and cryopreserved cadaveric external iliac veins are most commonly used as interposition vessel grafts.

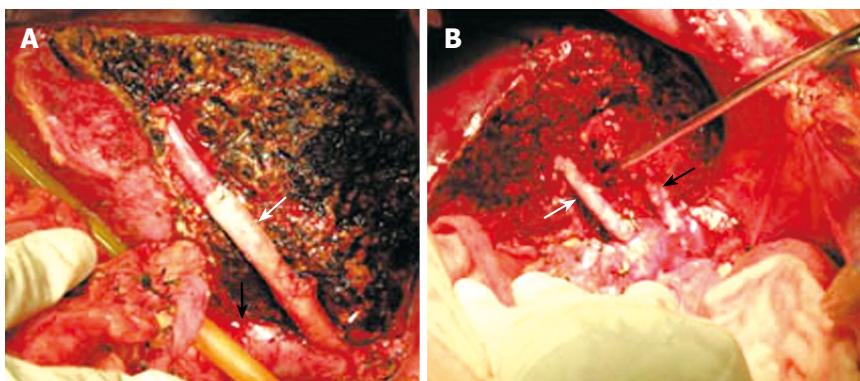
### Postoperative ultrasound examination

To prevent thrombosis of hepatic artery and vessel grafts, prophylactic anticoagulation therapy was routinely used after surgery. Blood flow in the graft or interposition vessel was checked by Doppler sonography using a HDI 5000 scanner (Philips Medical Systems, Bothell, WA) or a LOGIQ 9 scanner (GE Medical Systems, Milwaukee, Wis). Contrast enhanced ultrasound examinations were done with a Sequoia 512 ultrasound scanner (Acuson Siemens, Erlangen, Germany). The contrast agent was SonoVue (Bracco Imaging, Milan, Italy) consisting of sulphur hexafluoride microbubbles stabilized by a phospholipid shell, 2.4 mL of SonoVue per exploration was injected through a peripheral vein. A low mechanical index mode was used. Scans were performed by an ultrasound doctor with more than 5-year experience in liver transplantation ultrasound. Patients received ultrasound examination daily until postoperative day 14 and once a week thereafter until hospital discharge. Intercostal views of the right upper quadrant of abdomen were obtained to examine the full extension of the hepatic parenchyma. Thick tributaries of the MHV, identified at the left margin of the graft, were used as extrahepatic interposition VG. Fundamental gray scale, color flow images, and Doppler spectra were obtained. Angle-corrected velocities were obtained with the Doppler angle less than 60°. Contrast enhanced ultrasound study was conducted in 5 patients and late parenchymal phase (60 s after injection of SonoVue) was used to observe the patency of VG. All images were acquired when the patients did not hold their breath.

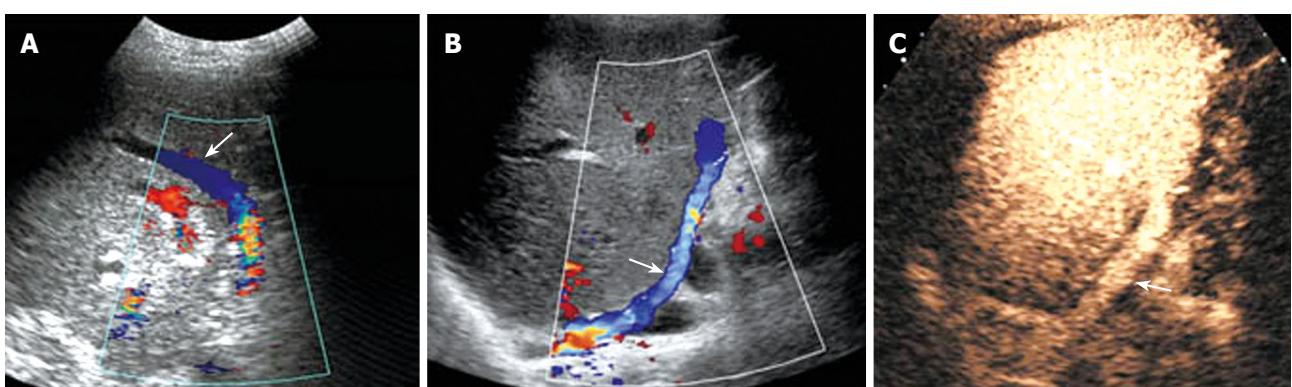
VG were divided into long term patent group and occluded group according to the vessel patency 3 mo after surgery. Student's *t* test was used to compare the velocity in two groups.

## RESULTS

Thirty-one vessel grafts were used to reconstruct the MHV tributaries, including V5 (*n* = 13), V8 (*n* = 4), both V5 and V8 (*n* = 7) (Figure 1). Ultrasonography identified 96.7% (30/31) of large MHV tributaries and 90.3% (28/31) of vessel grafts (Figure 2). The diameter of VG was  $5.6 \pm 0.8$  mm and the velocity of VG was  $19.7 \pm 8.1$  cm/s. Two VG (6.5%) were occluded on the first day after surgery in one patient (Figure 3) who had a prolonged recovery of graft function and persistent ascites for 2 mo. Twenty-six VG (83.9%) were patent 2 wk after operation. Six VG (19.4%) were patent over 3 mo after operation. Intrahepatic venous communications between the MHV tributaries and the right hepatic vein were detected in 7 (29.2%) patients (Figure 4). The velocity in long term patent VG group and occluded VG group was  $30.1 \pm 5.6$  cm/s and  $16.5 \pm 5.8$  cm/s, respectively. The



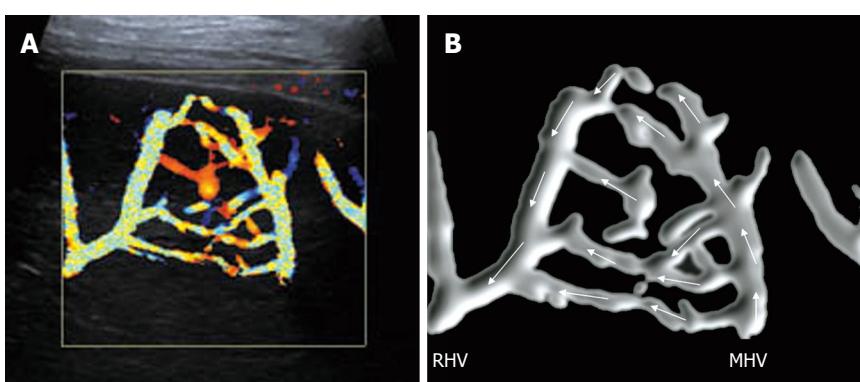
**Figure 1** Intraoperative photograph showing an interposition vessel graft (white arrow) draining venous flow from MHV tributaries in segments V-IVC (black arrow) (A) and interposition vessel grafts V5 (white arrow) and V8 (black arrow) (B) used to reconstruct MHV tributaries in segments V and VIII. MHV: Middle hepatic vein, IVC: Inferior vena cava.



**Figure 2** Color Doppler ultrasonography showing large MHV tributaries (arrow) in segment V containing hepatofugal venous flow (A), vessel grafts (arrow) draining the IVC along the surgical margin of liver graft (B), and vessel graft (arrow) filled with contrast agent on contrast enhanced ultrasound indicating the vessel graft patency (C).



**Figure 3** Color Doppler sonography showing MHV tributaries filled with hypoechoic substances representing thrombus (arrow) with no color signal in vessel grafts (A), contrast enhanced sonography showing no contrast agent in vessel grafts (arrow) and MHV tributaries (red arrow) indicating vessel occlusion (B), and contrast enhanced CT scan on postoperative day 2 showing an area of low attenuation in segment V corresponding to the draining territory of MHV (arrow) (C) in a 42-years old male who received a modified right lobe graft from his brother.



**Figure 4** Color Doppler ultrasonography (A) and its sketch (B) showing MHV tributaries draining into the RHV via collaterals after thrombosis of vessel grafts on postoperative day 7 in a 32 years old male who received a right lobe graft from his wife.

difference between two groups was statistically significant ( $P < 0.001$ ).

## DISCUSSION

The major limitation of AALDLT is the inadequacy of graft size. To guarantee the safety of the donor, many institutes perform AALDLT using a right liver graft without the MHV trunk<sup>[1,10,11]</sup>. However, this procedure might be complicated by severe congestion of the right paramedian sector, leading to tissue atrophy or necrosis. Therefore, the MHV tributaries larger than 5 mm in diameter should be preserved to achieve optimal outflow of the right paramedian sector.

Doppler sonography is generally regarded as a primary technique for vascular surveillance after LDLT and is, sometimes, the only available bedside imaging modality in the early postoperative period<sup>[12,13]</sup>. Good spatial resolution and high sensitivity to slow flow make ultrasound useful in depicting the MHV tributaries and interposition VD. In our study, Doppler sonography demonstrated 90.3% of the VG and 96.7% of the large MHV tributaries. However, ultrasound scan was interfered by bowel and lung gas, thus failing to identify the rest three VG and one MHV tributary.

Early occlusion of VG may result in an eventful recovery of liver function. One patient in whom the VG were occluded on the first postoperative day had a prolonged recovery of graft function and persistent ascites for 2 mo. Occlusion of the VG caused venous congestion of the right paramedian sector, thus delaying the recovery of liver function. Persistent ascites can be caused by an inadequate hepatic mass<sup>[14,15]</sup>. The regenerated liver cannot reach its adequate size in the early postoperative period and venous congestion of the right paramedian sector can further decrease the functional hepatic mass size.

Unlike the hepatic vein, since VG do not support the surrounding hepatic parenchyma, these VG are under a greater pressure from the neighboring structures due to liver regeneration. Moreover, because the diminished flow also attributes to the occlusion of VG, VG have a tendency to occlude with time. In our study, only 6 VG (19.4%) remained patent 3 mo after operation and had a higher velocity than the occluded VG.

Identification of intrahepatic collaterals between MHV tributaries and right hepatic vein is important, because the presence of intrahepatic collaterals indicates the outflow of the right paramedian sector is not occluded. Whether all patients have intrahepatic venous collaterals still remains controversial. De Cecchis *et al*<sup>[16]</sup> reported that intrahepatic communicating veins are sometimes observed. Kaneko *et al*<sup>[17]</sup> demonstrated that the right branch of MHV drains into the right hepatic vein via the collaterals between them. However, Cescon *et al*<sup>[18]</sup> described that the venous flow in MHV was eliminated without intrahepatic venous communication in 24 grafts of 30 living donor liver transplants when the MHV was clamped. Doppler ultrasound is sensitive to slow flow and provides information about the direction of blood flow. In our study, extensive subcapsular venous

communications were demonstrated in 7 patients (29.2%) after the occlusion of VG at follow-up ultrasound examination with no salient echogenicity change.

Contrast enhanced ultrasound is a newly emerged technique and can greatly improve the visualization of low velocity flow<sup>[19,20]</sup>. We did not routinely use it to evaluate VG due to the high price of contrast agent. In our series, 5 patients underwent contrast enhanced ultrasound study to exclude hepatic artery thrombosis and evaluate the patency of VG. The results show that it can greatly enhance the confidence of ultrasound doctors. However, further study is needed to evaluate the diagnostic value of this new technique.

In conclusion, Doppler sonography is useful in depicting thick MHV tributaries and VG. Most interposition VG are patent in the first week after surgery and trend to occlude with time with only a small portion remains patent 3 mo after operation. Intrahepatic venous collaterals can be observed after occlusion of VG.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Surgical reconstruction of major middle hepatic vein tributaries using interposition vessel grafts (VG) has been advocated to achieve optimal hepatic venous outflow of the paramedian sector in adult-to-adult living donor liver transplantation recipients using a modified right lobe graft. However, little attention has been given to postoperative radiological surveillance for thick MHV tributaries, VG and intrahepatic venous collaterals.

### Research frontiers

Doppler sonography and contrast enhanced ultrasound were used to investigate the patency and velocity of VG in study. The relation between VG velocity and long term patency was discussed. The issue of intrahepatic venous collaterals was also addressed.

### Innovations and breakthroughs

Although some studies have already discussed the issue of reconstructed vessels, our study focused on the patency of reconstructed vessels, which has received little attention.

### Applications

By displaying the relation between the velocity and patency of VG and the formation of intrahepatic venous collaterals, sonographic surveillance may represent a strategy for prediction of the VG patency and even the paramedian sector congestion of transplanted liver.

### Terminology

VG used to reconstruct middle hepatic vein (MHV) tributaries are vascular conduit between MHV tributaries and inferior vena cava. Autogenous great saphenous and cryopreserved cadaveric external iliac veins are the most frequently used VG.

### Peer review

The authors investigated the patency of vascular conduits (vessel graft) for MHV reconstruction with right liver graft in living donor liver transplantation. They used autogenous great saphenous and cryopreserved cadaveric external iliac veins as vascular conduit. Their data and experience are valuable because little evidence of the patency of reconstructed vessels is available, although similar studies are available.

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# Generalized megaviscera of lupus: Refractory intestinal pseudo-obstruction, ureterohydronephrosis and megacholedochus

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have termed this rare clinical syndrome generalized  
megaviscera of lupus (GML). Although the SLE  
disease-activity parameters responded to aggressive  
immunomodulative therapy in our patient, clinical  
evidence of peristaltic dysfunction persisted in all  
involved viscera. This is a variation from the favorable  
outcomes reported previously in SLE patients with  
GML and we attribute this poor clinical outcome to  
disease severity and, most importantly, delayed clinical  
presentation. Since inflammation followed by atrophy  
and fibrosis are key aspects in the pathogenesis  
and natural history of GML, the poor response in  
our patient who presented late in the clinical course  
may be the result of 'burnt out' inflammation with  
irreversible end-stage fibrosis. Thus, early recognition  
and timely initiation of treatment may be the key to  
recover visceral peristaltic function in patients with  
GML.

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**Key words:** Systemic lupus erythematosus; Intestinal  
pseudo-obstruction; Biliary tract diseases; Hydroureter;  
Hydronephrosis; Smooth muscle; Autoimmune myositis

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## INTRODUCTION

Chronic intestinal pseudo-obstruction (CIPO) is a  
rare but important clinical syndrome as it causes 20%  
of chronic intestinal failure in adults and 15% in  
children<sup>[1]</sup>. It is characterized by ineffective intestinal  
propulsion with signs and symptoms similar to

mechanical bowel obstruction including abdominal distension, pain, nausea, vomiting, obstipation and sluggish bowel sounds, but the absence of an occluding lesion of the intestinal lumen. CIPO is idiopathic in the vast majority of cases, but secondary causes can include virtually any disease process that affects structures involved in intestinal motility, including intestinal myocytes and the extrinsic and intrinsic neural networks. The management of CIPO has been challenging, and long-term outcomes often disappointing<sup>[1]</sup>. In contrast, CIPO in systemic lupus erythematosus (SLE) patients has shown an excellent response to immunosuppressive therapy when initiated early, consistent with an autoimmune etiology<sup>[2-5]</sup>. While the pathophysiology of CIPO in SLE remains unclear, the frequent concurrence of ureterohydronephrosis (67% in one case series)<sup>[3,4,6]</sup> and histopathologic evidence of intestinal leiomyocyte damage<sup>[3-5]</sup> suggest a systemic autoimmune process targeting smooth muscle cells. Here we describe a patient with SLE who presented with refractory intestinal pseudo-obstruction, ureterohydronephrosis and megacholedochus in the setting of delayed immunosuppressive therapy. This triad of gastrointestinal, genitourinary, and hepatobiliary hollow viscera dilatation and dysmotility has been described to co-manifest in lupus only once previously<sup>[6]</sup>. We term this rare and likely under-recognized clinical syndrome Generalized Megaviscera of Lupus (GML). An understanding of the pathophysiology of these processes is needed to avoid poor outcomes resulting from either unnecessary procedures and interventions or a delay in the diagnosis and/or initiation of treatment. We review the literature and discuss the pathophysiology.

## CASE REPORT

A 46-year-old Japanese-American female presented to our hospital with a 4-mo history of worsening arthralgias, abdominal discomfort, distension and obstipation. Her past medical history was notable for SLE diagnosed in 1993, which was complicated by disseminated encephalomyelitis, malabsorption syndrome, and biopsy-confirmed membranous glomerulonephritis. In the past, she has been treated with steroids and immunomodulators; however, because of adverse reactions to several of these medications, frequent changes were made to her treatment regimen. This resulted in poor compliance and resultant breakthrough episodes of disease exacerbations affecting various organ systems. Most recently, she was being treated with mycophenolate mofetil (CellCept<sup>®</sup>) and oral prednisone which she self-discontinued 8 mo prior to admission.

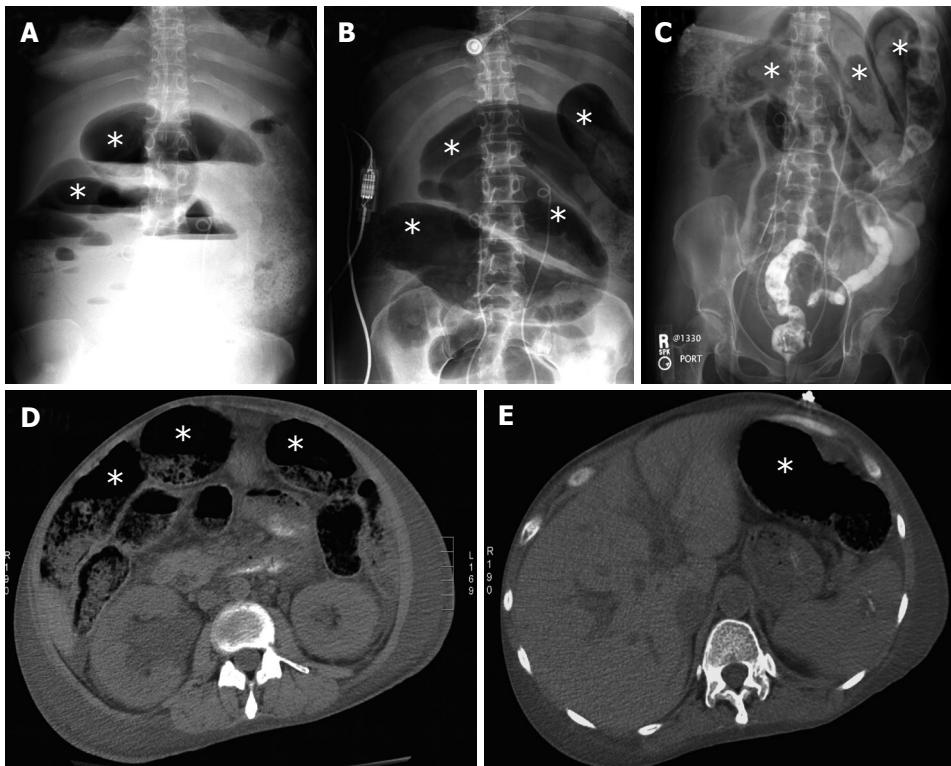
Physical examination revealed a distended tender abdomen with hypoactive bowel sounds. Laboratory tests were notable for elevated acute phase reactants, proteinuria, and acute renal insufficiency. An abdominal plain film X-ray series (Figure 1A-C) showed multiple air-fluid levels and distended loops of the large (up to

8 cm in diameter) and small (up to 3 cm in diameter) bowel without any obvious structural basis for luminal obstruction. Computed tomography (CT) of the abdomen (Figure 1D and E) confirmed the plain film findings but also showed ascites and fecalization of the luminal contents within the small bowel. Unlike lupus enteritis, the bowel wall in this case was neither thickened nor edematous<sup>[1]</sup>. Her symptoms and radiographic findings were consistent with an intestinal pseudo-obstruction. Supportive therapy was initiated with placement of a nasogastric tube in low intermittent suction, intravenous (IV) fluid, and pain medications. The patient was started on a course of tegaserod maleate (Zelnorm<sup>®</sup>) for 2 wk and 2 doses of neostigmine, with little improvement. In addition, she was also treated with enemas (glycerol, magnesium sulfate and water) and promotility agents (metoclopramide and erythromycin). A decompressive colonoscopy was attempted but the procedure was aborted at the level of the transverse colon because of poor visibility as a result of hard fecal matter and an increased risk of perforation. A gastrograffin enema was performed after the colonoscopic procedure, which demonstrated aperistaltic distal colon and contrast filling within a narrow lumen surrounded by stool that was cleared during the attempted colonoscopy (Figure 1C).

The abdominal CT scan also revealed intra- and extra-hepatic biliary tree dilatation (Figure 1E). To investigate this further, magnetic resonance cholangiopancreatography (MRCP) was performed and confirmed the presence of dilated bile ducts, without any structural basis for luminal obstruction (Figure 2). The common bile duct was dilated up to 2 cm without filling defects, calculi, or masses. The patient had no jaundice or right upper quadrant pain and her liver function tests remained within the normal limits throughout her hospital stay.

Regarding her acute renal insufficiency, she was started on IV fluid but her renal function did not improve despite adequate hydration. CT and MR images revealed bilateral hydroureters and hydronephrosis (Figure 1D, Figure 3B and C) without any structural obstruction. Obstructive uropathy resulting from lupus cystitis was entertained as a diagnosis and emergency placement of bilateral double-J ureteral stents was carried out in an attempt to decompress the genitourinary system. Despite optimal positioning of the stents, there was no improvement in the ureterohydronephrosis or her renal function. A voiding nephro-uretero-cystogram confirmed the persistence of dilated aperistaltic ureters bilaterally (Figure 3A and B). Treatment with cyclophosphamide (Cytoxan<sup>®</sup>) for presumed lupus cystitis also failed to produce any improvement. She continued to have modest urine output but nonetheless eventually required hemodialysis.

Full thickness biopsies from the genitourinary or gastrointestinal viscera were not performed in view of her poor clinical condition. She was treated with conventional immunomodulating therapies consisting of high dose IV methylprednisolone (Solu-Medrol<sup>®</sup>) and



**Figure 1** Megabowl: X-ray radiography (A-C) and computerized tomography scan (D, E) of the abdomen revealed the presence of distended loops (stars) of the small and large bowel (megabowl) in the absence of any luminal obstruction.



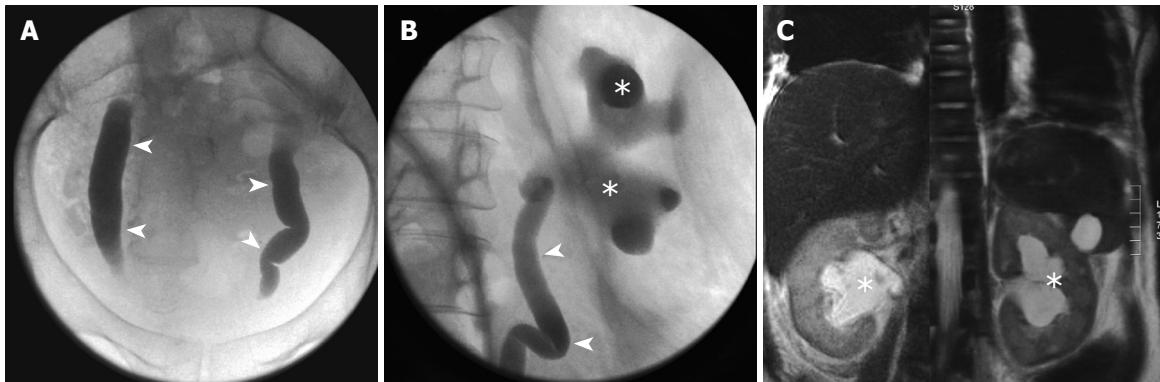
**Figure 2** Megacholedochus: magnetic resonance cholangiopancreatography revealed the presence of a distended common bile duct (megacholedochus, arrowheads) without any evidence of luminal obstruction.

mycophenolic acid (Myfortic<sup>®</sup>) along with parenteral nutrition, promotility agents, antibiotics, aggressive correction of electrolytes, and hemodialysis for presumed lupus flare with multiple organ involvement. She was discharged after improvement of her ascites, arthralgias, proteinuria and serum acute phase reactants. At 6 mo follow-up there was persistent dysfunction in all 3 organ systems without recovery of peristaltic function; however at no point during the clinical course was there any evidence of encephalopathy, cardiomyopathy, or any involvement of skeletal muscles either clinically or biochemically.

## DISCUSSION

We have described a patient with SLE who presented with abdominal distension, obstipation and renal

insufficiency with various imaging modalities revealing diffusely dilated hollow viscera in the absence of structural obstruction involving the genitourinary, hepatobiliary and gastrointestinal systems. Since these signs and symptoms are non-specific, it is important that all other possible diagnoses are entertained. The presentation as hydronephrosis in isolation deserves expert consultation and exclusion of other common causes e.g., lupus cystitis. Similarly, biliary tract dilation could occur as a result of coexisting liver diseases or as a direct consequence of an autoimmune phenomenon such as vasculitis. Hepatic artery vasculitis is known to give rise to a similar radiographic appearance but is usually accompanied by symptoms, resulting in abnormalities in liver function, and responds to systemic immunomodulation. Associated hepatobiliary diseases, e.g., autoimmune hepatitis, nodular regenerative hyperplasia, cryptococcal infection, should be considered in the differential diagnosis when isolated involvement of the biliary tract is seen. Involvement of multiple hollow visceral organs should raise suspicion of a generalized autoimmune smooth muscle injury, as in our case. Intestinal pseudo-obstruction is a very rare complication in SLE patients, but hydronephrosis are a frequent concurrent finding in these patients. The case presented here is among the first to show an additional visceral organ dilatation - megacholelithiasis. These findings suggest a truly rare, but likely under-recognized clinical syndrome we term generalized megaviscera of lupus (GML), which we define as hollow visceral dilatation and dysfunction present concurrently in more than one organ system in a patient with SLE, with intestinal pseudo-obstruction the most common manifestation.



**Figure 3** Hydronephrosis and megaureters: the presence of distended ureters (megaureters, arrowheads) was appreciated during a voiding nephro-uretero-cystogram (A, B) whereas hydronephrosis (stars) was detected by magnetic resonance imaging of the abdomen (C) as well as during the nephrogram (B).

We reviewed the anecdotal reports of patients with apparent GML in an attempt to further our understanding of the pathophysiology of this phenomenon. An overview of the pathological analysis of the gastrointestinal tract in lupus patients with CIPO revealed the following characteristics: widespread myocyte necrosis in the muscularis propria with active inflammatory cell infiltrate<sup>[5]</sup>, severe atrophy and fibrosis of the muscularis<sup>[3,5]</sup>, active serositis with serosal thickening and fibrosis<sup>[5]</sup>, little or no evidence of vasculitis or injury to bowel innervation<sup>[3-5]</sup>, and absence of thromboembolic disease<sup>[4,5]</sup>. It is notable that intestinal myonecrosis is observed without significant lupus vasculitis, often involving smooth muscle dysfunction in another organ system. These findings argue for the existence of a systemic circulating factor causing smooth muscle injury by a mechanism other than vasculitis (although the histopathologic basis for concomitant hydronephrosis and megacholedochus are yet to be determined). A good clinical response to immunosuppressive treatment has led some to hypothesize that the intestinal myopathy may be a direct result of an autoimmune phenomenon in the bowel wall<sup>[5]</sup>, and a common autoantibody against smooth muscle cells has been proposed<sup>[4]</sup>. It has been shown that autoantibodies against proliferating cell nuclear antigen (PCNA) have been detected exclusively in SLE patients and 2 cases were presented in which patients with this antibody in systemic sclerosis developed CIPO<sup>[7]</sup>; whether PCNA autoantibodies play a role in GML, however, remains unclear. Dense T-lymphocytic infiltrates with degeneration limited to the muscularis propria is considered the histopathologic hallmark of autoimmune enteric myositis causing CIPO in young children without lupus<sup>[8-10]</sup>. The location of intestinal myocyte damage is similar in SLE-associated CIPO, but dense T-cell infiltrates have not been observed; thus it is unclear whether the same disease process is involved. Autoimmune enteric neuropathy has been considered in the etiology of SLE-associated CIPO, but neuronal structures have generally been preserved on histopathology<sup>[3,5]</sup>, making this less likely. It has also been suggested that serositis can cause paralytic ileus,

and this may be a secondary cause of intestinal pseudo-obstruction in SLE<sup>[4]</sup>. Further studies will be needed to elucidate the pathogenic mechanism behind the development of GML.

In prior anecdotal reports, medical management that effectively led to improvement of GML, including remission of intestinal pseudo-obstruction and urinary symptoms, included a combination of high dose corticosteroids, immunomodulators and supportive care (parenteral nutrition, oral antibiotics, and pharmacological stimulation of small bowel motility)<sup>[2-5]</sup>. We had expected improvement and remission with similar management of our patient. Unfortunately, although there was improvement in the clinical and biochemical indicators of lupus exacerbation, e.g., ascites, arthralgias, proteinuria and serum acute phase reactants, she continued to show signs and symptoms of biliary dilatation, worsening of renal function, persistence of bowel distension and required parenteral nutrition and hemodialysis. This was concerning because of persistent generalized smooth muscle dysfunction causing aperistaltic megaviscera. While the clinical course is the best way to monitor treatment response, histopathologic reversal with immunosuppressive therapy has been documented for myositis-related intestinal pseudo-obstruction<sup>[11]</sup>. A delay in initiation of therapy has been associated with failure to regain functional peristalsis and was correlated with histopathologic progression to fibrosis and atrophy of the intestinal wall, and secondary impairment of the myenteric plexuses<sup>[12]</sup>. Progression to atrophy and fibrosis of the muscularis propria was also observed in a case of suspected non-compliance with immunosuppressive therapy in an SLE patient with CIPO<sup>[5]</sup>. Similarly, we presume that in the setting of delayed intervention and medication non-compliance, our patient developed advanced irreparable tissue destruction including myonecrosis, fibrosis and atrophy, resulting in failure of peristalsis to return despite treatment with high dose pulse steroids during her inpatient stay.

Our report emphasizes that timely diagnosis and intervention is crucial in the management of GML for the return of peristaltic activity in the various visceral organs involved. Appropriate imaging of the

gastrointestinal, genitourinary and hepatobiliary tracts should be obtained early in the investigation. It is also vital to recognize intestinal pseudo-obstruction as the cause of the symptoms and signs in order to avoid unnecessary and invasive interventions, which may confer extra risks and lead to further damage. Neostigmine was administered twice to our patient, and caused increased crampy abdominal pain and discomfort but failed to improve gut motility. Given that neostigmine acts at the myoneural junction upstream of the damaged myocyte, it is unlikely to help in this setting. Moreover, ureteral stenting has been utilized in lupus cystitis but is largely without benefit in the setting of aperistaltic ureters; this procedure caused multiple complications in our patient including ascending urinary tract infections and hematuria. The Gastroenterology service was consulted repeatedly to perform endoscopic retrograde cholangio-pancreatography (ERCP) for evaluation of her dilated biliary ducts. Biliary tree dilatation is without consequence, especially with normal liver function tests and reflects aperistalsis within the bile duct system. However, knowledge of this is essential to avoid unnecessary invasive procedures such as ERCP and/or biliary stenting. In our patient, a more conservative imaging modality, MRCP, was equally useful to rule out structural obstruction and avoided ERCP related risks. Overall, it seems that early recognition of generalized visceral organ dilatation in lupus patients, consistent with the syndrome GML, is helpful for gastroenterologists, urologists, and rheumatologists to initiate supportive care and early immunomodulation to restore peristaltic function as well as to avoid invasive procedures which may not address the basic pathophysiologic process involved.

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CASE REPORT

## Infiltrating adenocarcinoma arising in a villous adenoma of the anal canal

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### INTRODUCTION

The anal canal is comprised of several tissue types unique to this anatomic transition zone, ranging from colonic mucosa to keratinized epithelium with skin appendages. Anal neoplasms are a relatively uncommon occurrence in the context of all gastrointestinal tumors. Benign anal adenomas arising from the anus are particularly rare diagnoses. Furthermore, malignant neoplasms affecting the anal canal are most often of the squamous cell type and very seldom adenocarcinoma, especially in patients without known risk factors for the development of epithelial neoplasms, such as those with inflammatory bowel disease. The following describes an otherwise healthy octogenarian patient diagnosed with a well-differentiated adenocarcinoma. Although rarely reported, we concluded that this tumor may have arisen from epithelial components of the anal canal, following the sequence normal epithelium-adenoma-carcinoma (Vogelstein model) which, although has been well-described for colorectal tumors, it has only been reported twice previously.

### CASE REPORT

An 81 year-old Caucasian man presented to the emergency department with the chief complaint of continuous rectal bleeding of several months duration, which was particularly associated with bowel movements.

### Abstract

Primary neoplasms arising in the anal canal are relatively unusual. In particular, adenomas and adenocarcinomas are distinctly rare entities in this region. We describe an infiltrating, well-differentiated adenocarcinoma arising in a villous adenoma from the distal anal canal, in an otherwise healthy patient at low risk for gastrointestinal malignancy. This is the case of an octogenarian man with a several year history of hemorrhoids and intermittent rectal bleeding, more recently complaining of continuous hematochezia. Examination revealed a blood-covered pedunculated mass with a long stalk protruding from the anus. The lesion was amputated at the bedside. Microscopic evaluation revealed an infiltrating well-differentiated adenocarcinoma, arising from a villous adenoma. This was further evaluated under anesthesia and complete excision of distal anal tissue was performed. Our report is the first describing the possible malignant degeneration of a villous adenoma in the anal canal.

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**Key words:** Anal adenocarcinoma; Anal canal; Villous adenoma

He reported chronic intermittently bleeding hemorrhoids for about 20 years. He denied comorbid conditions and took no medications. He also denied recent weight loss, change in appetite or bowel habits. This patient reportedly underwent a colonoscopic evaluation about four years prior to the current presentation, with negative results. His family history was unremarkable for gastrointestinal benign or malignant problems. Physical examination revealed a gentleman who appeared younger than the stated age, in no acute distress. Perianal visual and digital examination showed a blood-covered 3 cm × 4 cm pedunculated mass protruding from the anus by a stalk. Significant laboratory data demonstrated microcytic anemia (hemoglobin of 6.4 mg/dL) and normal coagulation times. He was transfused with packed red blood cells and the lesion was amputated at the bedside. Microscopic analysis revealed an infiltrating well-differentiated adenocarcinoma arising from a villous adenoma (Figure 1). Computed tomography of the chest, abdomen and pelvis demonstrated no evidence of metastatic disease. The patient was subsequently taken to the operating room for examination under anesthesia and complete excision of the anal mass. Intraoperative findings revealed very large hemorrhoids, of 0.5 cm in diameter on average occupying the entire circumference of the anus (Figure 2). Gentle dilation of the anus revealed an ulcerated area of 2 cm in length at 5 o'clock in the supine position extending from the anal orifice to about 1 cm into the anal canal. Hemorrhoidectomy was performed and the ulcerated tissue was excised. Microscopic evaluation showed evidence of the prior procedure, but did not show residual neoplasm. This tumor was staged as T<sub>1</sub>N<sub>0</sub>M<sub>0</sub>.

Detailed microscopic evaluation of the mass showed villous fronds, lined by dysplastic, intestinal-type epithelium with scattered goblet cells. Several microscopic foci of invasion into the lamina propria were identified. The neoplasm demonstrated diffuse staining for cytokeratin (CK)-20 and was negative for CK7 (Figure 3). Histologic examination of the residual anal tissue excised in the operating room clearly showed reactive (not malignant) rectal-type glands present near the surface, in between areas of squamous epithelium, as well as ulceration of superficial mucosa, consistent with the prior excision. No residual adenoma or carcinoma was identified.

This case was discussed at our local tumor board conference. No consideration for further surgical or medical therapy was deemed appropriate for his optimal care. The patient was contacted two years after his intervention, and he is doing well, without recurrence of rectal bleeding or any other problems related to the tumor herein presented.

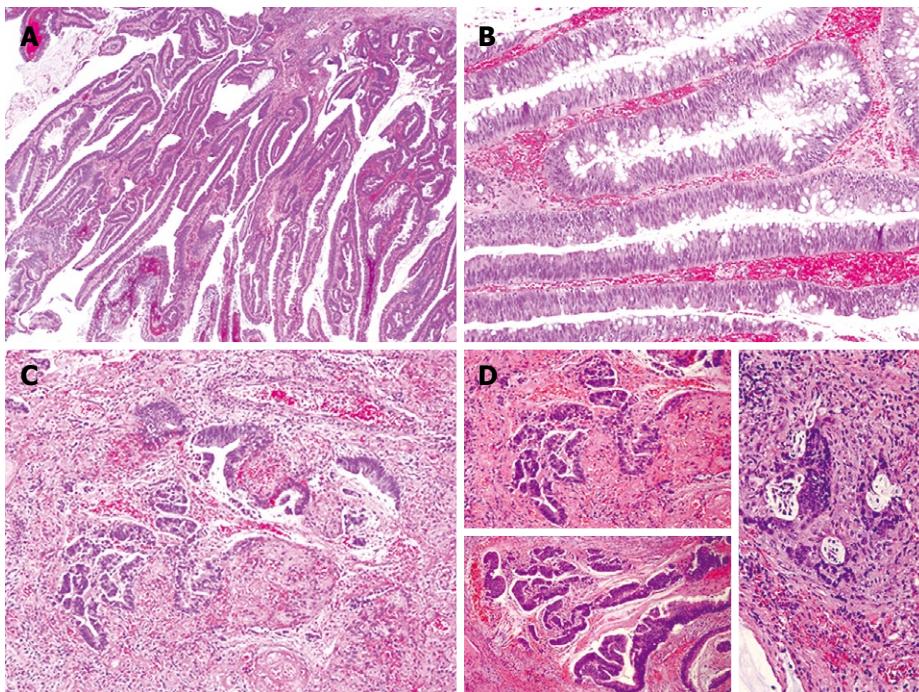
## DISCUSSION

Primary anal cancer is a rare occurrence. However, its incidence has been rising over the last 25 years. Moreover, despite the small number of patients affected by these malignancies, they remain as one of the most

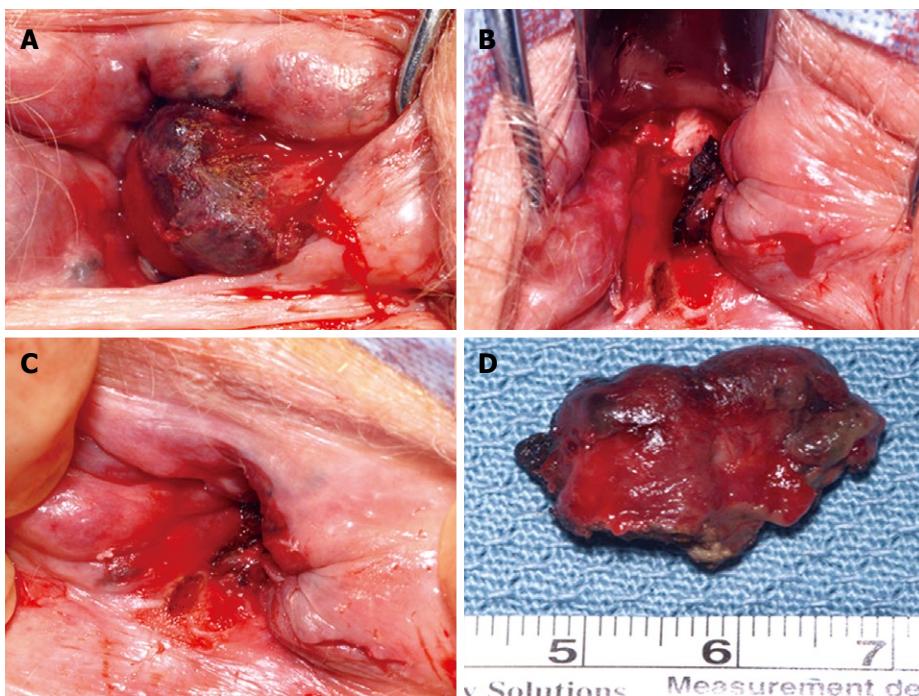
challenging cancers to treat. Once thought to be related to chronic irritation, multiple risk factors, including human papillomavirus or human immunodeficiency virus infection, anal sexual intercourse, smoking and immunosuppression, have relatively recently been identified<sup>[1]</sup>. Anal tumors represent 5% of anorectal cancers. They have been classified in tumors of the anal canal and those of the anal margin, with mainly two histological types described. Among them, squamous carcinoma comprises approximately 95% of anal tumors<sup>[2]</sup>.

Anal adenocarcinomas constitute about 5% of malignant anal neoplasms<sup>[2]</sup>. The latter tumors have been described in correlation with anal fistulae<sup>[3]</sup>, hemorrhoids<sup>[4]</sup>, after restorative proctocolectomy with a stapled ileal pouch-anal anastomosis without mucosectomy due to ulcerative colitis<sup>[5,6]</sup>, or with Crohn's disease<sup>[7]</sup>. Together, they account for 0.1% of all gastrointestinal cancers<sup>[8]</sup>.

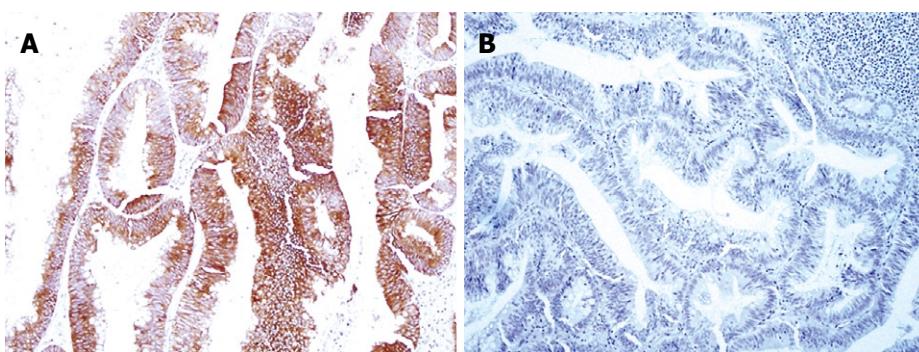
The progression of the normal colorectal epithelium to malignancy has been extensively studied in both experimental and clinical models, and a defined normal mucosa-adenoma-carcinoma sequence has been well recognized<sup>[9-11]</sup>. However, in the anal canal this information is lacking. Benign anal adenomas are extremely rare diagnoses. Multiple adenomatous polyps arising in the transitional zone of the anus in a patient with familial adenomatous polyposis (FAP) seven years after colon resection and ileo-anal anastomosis were described by Malassagne and colleagues<sup>[12]</sup>. Anand *et al*<sup>[13]</sup> reported a tubulo-villous adenoma which arose from either an anal gland or its duct that opens into the anus. These tumors are rarely encountered in patients without predisposing risk factors, such as FAP, ulcerative colitis or Crohn's disease<sup>[13]</sup>. The above-mentioned transformation from normal epithelium to malignant tumors in the anal canal has only been previously recognized twice to the best of our knowledge. MacNeill and colleagues<sup>[14]</sup> described the case of an invasive apocrine adenocarcinoma arising in a benign adenoma in the perianal region of a 45-year-old woman. Obaidat *et al*<sup>[15]</sup> reported the occurrence of anal adenocarcinoma *in situ* associated with a tubulopapillary apocrine hidradenoma. These reports suggest that adenomatous elements from the squamocolumnar junction may demonstrate hyperplastic or malignant transformation in patients without genetic predisposition. Intraoperative examination in our case made us believe that an adenomatous polypoid lesion originated from distal anal tissue in an area of chronic inflammation and ulceration consistent with the anal transition zone. Some investigators, however, have found evidence to support alternative pathways to the adenoma-carcinoma sequence<sup>[16]</sup>. Specific mutations in key onco-suppressor genes have been found to relate to the anatomical site of the tumor and therefore, a significant proportion of rectal cancers may arise *via* alternative pathways to the Vogelstein model. Polyp behavior along with malignant propensity may actually be site dependent. Rectal polyps are thought to harbor a more aggressive phenotype. In



**Figure 1 Microscopic sections.** A: The adenoma demonstrate villous architecture with long, delicate fronds (HE,  $\times 40$ ); B: The villous fronds are lined by dysplastic, intestinal-type epithelium with frequent goblet cells (HE,  $\times 100$ ); C and D: Multiple foci of invasive carcinoma are identified, with invasion confined to the lamina propria (HE,  $\times 100$ ).



**Figure 2 Intraoperative photographs.** A and D: Very large hemorrhoids up to 2 cm circumferentially located around the anal canal; B: The final appearance after complete excision of the hemorrhoids and the malignant mass; C: The area that contained the adenocarcinoma, previously excised in the emergency department, and prior to operating room excision.



**Figure 3 Immunohistochemical staining.** A: Cytokeratin 20 is diffusely positive within the epithelium ( $\times 100$ ); B: Cytokeratin 7 is negative ( $\times 100$ ).

the anal area, this remains uncertain.

CK-20 positivity with negative CK7 staining is an

immunohistochemistry profile most consistent with rectal-type mucosa and is not in keeping with the

apocrine or colloid types of adenocarcinoma that have been typically described as arising from the anal canal. In addition, the histologic appearance in this case of a well-formed villous adenoma with microscopic invasive carcinoma is different from typical anal gland carcinoma, which is characterized by small, tubular structures diffusely invading stroma. Clinical and pathologic findings in our case point to a true anal location for this pedunculated mass. Visual inspection showed this mass to arise distal to the dentate line, whereas histologic examination of the final excision specimen showed rectal-type glands present in between areas of normal squamous epithelium. The combination of these findings suggest the development of a tubulo-villous adenoma from rectal-type glands present in the anal mucosa, possibly as part of a reactive metaplastic phenomenon, similar to what was described in the case report from Anand and colleagues<sup>[13]</sup>. However, these authors did not report malignancy or immunohistochemistry findings. Therefore, whether the histologic profile of their case and ours is the same is unknown.

For anal adenocarcinoma, aggressive surgical resection remains the mainstay of therapy, with radiation therapy and chemotherapy used to aid in local disease control and for treatment of metastatic disease. A high rate of distant failure in this disease is responsible for the poor long-term prognosis<sup>[17]</sup>. Once invasive adenocarcinoma features were identified on pathologic examination of the polyp excised in the emergency department, this patient was taken to the operating room to ensure adequacy of resection. Some may argue that, to increase the chance of survival in this octogenarian, a larger extent of resection is needed. An analysis of 13 patients with primary anal adenocarcinoma who were treated over a 12-year period was performed<sup>[18]</sup>. With a median follow-up of 19 mo, the median survival was 26 mo, local failure rate was 37%, and the two-year actuarial survival was 62%. This study, although small, suggested that the combination of abdomino-perineal resection and combined modality therapy was a reasonable approach for this rare tumor<sup>[18]</sup>. However, this treatment strategy has to be individualized to both the patient and the tumor characteristics, as well as to the patients' wishes.

The need to perform lower gastrointestinal endoscopy in patients at low risk for colorectal malignancies is debatable. Patients such as the one presented herein, had rectal bleeding as the presenting symptom and without other alarming signs for colorectal cancer, such as recently altered bowel habit, weight loss and family or personal history of colorectal neoplasms, which were studied with colonoscopy by Sotoudehmanesh *et al*<sup>[19]</sup>. A number of unexpected findings were identified, such as adenomatous polyps (3%), ulcerative colitis (6%) and Crohn's disease (0.7%). Fissures were posterior in 106 cases (79.1%) and anterior in 27 cases (20.1%); one patient (0.7%) had both anterior and posterior fissures. The lower gastrointestinal endoscopy was abnormal in 10.4% of patients. No cases of adenocarcinoma were identified. Therefore, lower gastrointestinal endoscopy might be appropriate in this

selected group of patients, if these findings are confirmed by further studies<sup>[19]</sup>. Carlo *et al*<sup>[20]</sup> specifically studied patients with hematochezia but without risk factors for neoplasia, and concluded that those patients younger than 45 years of age do not need a total colonoscopy, whereas they recommended mandatory total colonoscopy in older patients even in the presence of anal pathology. In the latter group they were able to identify proximal colonic inflammatory bowel disease, neoplastic polyps and even carcinoma.

In conclusion, the transition between normal tissue-adenoma-carcinoma has been well studied in the colorectal arena but very infrequently reported in the anal canal. Our report supports the application of the same concept for the anal region, at least for patients without major risk factors for the development of gastrointestinal malignancies. The infrequent occurrence of anal adenocarcinoma and the paucity of reports in the literature documenting these tumors make conclusions about this subject difficult to reach. The literature seems to suggest further endoscopic studies of the rest of the gastrointestinal tract in elderly patients with rectal bleeding and no risk factors for colorectal cancer.

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## Extramedullary plasmacytoma associated with a massive deposit of amyloid in the duodenum

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associated with a massive deposit of amyloid in duodenum. A subsequent workup for multiple myeloma was completely negative. The patient showed no signs of local recurrence or dissemination of the disease after 12 mo follow-up. Because of the association of plasmacytoma and amyloidosis, the patient must be followed up because of the possible systemic involvement of the neoplasm and amyloidosis in future.

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### Abstract

We report a rare case of extramedullary plasmacytoma associated with a massive deposit of amyloid in the duodenum. A 72-year-old Japanese man was admitted to our hospital presenting with a 3-mo history of epigastric pain, vomiting and weight loss. On computed tomography (CT) a wall thickening of the fourth part of the duodenum was observed. Multiple biopsies obtained from the lesion showed infiltration of plasma cells and lymphocytes, but they were not conclusive. The patient underwent resection of the lesion and, on histopathological examination, the lesion consisted of a dense and diffuse infiltrate of plasma cells and a few admixed lymphocytes with reactive follicles extending to the muscular propria. An extensive deposition of amyloid was also observed. Immunohistochemical stains revealed that a few plasmacytoid cells showed  $\lambda$  light chain staining, though most were  $\kappa$  light chain positive. These cells also were positive for CD138 and CD56 but negative for CD20 and CD79. The findings were consistent with extramedullary plasmacytoma

### INTRODUCTION

Plasma cell neoplasms account for approximately 1%-2% of human malignancies and they are classically categorized into four groups: multiple myeloma (MM), plasma cell leukemias, solitary plasmacytomas of the bone (SPB), and extramedullary plasmacytomas (EMP). EMP represents 3% of these neoplasms and it is defined as a soft-tissue plasma cell tumor occurring in the absence of systemic signs of multiple myeloma<sup>[1]</sup>. Most cases of EMP arise in the upper aerodigestive tract (UAD); other sites of involvement include the gastrointestinal tract, breast, thyroid, testis, bladder, retroperitoneum, and lymph nodes. In non-UAD regions, the gastrointestinal tract represents 40% of cases<sup>[2]</sup>. We reported an unusual case of localized

plasmacytoma associated with massive local deposition of amyloid in the duodenum.

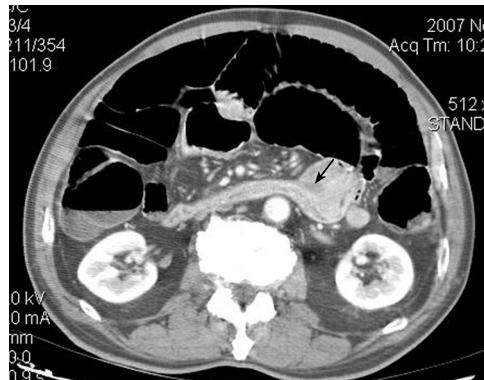
## CASE REPORT

A 72-year-old Japanese man was admitted to our hospital presenting with a 3-mo history of epigastric pain, vomiting and weight loss. No abdominal mass was palpated on physical examination. An upper endoscopy revealed a mass in the duodenum. CT showed wall thickening of the fourth part of the duodenum (Figure 1). Lymphadenopathy was not seen. Multiple biopsies obtained from the lesion showed infiltration of plasma cells and lymphocytes, but they were not conclusive. The patient underwent resection of the fourth part of the duodenum and proximal segment of jejunum. On gross examination of the surgical specimen, the involved segment measured 5 cm in length (Figure 2). On histopathological examination, the lesion consisted of a dense and diffuse infiltrate of plasma cells and a few admixed lymphocytes with reactive follicles extending to the muscular propria. An extensive deposition of hyaline amorphous eosinophilic extracellular material was also observed (Figure 3). With Congo red stain, amyloid appeared red in normal light and apple-green in polarized light. Immunohistochemical stains revealed that a few plasmacytoid cells showed  $\lambda$  light chain staining, while most were  $\kappa$  light chain positive (Figure 4A and B). These cells also were positive for CD138 and CD56 but negative for CD20 and CD79 (Figure 4C and D). The scattered lymphocytes represented a mixture of CD3-positive T cells and CD20-positive B cells. The extracellular hyaline materials also showed reactivity with anti-kappa (but not lambda) immunoglobulin light chains. The findings were consistent with a plasma cell neoplasm. A subsequent workup for MM (serum and urinary protein electrophoresis, radiographic examination of the axial skeleton and a bone marrow aspirate and biopsy) was completely negative. Anemia and hypercalcemia were not seen. The patient showed no signs of local recurrence or dissemination of the disease after 12 mo follow-up.

## DISCUSSION

The involvement of gastrointestinal tract is common in MM but a localized plasmacytoma is rare and not suspected clinically. In previous reports<sup>[3-6]</sup> of duodenal EMP, males were more affected than females and the disease was most commonly seen in patients older than 50 years of age. These patients presented with nonspecific symptoms such as dyspepsia, vomiting, epigastric pain and weight loss. As in our case, in these previous reports radiological features were not suggestive and the diagnosis was revealed only by the findings of histopathological and immunohistochemical examination of the sample biopsy or surgical specimen, mainly the light chain restriction (demonstrating the monoclonality) and positivity for CD138 (indicating plasma cell lineage).

Treatment options for EMPs include surgical resec-



**Figure 1** CT showing a mass on the fourth part of the duodenum (arrow).

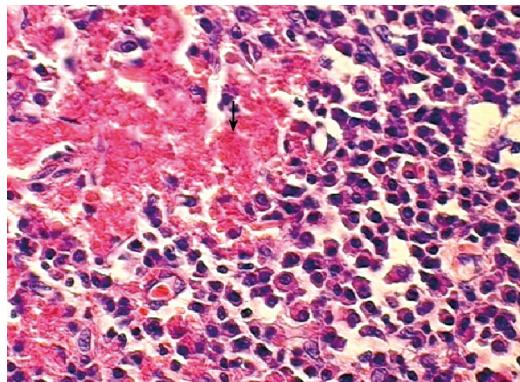
tion and radiation therapy, given the relative sensitivity of plasma cell neoplasms to radiation<sup>[2,7]</sup>. Alexiou *et al*<sup>[2]</sup> found no statistically significant difference in patient survival comparing surgery alone, surgery plus radiotherapy, or radiotherapy alone. Surgery alone, however, had the lowest recurrence rate. Our patient was not submitted to radiation after surgery. Of all of the plasma cell tumors, EMPs have the best prognosis. Progression to MM is more frequent in solitary osseous plasmacytoma than in EMP<sup>[8,9]</sup>. The prognosis of patients with EMP of the duodenum is uncertain as so few patients have been reported, but, of those cases reported in the literature, we found two that presented evolution to myeloma after surgery<sup>[10,11]</sup>.

Amyloidosis, a complication of plasmacytoma in the present case, is characterized histopathologically by the extracellular deposition of insoluble fibrillar proteins. In our case, amyloidosis was caused by the deposition of immunoglobulin light chains since both amyloid and plasma cells shared the same immunoglobulin light chain restriction (lambda-restriction). The absence of systemic amyloidosis in massive localized deposits, as in our case, may be explained by the secretion of abnormal, poorly soluble immunoglobulin molecules with a tendency toward local precipitation<sup>[12]</sup>. We did not find previous cases of amyloidosis associated with duodenal plasmacytoma, so the impact of associated amyloid accumulation on progression or response to treatment in our case cannot be determined. Amyloid deposition can be found in 15% to 40% of extramedullary plasmacytomas in the head and neck regions and, according to previous reports, without clinical significance<sup>[13]</sup>.

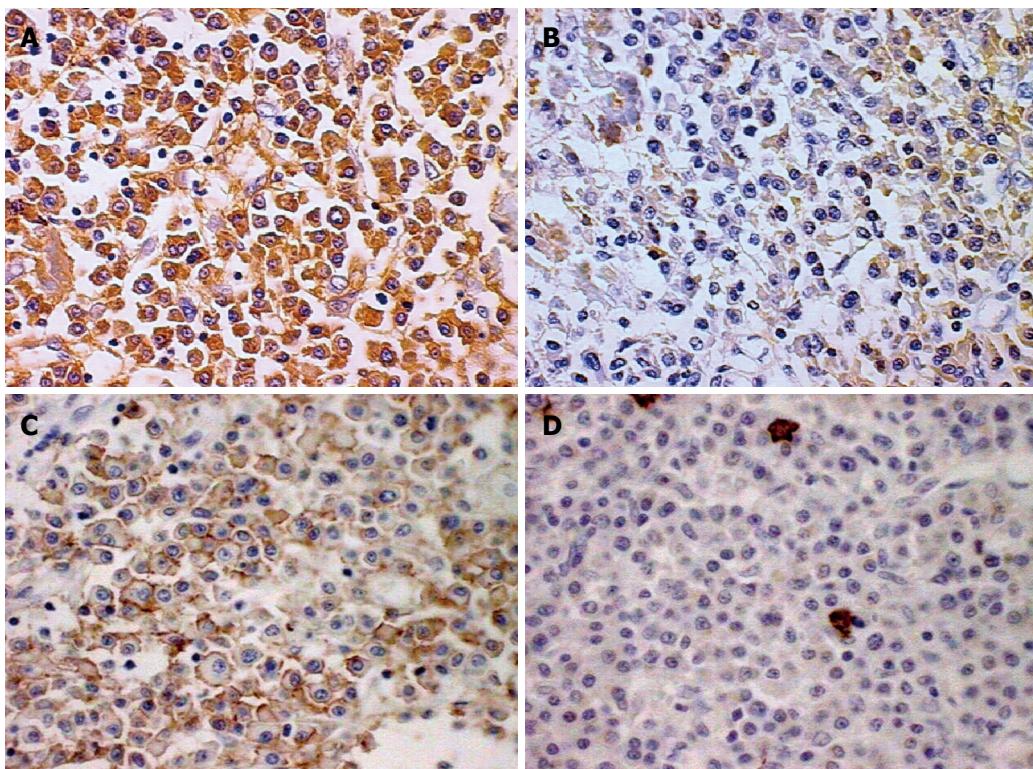
In view of their indolent biological nature and their frequent involvement of anatomic sites containing mucosal lymphoid tissues, it has been proposed that extramedullary plasmacytomas represent a low-grade lymphoma of mucosal lymphoid tissues (MALT) with extensive plasmacytic differentiation. Results from a previous study support this hypothesis<sup>[14]</sup>; morphologic features of MALT lymphoma, including centrocytic-like cells, reactive follicles, and lymphoepithelial lesions, are often found in extramedullary plasmacytomas. Besides this, amyloid deposits have also been described in lym-



**Figure 2** Gross appearance of the surgical specimen containing the tumor (arrow).



**Figure 3** Histopathological examination displaying a dense and diffuse infiltrate of plasma cells and amyloid deposit (arrow). (HE  $\times 400$ ).



**Figure 4** Immunohistochemical findings (positive cells in brown,  $\times 400$ ). A: Most plasmacytoid cells were positive for  $\kappa$  light chain; B: Few plasmacytoid cells showing  $\lambda$  light chain staining; Plasmacytoid cells were positive for CD56 (C) and negative for CD20 (D).

omas<sup>[15]</sup>. However, according to others authors, some immunohistochemical findings on plasmacytoid cells are helpful in differentiating plasmacytoma from MALT lymphoma<sup>[16,17]</sup>. These authors found that plasmacytoid cells were less likely to express CD56 in lymphoma than in myeloma. So, the positivity for CD56, in our case, suggests that it may indeed represent a true primary PC dyscrasia (plasmacytoma), with phenotypic feature similar to myeloma.

In conclusion, plasmacytoma localized in the duodenum is rare and may cause intestinal obstruction. The diagnosis is performed by histopathological examination and must be distinguished from lymphomas with extensive plasmacytic differentiation. In associations of EMP and amyloidosis, the patient must be followed up

because of the possible systemic involvement of the neoplasm and amyloidosis in future.

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# Laparoscopic diagnosis of pleural mesothelioma presenting with pseudoachalasia

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## Abstract

Pseudoachalasia due to pleural mesothelioma is an extremely rare condition. A 70-year-old woman presented with progressive dysphagia for solid and liquids and a mild weight loss. A barium swallow study revealed an esophageal dilatation and a smoothly narrowed esophagogastric junction. An esophageal manometry showed absence of peristalsis. Endoscopy demonstrated an extrinsic stenosis of the distal esophagus with negative biopsies. A marked thickening of the distal esophagus and a right-sided pleural effusion were evident at computed tomography (CT) scan, but cytological examination of the thoracic fluid was negative. Endoscopic ultrasound showed the disappearance of the distal esophageal wall stratification and thickening of the esophageal wall. The patient underwent an explorative laparoscopy. Biopsies of the esophageal muscle were consistent with the diagnosis of epithelioid type pleural mesothelioma. An esophageal stent was placed for palliation of dysphagia. The patient died four months after the diagnosis. This is the first reported case of pleural mesothelioma diagnosed through laparoscopy.

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**Key words:** Dysphagia; Achalasia; Pseudoachalasia; Mesothelioma; Laparoscopy

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## INTRODUCTION

Esophageal achalasia is an uncommon disorder, with an incidence of about one case per 100 000 persons per year. The term "pseudoachalasia" generally applies to a subgroup of less than 5% of patients presenting with clinical features of achalasia in whom an occult malignant tumor is the cause of dysphagia<sup>[1]</sup>. Benign pseudoachalasia is an even more unusual condition, accounting for less than 2% of patients with achalasia-like symptoms<sup>[2]</sup>. Adenocarcinoma of the esophagogastric junction represents the most frequent diagnosis in patients with malignant pseudoachalasia, accounting for 70% of cases<sup>[3]</sup>. Other causes include lymphoma<sup>[4-6]</sup> and primary neoplasms from the lung, esophagus, liver, pancreas, colon, kidney, prostate, and breast<sup>[7-14]</sup>.

Malignant mesothelioma has been infrequently associated with dysphagia. In some reports, dysphagia was secondary to direct extension of the cancer into the esophagus and was a late, usually terminal, event<sup>[15]</sup>. The first case of pleural mesothelioma presenting as dysphagia was described by Johnson in 1983<sup>[16]</sup>. We report the case of a patient presenting with pseudoachalasia in whom the diagnosis of pleural mesothelioma was performed by laparoscopy.

## CASE REPORT

A 70-year-old woman presented in December 2007 with a two-month history of progressive dysphagia for solids and liquids, and a 5-kg weight loss. There was no prior history of gastroesophageal reflux, odynophagia, or other gastrointestinal symptoms. Social history was negative for tobacco use and alcohol intake. Physical examination was unremarkable and routine laboratory tests were within normal limits. A barium swallow study revealed a mildly dilated esophagus and a smoothly narrowed esophagogastric junction (Figure 1). An



**Figure 1** Barium swallow showing dilatation of the esophageal body and narrowed esophago-gastric junction.

esophageal manometry showed absence of peristalsis (Figure 2), but evaluation of the lower esophageal sphincter was precluded due to the inability to advance the catheter into the gastric cavity. Subsequent upper gastrointestinal endoscopy showed no evidence of gross mucosal lesions, but a mild resistance to the passage of the instrument through the cardia was noted. On retroflexed view, the esophagogastric junction appeared normal. A pneumatic dilation was performed using a 30 mm Rigiflex balloon dilator inflated at 10 PSI for one minute, and multiple esophageal biopsies were performed, which showed no evidence of malignancy.

Three weeks later, due to the persistence of dysphagia, a thoraco-abdominal CT scan was performed that demonstrated the presence of a right pleural effusion, thickening of the mediastinal and parietal pleura, and a 4-cm long concentric thickening of the distal esophageal wall (Figure 3). A thoracentesis yielded 1500 cc of yellow fluid with a protein content of 2 g/dL and a pH of 9. Bacteriological and cytological examinations of the fluid were negative. Subsequent endoscopic ultrasound examination showed the disappearance of the distal esophageal wall stratification and thickening of the wall up to 9 mm. At that point, the decision to proceed with an exploratory laparoscopy was taken in an attempt to clarify the diagnosis. Upon incision of the peritoneal reflection, the distal esophageal wall appeared markedly thickened and tightly adherent to the diaphragm. No lymphadenopathy was found in the lesser sac. Peritoneal lavage cytology was negative. Intraoperative ultrasound confirmed the thickening of the distal esophagus.

Multiple biopsies from the esophageal muscle wall and from the contiguous diaphragm were taken. Histopathological findings from the esophageal wall were consistent with the diagnosis of pleural mesothelioma (epithelioid type). Immunohistochemistry was positive for calretinin, vimentin, Ckpan and CK 7 (Figure 4). Soon after surgery the patient complained of recurrent dysphagia and onset of pain in the right side of the chest.

Two weeks later, a right thoracoscopy in the prone position with single-lumen intubation was performed to complete the staging of the disease and to provide drainage of the recurrent pleural effusion. Multiple nodularities and plaques were noted along

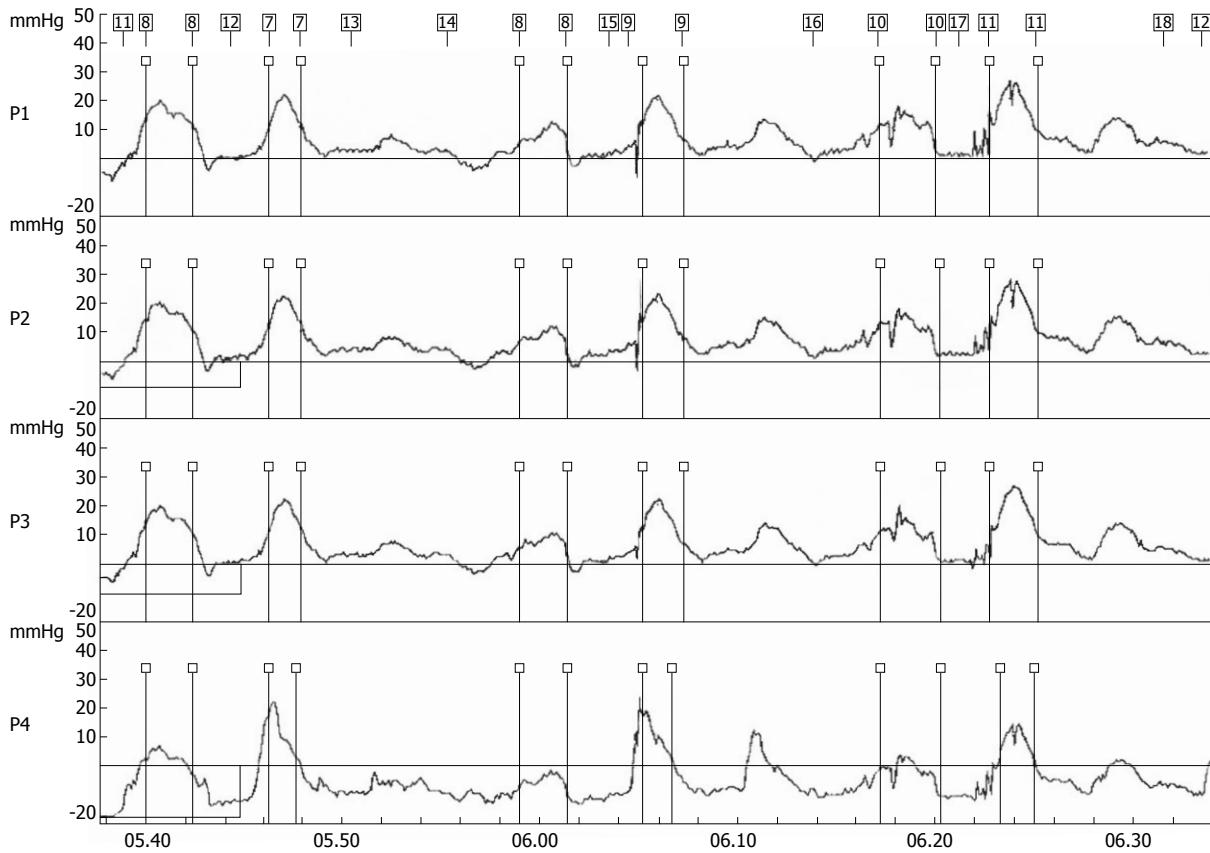
the diaphragmatic, parietal and mediastinal pleura surfaces, mostly in the lower half of the pleural cavity. Multiple biopsies were repeated, which confirmed the previous histopathological diagnosis. During the same operative session, a port-a-cath was inserted in the right subclavian vein, and a 10 cm self-expanding esophageal metal stent with antireflux valve (Hanarostent®) was deployed endoscopically. A gastrographin swallow study performed the next day showed a partial stent expansion that required a single endoscopic balloon dilation within the stent. As a result of this treatment, there was a marked improvement of dysphagia but worsening of the chest pain, requiring sustained analgesia. The patient was also started on a chemotherapy regimen. She died from septic shock four months after the diagnosis without stent related complications.

## DISCUSSION

Malignant pseudoachalasia is a term used to describe the clinical picture of gastroesophageal junction obstruction associated to an occult submucosal tumor or a non-contiguous tumor. Several mechanisms have been proposed to explain the pathogenesis of this type of secondary achalasia. First, the tumor might directly infiltrate the nerves within the myenteric plexus of the esophagus. Second, a paraneoplastic syndrome might affect the function of the distal esophagus<sup>[13]</sup>. Third, the tumor might replace the smooth muscle at the esophagogastric junction, reducing compliance of the esophageal wall to distension. In turn, the esophagus might generate high pressures to overcome this obstruction with dilatation as a compensatory response.

Patients with malignant pseudoachalasia are, as a group, older than patients with primary achalasia. In addition, there is a male predominance, in contrast to primary achalasia in which both sexes seem to be equally affected. Tucker<sup>[7]</sup> suggested that advanced age (> 50 years), short duration of symptoms (< 1 year) and marked weight loss (> 15 pounds) support the diagnosis of secondary achalasia over primary achalasia. When these criteria are met, patients should undergo additional imaging to rule out an occult malignancy. It should also be kept in mind that an occult malignancy cannot be reliably detected even during the course of a laparoscopy esophagomyotomy for presumed primary achalasia<sup>[3,17]</sup>.

Pleural mesothelioma is a rare cause of malignant pseudoachalasia, accounting for 7.5% of all diagnoses<sup>[3]</sup>. Goldschmiedt *et al*<sup>[18]</sup> reported of a 64-year-old male presenting with progressive dysphagia and radiological and manometric findings suggestive of achalasia. Thoracentesis and pleural biopsy were performed for a left pleural effusion and no malignant cells were found. The CT scan was negative. At left thoracotomy, multiple pleural plaques covered the diaphragmatic surfaces, as well as the pleura reflection over the lung. A distal esophageal myotomy was carried out and revealed an abnormal tissue consistency at the level of the esophagogastric junction. Biopsy and immunohistochemical studies showed a malignant epithelial mesothelioma.

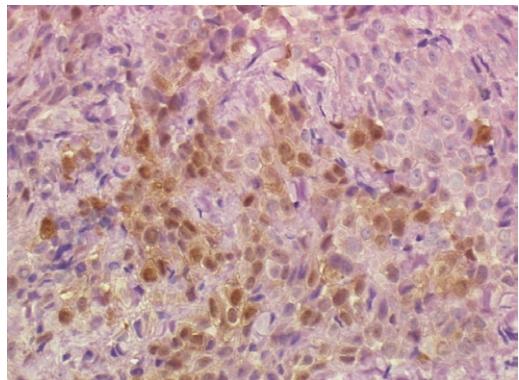


**Figure 2** Stationary esophageal manometry showing synchronous waves throughout the esophageal body indicating aperistalsis.



**Figure 3** CT scan showing right pleural effusion and concentric thickening of the esophageal wall.

The preoperative diagnosis of pseudoachalasia secondary to pleural mesothelioma is problematic. A CT scan is often inaccurate in diagnosing the extension of the disease through the diaphragm<sup>[19]</sup>. Thoracentesis is usually the initial diagnostic procedure because most patients present with a pleural effusion. However, pleural fluid cytology is positive for malignancy in less than 50% of patients. Percutaneous pleural biopsy is also unreliable because the small specimens obtained do not allow immunohistochemical studies to differentiate the disease from metastatic adenocarcinoma<sup>[20]</sup>. There is only one case report showing the efficacy of EUS-guided fine-needle aspiration to diagnose pleural malignant mesothelioma in a patient with CT and endosonographic evidence of a



**Figure 4** Biopsy of the distal esophageal wall taken at laparoscopy showing a neoplastic cell population consistent with the diagnosis of pleural mesothelioma (epithelioid type). Immunohistochemistry stains positive for calretinin (40 × HPF).

paraesophageal mass<sup>[21]</sup>. Video-assisted thoracoscopy is considered the diagnostic procedure of choice because it consistently yields a diagnosis without committing the patient to a major surgical procedure through a formal thoracotomy<sup>[22]</sup>. In our patient, laparoscopy was safe and effective in providing the correct diagnosis. To our knowledge, this is the first reported case of pleural mesothelioma diagnosed through laparoscopy.

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## Biliary cystadenoma

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## INTRODUCTION

Cystadenoma is a benign tumor, although prone to malignant degeneration<sup>[1]</sup>, supposedly originating in intrahepatic (and more rarely extrahepatic) embryonic tissue precursors of biliary epithelium. It is a non recurrent lesion, with only 125 cases reported in literature<sup>[2]</sup>. It may appear either as a uninodular or as a multinodular cystic lesion, and may attain large proportions.

Cystadenomas account for 4.6% of intrahepatic biliary cysts. They are more recurrent in middle-aged females (40-50 years old) with an incidence rate of 4:1 with respect to males. Cystadenomas are rarely found in extrahepatic bile ducts.

The etiology of cystadenomas remains unclear, although several theories have been suggested. Some authors consider this disease a premalignant lesion. Due to the usual absence of clinical symptoms, the most frequent diagnosis is by chance, as in the excision of a cystic lesion.

In this paper, we report a case of cystadenoma at the excision of a suspected choledochal cyst in an adult female.

## CASE REPORT

Our patient was a 60-year-old woman with a history of high blood pressure under treatment. While a laparoscopic cholecystectomy was performed for recurrent episodes of biliary colic, a dilated bile duct was evidenced intraoperatively.

Upon this finding, an informed magnetic cholangiography was requested due to dilatation of the choledochal duct. However, it did not show any proximal or distal bile duct dilatation, which was most likely a normal variant (type-1 choledochal cyst according to Todany's classification) (Figure 1). Blood tests with tumor markers were requested, and a CA19.9 of 51.8 was shown, whereas the rest of the tests were normal.

Surgery was performed as planned for the diagnosis of choledochal cyst. The patient underwent resection of the bile duct up to the pancreas joint and before the bifurcation of the hepatic duct, with terminolateral transmeso-

## Abstract

The diagnosis of cystadenoma is rare, even more so when located in the extrahepatic bile duct. Unspecific clinical signs may lead this pathology to be misdiagnosed. The need for pathological anatomy in order to distinguish cystadenomas from simple biliary cysts is crucial. The most usual treatment nowadays is resection of the bile duct, together with cholecystectomy and Roux-en-Y reconstruction.

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**Key words:** Cystadenoma; Extrahepatic bile duct tumors; Choledochal cyst; Jaundice; Biliary surgery

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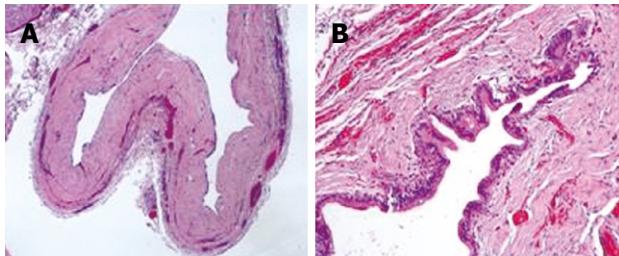
Hernandez Bartolome MA, Fuerte Ruiz S, Manzanedo Romero I, Ramos Lojo B, Rodriguez Prieto I, Gimenez Alvira L, Granados Carreño R, Limones Esteban M. Biliary cystadenoma. *World J Gastroenterol* 2009; 15(28): 3573-3575 Available from: URL:



**Figure 1** X-ray image showing a choledochal cyst in the bile ducts (Cholangio-NMR).



**Figure 2** Macroscopy displaying significant dilatation of the bile duct and an evident cyst in the sample.



**Figure 3** Microscopic view of the sample revealing a multiloculated cystic lesion (A) and a fibrous cyst wall is fibrous lined by epithelium (B).

colic Roux-en-Y hepaticojejunal anastomosis. A type-1 choledochal cyst was discovered macroscopically (Figure 2). The sample was sent to the Pathological Anatomy Service, where it was reported as a 1.4 cm extrahepatic bile duct cystadenoma, with chronic inflammation and bile duct dilatation with regenerative epithelial atypia. No evidence of malignant degeneration was found (Figure 3).

The postoperative course was eventful, with anastomotic leak noted on hepatobiliary iminodiacetic acid (HIDA) scan, central venous catheter-related septicaemia and abscess formation shown by computed tomography (CT), which were solved with percutaneous drainage. Finally, the patient was discharged from the hospital asymptotically.

## DISCUSSION

Biliary cystadenomas arise in the liver in 90% of the cases, and they are much less frequent in the extrahepatic bile ducts, as in our patient. Intra and extrahepatic concomitant cases have also been found. Despite the report of some unilocular cystadenomas, multilocular ones are by far more common. Cystadenomas are usually located in the right lobe of liver, although they may also be present in both lobes or only in the left one of liver.

Macroscopically, their surface is usually flat, and may reach great proportions. The contents are mostly liquid, tending to become mucinous texture, including biliary pigment, hemosiderine and even purulent material if overinfected. Biliary communication has been rarely reported. In some patients, the tumor projects into the bile ducts. In our case, the projection was located in the choledochal wall.

On light microscopy, the inner surface was covered with a cuboidal-to-tall epithelium and some papillary and polypous excrescences, basally oriented nuclei with prominent nucleus and thick chromatin fibers, pale acidophil cytoplasm with mucine-filled vacuoles. According

to some authors, this epithelium must be surrounded by a densely cellular mesenchymal stroma with plain muscular fibers and oval cells, which are typical of epithelia<sup>[3,4]</sup>. However, others claim that hepatic cystadenomas with such features are consistent only with females, males being different in stroma formation. That is why the latter scholars suggest the name of cystadenoma with mesenchymal stroma. Outside this cellular stroma, a dense layer of collagenous tissue separates it from the hepatic parenchyma<sup>[5]</sup>.

The etiology of cystadenomas is unclear. Cystadenomas without mesenchymal stroma have been induced experimentally with aflatoxins in an animal model. This might lead to a possible malignant transformation of simple hepatic cystic lesions<sup>[6,7]</sup>. Coincidences between cystadenoma, gallbladder embryonic tissue, and main bile ducts tissue have also been found<sup>[3]</sup>. Stimuli such as ischemia or carcinogenic elements also produce this kind of lesions.

Cystadenoma may display a wide range of symptoms, although it is mainly asymptomatic. The most typical symptoms are a slowly growing abdominal mass, upper abdomen pain, dyspepsia, anorexia, nausea and fever. Jaundice by compression, protrusion, invasion of bile ducts or by secretion of dense mucinous material has been reported<sup>[8]</sup>. Invasion of the bile ducts may result in pancreatitis episodes. In our patient, we could only reach a diagnosis by the anatomopathological study of the sample, as the patient was asymptomatic except for recurrent biliary colics due to gallbladder lithiasis.

The most widely used diagnosis methods are ecography and tomography. They allow us to observe the cyst formation walls, intracystic projections and possible multilocular arrangement. Since magnetic cholangioscopy provides precise images of the lesion, it is thus the current reference test for tumor study<sup>[8-10]</sup>.

For some scholars, ecologically-guided fine-needle aspiration puncture (FNAP) may be a good diagnostic method, but it may present drawbacks such as the danger of dissemination and its low diagnostic value<sup>[11]</sup>. CEA levels in cyst liquid help to differentiate cystadenomas from cystadenocarcinomas. Other tests, such as endoscopic retrograde cholangiopancreatography (ERCP), gammagraphy and angiography, may give indirect signs for diagnosis. In blood tests, high levels of CA 19.9 are inconsistent with relation to the lesion. In our case, the rise of this marker occurred inside a cystadenoma<sup>[12]</sup>.

Treatment must be surgical whenever possible, due to a potential malignant degeneration of these lesions. The technique chosen for bile duct sites is complete resection of the bile duct, associating cholecystectomy and recon-

struction with hepatic-jejunostomy in Roux-en-Y. When a partial resection has been done for other reasons and the sample shows evidence for a cystadenoma, complete resection of the bile duct and its reconstruction must be performed. However, this was not necessary for our case, as the bile duct was properly fully removed, and the gallbladder was previously removed.

In the hepatic lobes, enucleation must be the objective. The technique used should be personalised taking into account the placement and the patient in context<sup>[13,14]</sup>.

The patient follow-up is justified in order to avoid possible surgical complications in the bile duct, such as cholangitis, gallstones, estenosis of the anastomosis, and malignant degeneration. In hepatic cystadenomas, the high level of recurrence should be monitored in the postoperative follow-up<sup>[13,15,16]</sup>.

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CASE REPORT

## Ascending retrocecal appendicitis presenting with right upper abdominal pain: Utility of computed tomography

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### Abstract

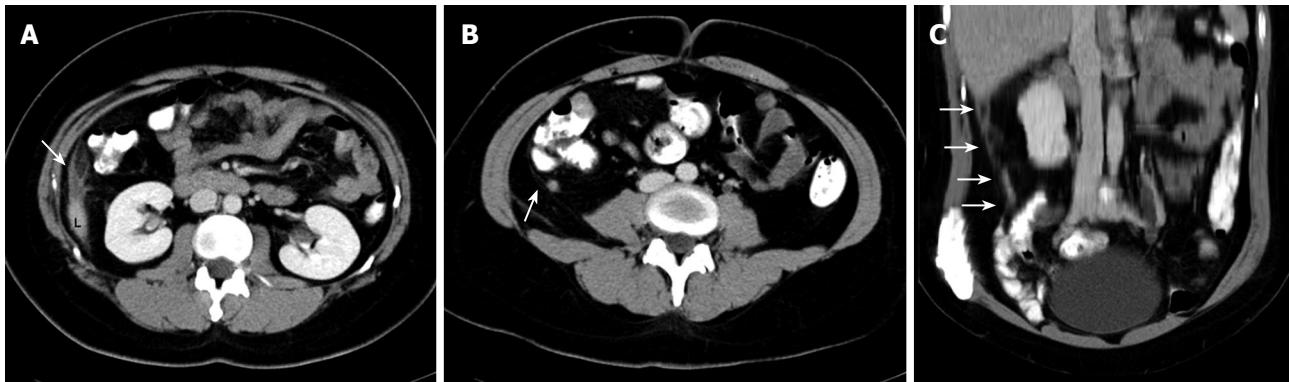
Acute appendicitis is a common surgical condition that is usually managed with early surgery, and is associated with low morbidity and mortality. However, some patients may have atypical symptoms and physical findings that may lead to a delay in diagnosis and increased complications. Atypical presentation may be related to the position of the appendix. Ascending retrocecal appendicitis presenting with right upper abdominal pain may be clinically indistinguishable from acute pathology in the gallbladder, liver, biliary tree, right kidney and right urinary tract. We report a series of four patients with retrocecal appendicitis who presented with acute right upper abdominal pain. The clinical diagnoses at presentation were acute cholecystitis in two patients, pyelonephritis in one, and ureteric colic in one. Ultrasound examination of the abdomen at presentation showed subhepatic collections in two patients and normal findings in the other two. Computed tomography (CT) identified correctly retrocecal appendicitis and inflammation in the retroperitoneum in all cases. In addition, abscesses in the retrocecal space ( $n = 2$ ) and subhepatic collections ( $n = 2$ ) were also demonstrated. Emergency appendectomy was performed in two patients, interval appendectomy in one, and hemicolectomy in another. Surgical findings confirmed the presence of appendicitis and its retroperitoneal extensions. Our case series illustrates the usefulness of CT in diagnosing ascending retrocecal appendicitis and its extension, and excluding other inflammatory conditions that mimic appendicitis.

### INTRODUCTION

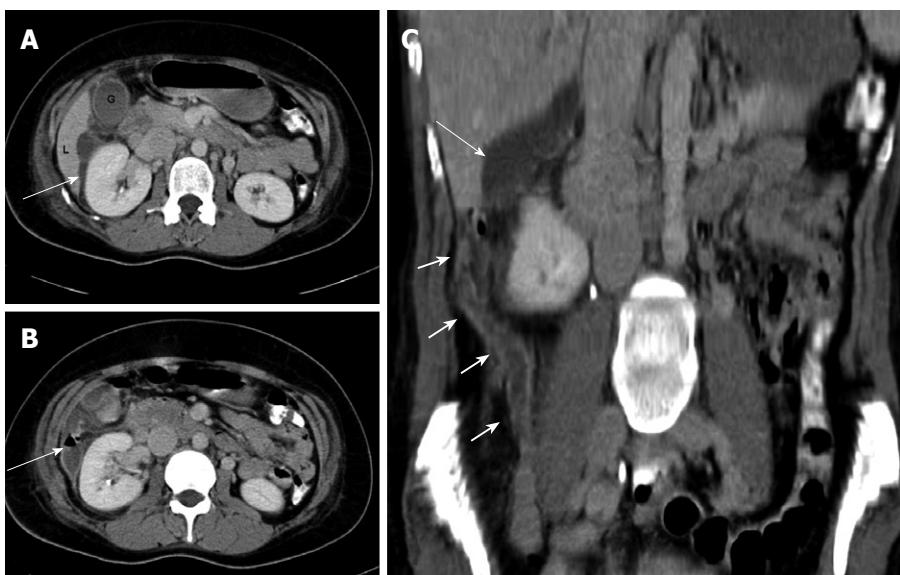
The veriform appendix may occupy several positions in relation to the cecum. The most common positions are descending intraperitoneal (31%-74%) and retrocecal (26%-65%)<sup>[1,2]</sup>. The location and spread of inflammation from acute appendicitis depends on the location of the appendix. If the appendix is located retroceally, it may give rise to an abscess in the pararenal space and spread to bare area of the liver<sup>[3]</sup>, or it may spread along the right paracolic gutter, and extend to the right posterior subhepatic and right subphrenic spaces<sup>[4]</sup>. More than half of the patients with ascending retrocecal appendicitis may have an atypical clinical presentation<sup>[5]</sup>. We present a case series of ascending retrocecal appendicitis with atypical clinical presentation, and highlight the utility of computed tomography (CT) in diagnosing the condition.

### CASE REPORT

During the period January 2003 to December 2006, a computerized search for patients with surgically confirmed retrocecal appendicitis was made. Patients with retrocecal appendicitis and preoperative CT were selected. There were four patients who had atypical clinical presentation with right upper abdominal pain, who formed the study group. The hospital records were reviewed for clinical features, laboratory investigations, surgical findings and follow-up. Preoperative ultrasound and CT were reviewed.



**Figure 1** A 30-year-old woman presenting with a clinical diagnosis of acute cholecystitis. A and B: Contrast-enhanced computed tomography (CT) sections showing inflammatory changes (arrow) adjacent to the inferior tip of the liver (L); B: Thickened appendix (arrow) with mild inflammatory changes in the retrocecal region; C: Coronal reconstruction showing the extent of inflammatory changes (arrows) from the retrocecal region to the tip of the liver.



**Figure 2** A 31-year-old woman presenting with right hypochondrial pain and a clinical diagnosis of pelvic inflammatory disease and right pyelonephritis. A: Contrast-enhanced CT scan showing fluid collection (arrow) in the subhepatic region, extending anteriorly to the gallbladder fossa with inflammatory stranding; B: Note the air fluid level in the collection adjacent to the right kidney; C: Coronal reconstruction showing the long thickened and inflamed appendix (short arrows) reaching the subhepatic region, and the subhepatic collection (arrow) is seen extending more cranially.

### Case 1

A 30-year-old woman presented with a 3-d history of right hypochondrial pain that was constant and radiated to the back. There was right hypochondrial tenderness with negative Murphy's sign and renal punch. She had leukocytosis (13 200 cells/ $\mu$ L). A clinical diagnosis of acute cholecystitis was made. Ultrasound showed a normal liver and gallbladder with no significant abnormality. She was treated initially with antibiotics. A CT scan performed 2 d after presentation showed an inflamed appendix with inflammatory changes in the retrocecal and subhepatic regions (Figure 1). Open appendectomy revealed a moderately inflamed retrocecal appendix with no perforation. Postoperative recovery was uneventful.

### Case 2

A 31-year-old woman presented with a 2-d history of right hypochondrial pain that radiated to the back and right shoulder tip, fever with chills, and vomiting. She also complained of foul-smelling urine and vaginal discharge. She had a termination of pregnancy 4 d earlier. The pain was increasing in severity and aggravated by movement and coughing. She was febrile with right hypochondrial

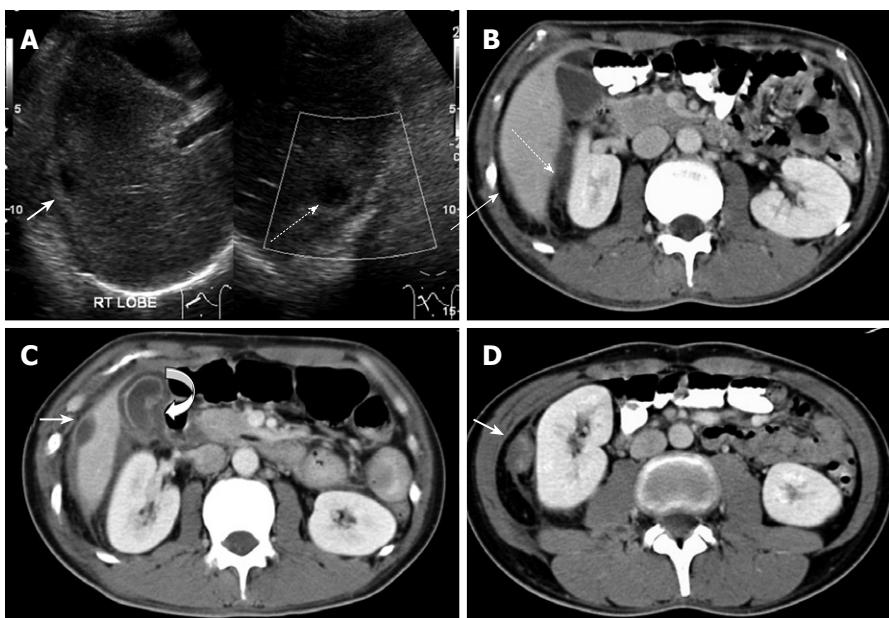
rebound tenderness and a positive right renal punch. She had leukocytosis (14 080 cells/ $\mu$ L). The clinical impression was pelvic inflammatory disease with pyelonephritis and hepatobiliary sepsis. Focused assessment with sonography for trauma (FAST) showed no fluid collection in Morrison's pouch, and pelvic ultrasound did not show any collections. CT (Figure 2) at 3 d after admission showed loculated collections adjacent to segment 6 of the liver. A long inflamed retrocecal appendix was seen with surrounding inflammation that extended to involve the hepatic flexure and anterior pararenal space. There was also consolidation of the right lung base. At surgery, there was retrocecal appendicitis with perforation that caused a subhepatic collection. The patient made a good recovery.

### Case 3

A 34-year-old man presented with right flank pain of 6 d duration. The pain was colicky and radiated to the back. There was no history of dysuria or urinary frequency, but he also had nausea and vomiting with loss of appetite for 2 d. He had leukocytosis (13 620 cells/ $\mu$ L). The abdomen was soft but the right renal punch was positive. The clinical impression was of right-sided ureteric colic and urinary



**Figure 3 A 34-year-old man with colicky right flank pain and clinical diagnosis of right ureteric colic.** A: Ultrasound showed a subhepatic fluid collection (arrows) and no other significant abnormality; B: CT scan performed 2 d later showed the collection in the subhepatic region (short arrow). Note the air-fluid level in the anterior collection (long arrow) with inflammatory changes; C: The section at the level of the cecum and appendix shows inflammatory changes in the retrocecal region (short arrow) and thickened appendix (long arrow).



**Figure 4 A 27-year-old man with recurrent right upper abdominal pain.** A: Ultrasound showed a hypoechoic area in the subphrenic (straight arrow) and subhepatic (broken arrow) region; B: Confirmation by contrast-enhanced CT; C: CT also showed a thickened gallbladder wall (curved arrow), subhepatic collection (white arrow) and inflammation in the perinephric region; D: Another caudal section shows a thickened appendix with inflammatory stranding in the perinephric region.

tract infection. Ultrasound showed no urinary calculi but there was a subhepatic collection (Figure 3) with hyperechoic areas that were suggestive of an abscess. CT (Figure 3) performed on the next day showed the appendix to be swollen and inflamed. There were collections in the subhepatic, rectovesical and paravesical regions. Surgery on the same day confirmed an inflamed and perforated retrocecal appendix with extensive retrocecal collections and abscesses, and a hemicolectomy was performed. The patient recovered uneventfully.

#### Case 4

A 27-year-old man presented with fever and right hypochondrial pain for 1 d. There was tenderness in the right hypochondrium. He was treated nonsurgically for acute cholecystitis but defaulted from treatment and follow-up. He presented again 2 mo later with fever, vomiting and right hypochondrial pain of 2 d duration. There was tenderness in the right hypochondrium with a positive right renal punch. The total white cell count was elevated at 24700 cells/ $\mu$ L but liver function tests were normal. Ultrasound showed two abscesses in the right hepatic lobe and a thickened gallbladder wall, but no gallstones were

seen. He was treated for liver abscesses with intravenous antibiotics and made an excellent recovery. Follow-up ultrasound scans showed resolution of the liver abscesses.

Four months later, he presented again with fever and right hypochondrial pain. Ultrasound showed no significant abnormality. He was treated with a further course of intravenous antibiotics and discharged. He was readmitted 5 mo later. CT showed subphrenic and subhepatic collections with a thickened inflamed appendix (Figure 4). In view of the recurrent collections, diverticulitis was suspected. Colonoscopy was normal and he was managed conservatively. Follow-up CT 3 mo later showed resolution of the collection, but the appendix was still thickened with periappendiceal inflammatory changes. Elective laparoscopic appendectomy was then performed. This showed a high retrocecal appendix with dense adhesions between the appendix and the liver. A final diagnosis of recurrent retrocecal appendicitis was made. The patient made a good recovery with no further episodes.

#### DISCUSSION

Acute appendicitis may be diagnosed easily and treated

in children and adults if there is a classical history with typical clinical signs<sup>[5]</sup>. When the appendix is in the retrocecal position, the signs and symptoms of acute appendicitis may be atypical and mimic pathology in the right flank and hypochondrium, such as acute cholecystitis, diverticulitis, acute gastroenteritis, ureter colic, acute pyelonephritis, colon cancer and irritable bowel syndrome<sup>[6]</sup>.

When the clinical impression is of gallbladder, hepatobiliary or urinary tract pathology, ultrasound is often performed. This may show liver abscesses and collections in the subhepatic and right flank regions. Although ultrasound is used frequently in the assessment of suspected acute appendicitis in young children, it requires expertise and dedicated techniques, such as graded compression. The appendix could be visualized in up to 99% of suspected cases of appendicitis in children in one series<sup>[7]</sup>. However, in adults, un-enhanced CT has been shown to be more sensitive in diagnosing acute appendicitis than ultrasound is<sup>[8]</sup>. In our series, acute appendicitis was not suspected in any of the patients, therefore, the ultrasound scan performed was not dedicated to rule out appendicitis.

CT is very sensitive for evaluating the appendix, and a thickened appendix, inflamed periappendiceal fat, collections, and presence of free gas in ruptured appendix are detected readily by CT. The inflammatory changes that result from an acutely inflamed ascending retrocecal appendix may extend to the perirenal, adrenal and subhepatic regions, and on rare occasions, inferior extension along the psoas muscle into the thigh has been reported<sup>[9,10]</sup>. The inflammatory changes are seen most commonly in the retrocolic space (88%), followed by paracolic gutter (30%), pararenal space (27%), mesentery (24%), perirenal space (18%), and less often, in the subhepatic space (3%)<sup>[6]</sup>. In a recently published series of 33 patients<sup>[6]</sup> with ascending retrocecal appendicitis who were investigated with CT, only one was found to have a subhepatic collection.

It is interesting that all four of our cases involve young adult patients rather than children. A retrocecal appendix is common and one series showed the appendix to be retrocolic and retrocecal in 58% of cases<sup>[11]</sup>. A retrocecal appendix has been described also in families and is thought to be inherited as a simple dominant unit character<sup>[11]</sup>. Studies of the fetal appendix, however, show that it is almost always subcyclic during this period<sup>[12]</sup>. It is possible therefore that the appendix continues to grow during childhood, extending further from the cecum later in life, although there have been no published studies documenting this process. Therefore, retrocecal appendicitis with symptoms remote from the right iliac fossa may occur also in an older age group.

Our case series illustrated a spectrum of uncommon

clinical and radiological manifestations of ascending retrocecal appendicitis. This emphasizes the importance of considering the possibility of ascending retrocecal appendicitis in cases in which the signs and symptoms are referred to areas along the possible location of a retrocecal appendix, especially when initial investigations like ultrasound do not support other diagnoses, such as cholecystitis, or hepatobiliary or urinary tract pathology. CT is helpful to establish rapidly the correct diagnosis, as delays in appendectomy for over 24–36 h have been shown to increase the complication rate<sup>[13,14]</sup>.

In summary, CT is useful for evaluation of patients with atypical right upper abdominal pain and nonspecific clinical findings, to rule out the possibility of retrocecal appendicitis.

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## Meetings

### Events Calendar 2009

January 12-15, 2009  
Hyatt Regency San Francisco, San Francisco, CA  
Mouse Models of Cancer

January 21-24, 2009  
Westin San Diego Hotel, San Diego, CA  
Advances in Prostate Cancer Research

February 3-6, 2009  
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)  
Second AACR Conference  
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009  
Hyatt Regency Boston, Boston, MA  
Translation of the Cancer Genome

February 8-11, 2009  
Westin New Orleans Canal Place, New Orleans, LA  
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009  
Hong Kong Convention and Exhibition Centre, Hong Kong, China  
19th Conference of the APASL  
<http://www.apasl2009hongkong.org/en/home.aspx>

February 27-28, 2009  
Orlando, Florida  
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009  
Vienna, Austria  
EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease  
[www.easl.ch/vienna2009](http://www.easl.ch/vienna2009)

March 13-14, 2009  
Phoenix, Arizona  
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009  
Marriott Wardman Park Hotel  
Washington, DC  
13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009  
Glasgow, Scotland  
British Society of Gastroenterology (BSC) Annual Meeting  
Email: bsg@mailbox.ulcc.ac.uk

April 8-9, 2009  
Silver Spring, Maryland  
2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009  
Colorado Convention Center, Denver, CO  
AACR 100th Annual Meeting 2009

April 22-26, 2009  
Copenhagen, Denmark  
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)  
<http://www.easl.ch/>

May 17-20, 2009  
Denver, Colorado, USA  
Digestive Disease Week 2009

May 29-June 2, 2009  
Orange County Convention Center Orlando, Florida  
45th ASCO Annual Meeting  
[www.asco.org/annualmeeting](http://www.asco.org/annualmeeting)

May 30, 2009  
Chicago, Illinois  
Endpoints Workshop: NASH

May 30-June 4, 2009  
McCormick Place, Chicago, IL  
DDW 2009  
<http://www.ddw.org>

June 17-19, 2009  
North Bethesda, MD  
Accelerating Anticancer Agent Development

June 20-26, 2009  
Flims, Switzerland  
Methods in Clinical Cancer Research (Europe)

June 24-27 2009  
Barcelona, Spain  
ESMO Conference: 11th World Congress on Gastrointestinal Cancer  
[www.worldgicancer.com](http://www.worldgicancer.com)

June 25-28, 2009  
Beijing International Convention Center (BICC), Beijing, China  
World Conference on Interventional Oncology  
<http://www.chinamed.com.cn/wcio2009/>

July 5-12, 2009  
Snowmass, CO, United States  
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009  
Aspen, CO, United States  
Molecular Biology in Clinical Oncology

August 1-7, 2009  
Vail Marriott Mountain Resort, Vail, CO, United States  
Methods in Clinical Cancer Research

August 14-16, 2009  
Bell Harbor Conference Center, Seattle, Washington, United States  
Practical Solutions for Successful Management  
<http://www.asge.org/index.aspx?id=5040>

September 23-26, 2009  
Beijing International Convention Center (BICC), Beijing, China  
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists (IASGO)  
<http://iasgo2009.org/en/index.shtml>

September 27-30, 2009  
Taipei, China  
Asian Pacific Digestive Week  
<http://www.apdwcongress.org/2009/index.shtml>

October 7-11, 2009  
Boston Park Plaza Hotel and Towers, Boston, MA, United States  
Frontiers in Basic Cancer Research

October 13-16, 2009  
Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States  
Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009  
Versailles, France  
Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009  
Boston, MA, United States  
The Liver Meeting

November 15-19, 2009  
John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States  
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009  
London, UK  
Gastro 2009 UEGW/World Congress of Gastroenterology  
[www.gastro2009.org](http://www.gastro2009.org)



### Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.



## Instructions to authors

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*World Journal of Gastroenterology* (*World J Gastroenterol* ISSN 1007-9327 CN 14-1219/R) is a weekly open-access (OA) peer-reviewed journal supported by an editorial board consisting of 1179 experts in gastroenterology and hepatology from 60 countries.

The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

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The major task of *WJG* is to rapidly report the most recent results in basic and clinical research on gastroenterology, hepatology, endoscopy and gastrointestinal surgery fields, specifically including autoimmune, cholestatic and biliary disease, esophageal, gastric and duodenal disorders, cirrhosis and its complications, celiac disease, dyspepsia, gasteroesophageal reflux disease, esophageal and stomach cancers, carcinoma of the colon and rectum, gastrointestinal bleeding, gastrointestinal infection, intestinal inflammation, intestinal microflora and immunity, irritable bowel syndrome, liver biology/pathobiology, liver failure, growth and cancer, liver failure/cirrhosis/portal hypertension, liver fibrosis; *Helicobacter pylori*, hepatitis B and C virus, hepatology elsewhere; pancreatic disorders, pancreas and biliary tract disease, pancreatic cancer; transplantation, genetics, epidemiology, microbiology and inflammatory disorders, molecular and cell biology, nutrition; geriatric gastroenterology, pediatric gastroenterology, steatohepatitis and metabolic liver disease; diagnosis and screening, endoscopy, imaging and advanced technology.

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### Acknowledgments

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### Format

#### Journals

*English journal article (list all authors and include the PMID where applicable)*

- 1 Jung EM, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

*Chinese journal article (list all authors and include the PMID where applicable)*

- 2 Lin GZ, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

*In press*

- 3 Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

*Organization as author*

- 4 Diabetes Prevention Program Research Group. Hypertension,

insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

*Both personal authors and an organization as author*

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

*No author given*

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

*Volume with supplement*

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

*Issue with no volume*

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900 DOI:10.1097/00003086-20020800-00026]

*No volume or issue*

- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

## Books

*Personal author(s)*

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

*Chapter in a book (list all authors)*

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

*Author(s) and editor(s)*

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

*Conference proceedings*

- 13 **Hardden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

*Conference paper*

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming-EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

*Electronic journal (list all authors)*

- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

*Patent (list all authors)*

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc, assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

## Statistical data

Write as mean  $\pm$  SD or mean  $\pm$  SE.

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Express  $t$  test as  $t$  (in italics),  $F$  test as  $F$  (in italics), chi square test as  $\chi^2$  (in Greek), related coefficient as  $r$  (in italics), degree of freedom as  $v$  (in Greek), sample number as  $n$  (in italics), and probability as  $P$  (in italics).

## Units

Use SI units. For example: body mass,  $m$  (B) = 78 kg; blood pressure,  $p$  (B) = 16.2/12.3 kPa; incubation time,  $t$  (incubation) = 96 h, blood glucose concentration,  $c$  (glucose)  $6.4 \pm 2.1$  mmol/L; blood CEA mass concentration,  $p$  (CEA) = 8.6 24.5  $\mu$ g/L;  $CO_2$  volume fraction, 50 mL/L  $CO_2$ , not 5%  $CO_2$ ; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23243641.

The format for how to accurately write common units and quanta can be found at: <http://www.wjgnet.com/wjg/help/14.doc>.

## Abbreviations

Standard abbreviations should be defined in the abstract and on first

mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

## Italics

Quantities:  $t$  time or temperature,  $c$  concentration,  $A$  area,  $l$  length,  $m$  mass,  $V$  volume.

Genotypes:  $gyrA$ ,  $argI$ ,  $cmyc$ ,  $c fos$ , etc.

Restriction enzymes: *Eco*RI, *Hind*II, *Bam*HI, *Kbo*I, *Kpn*I, etc.

Biology: *H pylori*, *E coli*, etc.

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# Hepatic portal venous gas: Physiopathology, etiology, prognosis and treatment

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## INTRODUCTION

Hepatic portal venous gas (HPVG), an ominous radiologic sign, was first described by Wolfe and Evens in infants with necrotizing enterocolitis (NEC)<sup>[1,2]</sup>. HPVG is associated with numerous underlying abdominal diseases, ranging from benign causes to potentially lethal diseases that require prompt surgical intervention<sup>[3-6]</sup>.

The mechanism for the appearance of gas in the portal vein is not well understood. The proposed factors predisposing the portal venous system to the accumulation of gas include the following: (1) escape of gas produced by gas-forming organisms in the bowel lumen or in an abscess which then circulate into the liver or (2) the presence of gas-forming organisms in the portal venous system with passage of gas into the circulation<sup>[7]</sup>.

The diagnosis of HPVG is usually made by plain abdominal radiography, sonography, color Doppler flow imaging, or computed tomography (CT) scan. The characteristic finding on abdominal plain film is a branching radiolucency extending to within 2 cm beneath the liver capsule. This is because of the centrifugal flow of portal venous blood, which carries portal venous gas peripherally, in contrast to biliary gas, which tends to collect centrally as a result of the centripetal movement of bile. Although HPVG may be diagnosed by conventional radiography, detection is difficult and it is easily overlooked<sup>[8]</sup>. Sonography, color Doppler flow imaging, and CT scan have been reported to be superior to abdominal radiographs in identifying HPVG. Sonography coupled to Doppler is very sensitive for HPVG detection and its follow-up, and can be useful as an initial screening examination<sup>[9,10]</sup>. However, use of ultrasound is limited because of its high inter-operator variability and lack of availability at all times. The typical ultrasonographic features of HPVG are (1) highly echogenic particles, flowing within the portal vein, or (2) poorly defined, highly echogenic patches within the hepatic parenchyma, which are most apparent in the non-dependent part<sup>[11]</sup>.

## Abstract

Hepatic portal venous gas (HPVG), an ominous radiologic sign, is associated in some cases with a severe underlying abdominal disease requiring urgent operative intervention. HPVG has been reported with increasing frequency in medical literature and usually accompanies severe or lethal conditions. The diagnosis of HPVG is usually made by plain abdominal radiography, sonography, color Doppler flow imaging or computed tomography (CT) scan. Currently, the increased use of CT scan and ultrasound in the inpatient setting allows early and highly sensitive detection of such severe illnesses and also the recognition of an increasing number of benign and non-life threatening causes of HPVG. HPVG is not by itself a surgical indication and the treatment depends mainly on the underlying disease. The prognosis is related to the pathology itself and is not influenced by the presence of HPVG. Based on a review of the literature, we discuss in this paper the pathophysiology, risk factors, radiographic findings, management, and prognosis of pathologies associated with HPVG.

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**Key words:** Hepatic portal venous gas; Bowel ischemia/necrosis; Diverticulitis; Gastric pathologies; Ulcerative colitis; Abdominal computed tomography scan; Crohn's disease; Liver transplantation; Chemotherapy

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Color Doppler flow imaging shows hyperechogenic foci moving within the lumen of the portal vein, producing sharp bidirectional spikes superimposed on the normal monophasic portal vein wave pattern. The CT scan has a high sensitivity for detection of HPVG and can detect the underlying pathology<sup>[12,13]</sup>. On scanographic images, HPVG is characteristically associated with peripheral gas lucencies, which branch out and are noted even within the last 2 cm beneath the liver capsule. This peripheral gas distribution is related to the direction of blood flow into the liver. It is crucial to differentiate it from pneumobilia, which is centrally located because of the biliary anatomy and the direction of bile flow<sup>[14,15]</sup>. Also, a CT scan can disclose gas in the bowel wall (pneumatosis intestinalis) and in the extrahepatic portal vein or its splanchnic vasculature.

The underlying clinical events associated with HPVG might be important factors contributing to patient survival and prognosis. Liebman *et al*<sup>[7]</sup> reported that HPVG is associated most commonly with bowel necrosis (72%), followed by ulcerative colitis (8%), intra-abdominal abscess (6%), small bowel obstruction (3%) and gastric ulcer (3%). This explains the high mortality rate (56%-90%) reported in association with HPVG<sup>[16,17]</sup>. Another factor affecting the outcome of these patients is the coexistence of a long-term chronic disease, such as chronic renal failure, diabetes mellitus or hypertension<sup>[13]</sup>, which decreases immune functions and alters the intestinal microbial flora.

However, currently, the increased use of CT scan and ultrasound in the inpatient setting allows early and highly sensitive detection of such severe illnesses<sup>[16,18-20]</sup> and recognition of an increasing number of benign and non-life threatening causes of HPVG<sup>[20,21]</sup>. The prognosis is related to the pathology itself and is not influenced by the presence of HPVG<sup>[12]</sup>.

In this paper, we discuss the pathophysiology, risk factors, management, and prognosis of pathologies associated with HPVG.

## NEC

NEC is a disease of premature neonates, with 90% of cases occurring in infants born before 36 wk gestational age. In 10% of cases it occurs in full-term infants who have comorbidities predisposing them to decreased mesenteric perfusion<sup>[22]</sup>. This pathognomonic radiographic sign can be missed in extremely low birth weight (less than 1000 g) neonates, despite the gravity of the illness, because of absence of enteral feeding.

NEC is a multifactorial disease process resulting from the association of a hypoxic ischemic injury of the immature gastrointestinal tract and alterations in the microbiological intestinal flora<sup>[1]</sup>. Hypoperfusion in preterm infants has many etiologies (Table 1). It induces blood to shunt away from the bowels towards critical organs which may cause alterations in the mucosal barrier. These alterations, in combination with pathogenic microbiological intestinal flora proliferation exaggerated by

**Table 1 Pathologies associated with hypoperfusion in neonates**

**Etiologies of hypoperfusion in preterm infants**

Patent ductus arteriosus
Sepsis
Polycythemia
In utero cocaine exposure
Peri- or postnatal asphyxia
Respiratory distress syndrome
Congenital heart disease
Umbilical catheters use and exchange transfusion

feeding and antibacterial use, result in mucosal and/or transmural necrosis.

The bacterial translocation and production of hydrogen gas into the bowel wall cause pneumatosis intestinalis<sup>[1]</sup> which can be seen radiographically as linear or circular lucencies within the intestinal wall<sup>[23]</sup>. As a consequence, the gas can embolize from the bowel wall through the mesenteric veins to the portal venous system and the non-dependent parts of the liver, particularly the left lobe and anterior segment of the right lobe.

Abdominal sonography is very specific and sensitive for early detection of portal and hepatic gas thus allowing early diagnosis and efficacious treatment of NEC. The micro-bubbles of gas appear as highly echogenic particles flowing within the portal vein and highly echogenic patches within the hepatic parenchyma. At a more advanced stage, HPVG can be seen on abdominal radiographs. Furthermore, HPVG may only be a transitional sign or can persist for longer than 2 d<sup>[24]</sup>.

HPVG alone is not an indication for surgery<sup>[24]</sup>, since 47% of infants with NEC and HPVG survive without operative procedure. Surgery is indicated when infants do not improve significantly despite medical treatment for several days, as well as when radiographs show persistent fixed dilated bowel loops or pneumoperitoneum which are, respectively, signs of bowel necrosis and perforation. In infants with a birth weight > 1500 g, laparotomy and resection of the necrotic intestine is generally the preferred approach. In very low birth weight infants < 1500 g, and unstable neonates, surgery is associated with a high rate of mortality and morbidity<sup>[22]</sup>. In this case, peritoneal drainage can be indicated as a definitive procedure or as part of the resuscitation phase prior to definitive laparotomy.

HPVG has been associated, in some studies, with a poorer survival rate. In their prospective study, Sharma *et al*<sup>[24]</sup> found that infants with HPVG were 1.4 times more likely to have severe NEC, but mortality rates did not differ from those of infants without HPVG.

HPVG is associated with severe lesions of the bowel wall and intra-mural gas that leads to muscular disruption and secondary stricture formation in up to 20% of all patients with NEC<sup>[22,23]</sup>.

## BOWEL ISCHEMIA

Bowel ischemia and/or infarction is a common and

**Table 2 Etiologies of bowel ischemia**

<b>Etiologies</b>	
Thromboembolism	Atherosclerosis Arterial dissection Aortic surgery Neoplasm, inflammatory or infectious causes
Vasculitis	Producing occlusion of large, medium and small arteries
Segmental mediolytic arteriopathy	Characterized by a non inflammatory arteriopathy causing lysis of adult visceral arteries
Bowel obstruction	Distension of proximal bowel loops resulting in venous congestion Strangulation of mesenteric vessels
Abdominal trauma	Blunt abdominal trauma causing intestinal stenosis with late chronic presentation Penetrating trauma with direct injury to the major mesenteric vessels
Neoplasms	Invasion of the major mesenteric vessels by the tumor Over distension and fecal material stagnation above an obstacle
Abdominal inflammatory conditions	Mesenteric, portal and splenic vein thrombosis
Chemotherapy, drugs and corrosive injury	Vasoconstriction Hypotension Thromboembolism Liquefaction necrosis (Alkalies) Coagulative necrosis (Acids)
Radiation	Obliteration of small arterioles producing a progressive occlusive vasculitis

dangerous abdominal condition, especially in elderly patients<sup>[25,26]</sup>. It is associated with a high mortality rate that ranges from 75% to 90% of cases<sup>[15]</sup>.

Bowel ischemia is produced by insufficient blood flow to or from the intestines. It may have an acute or chronic setting depending on the underlying disorder (Table 2). The extent of bowel ischemia in the bowel wall is divided into three stages<sup>[26]</sup>: Stage I : the ischemic lesions are confined to the mucosa and are reversible (known as reversible ischemic enteritis); Stage II: characterized by necrosis of the mucosal and submucosal tissues, which may lead to fibrotic stricture development; Stage III: the entire wall is affected by ischemia. It is associated with a high mortality rate.

Intestinal ischemia results in damage to the mucosal barrier which, in association with over-distension of the bowel loops and gas-forming bacterial proliferation, leads to gas moving from the intestinal lumen to the mesenteric veins and flowing through it to the portal system and hepatic parenchyma.

Bowel ischemia is the primary etiology of HPVG (70% of cases) and when associated, they are related to transmural necrosis in 91% of cases and to a high mortality rate (85% of patients)<sup>[15]</sup>. These facts signify that HPVG is an absolute indication for surgery in the context of mesenteric ischemia.

Abdominal radiographs are not sensitive for early stage bowel ischemia detection, but they predict bowel infarction and a poor prognosis when intramural gas and HPVG are seen<sup>[23]</sup>.

Currently, the multidetector row CT angiography (MDCT) has become the first choice for HPVG detection and determination of the underlying process<sup>[20]</sup>. MDCT sensitivity has been markedly increasing over time from a low of 39% to a current high of 82%, and it has reached a similarly high sensitivity in diagnosing acute bowel ischemia as that of angiography.

HPVG is often associated with pneumatosis intestinalis, posing a grave prognosis, especially in the ischemic intestine<sup>[27-31]</sup>. The CT scan alone cannot predict which patients are experiencing true intestinal ischemia and which simply have benign pneumatosis. The presence of HPVG does not provide any information concerning the extent of bowel necrosis. In all cases, CT findings should be correlated with the clinical signs and with laboratory parameters to reach a high sensitivity and specificity level for intestinal necrosis. When HPVG associated with ischemic bowel disease is encountered, coexisting other abdominal conditions should be considered pre- and intraoperatively. Intestinal resection is performed when bowel necrosis is found on laparotomy. Nowadays, with the development of highly advanced imaging techniques, potentially severe pathologies, such as bowel ischemia, are diagnosed at much earlier stages, allowing prompt treatment and significantly reducing mortality rates.

## DIVERTICULITIS

Hepatic portal venous gas is a rare complication of diverticulitis<sup>[16,32]</sup>. However, Sellner *et al*<sup>[16]</sup> found that complicated diverticulitis is the second most frequently reported cause of HPVG, which can be due to two mechanisms: The first is a septic thrombophlebitis of the inferior mesenteric vein complicated by gas-forming pathogens. The second is a direct communication between the intestinal lumen and the portomesenteric vein system. This is caused by intramesocolic intestinal perforation, dissecting between the peritoneal leaflets of the mesocolon and creating access to mesocolic veins<sup>[33]</sup>.

Patients with mesocolic abscess have better prognosis than patients with septic pylephlebitis<sup>[16]</sup>.

Diverticulitis associated with HPVG necessitates a selective surgical approach after adequate reanimation with intravenous fluids and antibiotics<sup>[34]</sup>. However, Nobili *et al*<sup>[35]</sup> suggested that if medical conservative therapy is effective and the clinical status improves, the surgery could be delayed.

In one case, Negro *et al*<sup>[36]</sup> reported a patient with sigmoid diverticulitis who developed a massive embolism of the intra- and extra-hepatic portal systems due to an enterovascular fistula, and who was treated with fistula embolization and subsequent sigmoidectomy.

## GASTRIC PATHOLOGIES

HPVG has been reported in association with either gastric dilatation or gastric emphysema<sup>[37,38]</sup>. The treatment is conservative or surgical depending on the underlying process.

Gastroparesis is a frequent complication after a polytraumatic event, leading to gastric emphysema and subsequent HPVG<sup>[37]</sup>. In these cases, gastric decompression with a naso-gastric tube, nil by mouth and observation is sufficient treatment.

Furthermore, HPVG has been described in a patient with hypertrophic pyloric stenosis<sup>[39,40]</sup>, and in a patient with peptic ulcer<sup>[41]</sup>. A gastric volvulus in a diaphragmatic hernia, without necrosis, was also reported in one patient<sup>[42]</sup>. These observations prove that raised intra-luminal pressure results in gastric pneumatosis and HPVG in the absence of bowel ischemia and gas-forming organism proliferation.

Several cases of HPVG have been described after accidental ingestion of caustics, hydrogen peroxide and chronic toluene inhalation<sup>[30,43]</sup>, which lead to mucosal ulcerations and acute gastric distension by oxygen production. Generally, the patient will be stable and can be managed by symptomatic treatment. In cases of massive gas embolization to the portal venous system, and the presence of cardiac and neurological symptoms, hyperbaric oxygen should be used and can be a successful treatment<sup>[44]</sup>.

## INFLAMMATORY BOWEL DISEASES

Kinoshita *et al*<sup>[3]</sup> reported that in 182 cases of HPVG, 4% were associated with ulcerative colitis, and 4% with Crohn's disease.

HPVG, in patients presenting inflammatory bowel disease, can be caused by mucosal damage alone or in combination with bowel distension, sepsis, invasion by gas-producing bacteria<sup>[45,46]</sup>, or after colonoscopy, upper gastrointestinal barium examination, barium enema or blunt abdominal trauma<sup>[47]</sup>. Therefore, a finding of HPVG associated with Crohn's disease does not mandate surgical intervention especially in the absence of peritoneal signs or free intraperitoneal gas<sup>[48]</sup>.

## LIVER TRANSPLANTATION

Hepatic portal venous gas is a common finding on Doppler sonography in the early postoperative period after liver transplantation. Chezmar *et al*<sup>[3]</sup> concluded in their study that HPVG alone, in the absence of bowel necrosis, intra-abdominal abscess, small-bowel obstruction or sepsis, is a transient finding without clinical significance. Furthermore, there was no correlation between the finding of portal venous air and transplant rejection, the need for retransplantation, the cause of hepatic failure, the type of biliary anastomosis, ventilator dependence, or subsequent death.

In pediatric liver transplant recipients, Wallot *et al*<sup>[49]</sup> suggested that the detection of HPVG beyond the early postoperative period may be a sign of intra-abdominal post-transplant lymphoproliferative disease, leading to the loss of bowel wall integrity.

## CHEMOTHERAPY

Two cases of HPVG after chemotherapy have been

reported. In the first case, Kung *et al*<sup>[50]</sup> described HPVG to be secondary to pneumatosis intestinalis in a patient who received irinotecan and cisplatin. On laparotomy, the colon and the small bowel were normal, and the patient was managed supportively with success. Gastrointestinal toxicity is a common side effect of irinotecan<sup>[51]</sup> which may be exacerbated by the adjunction of cisplatin leading to mucosal ulceration, bowel distension and gas-forming anaerobic bacterial proliferation.

In the second case, Zalinski *et al*<sup>[52]</sup> reported localized HPVG in the right liver after complete colorectal cancer liver metastasis necrosis in a patient receiving a treatment of oxaliplatin and cetuximab. Infection of the necrotized metastasis was promoted by the tumor which subsequently turned into a liver abscess, and fistulized to the right portal vein.

One case of HPVG was reported after chemoradiation therapy for an advanced esophageal cancer<sup>[53]</sup>.

## OTHER CONDITIONS

HPVG has been reported in association with acute pancreatitis<sup>[54,55]</sup>, obstructive pyelonephritis after extracorporeal shock wave lithotripsy<sup>[56]</sup>, acute appendicitis<sup>[57]</sup>, cholangitis<sup>[20]</sup>, gastro-jejunal anastomotic leak after laparoscopic gastric bypass<sup>[58]</sup>, uterine gangrene<sup>[31]</sup>, and percutaneous endoscopic gastrostomy tube placement<sup>[59]</sup>. HPVG has also been seen in the presence of a jejunal feeding tube<sup>[60]</sup>, following esophageal variceal band ligation<sup>[61]</sup>, gastrointestinal perforation with amyloidosis<sup>[62]</sup>, with severe hyperglycemic shock<sup>[63]</sup>, and in superior mesenteric artery syndrome<sup>[64-66]</sup>.

These conditions may lead to bowel hypoperfusion and subsequent intestinal ischemia, or may induce an ileus and intestinal distention with mucosal damage and bacterial proliferation producing pneumatosis intestinalis and HPVG. In these cases, surgical treatment after adequate medical resuscitation is indicated depending on the underlying process.

Occasionally, in cases of abdominal blunt trauma, the increased intra-luminal pressure causes mucosal tears within the intestine, which allows gas to enter submucosal veins and flow to the hepatic portal vein<sup>[19]</sup>. However, a severe blunt abdominal trauma may lead to intestinal necrosis and eventual bowel rupture with peritonitis. Thus, when HPVG after abdominal trauma is associated with free intra-abdominal gas, pneumatosis intestinalis and signs of peritonitis, surgical intervention is mandatory.

## CONCLUSION

A radiologic finding of HPVG does not necessarily indicate a severe underlying pathology. It can be seen in relatively benign situations such as following endoscopic procedures and gastric dilatation which only necessitate conservative therapy. Traditionally, HPVG was considered as being an indicator of bad prognosis and as being associated with a particularly high mortality rate. Nowadays, with the development of highly advanced

imaging techniques, potentially severe pathologies, such as bowel ischemia, are diagnosed at much earlier stages, allowing prompt treatment and significantly reducing mortality rates. HPVG is not by itself a surgical indication and the treatment depends mainly on the underlying disease. The prognosis is related to the pathology itself and is not influenced by the presence of HPVG.

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TOPIC HIGHLIGHT

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## Post-infectious irritable bowel syndrome

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### INTRODUCTION

Irritable bowel syndrome (IBS) is a common gastrointestinal disorder characterized by abdominal discomfort and altered bowel habit with no abnormality on routine diagnostic tests<sup>[1,2]</sup>. In some patients, IBS symptoms arise *de novo* following an exposure to acute gastroenteritis (GE). This phenomenon, known as post-infectious IBS (PI-IBS), denotes the persistence of abdominal discomfort, bloating and diarrhea that continue despite clearance of the inciting pathogen<sup>[3-15]</sup>. A recent systematic review and meta-analysis demonstrated that the risk of developing IBS increases six-fold after gastrointestinal infection<sup>[16]</sup> and remains elevated for at least 2-3 years post-infection. The current conceptual framework regarding the pathophysiologic mechanism for PI-IBS suggests that PI-IBS is associated with altered motility, increased intestinal permeability, increased numbers of enterochromaffin cells and persistent intestinal inflammation, characterized by increased numbers of T-lymphocytes and mast cells, and increased expression of proinflammatory cytokines<sup>[3,12,17,18]</sup> (Figure 1). This therefore suggests that an exposure to pathogenic organisms disrupts intestinal barrier function, alters neuromuscular function and triggers chronic inflammation which sustain IBS symptoms. We provide herein a review of PI-IBS epidemiology, pathophysiology, and management.

### EPIDEMIOLOGY

A link between IBS and enteric infection was first proposed by Stewart more than five decades ago<sup>[19]</sup>. A subsequent retrospective study by Chaudhary and Truelove found that a substantial proportion of patients with IBS reported the onset of their symptoms after an acute episode of GE<sup>[20]</sup>. Since then, various prospective and retrospective studies from the United Kingdom, North America, Spain, Korea, Israel and New Zealand have reported the incidence or prevalence of PI-IBS to range from 5% to 32%<sup>[4-13,21-25]</sup>. Consistent among these studies is the suggestion that PI-IBS is a global phenomenon, and not unique to any ethnic group or

### Abstract

Post-infectious irritable bowel syndrome (PI-IBS) is a common disorder wherein symptoms of IBS begin after an episode of acute gastroenteritis. Published studies have reported incidence of PI-IBS to range between 5% and 32%. The mechanisms underlying the development of PI-IBS are not fully understood, but are believed to include persistent sub-clinical inflammation, changes in intestinal permeability and alteration of gut flora. Individual studies have suggested that risk factors for PI-IBS include patients' demographics, psychological disorders and the severity of enteric illness. However, PI-IBS remains a diagnosis of exclusion with no specific disease markers and, to date, no definitive therapy exists. The prognosis of PI-IBS appears favorable with spontaneous and gradual resolution of symptoms in most patients.

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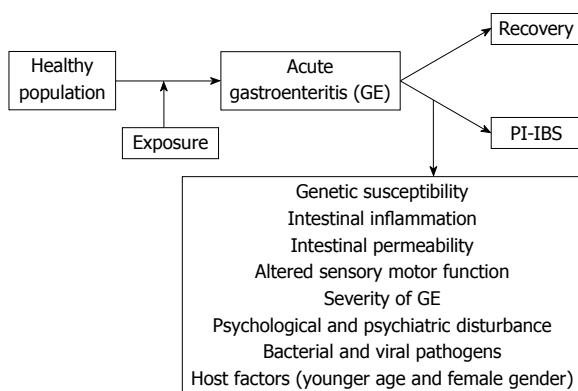
**Key words:** Irritable bowel syndrome; Functional colonic disease; Gastroenteritis; Functional bowel disorder

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**Table 1** Estimates of incidence of PI-IBS<sup>1</sup>

Author	Study type	Control group	Type of exposure	Follow-up (mo)	Criteria for diagnosis of IBS	Mean quality assessment score	Incidence of IBS in exposed cohort	Country
McKendrick <sup>[24]</sup>	Prospective	None	Confirmed <i>Salmonella</i>	12	Rome I	14.5	12/38 = 31.6%	United Kingdom
Neal <sup>[13]</sup>	Prospective	None	Confirmed-bacterial GE	6	Modified Rome I	16.0	23/366 = 6.3%	United Kingdom
Gwee <sup>[22]</sup>	Prospective	None	Confirmed gastroenteritis	6	Clinical assessment	21.0	9/86 = 10.5%	United Kingdom
Gwee <sup>[28]</sup>	Prospective	None	Confirmed <i>Shigella</i> , <i>Campylobacter</i> , <i>Salmonella</i>	12	Rome I	21.5	22/109 = 20.2%	United Kingdom
Rodríguez <sup>[6]</sup>	Prospective	Matched from database	Confirmed bacterial GE	12	Physician diagnosis	19.0	14/318 = 4.4%	United Kingdom
Ilnyckyj <sup>[5]</sup>	Prospective	Uninfected contemporaneous	Self reported (traveler's diarrhea)	3	Rome I	22.5	2/48 = 4.2%	Canada
Dunlop <sup>[3]</sup>	Prospective	None	Self reported (presumed <i>Campylobacter</i> )	3	Rome I	21.0	103/747 = 13.8%	United Kingdom
Parry <sup>[27]</sup>	Prospective Case control	Matched from database	Confirmed <i>Campylobacter</i> , <i>Salmonella</i>	3-6	Rome II	23.5	18/128 = 14.1%	United Kingdom
Wang <sup>[12]</sup>	Prospective	Uninfected family	Confirmed <i>Shigella</i>	12, 24	Rome II	23.5	24/295 = 8.1%	China
Okhuyzen <sup>[15]</sup>	Prospective	None	Self-reported travelers' diarrhea	6	Rome II	20.0	6/169 = 3.6%	United States
Kim <sup>[4]</sup> , Ji <sup>[23]</sup> , Jung <sup>[41]</sup>	Prospective	Uninfected contemporaneous	Self reported (presumed <i>Shigella</i> )	12, 36, 60	Modified Rome I & II	24.5	15/143 = 10.5%	South Korea
Mearin <sup>[25]</sup>	Prospective	Uninfected contemporaneous	Self reported (presumed <i>Salmonella</i> )	3, 6, 12	Rome II	22.5	27/467 = 5.8%	Spain
Marshall <sup>[11]</sup>	Prospective	Uninfected contemporaneous	Self reported (presumed <i>E. coli</i> , <i>Campylobacter</i> )	24-36	Rome I	26.5	417/1368 = 30.5%	Canada
Borgaonkar <sup>[21]</sup>	Prospective	None	Confirmed (any bacterial pathogen)	3	Manning & Rome I	20.0	7/191 = 3.7%	Canada
Moss-Morris <sup>[26]</sup>	Prospective	Mononucleosis	Confirmed <i>Campylobacter</i>	3, 6	Rome I & II	21.5	59/592 = 10.0%	New Zealand
Stermer <sup>[14]</sup>	Prospective	Uninfected contemporaneous	Self reported (traveler's diarrhea)	6	Rome II	13.6	13/118 = 11.0%	Israel
Marshall <sup>[10]</sup>	Prospective	Uninfected contemporaneous	Self reported (presumed viral)	3, 6, 12, 24	Rome I	23.5	15/92 = 16.3%	Canada
Spence <sup>[30]</sup>	Prospective	None	Confirmed <i>Campylobacter</i>	3, 6	Rome I & II	24.0	63/620 = 10.2%	New Zealand

<sup>1</sup>Thabane et al. APT 2007; 26: 535-544.**Figure 1** Conceptual model for post-infectious irritable bowel syndrome.

environment. Reported estimates of the prevalence/incidence of PI-IBS vary in part because of differences in study methodology, including the criteria used to define IBS (Table 1). In general, Rome II criteria generate lower estimates than Rome I or III.

Unlike sporadic IBS, PI-IBS has a defined moment of onset. Features of the inciting infectious illness such as diarrhea, abdominal cramps, increased stool frequency, bloody or mucous stools, positive stool culture and weight loss are potent predictors of long term outcome. The risk of PI-IBS appears to correlate with the severity of the acute enteric infection, increasing at least two-fold if diarrhea lasts more than 1 wk and over threefold if diarrhea lasts more than 3 wk<sup>[11,13]</sup>. Abdominal cramps, weight loss and bloody stools are also associated with increased risk, with abdominal cramps increasing the risk fourfold<sup>[11,12]</sup>. Various bacterial pathogens including *Campylobacter*, *Shigella*, *Salmonella* and *Escherichia coli* (*E. coli*) O157:H7<sup>[11,12,22-24,26,27]</sup> have been implicated in the development of PI-IBS but it remains unclear whether all organisms confer an equivalent risk. Viral GE appears to cause a more transient form of PI-IBS than bacterial pathogen dysentery<sup>[10]</sup>.

Other reported risk factors for PI-IBS include host factors, and psychological disorders<sup>[11-13,22,24,28]</sup>. Despite the fact that there are no reported gender differences

in the severity of initial infectious illness or immune response, the reported risk of developing PI-IBS is higher among females than males with an adjusted relative risk ranging from 1.47 to 2.86<sup>[11,13,29,30]</sup>. The female predisposition for PI-IBS may be confounded by a female preponderance of psychological distress. In a study by Gwee *et al*<sup>[22]</sup>, female gender was no longer a significant risk factor when psychological variables were controlled in a multivariate analysis. In two studies, the risk of developing PI-IBS decreased with increasing age<sup>[11]</sup> and age above 6 years was reported to have a protective effect with adjusted relative risk of 0.36<sup>[13]</sup>. Dunlop *et al*<sup>[31]</sup> demonstrated that older individuals have fewer lymphocytes and mast cells in the rectal mucosa, which may attenuate the inflammatory response to luminal antigens and yield a reduced risk of IBS.

Just as psychological disorders have been associated with development of sporadic IBS, Sykes *et al*<sup>[32]</sup> observed that people with premorbid psychiatric diagnoses, particularly anxiety disorders, are also at increased risk of PI-IBS after acute GE. In addition, depression, neuroticism, somatisation, stress and negative perception of illness have all been linked to PI-IBS<sup>[22,27,30,33]</sup>. In a recent study, patients who developed PI-IBS had significantly higher levels of perceived stress (OR 1.10, 95% CI: 1.02-1.15), anxiety (OR 1.14, 95% CI: 1.05-1.23), somatisation (OR 1.17, 95% CI: 1.02-1.35) and negative illness beliefs (OR 1.14, 95% CI: 1.03-1.27) at the time of infection than those who did not develop PI-IBS<sup>[30]</sup>. Furthermore, Gwee *et al*<sup>[22]</sup> showed that patients who developed IBS reported more life events and had higher hypochondriasis scores. These observations suggest a psychological-environmental interaction wherein exposure to GE may trigger symptoms that are sustained by psychological disturbances<sup>[30]</sup>. This paradigm provides support for cognitive-behavioral therapy as a treatment for PI-IBS.

## PATHOPHYSIOLOGY

PI-IBS patients are more likely than sporadic IBS patients to exhibit a diarrhea-predominant phenotype<sup>[11,17,34]</sup>. Spiller found that serial intestinal biopsies from patients recovering from *Campylobacter jejuni* (*C. jejuni*) showed persistent inflammation with elevated T lymphocytes and calprotectin-positive macrophages, possibly as a response to mucosal injury and inflammation<sup>[18]</sup>. Dunlop *et al*<sup>[3]</sup> noted a 25% increase in rectal enterochromaffin cells in patients with PI-IBS, compared to those with sporadic IBS or healthy controls. Significant increases in postprandial plasma serotonin levels have also been seen in PI-IBS and in sporadic constipation-predominant IBS<sup>[35]</sup>. Gwee *et al*<sup>[36]</sup> reported increased expression of IL-1 $\beta$  in rectal biopsies from patients with PI-IBS, when compared with those who suffered infectious enteritis but did not develop PI-IBS. Wang *et al*<sup>[37]</sup> also observed increased IL-1 $\beta$  in patients with PI-IBS after *Shigella* infection when compared with patients with sporadic IBS<sup>[12]</sup>.

Patients with PI-IBS also demonstrate increased small

intestinal permeability compared to non-IBS controls, suggesting a defect in epithelial integrity that might promote intestinal inflammation<sup>[18]</sup>. In one study, an increased lactulose-mannitol fractional excretion ratio was observed among patients with IBS 2 years after a waterborne outbreak of GE involving *C. jejuni* and *E. coli* 0157:H7. In this cohort, increased permeability was associated with increased stool frequency<sup>[37]</sup>. Similar elevations in gut permeability were also observed by Spiller *et al*<sup>[18]</sup>. Increased intestinal permeability can promote inflammation by facilitating exposure of the submucosa to luminal antigens with subsequent disturbance of enteric sensation and motility.

There is evidence that genetic risk factors may contribute to PI-IBS pathophysiology. A recent study by Villani *et al*<sup>[38]</sup> identified three candidate gene variants, namely *TLR9*, *CDH1* and *IL6*, which were associated with development of IBS following acute GE. These observations suggest that PI-IBS might result from abnormalities in genes encoding epithelial barrier functions and innate immune responses to enteric bacteria. This discovery is consistent with a paradigm of PI-IBS pathogenesis involving decreased mucosal barrier function, low grade inflammation, and immune activation in the colonic mucosa. Future studies are needed to confirm these potential candidate gene variant associations.

## PROGNOSIS

The prognosis of PI-IBS appears favorable with spontaneous and gradual resolution of symptoms in most patients. In the largest and longest prospective cohort study of PI-IBS, the prevalence of PI-IBS in a Canadian cohort dropped from 31% at 2 years to 23% after 4 years and 17% after 6 years<sup>[39,40]</sup>. After 6 years, the incidence of new IBS was no longer increased among those exposed to the initial infection when compared to unexposed controls. A similar decline was reported in an Asian and in a British study, where half of patients recovered 5 and 6 years respectively, after an exposure to acute GE<sup>[41,42]</sup>. In the same Asian follow up study, a 25% recovery rate was reported 3 years after infection<sup>[4]</sup>. In studies that have followed patients for only 1 year after outbreaks of bacterial dysentery, the prevalence of IBS has remained relatively stable<sup>[23,43]</sup>. Of note, in the only study to date which has followed an outbreak of viral GE, the prevalence of IBS symptoms remained elevated for only 3 mo after infection<sup>[10]</sup>. This suggests that viral GE may be associated with a more transient functional disturbance.

## THERAPY

To date there exist no therapies proven to be effective specifically for the management of PI-IBS. Because the phenomenon of PI-IBS is clinically indistinguishable from sporadic IBS, conventional approaches to the management of functional bowel disorders should be adopted. Given a limited insight into the pathogenesis of

sporadic IBS, these approaches consist largely of non-specific measures intended to relieve symptoms. However, our enhanced understanding of the pathogenesis of PI-IBS provides a compelling rationale for some specific approaches that target key mechanisms. These warrant further study in clinical trials.

Acute GE can shift colonic flora, which may in turn induce or promote many of the changes in physiology noted above. Modulation of the flora with probiotics, antibiotics or prebiotics can down-regulate inflammation, improve barrier function and reduce visceral sensitivity<sup>[44-50]</sup>. Probiotics have been proven effective in preventing or attenuating acute GE<sup>[51-53]</sup>. However, no study has yet assessed the efficacy of interventions that modulate gut flora for preventing or treating PI-IBS.

The observed increases in enterochromaffin cell numbers and post-prandial serotonin release in PI-IBS<sup>[18,53]</sup> suggest that serotonergic therapies might prove particularly effective in this population. While the therapeutic gain associated with 5-HT<sub>3</sub> antagonists<sup>[54-62]</sup> and 5-HT<sub>4</sub> agonists<sup>[63-72]</sup> in treatment of sporadic IBS has been small, patients with PI-IBS are relatively more homogeneous and might demonstrate enhanced efficacy. No study of such interventions in the management of PI-IBS has been reported.

There remains little doubt that PI-IBS is associated with persistent intestinal inflammation, and that inflammation itself can disturb gut function and generate symptoms. An underpowered randomized trial of prednisolone for treatment of established PI-IBS failed to show a significant improvement in symptoms, but did show a reduction in intestinal enterochromaffin cell and lymphocyte counts<sup>[73]</sup>. In animal models, early corticosteroid therapy has been shown to attenuate post-infectious neuromuscular dysfunction<sup>[74,75]</sup>. Hence it remains plausible that corticosteroids given to patients with GE at high risk of PI-IBS would be effective in preventing or attenuating chronic symptoms. Locally active steroids with reduced systemic toxicity, such as budesonide, are particularly attractive for this indication.

## CONCLUSION

PI-IBS is a common complication of acute enteric infection. While the epidemiology and natural history of this clinical phenomenon have been well characterized, our understanding of its pathophysiology remains limited. Future research is needed to further define this complex host-microbe interaction and provide new tools for prevention and management.

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## Irinotecan therapy and molecular targets in colorectal cancer: A systemic review

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limited by irinotecan toxicity levels. To conclude, irinotecan improves the patient's quality of life and the survival rates of patients with advanced CRC. p53 and VEGF status of the patients' tumour is likely to affect the responsiveness of CRC to irinotecan. It is recommended that studies of the expression of these molecular markers in relation to chemoresponsiveness of irinotecan should be carried out for better management of patients with advanced CRC.

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### Abstract

Irinotecan is the second line chemotherapy for advanced stage colorectal cancer (CRC) after failure of first line chemotherapy with oxaliplatin and 5-fluorouracil. The aim of this review is to analyse the data on irinotecan as second line chemotherapy for advanced CRC and the potential roles of the molecular markers, p53 and vascular endothelial growth factor (VEGF) in the management of advanced CRC. Thus, the English literature from 1980 to 2008 concerning irinotecan, p53, VEGF and CRC was reviewed. On review, Phase II and III clinical trials showed that irinotecan improves pain-free survival, quality of life, 1-year survival, progression-free survival and overall survival in advanced CRC. p53 and VEGF were expressed in CRC and had a predictive power of aggressive clinical behaviour in CRC. Irinotecan sensitizes p53 wild type, mutant and null cells to Fas-mediated cell apoptosis in CRC cells. Wild type p53 cells were more sensitive to irinotecan than mutated p53. Irinotecan has an anti-VEGF effect inhibiting endothelial cell proliferation, increasing apoptosis and reducing microvascular density which is only

### INTRODUCTION

Colorectal carcinoma (CRC) is one of the most common types of cancer in Western countries<sup>[1]</sup>. Five-year survival in CRC patients is related to diagnostic staging of CRC<sup>[2]</sup>. Due to the late onset of symptoms, the majority of cases are diagnosed in Duke's stages C or D. Most patients with CRC undergo surgical resection and then commence adjuvant chemotherapy, with the exception of stage A and some stage B cancers where side effects outweigh potential benefits. In patients who develop recurrent or metastatic CRC, oxaliplatin and 5-fluorouracil (5-FU) combined are the most extensively used first line treatment, with a response in approximately half of patients<sup>[3]</sup>. If the cancer progresses after this treatment, irinotecan is commenced. This review was undertaken to study the impact of irinotecan in the treatment of advanced CRC and to identify advanced CRC molecular markers that predict response to irinotecan. The aims are to influence patient selection and to recommend

evidence-based treatment options for patients with advanced CRC and to reduce patient morbidity and mortality.

## DATA COLLECTOIN

The English literature between 1980 and 2008 on the treatment of advanced CRC with irinotecan was reviewed; the effects of irinotecan on CRC, p53, vascular endothelial growth factor (VEGF) and CRC in Australia. Studies not including irinotecan as the variable were excluded, as were those without CRC. The studies were statistically significant when  $P < 0.05$ . Only original full text publications were reviewed. The selection criteria included adequate follow-up, sample numbers, second line irinotecan therapy and clinicopathological features: grading and staging of the CRCs, histological subtype, nodal and metastatic status, threshold cut off values and complete reference lists. The blinded status and number of experimental observers were noted. The review was limited by suboptimal cohort sizes as it focuses on recently emerging research. Whilst the methodology and statistical analysis varied between studies, their designs were similar.

## LITERATURE OVERVIEW

### *Irinotecan therapy*

Irinotecan is activated by hydrolysis to SN-38, an inhibitor of topoisomerase I. This is then inactivated by glucuronidation by uridine diphosphate glucuronosyltransferase 1A1<sup>[4]</sup>. The inhibition of topoisomerase I by the active metabolite SN-38 eventually leads to inhibition of both DNA replication and transcription. Unlike hepatocytes, other cells in the body have no way of detoxifying SN-38 through glucuronidation, thus contributing to its high cytotoxicity. The most frequent irinotecan toxicities are severe diarrhoea and suppression of the immune system (neutropenia). Other side effects include nausea, hyperbilirubinaemia, fatigue, emesis, fever, weight loss, alopecia, oedema, dyspnoea and thromboembolism.

A Phase II study on irinotecan therapy was performed by Cunningham and colleagues in 289 patients with advanced CRC<sup>[5]</sup>. It was shown that the survival rates were much better in 189 patients who received chemotherapy than the 90 patients who received supportive care only (36.2% vs 13.8%). In addition, both pain-free survival and quality of life were higher with irinotecan 2nd line chemotherapy. Also, irinotecan increased survival of patients without World Health Organisation (WHO) performance status deterioration and without weight loss exceeding 5%.

A Phase III study by Rougier and colleagues concluded that irinotecan produced better 1-year survival of 45% and median survival of 10.8 mo than the comparison groups treated with three different regimes of infused FU, where a survival rate of 32% and a median survival of 8.5 mo were recorded<sup>[6]</sup>. These were both well designed randomised non-blinded studies with large cohorts both in excess of 265 patients. The patients

were assessed every 3 wk with imaging and CEA levels to detect progression of disease. All the patients had metastatic CRC and had received treatment within the preceding 6 mo with 5-FU.

Clinicopathological features were a major confounding factor, however, the results of Cunningham *et al*<sup>[5]</sup> and Rougier *et al*<sup>[6]</sup> remained significant after the WHO baseline performance status of the patients was analysed with multivariate analysis. These studies support the use of second line irinotecan monotherapy to increase pain-free survival, quality of life, 1-year survival, progression-free survival and overall survival in metastatic CRC, both in comparison to best supportive care and to 5-FU.

Rothenberg and colleagues conducted a Phase II trial, involving 166 patients with metastases involving either liver, lung, lymph nodes or other soft tissue, treated with second line irinotecan monotherapy<sup>[7]</sup>. They found a significant response to irinotecan with 10.8% of patients achieving complete or partial response and 40.4% with stabilised disease. The median progression-free survival was 3.9 mo with a median survival of 9.9 mo. Unlike the above studies which examined the effect of clinicopathological variants through multivariate analysis, Rothenberg *et al*<sup>[7]</sup> directly examined the effect of symptomatic disease on overall survival. They found that asymptomatic patients had a longer overall survival than those patients with symptoms prior to irinotecan therapy. However, on further analysis the 1-year survival was 42.4%, with no significant effect from clinicopathological factors or baseline variables. There were several limitations in this study. Most importantly there was no control or comparison group. Despite the tumors being assessed every 12 wk, the assessment criteria states the tumors must decrease or remain stable for 4 wk or more to be classified as responding to therapy. Therefore the tumors needed to respond for 12 or more weeks. Such a long time lapse between monitoring affects progression-free results, leading to an underestimation of true progression-free survival. Also, the study was not blinded. However, the authors overcame this by the use of two teams assessing the imaging and CEA results, one of which involved radiologists and oncologists independent of the study.

These three major studies show statistically that clinicopathological features have no effect on overall survival, and that the presence of symptomatic disease is not an independent predictor for outcome in a multivariate analysis. Overall, irinotecan is an effective second line chemotherapeutic agent for CRC. In Phase II and III clinical trials, it has been shown to increase 1-year survival, quality of life and symptom control despite a high side effect profile.

### *Molecular targeted therapy*

Molecular targeted therapy is a “drug or therapeutic strategy with a focused mechanism specifically acting on a well-defined target or biological pathway that, when inactivated, causes the regression or destruction of cancer”. The advantages of molecular targeted therapy include (1) providing evidence-based treatment options

for patients, (2) treating patients most likely to respond to the therapy (3) sparing patients with poor response profiles from further harm in terms of side effects, (4) minimising side effects (5) streamlining healthcare resources and finally (6) to promote further research into molecular targeted therapy.

This review focused on two common genes that are commonly investigated in cancer, namely VEGF and p53. Being a second line chemotherapeutic agent, it is important to research the effects of irinotecan on such mutations as these antigens are key determinants in the survival of advanced CRCs. By researching irinotecan it is hoped that results will show different molecular targeted pathways by which irinotecan can potentially inhibit CRCs so that patients with the most responsive marker profile may be treated with a molecular targeted drug that acts on different sites rather than just one site.

### **p53 in CRC**

p53 is a tumour suppressor gene<sup>[8]</sup>. Alterations in p53 are the most common genetic changes noted in human cancer. p53 senses DNA damage in the G1 stage of the cell cycle and either prevents cell cycle progression until DNA is repaired or induces apoptosis. p53 acts as a central mediator of the cellular response to stressful stimuli. The growth-suppressive function of p53 is lost with mutation and this occurs commonly in human cancer. In addition to suppressing cancer development and progression, wild-type p53 further confers chemo-sensitivity and radio-sensitivity in tumour cells.

Our recent study, involving 188 patients, showed that p53 is over-expressed in 63% of Australian patients with CRC<sup>[9]</sup>. The survival of the CRC patients was related to staging and p53 protein nuclear expression in the tumors. In our other study, p53 was also noted to predict poorer survival in a subset of CRC patients with mucinous adenocarcinomas<sup>[10]</sup>.

Bosari *et al*<sup>[11]</sup> conducted a 5-year retrospective study on p53 immunostaining in 206 CRC specimens from patients with no neoadjuvant therapy or history of other malignancies and with clear resection margins. 65 specimens stained positive for nuclear p53 and 99 for cytoplasmic p53 accumulation. Cytoplasmic p53 accumulation correlated with reduced overall survival and reduced disease-free survival. In addition, cytoplasmic accumulation of p53 was a significant prognostic factor for poorer overall survival and disease-free survival in left sided CRC. The study could have been strengthened by the identification of the type of p53, rather than relying on past research that indicates cytoplasmic accumulation of p53 is usually WTp53. WTp53 and Mp53 respond differently in CRC and induce cell death *via* different mechanisms.

Flamini and colleagues studied 96 CRC patients, of which 47% were p53 positive<sup>[12]</sup>. The patients were studied for 3 years and treated exclusively by surgical resection of the CRC and metastases. Compared to other stages, Duke's D CRC had increased cytoplasmic p53 expression, whilst nuclear p53 was over expressed in Duke's B CRC. This study had unique findings

and is unlikely to ever be repeated due to the ethical restrictions of not treating patients with either adjuvant or neoadjuvant therapy.

Diez and colleagues studied 174 patients and concluded that p53 positivity in the primary CRC increased the risk of recurrence only after the first year of follow-up<sup>[13]</sup>. Lanza and colleagues studied 204 CRCs by IHC, of which 60.4% were p53 positive<sup>[14]</sup>. The study concluded that there was no statistical significance with regard to age, gender, tumour site, tumour stage or grade of differentiation. Of the 141 patients with TNM I - III disease who underwent curative resection, positive p53 staining was associated with poorer overall survival.

Adrover and colleagues randomly selected 111 patients with sporadic CRCs TNM stages I - IV, and quantitatively measured the cytoplasmic WTp53 and Mp53 in both the cancerous and non-neoplastic tissue of all patients using immunoassay with the p53 antibodies Ab1801 and DO1 as markers<sup>[15]</sup>. High p53 expression was defined as having  $\geq 2.7$  ng/mg cytosolic protein. TNM stage III and high p53 expression correlated with increased disease-free survival. In multivariate analysis, p53 expression is related to a survival advantage in stage III CRCs. These results significantly contradict previous studies. However, Adrover *et al*<sup>[15]</sup> were the first to identify the difference in normal p53, wild type and mutant p53. The definition of high levels of p53 is debatable as the p53 expression in adjacent non-neoplastic tissue has not been evaluated.

To conclude, p53 expression is important in the prognosis of CRC. The identification of WTp53 and Mp53 is controversial due to the half life of WTp53. Based on the larger studies, the overexpression of p53, especially cytoplasmic p53, is a crucial target for molecular targeted chemotherapy.

### **Vascular endothelial growth factor in CRC**

The VEGF family of genes are key regulators of angiogenesis<sup>[16]</sup>. VEGF expression correlated with clinical and pathological parameters in cancers<sup>[17-19]</sup>. For instance, we have shown that strong immunohistochemical VEGF expression levels tended also to have higher serum VEGF level than those with low expression levels. In addition, elevated serum VEGF levels are strongly correlated to the recurrence of thyroid cancer and the presence of lymph node metastases. VEGF expression was noted in the non-cancerous tissue adjacent to the cancer indicating that genetic changes may occur before the morphological appearance of cancer.

Colorectal mucosa contains all the subtypes of the VEGF family A-D<sup>[20]</sup>. VEGF mRNA is expressed in higher levels in human CRC cells compared to adjacent normal tissue<sup>[21]</sup>. VEGF mRNA expression rises most between CRC stages Tis to T1.

A meta-analysis involving 27 studies of VEGF expression and overall survival of CRC observed a 1.65 times poorer survival in those with higher ratios of VEGF in cancer tissue<sup>[22]</sup>. Kondo *et al*<sup>[23]</sup> found VEGF mRNA and protein only in CRCs (15 of 26 studied) compared to no expression in adenomas. They

suggested that mutant p53 induced VEGF expression, which coincides with the progression from adenoma to carcinoma in CRC.

Of the 31 human CRC cell lines examined with IHC, Kuramochi *et al*<sup>[24]</sup> concluded there was no significant difference in median VEGF mRNA levels of expression in the primary CRC in patients with or without hepatic metastases. However, the level of VEGF expression is significantly less in the hepatic metastatic tissue compared to the adjacent non-cancerous hepatic tissue. Patients with more than one site of metastasis expressed higher levels of mRNA VEGF compared to those with one metastasis site<sup>[22]</sup>.

Comparing CRC (T) to the adjacent colorectal mucosa (N), Hanrahan *et al*<sup>[25]</sup> concluded that VEGF-A and VEGF-B play a significant role in the early development of CRC from adenomas, whilst VEGF-C is expressed at higher levels in metastatic CRC. They concluded that VEGF A and C mRNA levels were correlated to tumour grade and tumour size, but not significantly related to the staging of CRC.

Ottaiano and colleagues concluded from their study of 71 patients that CXCL12 stimulates ICAM-1 and VEGF expression and clonogenic growth of CRC cells, which all lead to metastases, and that over expression of VEGF was an independent predictor of early metastases in CRC patients<sup>[26]</sup>. Ishigami *et al*<sup>[27]</sup> further concluded that over expression of VEGF mRNA also correlated with poor overall survival.

Saad and colleagues showed that VEGF expression correlated with the presence of angiolymphatic invasion, lymph node metastasis and the depth of invasion<sup>[28]</sup>. They found no significant correlation between VEGF expression and tumour grade and development of liver metastases.

### Molecular markers and irinotecan

**p53:** Yu *et al*<sup>[29]</sup> profiled the 24 genes associated with the mechanism of action of irinotecan in 52 CRC specimens from humans with no previous neoadjuvant therapy. Irinotecan is converted to SN-38 which acts on topoisomerase-1 then subsequently *via* a cascade effect on TNFSF6 to FDXR and then p53 which finally induces cell apoptosis. Through *in vitro* gene profiling and cluster analysis, Yu *et al*<sup>[29]</sup> found that the pathway leading to p53 was expressed at higher levels in tumourous tissues (T) compared to adjacent normal tissue (N), however, none as high as p53. Whilst the level of p53 RNA expression was not identical for each specimen, the general trend remained. The study showed that irinotecan is more effective in inducing apoptosis in CRC when the p53 T:N is high, despite the type of mutation in p53.

*In-vitro* studies concluded that irinotecan sensitizes p53 wild type, mutant and null cells to Fas-mediated cell apoptosis in CRC cells<sup>[30]</sup>. Irinotecan caused a significant rise in Fas mRNA in WTp53 cells<sup>[31]</sup>. However, irinotecan also caused a small increase in Fas mRNA in p53 mutant and null cells. This indicates that not only does p53 have a major role in Fas cell surface expression, but

that irinotecan also increases Fas cell surface expression independent of p53. Irinotecan induces signal transducer and activator of transcription 1 (STAT1) phosphorylation in the p53-null cell lines and increases the expression of genes involved in cell surface trafficking of Fas, despite STAT1 not being identified in the promoter of Fas. Absence of STAT1 decreases Fas expression.

McDermott and colleagues found a significant increase in STAT-1 Ser727 phosphorylation in irinotecan treated null cells<sup>[32]</sup>. The experiment was repeated with the addition of STAT1 small interfering RNA which caused a down regulation of STAT1 expression in the WTp53 and null cell lines. Subsequent treatment with irinotecan produced no change in the level of Fas mRNA, however, down regulation of STAT1 resulted in a significant decrease in Fas cell surface expression in the p53-null cell line. They proposed that STAT1 silencing was incomplete or that STAT-1 independent pathways regulate Fas ligand expression in response to irinotecan

p53 wild type cells were also more sensitive to irinotecan treatment compared to mutant p53 at low dose and high dose treatment<sup>[33]</sup>. No significant response to irinotecan treatment was recorded in the mutant p53 cell lines As irinotecan is known to have a higher response in both cytoplasmic accumulation of p53 and of WTp53, this study further suggests that WTp53 accumulates in the CRC cell cytoplasm and that WTp53 in CRC is a positive predictive factor for response to irinotecan.

Irinotecan was added to cells from a single Duke's B CRC with mutant p53 and its sub-clone transfected with wide type p53. The cells were synchronised to G0/G1 stage of the cell cycle by starvation. Following the addition of irinotecan, the wild type p53 cells were arrested in S phase whilst the mutated p53 cells continued to progress through the cell cycle, indicating a lack of functionality of p53. The functional response of irinotecan in the wide type p53 line and not the mutant p53 was observed during *in-vivo* studies of nu/nu mice with xenografted human CRC, whereby there was increased apoptosis and decreased proliferation.

Overall, irinotecan reduces progression in WTp53 CRCs, and plays a less significant role in inhibiting mutant and null p53 CRCs *via* the STAT1 pathway. Having identified p53 as the key component of the apoptotic pathway and the significance of the T:N p53 ratio in predicting a positive response to irinotecan, it is necessary that these findings are implemented in practice. Thus, further research should be conducted using *in-vivo* research methods on a large scale to confirm the clinical significance of such results.

**Vascular endothelial growth factor:** In CRCs, high levels of EGFR correlated with response to irinotecan and progression-free survival<sup>[34]</sup>. However, this did not prove that VEGF expression is correlated with response to irinotecan. The study had a small sample size, all were treated with irinotecan, 5-FU and leucovorin, and no control groups treated with single agent therapy. Whilst there was an adequate follow up period of 23 mo, there were several variables.

Koizumi and colleagues developed NK012, a SN-38 (irinotecan metabolite) releasing nanodevice and tested its efficacy in xenografted CRCs in mice<sup>[35]</sup>. A comparison of tumour size reduction with NK012 (doses 15 and 30 mg/kg per day) *vs* irinotecan (66.7 mg/kg per day) showed a significant reduction in tumour size compared to the irinotecan treatment. Compared to irinotecan, NK012 was more cytotoxic with potent anti-tumour activity, thought to be secondary to the enhanced and prolonged distribution of free SN-38 in the tumour. This showed that irinotecan and its active metabolite has an anti-VEGF effect. Thus, it can be proposed that these activities should be reflected in VEGF positive CRCs if adequate levels of SN-38 are produced, which is only limited by the irinotecan dose side effects.

Bocci and colleagues compared the effects of irinotecan metronomic monotherapy against irinotecan combined with semaxinib on xenografted CRCs in mice and in *in-vivo* CRC cell lines<sup>[36]</sup>. They analysed *in-vitro* proliferation of cells, apoptosis and thrombospondin-1 (TSP-1)/VEGF expression and concluded that in the cells treated with SN-38 monotherapy, there was inhibited endothelial cell proliferation alone, and that irinotecan worked synergistically with semaxinib to increase apoptosis and increase expression and secretion of TSP-1 and to reduce microvascular density<sup>[35]</sup>.

In *in-vitro* studies, SN-38 increased TSP-1 expression and in *in-vivo* studies, SN-38 reduced tumour and microvessel growth<sup>[37]</sup>. Higher levels of TSP-1, an anti-angiogenic factor are linked with low levels of VEGF<sup>[37]</sup>. TSP-1 inhibits angiogenesis through the inhibition of matrix metalloproteinase-9 (MMP9). TSP-1 null mice have both increased levels of MMP9, whilst over expression of TSP-1 in mammary tissue reduced the levels of active MMP9. The levels of MMP9 correlated with the level of expression of VEGF binding to VEGFR-2<sup>[38]</sup>. Gupta *et al*<sup>[39]</sup> concluded that TSP-1 inhibits VEGF mobilisation from the extracellular matrix by inhibiting active MMP9 and that TSP-1 also has a direct role in inhibiting VEGF activity.

Irinotecan increases TSP-1 expression and secretion and TSP-1 reduces VEGF expression and activity<sup>[36]</sup>. CRCs expressing high levels of VEGF respond more significantly to irinotecan as the stimulus for angiogenesis and thus subsequent growth of the tumour is reduced.

## CONCLUSION

Increased expression of p53, especially cytoplasmic WTp53 is associated with poor overall survival, decreased disease-free survival, increased relapse and metastases. However, cytoplasmic accumulation of wild type p53 is a positive predictive factor for irinotecan response. Irinotecan induces apoptosis in both mutant and null p53 CRCs to a lesser extent *via* the STAT1 pathway.

Increased expression of VEGF is correlated with multiple metastases, tissue invasion and lymph node invasion. Whilst there is evidence that irinotecan increases TSP-1 expression which in turn reduces VEGF expression and the angiogenic growth of tumors, there

is no conclusive evidence linking irinotecan with VEGF expression and response. Irinotecan has the potential for multiple effects within CRC cells which may reduce the number of prescribed drugs and side effects for patients.

As a second line therapy, irinotecan improves patient quality of life, 1-year survival and progression-free survival. It would be beneficial to all patients to determine the molecular profiles of CRC most likely to respond to irinotecan and to spare those unlikely to respond from unnecessary side effects.

A standardised criteria needs to be developed to compare marker expression in normal colorectal mucosa to that of cancerous mucosa in order to define overexpression of markers. Further research would also benefit from a standard CRC staging system so that studies can be compared directly, as well as 12 mo follow-up periods for irinotecan treatment. Areas of potential research focus include: (1) large studies on the effects of irinotecan with regard to VEGF and p53 expressions in CRC and (2) determine whether the expression of molecular markers can predict response to irinotecan.

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# Transcriptional down regulation of hTERT and senescence induction in HepG2 cells by chelidonine

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## Abstract

**AIM:** To investigate the potential effects of chelidonine, the main alkaloid of *Chelidonium majus*, on telomerase activity and its regulation in HepG2 cells.

**METHODS:** Cytotoxicity of chelidonine for HepG2 cells was determined by neutral red assay. A modified polymerase chain reaction (PCR)-based telomerase repeat amplification protocol was used to estimate relative telomerase activity in chelidonine-treated cells in comparison with the untreated control cells. Relative expression level of the catalytic subunit of telomerase (hTERT) gene and P-glycoprotein (pgp) were estimated using semi-quantitative real-time reverse transcription-PCR (RT-PCR). Cell senescence in treated cells was demonstrated using a  $\beta$ -galactosidase test.

**RESULTS:** Cytotoxicity of chelidonine in HepG2 cells was not dose-dependent and tended to reach plateau immediately after the living cells were reduced in number to slightly higher than 50%. However, 12  $\mu$ mol/L concentration of chelidonine was considered as LD<sub>50</sub>, where the maximal attainable effects were realized. Real-time RT-PCR data showed that the expression of pgp increased three-fold in chelidonine treated HepG2 cells in comparison with the untreated controls. Morphologically, treated HepG2 cells showed apoptotic features after 24 h and a small fraction of cells appeared with single blister cell death. The relative expression

level of Bcl-2 dropped to less than 50% of control cells at a sub-apoptotic concentration of chelidonine and subsequently increased to higher than 120% at LD<sub>50</sub>. Telomerase activity was reduced considerably after administration of very low doses of chelidonine, whereas higher concentrations of chelidonine did not remarkably enhance the effect. Real-time RT-PCR experiments indicated a drastic decrease in expression level of hTERT subunit of telomerase under treatment with chelidonine. Repeated treatment of cells with very low doses of chelidonine caused a decline in growth rate by 4 wk and many of the cells appeared to be aged with large volume and dark staining in the  $\beta$ -galactosidase assay.

**CONCLUSION:** Chelidonine reduces telomerase activity through down-regulation of hTERT expression. Senescence induction might not be directly caused by reducing telomerase activity as it occurs after a few population doublings.

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**Key words:** Chelidonine; Telomerase; Inhibition; Apoptosis; Senescence

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## INTRODUCTION

*Chelidonium majus* (*C. majus*), the greater celandine (family Papaveraceae), has a long history in phytomedicine for the treatment of many diseases or health disturbances. It contains isoquinoline alkaloids, especially protoberberine and benzophenanthridine alkaloids<sup>[1]</sup>. Extracts of this plant exhibit interesting antitumor and antiviral activities<sup>[2]</sup> in addition to hepato-protective and anti-genotoxic

effects in induced hepatocarcinogenesis in mice<sup>[3]</sup>. Ukrain, a semisynthetic derivative of *C. majus* alkaloids has been reported to be selectively toxic to malignant cells without toxic side effects to normal cells<sup>[4,5]</sup>. Ukrain has been used in the therapy of several solid tumors. It is known to induce apoptosis in glioblastoma cells<sup>[6]</sup> and in cell cultures<sup>[7]</sup>. Data from randomized clinical trials based on numerous pre-clinical and clinical investigations has established Ukrain as an anticancer drug<sup>[8]</sup>.

Chelidoneine, the tertiary hexahydro-benzophenanthridine alkaloid, is the major component of Ukrain. It primarily causes apoptosis in primary human uveal melanoma cell lines as shown by cellular morphology studies and flow cytometric analysis<sup>[9]</sup>. This alkaloid arrests mitosis by inhibition of tubulin polymerisation and activation of the stress activated protein kinase/jun kinase pathway (SAPK/JNK)<sup>[10]</sup>. A mitochondrial cell death pathway induced by chelidoneine in MT-4 cells has also been realized with an increased ratio of cytosolic to mitochondrial cytochrome c and increased percentage of cells with active caspase-3 and pro-apoptotic Bax protein<sup>[11]</sup>.

In this communication telomerase activity has been investigated in HepG2 cells after treatment with chelidoneine, using a modified telomerase repeat amplification protocol (TRAP). Telomerase, basically a reverse transcriptase, compensates telomere attrition, which occur in proliferating cells due to incomplete genome replication by DNA polymerases. This enzyme, while detectable, is diminished and shows very low activities in many adult somatic cells but is drastically overexpressed in almost 90% of different kinds of human cancer cells by up to 100-fold over the normal counterpart cells<sup>[12]</sup>, and confers a strong selective advantage for continued growth of malignant cells<sup>[13]</sup>. This enzyme is considered as a key player in cell immortality<sup>[14]</sup> as it protects chromosome ends from degradation and also plays an important role in the telomere-capping function<sup>[15]</sup>. It has been proposed that telomerase inhibitors can be utilized as complementary drugs in cancer therapy<sup>[16,17]</sup>. Critically shortened telomere(s) induce proliferative senescence, apoptosis or continued proliferation accompanied by genomic instability<sup>[18]</sup>. Many studies have indicated that inhibition of telomerase can affect the survival of cancer cells through apoptosis induction without long-term treatment of the cells<sup>[19,20]</sup>.

There is currently much interest in the inhibition of telomerase by various strategies such as antisense oligonucleotides<sup>[21]</sup>, small-molecule inhibitors<sup>[22]</sup>, ribozymes<sup>[23]</sup> and RNA interference<sup>[24]</sup>, which in turn initiate the onset of growth inhibition and antitumor activity.

This study was focused mainly on the effect of chelidoneine on telomerase activity and its regulation. The focus on drugs such as natural benzophenanthridines that may possibly inhibit telomerase may help in use of these alkaloids specifically against cancer. The expression levels of the catalytic subunit of telomerase (hTERT) gene and morphological changes were

studied as well. Expression of Bcl-2, the related gene to suppress apoptosis, P-glycoprotein (pgp) involved in drug resistance, and  $\beta$ -galactosidase assay as a biomarker to identify cell senescence were also analyzed.

## MATERIALS AND METHODS

### Cell culture

HepG2 cell line (ACC 180 from DSMZ) was maintained in 75 cm<sup>2</sup> culture flasks in RPMI-1640 culture medium (Gibco, Invitrogen), supplemented with 10% heat-inactivated fetal bovine serum (Biochrome, Berlin, Germany), 100 000 U/L penicillin, and 100 mg/L streptomycin. Cells were grown in 5% CO<sub>2</sub> and 95% air atmosphere at 37°C and 100% humidity. Media were changed every 48 h, and the cells were subcultured after 5-6 d using trypsin-EDTA. Cell viability was evaluated by staining with 0.1% trypan blue exclusion method using a hemocytometer. To estimate the cytotoxicity of chelidoneine a neutral red uptake test was employed<sup>[25]</sup>.

For long-term growth of HepG2 cells, chelidoneine-treated or untreated cells were seeded into 75 cm<sup>2</sup> tissue culture flask at 1 × 10<sup>6</sup> for 4-5 d until 70%-80% confluence, then trypsinized and counted. Each time 1 × 10<sup>6</sup> cells were replated into new culture flasks but in each passage the chelidoneine series was treated at the desired concentration for 48 h and afterward cells were fed with fresh normal medium. The rest of the cells in each passage were plated for  $\beta$ -galactosidase assay. All the treatments were done in duplicate.

### Cytotoxicity test

Exponentially growing HepG2 cells were seeded in 96 well plates with 10 000 cells per well. The medium was changed after 24 h with the fresh medium plus various concentrations of chelidoneine. After 48 h the cells were washed with PBS and incubated in fresh medium including 50 mg/L neutral red. The medium was removed after 3 h incubation and 200  $\mu$ L isopropanol containing 0.4 mol/L hydrochloric acid was added to each well and shaken for 15 min at room temperature. The absorbance of neutral red was measured at 570 nm. The test was repeated four times each in triplicate and the 50% lethal dosage, LD<sub>50</sub>, values were calculated from dose-response curves.

### Assay of telomerase activity

Subconfluent HepG2 cells were seeded in six well plates with 100 000 cells per well and incubated for 24 h at 37°C, in 5% CO<sub>2</sub> and 100% humidity. The media were then changed for fresh ones containing various concentrations of chelidoneine and followed by 48 h incubation. After trypsinizing and washing with cold PBS, lysis buffer was added to the cell pellet according to Gelmini's method with small modifications<sup>[26]</sup>. Cell lysates were incubated on ice for 30 min and then centrifuged at 16 000 g for 30 min. The protein concentration of the supernatant was measured based on the Bradford assay<sup>[27]</sup>. For each of control and/or treated cells 0.5  $\mu$ g of extracted total protein was used for TRAP assay based on Gelmini's

method<sup>[26]</sup>. Telomerase activity was determined for each concentration of chelidone in at least three repetitions, each one in duplicate. Two controls were used: a positive control using cell extract containing 0.5 µg protein of untreated HepG2 cells, and a negative control which was the same as the positive control but treated with boiling heat and/or RNase A at 37°C for 20 min.

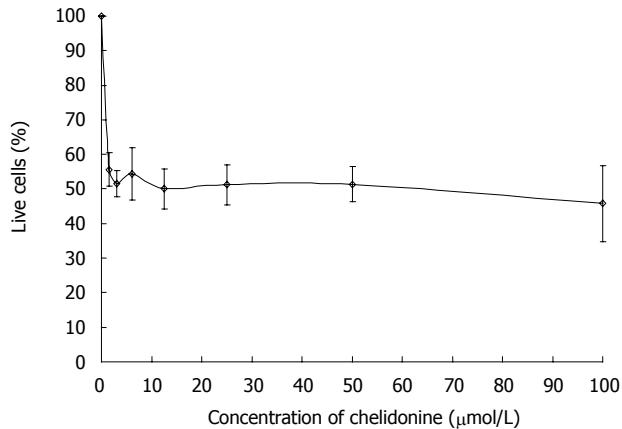
#### **RNA isolation, reverse transcription and real-time RT-PCR**

HepG2 cells were seeded in 12-well plate culture dishes with 50 000 cells per well. After 24 h incubation the medium was replaced with fresh medium including various concentrations of chelidone lower than the LD<sub>50</sub> and incubated for another 48 h. For RNA extraction, the cells were harvested and total RNA was isolated using RNeasy Mini Kit (Qiagen; Germany) according to the manufacturer's instruction and treated with DNaseI (Rnase-Free DNase Set, Qiagen; Germany) and stored at -80°C. Purity and quality of the extracted RNA were checked by gel electrophoresis, and the concentrations were measured using UV spectrophotometry.

First strand cDNA synthesis was performed according to the protocol suggested for the Reverse Transcription System (Promega Corporation; Madison, USA) using oligo(dT)<sub>16</sub> primers (Pharmacia Biotech; Uppsala, Sweden). Quantification of mRNA levels of the genes was achieved by quantitative real-time RT-PCR (Light Cycler, Roche Applied Science; Mannheim, Germany) using β2-microglobulin as the housekeeping gene. PCR amplification was carried out in 20 µL reaction volume containing 0.2 µg of cDNA, 1 × Light Cycler Fast Start DNA Master SYBR Green I (Roche Applied Science; Mannheim, Germany), 0.5 µmol/L of each primer set. MgCl<sub>2</sub> concentration was 3.25 mmol/L for β2-microglobulin gene and 3-4 mmol/L for other genes. The sequence of the primer sets used was: forward 5' GCTGCTCAGGTCTTCTTTATGTC3' and reverse 5'TCAAGTGCTGCTGATTCCAATG3' for hTERT, forward 5'AGGAAGTGAACATITCGGTGAC3' and reverse 5'GCTCAGTTCCAGGACCAGGC3' for Bcl-2. Primers used for β2-microglobulin and pgp (MDR1) were chosen as described by Albermann *et al.*<sup>[28]</sup>. Data analysis was performed using a calibrator and a standard curve for each gene and based on measurements of at least two repetitions. Each repetition includes two measurements for each of the duplicated samples for the defined concentrations of chelidone.

#### **β-galactosidase assay**

After removing medium, control and treated HepG2 cells with chelidone were washed in PBS, fixed for 3-5 min at room temperature in 2% formaldehyde/0.2% glutaraldehyde. Cells were washed twice with PBS and incubated at 37°C (no CO<sub>2</sub>) with fresh senescence-associated β-gal (SA-β-gal) stain solution containing 0.4 g/L X-gal, 40 mmol/L citric acid/sodium phosphate, pH 6.0, 5 mmol/L potassium ferrocyanide, 5 mmol/L potassium ferrocyanide, 150 mmol/L NaCl and 2 mmol/L MgCl<sub>2</sub> as described by Dimri *et al.*<sup>[29]</sup>. Staining was achieved in 2-4 h.



**Figure 1** The effect of chelidone on the growth of HepG2 cells after 48 h exposure. Viability of cells at each concentration of chelidone was evaluated by neutral red uptake test and is expressed as a percentage relative to untreated control cells. The values presented are the mean ± SD of six repeats each one in triplicate.

#### **DNA ladder formation**

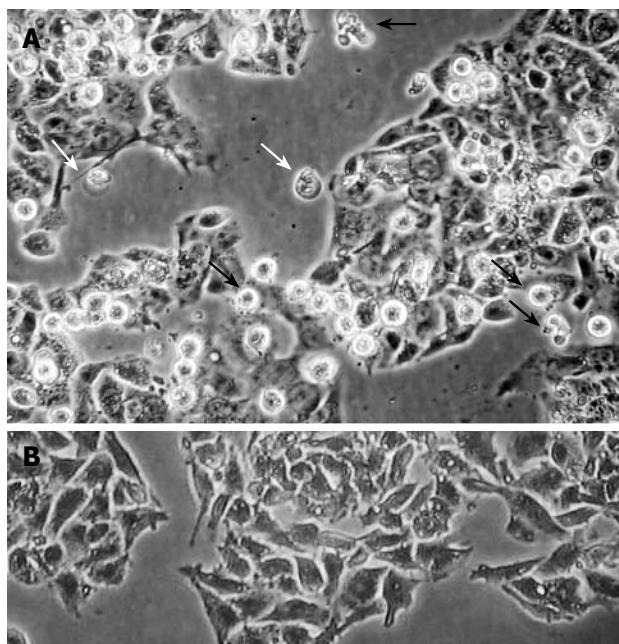
The cells were treated with chelidone at the specified concentrations for 48 h and harvested; cellular DNA was isolated and subjected to agarose gel electrophoresis followed by visualization of bands.

## **RESULTS**

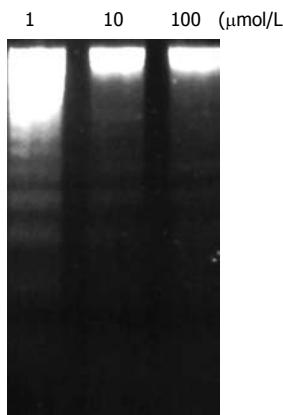
The effect of chelidone on HepG2 cell survival tested with neutral red uptake assays after 48 h treatment is shown in Figure 1. The cell viability curve of HepG2 cells under chelidone treatment had two distinct parts with quite different slopes. The first part with a sharp decline indicated its high toxicity, even at very low concentrations. The second part was a plateau. The fraction of live cells treated with 1.5-6 µmol/L chelidone was slightly more than 50% and the maximal effect of chelidone in reducing the live cells by 50% was at 12 µmol/L. By increasing the concentration of chelidone up to 100 µmol/L only a negligible increment in cytotoxicity was seen. This may suggest a potential mechanism involved in drug resistance.

Chelidone treated HepG2 cells showed apoptotic features after 24 h and a small fraction of cells appeared with single blister cell death<sup>[30]</sup>. Figure 2 shows the cells at 48 h after treatment with 12 µmol/L chelidone (LD<sub>50</sub>). DNA fragmentation due to apoptosis, as shown in Figure 3, represent the ladder formation in apoptotic HepG2 cells at different concentration after 48 h treatment.

Telomerase activity in HepG2 cells (control and cells treated with different concentration of chelidone for 48 h) is illustrated in Figure 4, which shows the resolving TRAP assay products on a native polyacrylamide gel electrophoresis. It clearly indicated decreased activity of telomerase in treated cells compared to untreated control cells. Considerable reduction of telomerase activity occurred at very low concentrations of chelidone, whereas increasing the chelidone concentration did not strengthen the effect significantly. The inhibitory ef-



**Figure 2** Morphological changes in HepG2 cells after 48 h treatment with 12  $\mu\text{mol/L}$  chelidone ( $\text{LD}_{50}$ ) visualized by phase contrast microscopy. A: Apoptotic cells (black arrows) and a few cells undergoing blister cell death (white arrows) are clearly seen; B: Untreated control HepG2 cells.

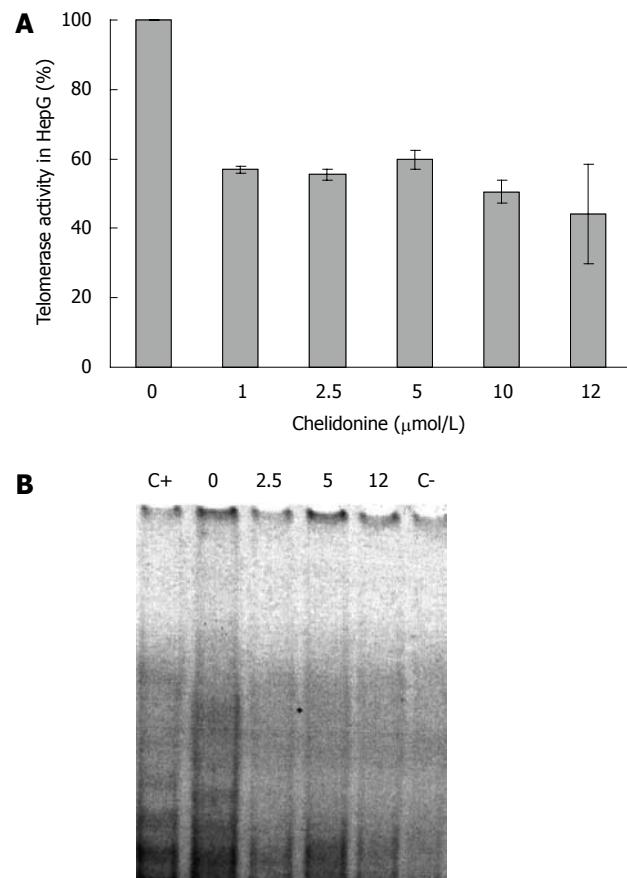


**Figure 3** Induction of DNA fragmentation in HepG2 cells after 48 h treatment with different concentrations of chelidone. The concentration of chelidone is indicated at the top of each well.

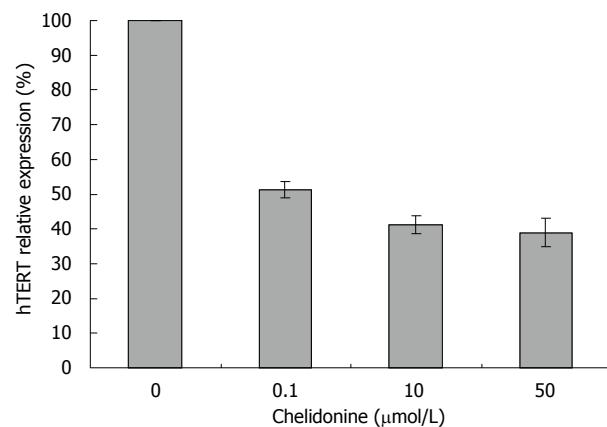
fect of chelidone on telomerase activity was not dose dependent. Moreover the 50% activity of telomerase in treated cells compared to the untreated control cells occurred almost at the same concentration ranges of chelidone as  $\text{LD}_{50}$  in cytotoxicity tests.

Real-time RT-PCR experiments showed a drastic decrease in the expression level of the hTERT subunit of telomerase by 0.1  $\mu\text{mol/L}$  chelidone. Again higher concentrations of chelidone (even up to 50  $\mu\text{mol/L}$ ) showed only slight increments of the effect (Figure 5).

Long-term treatment of HepG2 cells with 0.1  $\mu\text{mol/L}$  chelidone for 48 h in every passage showed effective growth inhibition at very low concentrations of chelidone. Figure 6 illustrates the mean number of cell population doubling in relation to different modes of treatments. The growth rate of cells that had been treated four times with 0.1  $\mu\text{mol/L}$  chelidone for 48 h per passage (re-treated 3 in Figure 6) showed a decline after 12 d and the growth rate tended to reach a plateau by 4 wk

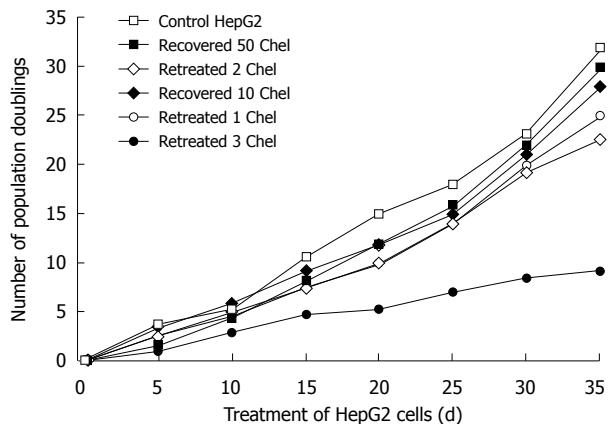


**Figure 4** Telomerase activity using TRAP assay in HepG2 control and 48 h treated cells with different concentration of chelidone up to  $\text{LD}_{50}$ . A: The relative activity of treated cells against untreated control cells are indicated as mean  $\pm$  SD; B: TRAP products resolved on non-denaturing PAGE and stained with SYBR Green. The concentration of chelidone is indicated on top of each well in  $\mu\text{mol/L}$ . C+: Positive control; C-: Negative control.

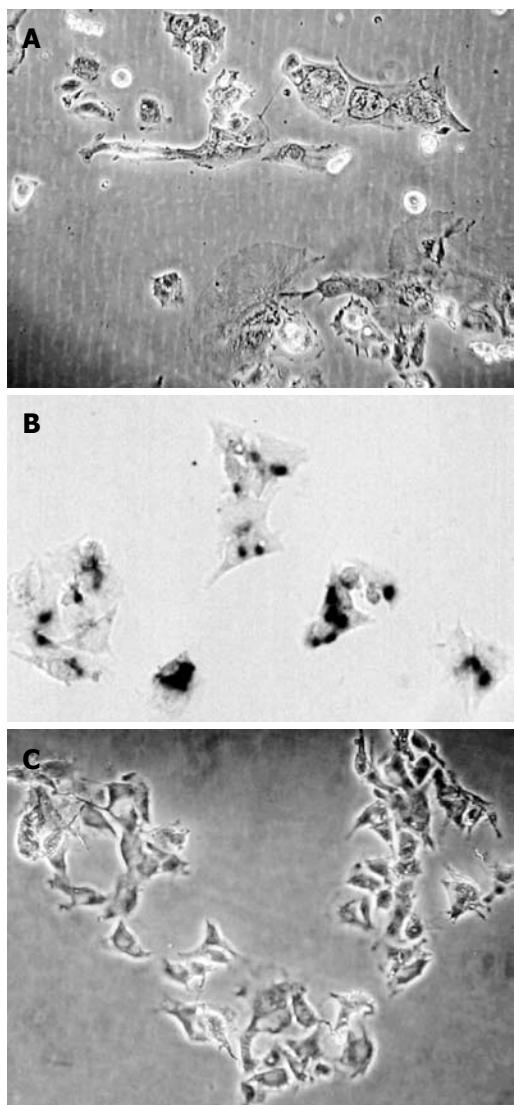


**Figure 5** HepG2 cells were treated for 48 h with medium supplemented with solvent (ethanol, not exceeding 0.1%) or 0.1, 10 and 50  $\mu\text{mol/L}$  chelidone. Relative hTERT gene expression as measured by real-time RT-PCR which was calibrated with the constitutive expression of  $\beta 2$ -microglobulin gene. hTERT level in control samples was considered as 100%. The values indicated as mean  $\pm$  SD ( $n \geq 6$ ).

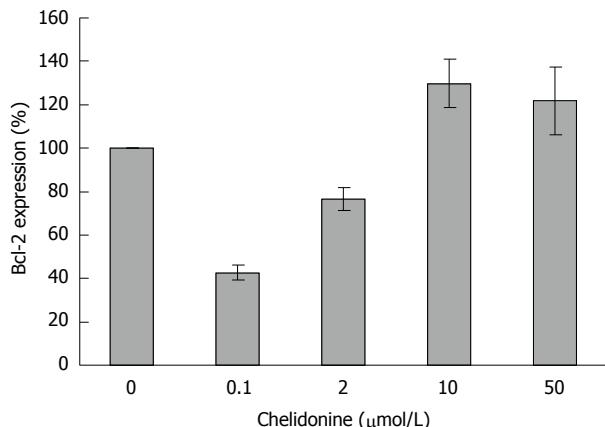
in comparison with untreated controls. Morphologically, after 28 d many of the cells appeared to be aged with a large cell volume and high cytoplasmic to nuclear ratio, which could be stained in  $\beta$ -galactosidase assay (Figure 7).



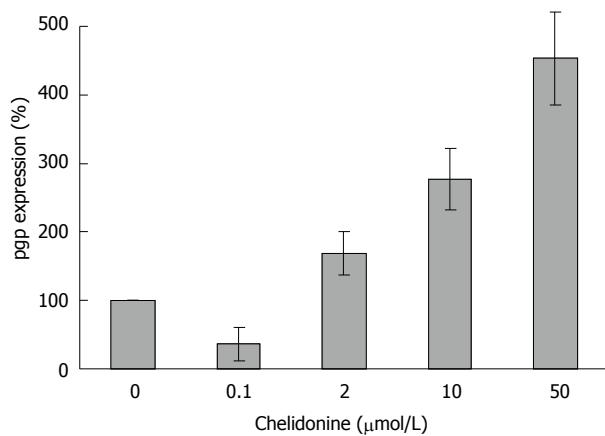
**Figure 6** Number of population doublings after long-term treatment with a sub-apoptotic dose of chelidone (0.1  $\mu\text{mol/L}$ ) or recovered cells after 48 h exposure to LD<sub>50</sub> concentration of chelidone in comparison with control HepG2 cells. In re-treatment experiments (long-term treatment) HepG2 cells were exposed to 0.1  $\mu\text{mol/L}$  only 48 h in each passage and followed by recovery with normal medium devoid of chelidone. Each point indicates the mean value of a duplicated experiment.



**Figure 7** The morphological changes and induction of senescence in HepG2 cells in a long-term treatment experiment. A: HepG2 cells after treatment five times with 0.1  $\mu\text{mol/L}$  chelidone; B: Cells retreated five times stained for  $\beta$ -galactosidase activity; C: Stained control cells.



**Figure 8** Relative expression of *Bcl-2* gene in HepG2 cells after 48 h treatment with different concentrations of chelidone in relation to untreated control cells assessed by real-time RT-PCR and calibrated with  $\beta$ -microglobulin gene expression. The expression level of *Bcl-2* in control cells was considered as 100%. mean  $\pm$  SD are indicated ( $n = 3$ ).



**Figure 9** Relative expression level of P-glycoprotein in HepG2 cells 48 h after treatment with different concentrations of chelidone. Each bar indicates the mean  $\pm$  SD ( $n = 3$ ).

The long-term treated cells showed distinctive morphological features associated with senescence of aged human cells. In contrast to control cells, the treated cells became enlarged and vacuolated in the cytoplasm.

The relative expression level of *Bcl-2* in HepG2 treated cells as compared to controls dropped to less than 50% at 0.1  $\mu\text{mol/L}$  and subsequently increased to higher than 120% in LD<sub>50</sub> values of chelidone concentration (Figure 8).

Furthermore real-time RT-PCR data showed (Figure 9) that the expression of pgp which is involved in general drug resistance increased 3-fold in chelidone treated HepG2 cells in comparison with untreated control cells. However it is interesting that at 0.1  $\mu\text{mol/L}$  chelidone expression of pgp was reduced by 40%.

## DISCUSSION

Several potent natural products, mainly secondary plant metabolites, have so far been reported as anticancer agents. Among alkaloids, derivatives of Ukrainian are used in chemotherapy<sup>[31]</sup>. An inhibition of telomerase by

berberine, an alkaloid related to chelidone, has already been reported<sup>[32]</sup>. Here for chelidone, the main compound of *Ukrain*, the mechanism of action in growth inhibition and its potential effect on telomerase inhibition and senescence induction has been analysed. Cytotoxicity of chelidone in HepG2 cells is not dose-dependent within the range examined and tends to reach a plateau immediately after reduction of live cells to a point slightly higher than 50%. Therefore several cytotoxicity methods were tested to gain the best curve. The best results were attained from the neutral red uptake assay. Even with six repeats each in triplicate, it is hard to accept 12 μmol/L chelidone as the LD<sub>50</sub> in this cell line as cell viability does not change considerably above this concentration. However, this dose was considered to be LD<sub>50</sub>, where the maximal attainable effects of chelidone were realized. The negligible increment of cytotoxicity of chelidone at higher doses could be due to stimulation of a drug resistance mechanism in this cell line. Therefore pgp as a protein involved in the general mechanism of drug resistance was analyzed. Up-regulation of the gene tested using quantitative real-time RT-PCR in comparison with the β2-microglobulin gene was seen at 2, 10 and 50 μmol/L after 48 h treatment. Enhancement of the expression of pgp looks to be dose-dependent at concentrations above 2 μmol/L. This cell response could be important in dose adjustment of the alkaloid in cancer therapy to avoid chemoresistance as well as side effects.

Chelidone showed a very strong inhibitory effect on telomerase activity in HepG2 cells after 48 h incubation. Several mechanisms need to be discussed. Some of them could include alteration of transcription, interaction with the telomerase subunits and probably interaction with telomere structure or telomere binding proteins. The DNA binding capacity (intercalation) of chelidone was already shown to be negligible<sup>[33]</sup>. Here, we focused on the expression of hTERT as the most effective component of telomerase in activity and gene regulation. As changed patterns in expression level of hTERT are very similar to TRAP assay results, it seems that chelidone modulates telomerase activity, at least partially, by down-regulation of the transcription of its catalytic subunit gene. Surprisingly expression of P-glycoprotein in 0.1 μmol/L chelidone treated cells was reduced by 40%, indicating that it may sensitize cells to chelidone toxicity, while higher concentrations close to LD<sub>50</sub> boost the drug resistance response. This could be a possible explanation of the plateau shape of the cell viability curve attained in cytotoxicity tests as well. A very sharp reduction of the survived cell number in very low concentration of chelidone is accompanied with a lower level of pgp whereas increased concentrations of chelidone probably enforce drug resistance through up regulation of pgp and reduce cell death via apoptosis.

Obvious morphological changes toward cell death appeared after 24 h treatment with chelidone by at least two different modalities of cell death; apoptosis and blister cell death. In this cell line the classical morphology of apoptosis manifests at 1 μmol/L chelidone

while single blister formation or blister cell death is also visible in a smaller number of cells. These data are in good concordance with several reports concerning the involvement of programmed cell death *via* the mitochondrial pathway induced by *C. majus* and its alkaloids in cancer cells<sup>[34]</sup>.

It seems that primary down regulation of the anti-apoptotic gene Bcl-2 in the cells treated by a lower concentration of chelidone (almost 40% in comparison with untreated controls) support apoptosis, while its return to normal expression level and even higher (about 120% in comparison with control) in LD<sub>50</sub> concentration restrains apoptosis. This could in part explain the stronger toxicity of chelidone at relatively lower concentrations.

Our data demonstrate apoptotic cell death in very low concentrations of chelidone (1 μmol/L) and down-regulation of the hTERT subunit of telomerase in sub-apoptotic concentrations (0.1 μmol/L). Both of these effects seem to be in much lower concentrations of mitotic arrest of chelidone through inhibition of tubulin polymerization, which has a LD<sub>50</sub> of 24 μmol/L in different cells including HeLa<sup>[9]</sup>. It is also noteworthy that the antimitotic effect of low doses of alkaloids of *C. majus* is reversible but the effect at higher doses is not<sup>[35]</sup>.

On the other hand long-term treatment of HepG2 cells with sub-apoptotic concentrations of chelidone is also accompanied by induction of senescence which was confirmed by β-galactosidase activity, a commonly used biomarker in such studies. The cells treated repeatedly with 0.1 μmol/L showed a cell growth plateau, which appeared at day 28 in comparison with control untreated cells that corresponded to the failure of replating the treated cells. At this time, growth of treated cells shows an almost 60% decline in population doubling without affecting cell viability, suggesting an increase in cell cycle duration. Few apoptotic cells are visible in long-term treatments with 0.1 μmol/L chelidone, whereas aged cells according to the β-galactosidase test are remarkably increased. In long-term treatment there are signs of differentiation visible in viable cells as well; some features of cell shape similar to fibroblasts, including long extensions attached to the substratum and a flat relatively large cytoplasm.

In conclusion, chelidone shows a promising effect on proliferation inhibition of cancer cells, compatible with a recent suggestion of the *Chelidonium majus* extract as an anti-tumor and hepatoprotective agent against induced hepatocarcinoma in mice<sup>[3]</sup>. Our data showed that chelidone inhibits telomerase in tumor cells strongly and may provide a basis for the development of more specific anticancer agents. Although possible selectivity effects of chelidone on cancer cells needs further investigation, in a recent study on primary mouse spleen cells and mouse lymphocytic leukemia cells, L1210, chelidone completely arrested L1210 cells with minimal cytotoxicity<sup>[36]</sup>.

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## COMMENTS

### Background

Telomerase, the key enzyme in most cancer cells for overcoming the proliferation limitation, is a promising target for chemotherapy. Among other strategies including PNA, antisense, ribozymes and RNA interference, chemotherapy using natural bio-products could be helpful in inhibition/down-regulation of telomerase. Several other plant secondary metabolites and their derivatives have been introduced in this proposal. *Chelidonium majus* (*C. majus*), which has been used as anti-tumor agent, in traditional medicine has been considered here and the main alkaloid of this plant was investigated for telomerase inhibitory potencies.

### Research frontiers

*C. majus* and its derivative Ukrain have been used for cancer therapy, but little is known about the mechanism of action. Whether it has telomerase inhibitory effects is the main subject of this research. This is the first published data concerned with alteration of telomerase activity in cancer cells after treatment with chelidoneine. Senescence induction is another aspect of chelidoneine effects on the cancer cell culture model.

### Innovations and breakthroughs

The molecular mechanism of chelidoneine's anti-proliferative effect on cancer cells was investigated by measuring telomerase activity and the transcription level of the catalytic subunit of the enzyme. Meanwhile population doubling and senescence induction was under consideration. All the data and viewpoints are original and never been published elsewhere.

### Applications

Chelidoneine shows strong inhibitory effects on telomerase activity and induces senescence in a cell culture model, so that it could be a promising agent in development of effective anticancer agents with higher specificities.

### Terminology

Telomerase: A special kind of reverse transcriptase which extends telomeres in chromosomes using its own RNA components as the template. It is almost completely switched off in most normal somatic cells, while it is up-regulated in more than 90% of different cancers. Chelidoneine: A benzophenanthridine alkaloid of *Chelidonium majus* - a well known plant with anti-tumor effects in traditional medicine.

### Peer review

This article shows the effect of chelidoneine on telomerase, which has therapeutic potential against cancer. The research topic is interesting and identifies the molecular target of chelidoneine.

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ORIGINAL ARTICLES

# Phenotypic changes of human cells in human-rat liver during partial hepatectomy-induced regeneration

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during liver regeneration were examined by flow cytometry, *in situ* hybridization and immunohistochemistry.

**RESULTS:** ISH analysis demonstrated human Alu-positive cells in hepatic parenchyma and stroma of recipient liver. Functional human hepatocytes generated in this model potentially constituted human hepatic functional units with the presence of donor-derived human endothelial and biliary duct cells in host liver. Alpha fetoprotein (AFP)<sup>+</sup>, CD34<sup>+</sup> and CD45<sup>+</sup> cells were observed in the chimeric liver on day 10 after PHx-induced liver regeneration and then disappeared in PHx group, but not in non-PHx group, suggesting that dynamic phenotypic changes of human cells expressing AFP, CD34 and CD45 cells may occur during the chimeric liver regeneration. Additionally, immunostaining for human proliferating cell nuclear antigen (PCNA) showed that the number of PCNA-positive cells in the chimeric liver of PHx group was markedly increased, as compared to that of control group, indicating that donor-derived human cells are actively proliferated during PHx-induced regeneration of HRC liver.

**CONCLUSION:** HRC liver provides a tool for investigating human liver regeneration in a humanized animal model.

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**Key words:** Human-rat chimera; Humanized liver; Human hepatocyte-like cells; Humanized hepatic functional unit; Partial hepatectomy model; Liver regeneration

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## Abstract

**AIM:** To examine the human hepatic parenchymal and stromal components in rat liver and the phenotypic changes of human cells in liver of human-rat chimera (HRC) generated by *in utero* transplantation of human cells during partial hepatectomy (PHx)-induced liver regeneration.

**METHODS:** Human hepatic parenchymal and stromal components and phenotypic changes of human cells

## INTRODUCTION

The complexity of a biologic network can only be

reproduced using an *in vivo* system, and complex biological and pathological processes often require an *in vivo* analysis. However, biomedical researches in humans are largely performed in *in vitro* models lacking of the components and complexity of a living organism because of scientific, technical and ethical considerations. Since there is a certain level of similarity between animals and humans, various laboratory animals including small (e.g. mice and rats) and large animals (e.g. pigs, dogs and non-human primates) are instrumental in increasing the understanding of human biology and disease. However, laboratory animals cannot fully replicate human physiology and disease because animal models are enormously limited by the practical considerations, physiological and genetic diversity, *etc.*

Since these findings derived from mice and *in vitro* human models cannot always be extrapolated to precisely reflect the true situations in humans, a preclinically and/or clinically relevant human-animal chimera (HAC) carrying various humanized organs, such as liver, brain, heart, kidney, *etc.*, constituted by a wide variety of transplanted donor-derived human cells with different cell phenotypes engrafted into the recipient organs, has been developed by performing *in utero* transplantation or blastocyst transplantation of various human stem cells (hSCs) during the preimmune development stage, which can imitate the *in vivo* situations in humans, thus greatly facilitating related researches based on HAC harboring humanized organs within the xenogeneic competitive settings<sup>[1-24]</sup>.

*In utero* transplantation of hSCs, such as human hematopoietic stem cells and mesenchymal stem cells, into fetal sheep<sup>[2,6]</sup>, goats<sup>[24]</sup>, rats<sup>[19,20]</sup>, and mice<sup>[18,22]</sup> or blastocyst transplantation of hSCs into mice<sup>[22]</sup>, has led to the establishment of non-injury human-animal xenograft models carrying humanized liver, in which a significant number of functional donor-derived human mature hepatocyte-like cells (HLCs) stained positively for human albumin (Alb), alpha fetoprotein (AFP) and hepatocyte nuclear factor-4 can be found. Moreover, such a "HAC liver" can also produce and secrete human Alb, alanine aminotransferase (ALT), aspartic acid aminotransferase (AST) and alkaline phosphatase (ALP) in the circulation of host mice<sup>[18]</sup> and sheep<sup>[2,6]</sup> that have undergone transplantation.

Compared with the general laboratory animals including mice, rats, pigs, dogs, non-human primates, and immune-deficient mice (*in vivo* injury model) carrying humanized liver reconstructed with human hepatocytes<sup>[5,25-28]</sup> or hSCs<sup>[29,30]</sup>, such a HAC harboring humanized liver with a relatively large number of donor-derived human liver cells clustered to form functional human liver units in host animal liver is an *in vivo* non-injury human-animal xenograft animal model with normal physiological conditions, and will become an ideal *in vivo* system for studies of the mechanisms underlying human liver development, repair and regeneration; the pathogenesis of human liver-related diseases including viral hepatitis, liver cirrhosis, hepatocellular carcinoma (HCC), *etc.*; and drug discovery and preclinical assessment of ADME-Tox<sup>[17,19,20,25,31]</sup>.

From a scientific perspective, human-rat chimera (HRC) and human-mouse chimera (HMC) carrying humanized organs are the suitable models for mechanistic research. Thus, attention has been paid to the new generation of non-injury models, such as HRC and HMC (data not shown) carrying humanized liver, and their potential applications<sup>[19,20]</sup>.

Our previous data demonstrate that donor-derived human liver cells with different cellular phenotypes are formed in chimeric liver of some animals after *in utero* transplantation of hSCs, including human liver cells stained positively for CD34 (markers for hematopoietic stem/progenitor cells and oval cells), CD45 (markers for oval cells and nucleated cells of hematopoietic lineage), AFP (embryonic hepatocyte marker), CK8 and CK18 (hepatocyte markers), CK19 (markers for cholangiocyte and bile duct cells), and Alb (hepatocyte marker), suggesting that donor-derived human hepatocyte and cholangiocyte lineages exist in host liver<sup>[19,20]</sup>. Moreover, human hepatic cell differentiation in rat liver appears to partially follow the process of hepatic ontogeny<sup>[20]</sup>.

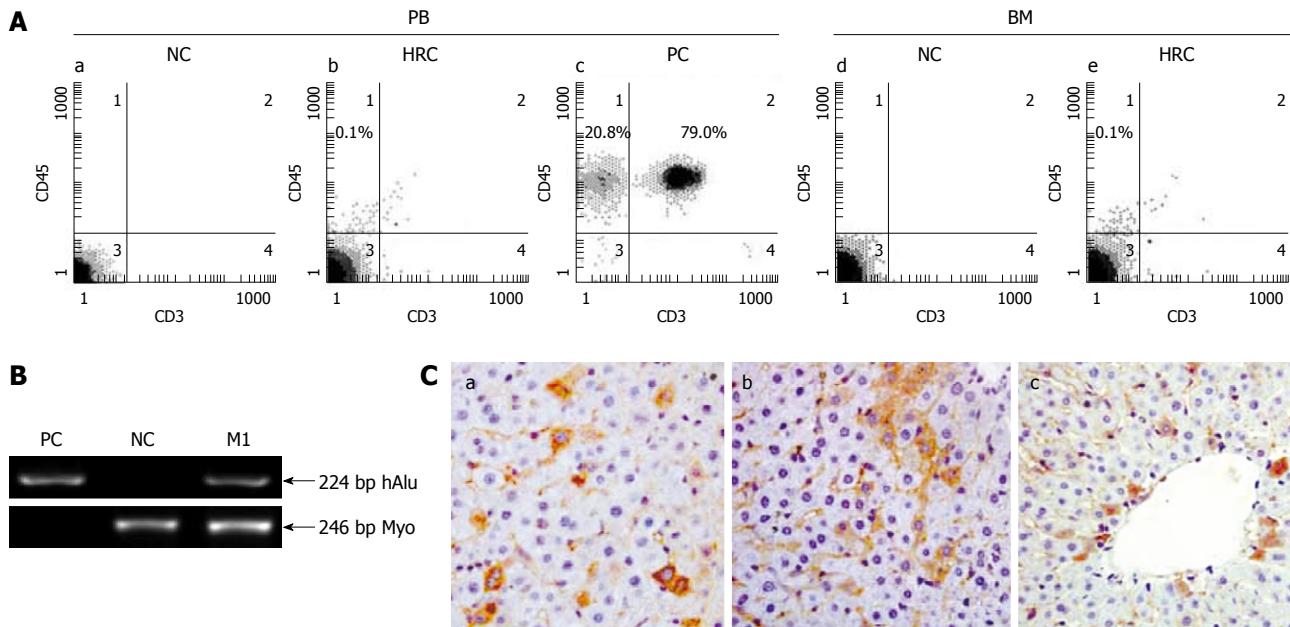
Furthermore, donor-derived functional human mature HLCs in parenchyma of human-sheep chimeric liver constitute the "humanized hepatic functional units" with the presence of donor-derived human hepatic stromal cells (endothelial and biliary duct cells) integrated into the chimeric liver stroma<sup>[2]</sup>, which remains to be confirmed in HRC liver<sup>[19,20]</sup>.

Based on the HRC carrying newly developed humanized liver after *in utero* transplantation of hSCs<sup>[19,20]</sup>, this study was to further identify the donor-derived human stromal components of HRC liver by *in situ* hybridization (ISH) for detecting donor-derived human cells in HRC liver, and to examine the changes in cellular phenotypes of donor-derived human cells during partial hepatectomy (PHx)-induced liver regeneration in the xenogeneic competitive environment, which helps to establish a solid foundation for the further research and potential applications based on such a "humanized liver".

## MATERIALS AND METHODS

### HRC generation

Human-rat hybrid animals were produced by *in utero* transplanting low-density mononuclear cells (MNCs) from human umbilical cord blood (hUCB) into fetal rats at the gestation of 9-11 d. The engraftment and long-term survival of donor-derived human cells in the HRC liver were determined by human gene-specific PCR (Figure 1B) for human Alu repetitive sequence (hAlu) on genomic DNA prepared from the liver of MNC-transplanted rats (MTRs) which were positive for human CD45 cells in peripheral blood (PB). PCR-positive organs were subjected to ISH for human Alu sequences and immunohistochemistry (IHC) using antibodies specific for human β2-microglobulin (β2-M), CK18 and CK19. Chimerism was accepted if the liver samples were positive for β2-M, CK18, CK19 and ISH. Protocols for producing and identifying HRC have been described elsewhere<sup>[19]</sup>.



**Figure 1** Screening for human-rat chimera (HRC) derived from *in utero* transplantation of hUCB-MNCs. Human-rat hybrid animals were produced by *in utero* transplantation of low-density mononuclear cells (MNCs) from human umbilical cord blood (hUCB) into fetal rats at the gestation of 9–11 d. A: Representative flow cytometric analysis of human CD45-positive cell engraftment in PB and BM of HRC. After birth, peripheral blood (PB) was collected at the indicated time points. The collected blood cells were stained with anti-human CD45 and CD3 antibodies and analyzed by flow cytometry. MNC-transplanted rat (MTR) was killed at end of each experiment, with bone marrow (BM) obtained and assessed for human CD45<sup>+</sup> cell engraftment by 2-color flow cytometry. Human PB from healthy volunteers was used as a positive control (PC) and normal rat PB was used as a negative control (NC); B: Identification of human genes in liver of MTR by human gene-specific PCR. To determine the human donor contribution in the liver of MTR, PCR was carried out on genomic DNA with primers specific for hAlu gene<sup>[19]</sup>. The PCR products were analyzed by agarose gel electrophoresis from positive-control human blood genomic DNA (lane PC), normal/non-transplanted rat genomic DNA (lane NC) and genomic DNA prepared from liver of one MTR was shown. Rat myogenin (Myo)-specific PCR was carried out for quality and quantity controls of genomic DNA with primers specific for rat Myo gene<sup>[19]</sup>. Data are representative of three independent PCRs yielding similar results. Arrow indicates the position of PCR products amplified by the primers for hAlu<sup>[19]</sup>. M1: Molecular weight DNA marker (DL2000, TaKaRa); C: Immunohistochemical analysis of various human antigens in liver of MTR. In the receipt liver, donor-derived human cells with different cellular phenotypes were detected by IHC for different human markers, such as β2-microglobulin [β2-M (a), CK18 (b) and CK19 (c)]. Brown staining shows positive human cells in various tissues from MTR and human samples (data not shown), whereas no positive human cells were found in normal control (NC) rats (data not shown).

All time points referred to the time length after birth. Animal care and experiment were performed according to the Ethical Guidelines for Animal Care, Handling and Termination established by the Subcommittee of Sun Yat-Sen University. The study was approved by the Ethics Review Committee of Sun Yat-Sen University. hUCB samples were obtained from normal full-term deliveries and informed consent was given by the participants.

#### ISH for detecting donor-derived human cells in HRC liver

After evaluation of the donor-derived human cell distribution in HRC liver by human gene-specific PCR on genomic DNA, human donor contribution in the formalin-fixed, paraffin-embedded liver tissues (from HRC) proved by PCR for hAlu gene prior to immunohistochemical assessment<sup>[19]</sup>, was further determined by ISH for human DNA Alu sequences. Digoxigenin (DIG)-labeled Alu probes (DNA probes) were obtained from PanPath B.V. (Amsterdam, Netherlands). Hybridization was performed according to the manufacturer's instructions. DIG-labeled hybrids were detected with an anti-DIG-alkaline phosphatase conjugate and a BCIP/NBT substrate giving a light-blue precipitate, and counterstained (pink) with nuclear fast red.

#### Experimental design

Seventy percent of PHx rat liver model provides an effective medium for study of the transition and regulation of hepatocytes from quiescent to proliferating phase. Ten selected rats, at the age of 2 mo, with CK18-positive and/or CK19-positive hHLC engraftment in the HRC liver were randomly divided into 70% PHx group (PHx group) and control group (non-PHx group) ( $n = 5$ ). Before (day 0) and after 10 and 20 d of PHx, minimal liver tissues were dissected from each rat of PHx group and control group, and used to detect donor-derived human cells stained positively for CK18, CK19, CD34, CD45 and AFP with IHC.

#### Immunohistochemical detection of donor-derived human cells

The minimal chimeric liver samples were immediately harvested from HRC containing donor-derived human cells in rat liver at the indicated time points after birth, fixed in 10% formalin, and paraffin embedded. The sections (4-μm) were heated in a 10 mmol/L Na-citrate buffer (pH 6.0) at 95°C for 20 min and cooled at room temperature for antigen detection. An envision system was used for immunohistochemical analysis. Human cells with different cell phenotypes in chimeric liver were detected

with antibodies to CD45, CD34, CK18, CK19 and AFP (DAKO), respectively. The complex was visualized with diaminobenzidinetetrahydrochloride (DAB) and counterstained with hematoxylin. Anti-human CD45, CK18 and AFP antibodies used as primary antibodies could specifically react with human CD45, CK18 and AFP antigens, respectively. Data from the Antibody Company indicate that anti-human CD34 and CK19 antibodies could cross-react very mildly with rat CD34 and CK19 antigens, respectively. However, our previous study strongly indicated that CD34 and CK19 antibodies do not cross-react with their corresponding antigens, CD34 and CK19, in IHC<sup>[20]</sup>. Since anti-human CD45, CK18, AFP, CD34 and CK19 antibodies have been confirmed to specifically react with human CD45, CK18, AFP, CD34 and CK19 antigens, but not with rat CD45, CK18, AFP, CD34 and CK19 antigens, thus MNC-derived human CD45<sup>+</sup>, CK18<sup>+</sup>, AFP<sup>+</sup>, CD34<sup>+</sup> and CK19<sup>+</sup> cells engrafted into rat liver can be easily distinguished from rat CD45<sup>+</sup>, CK18<sup>+</sup>, AFP<sup>+</sup>, CD34<sup>+</sup> and CK19<sup>+</sup> cells, respectively, by IHC performed on chimeric liver sections of HRC. For each staining, tissue sections were prepared from a normal rat as a negative control (NC). To identify donor-derived human cells in rat liver sections, we performed IHC on six different sections from each rat that underwent transplantation. The variability in percentage of donor-derived human cells was not significant between sections, but significant between animals which is consistent with the reported findings<sup>[19,20]</sup>.

#### IHC for proliferating cell nuclear antigen (PCNA)

To perform immunostaining for PCNA, liver tissue sections were autoclaved in a 10 mmol/L citrate buffer at 121°C for 5 min and cooled to room temperature. After washed in PBS with 0.05% Tween 20 (PBS-T), the sections were primarily incubated with anti-PCNA antibody (Sigma, St. Louis, MO, USA) overnight at 4°C. After washed three times in PBS-T, the sections were incubated with secondary HRP-conjugated anti-mouse antibody (Chemicon, Temecula, CA) for 1 h at room temperature following its manufacturer's instructions. The complex was visualized with DAB and counterstained with hematoxylin.

## RESULTS

#### **Human donor contributions in MTR liver analyzed by ISH for human Alu sequences**

Human donor contribution in recipient liver was determined by human Alu gene-specific PCR. The presence of donor-derived human cells in PCR-positive rat liver was confirmed by IHC using antibodies specific for human β2-M which is present in almost all cells of the body except for red blood cells<sup>[19]</sup> (Figure 1B and C). Moreover, 0.1%-10.7% of β2-M-positive human cells in the liver sections analyzed at the indicated time points were of human origin which is consistent with the reported data<sup>[20]</sup>.

Since human Alu (hAlu) repetitive sequence constitutes 10% of human genomes, the results of this study, obtained by PCR and IHC for β2-M, were further confirmed by ISH with a human Alu-specific probe

to detect donor-derived human cells containing Alu sequences in chimeric liver. ISH analysis indicated that a large number of human Alu-positive cells with black/dark brown nuclei were found in the hepatic parenchyma and stroma of recipients (Figure 2B-D), but not in normal control rats (NCR) (data not shown). Moreover, all human cells in human liver samples demonstrated black/dark brown nuclei (Figure 2A). These findings illustrate that there are donor-derived cells of human origin in the liver of host rats.

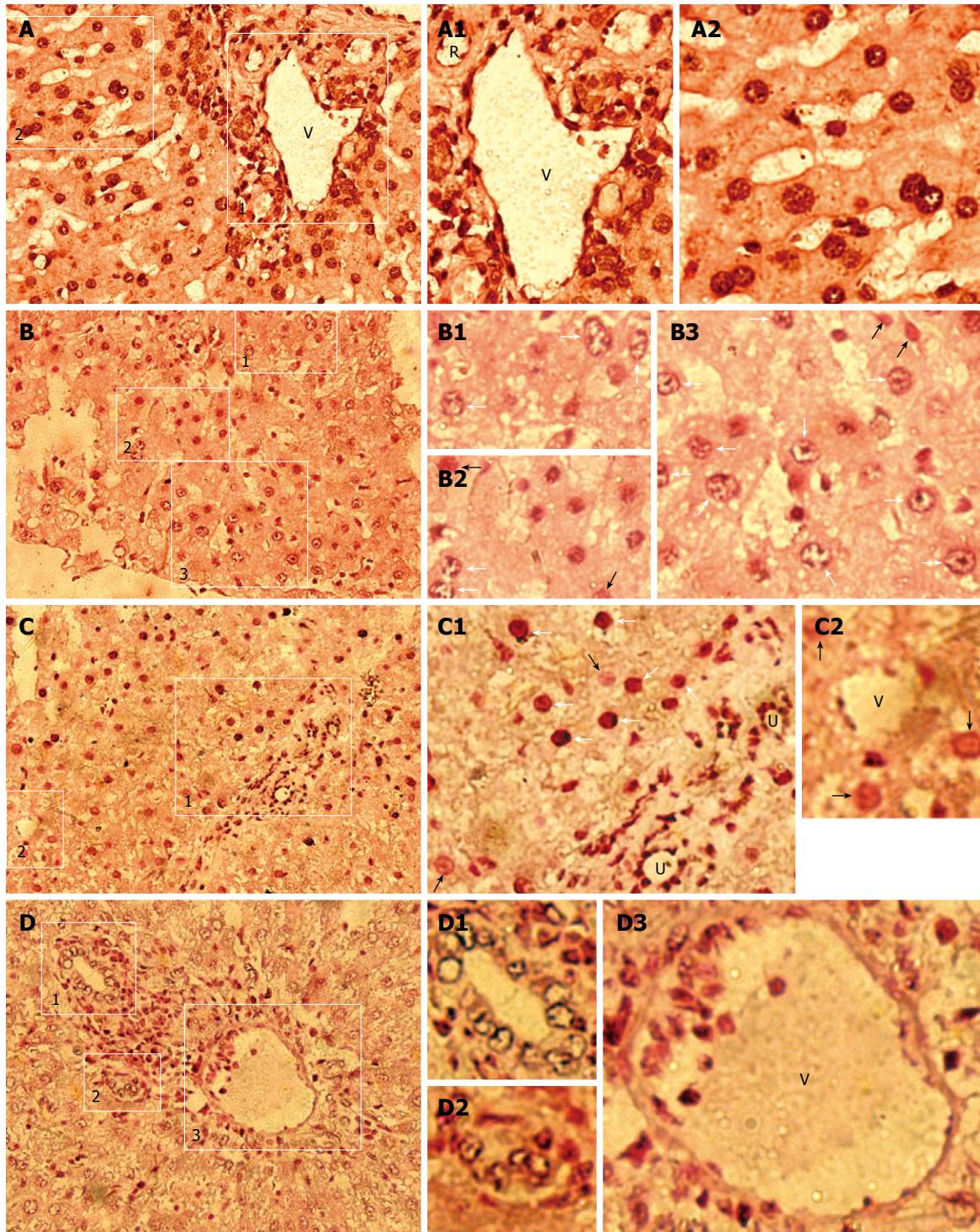
#### **Formation of humanized liver with donor-derived human hepatic parenchymal and stromal components in HRC**

Mammalian liver is composed of hepatic parenchymal components, such as stem/progenitor cells including oval cells, and cells expressing AFP, CK18, CK8, Alb, etc, in hepatocyte lineage, and hepatic stromal components including endothelial and biliary duct cells, etc<sup>[31]</sup>. Our previous data fully demonstrate that donor-derived human cells positive for CD34, CD45, AFP, CK8, CK18, CK19 and Alb are formed in the chimeric liver of HRC, suggesting that donor-derived human hepatic parenchymal components are formed in rat liver<sup>[19,20]</sup>.

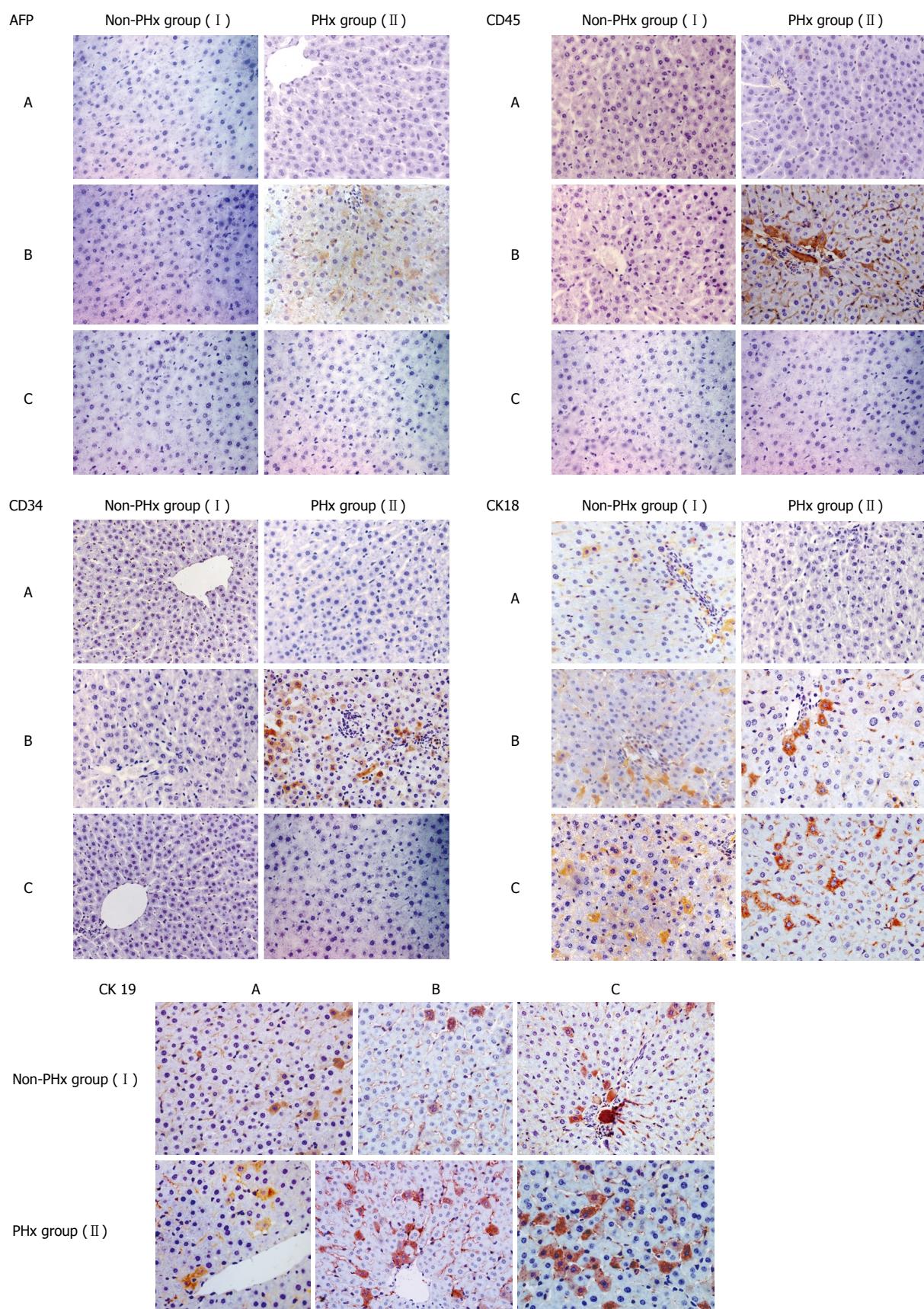
To further evaluate the *in vivo* differentiation potential of transplanted human cells into hepatic stromal cells, including endothelial and biliary duct cells, etc, in host liver, we performed ISH on liver sections from rats that underwent transplantation using a probe specific for the human Alu sequence. hAlu-positive vein (V) containing hAlu-positive human endothelial cell-like cells (hECLs), hAlu-positive artery (R) containing hAlu-positive human cells, hAlu-positive human hepatocytes, and other human cells, were found in the same liver section of human liver samples (Figure 2A). Moreover, no hAlu-positive human cells with black/dark brown nuclei were observed in the sections from control group (data not shown). ISH showed that a large number of cell nuclei in vein walls containing hAlu-positive hECLs (Figure 2C, C2 and D3) and biliary ducts containing hAlu-positive human bile duct epithelial cells (Figure 2C, C1, D1 and D2) were of human origin, indicating that donor-derived human hepatic stromal components (i.e. donor-derived human endothelial and biliary duct cells) are formed in rat liver. Furthermore, hAlu-positive vein (V) and bile duct (U) were observed in hepatic stroma and hAlu-positive human cells in hepatic parenchyma of host rats (Figure 2C and D), suggesting that humanized liver with donor-derived human hepatic parenchymal and stromal components is formed in xenogeneic competitive settings.

#### **Changes in cellular phenotypes of donor-derived human cells during PHx-induced liver regeneration in HRC**

The well-characterized PHx model of rat liver regeneration was used to explore the evolution rules in cellular phenotypes of donor-derived human cells participating in PHx-induced chimeric liver regeneration, and IHC was used to detect the expression of cell markers, such as AFP, CD34, CD45, CK18 and CK19. IHC analysis showed phenotypic changes of donor-derived



**Figure 2 Human donor contributions in HRC liver analyzed by *in situ* hybridization (ISH) for human Alu sequences.** Black/dark brown Alu-positive human cells in nuclei (white arrow) were identified in receipt liver using a probe specific for human DNA Alu sequences, and counterstained (pink) with nuclear fast red. Black/dark brown hybridization signals (white arrow) shows Alu-positive human cells in liver of MTR and human samples, whereas no Alu-positive human cells were found in normal control (NC) rats (data not shown). White arrow and black arrow indicate the partially demonstrated Alu-positive and negative human cells in B1-B3, C1 and C2, respectively. A: Human liver (positive control); B-D: Detection of donor-derived human cells in the chimeric liver of HRC. R: Artery; U: Bile duct; V: Vein. A: Low-power magnification of hAlu-positive human cells; A1: High-power magnification of the region (1) in the right rectangle of (A) demonstrating hAlu-positive vein (V) containing hAlu-positive human endothelial cell-like cells (hECLs), hAlu-positive artery (R) containing hAlu-positive human cells, and hAlu-positive human hepatocytes and other human cells in human liver; A2: High-power magnification of the region (2) in the left rectangle of (A), hAlu-positive human hepatocytes and other human cells in human liver; B: Low-power magnification of hAlu-positive human cells; B1: High-power magnification of hAlu-positive human cells (white arrow) of the rectangle region (1) in (B); B2: High-power magnification of hAlu-positive human cells (white arrow) and hAlu-negative cells (white arrowhead) of the rectangle region (2) in (B); B3: High-power magnification of hAlu-positive human cells (white arrow) and hAlu-negative cells (white arrowhead) of the rectangle region (3) in (B); C: Low-power magnification of hAlu-positive human cells; C1: High-power magnification of the rectangle region (1) in (C) showing hAlu-positive human hepatocyte-like cells (hHLCs) (white arrow), hAlu-positive bile duct (U) epithelial cells and other human cells (white arrow) in chimeric liver; C2: High-power magnification of the rectangle region (2) in (C) showing hAlu-positive hECLs in vein (V) of chimeric liver; D: Low-power magnification of hAlu-positive small bile ducts (U) and hAlu-positive vein (V) in chimeric liver; D1, D2: High-power magnification of hAlu-positive small bile ducts (U) in (1, 2) of (D), respectively; D3: High-power magnification of vein (V) containing hAlu-positive hECLs in (3) of (D).



**Figure 3 Representative immunohistochemistry analysis of phenotypic changes of donor-derived human cells in HRC liver during PHx-induced liver regeneration.** Seventy percent partial hepatectomy (PHx), a procedure that removes 70% of the liver, is regarded as the preferred *in vivo* method to study liver growth due to its synchronized growth response. In the representative chimeric livers of animals 5 and 6 (Table 1), donor-derived human cells with different cellular phenotypes were detected by IHC for different human markers (AFP, CD34, CD45, CK18 and CK19). Minimal liver tissues used for IHC were collected before PHx, and after 10 and 20 d of PHx. Additionally, the untransplanted control group was also set up when we performed this experiment (data not shown). The results demonstrate that CD45<sup>+</sup>, CK18<sup>+</sup>, AFP<sup>+</sup>, CD34<sup>+</sup> and CK19<sup>+</sup> cells could not be detected in liver sections from untransplanted control rats by human-specific IHC, suggesting that human-specific IHC can show human specificity of staining (data not shown). PHx: 70% partial hepatectomy; I : Non-PHx group; II : PHx group. A: before PHx; B: 10 d after PHx; C: 20 d after PHx.

**Table 1** Phenotypic changes of donor-derived human cells in HRC liver during PHx-induced liver regeneration

Animal No. (age)	Cell markers									
	AFP		CK19		CK18		CD34		CD45	
	I	II	I	II	I	II	I	II	I	II
1 and 2 (2 mo)	A	-	-	+	-	-	+	-	-	-
	B	-	+	+	-	-	+	-	+	-
	C	-	-	+	-	-	+	-	-	-
3 and 4 (2 mo)	A	-	-	+	+	+	-	+	-	-
	B	-	-	+	+	+	-	+	+	-
	C	-	-	+	+	+	+	-	-	+
5 and 6 (2 mo)	A	-	-	+	+	+	-	-	-	-
	B	-	+	+	+	+	+	-	+	-
	C	-	-	+	+	+	+	-	-	-
7 and 8 (2 mo)	A	-	-	-	+	+	+	-	-	-
	B	-	-	-	+	+	+	-	-	-
	C	-	-	-	+	+	+	-	-	-
9 and 10 (2 mo)	A	-	-	-	-	+	+	-	-	-
	B	-	-	-	-	+	+	-	-	+
	C	-	-	-	+	+	+	-	-	-

Animals 1-10 at the age of 2 mo with the engraftment of donor-derived hHLCs in the liver of HRC were screened by human gene-specific PCR for hAlu as previously described<sup>[19,20]</sup> and ISH for human Alu sequences confirmed by detecting human  $\beta$ 2-M expression using IHC. Animals 2, 4, 6, 8 and 10 at the age of 2 mo underwent 70% PHx. Age-matched animals 1, 3, 5, 7 and 9 (not undergone PHx), were used as negative controls. In the human-rat chimeric liver, donor-derived human cells with different cellular phenotypes were detected by IHC for different markers (AFP, CD34, CD45, CK18 and CK19). Minimal liver tissues from PHx (II) and non-PHx (I) groups used for IHC were dissected and collected before PHx (A), and after 10 d (B) and 20 d (C) of PHx.

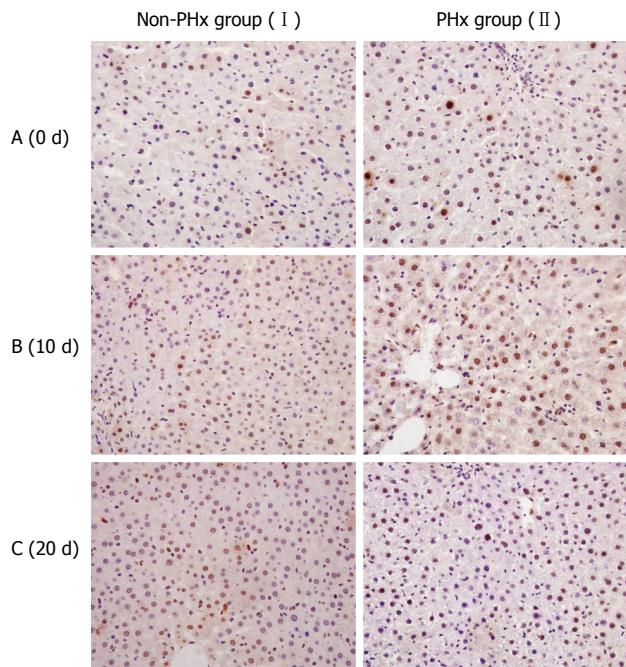
human cells during PHx-induced liver regeneration in HRC (Figure 3). The changes in the cellular phenotypes of donor-derived human cells during PHx-induced liver regeneration in five HRCs after PHx are summarized in Table 1. Donor-derived AFP<sup>+</sup>, CD34<sup>+</sup> and CD45<sup>+</sup> human cells in the portal area were found in the chimeric liver of HRC after 10 d of PHx in PHx group but not in control group (Figure 3 and Table 1), suggesting that dynamic changes of donor-derived human cells expressing AFP, CD34 and CD45 may occur during liver regeneration.

#### Proliferative activity of hHLCs during PHx-induced liver regeneration in HRC

Since PCNA is present specifically in nuclei of proliferating cells, it was employed to indicate the active proliferation of donor-derived human cells during human-rat chimeric liver regeneration in this study. Immunostaining for human PCNA showed that the number of PCNA-positive cells in chimeric liver was significantly higher in PHx group than in control group after 10 d of PHx (Figure 4), indicating that donor-derived hHLCs are actively proliferated during PHx-induced HRC liver regeneration.

## DISCUSSION

Adult mammalian liver contains different cell types, such as stem/progenitor cells including oval cells, hepatocytes, bile duct epithelial cells, vascular endothelial cells, stellate



**Figure 4** Proliferative activities of hHLCs during PHx-induced liver regeneration in HRC. Immunostaining for PCNA, a marker for cell proliferation, was performed to evaluate the proliferation of hHLCs. An antibody against PCNA antigen was used to evaluate the percentage of hepatocytes in the regenerative process after 70% hepatectomy. Ten days after PHx, PCNA staining demonstrated the active proliferation of hHLCs in the PHx group (II) compared with the control group (I).

cells, Kupffer cells (K), fibroblasts and leukocytes, etc, which constitute the functional units of liver<sup>[31]</sup>. Hepatocytes are responsible for most liver functions and account for 90% of liver weight.

Since *in vivo* study of human biology is severely limited by scientific, technical and ethical constraints, livers of small and large laboratory animals are routinely used to establish human liver biology and disease models, which, however, cannot fully replicate the complex biological and pathological processes of human liver. It is, therefore, necessary to develop a preclinically and/or clinically relevant HAC harboring a “humanized liver” with a relatively large number of different human liver and bile duct epithelial cells that cluster to form functional human liver units in host animals, which can imitate the *in vivo* situations in humans. Such a HAC harboring a “humanized liver” can be used in study of human liver development, repair and regeneration, the pathogenesis of human hepatitis virus infection, and human liver-specific metabolic responses to drugs, etc.

Transplantation of human hepatocytes into immuno-deficient mice can generate humanized mice carrying a humanized liver, and nearly 80%-90% of which can be replaced by transplanted human hepatocytes<sup>[25,27,32,33]</sup>. Furthermore, functional donor-derived human mature hepatocytes are positive for Alb, CK18 and CK8 in such a “humanized liver”. The presence of donor-derived human hepatic progenitor cells in liver parenchyma and donor-derived functional human bile canaliculi connected to mouse canaliculari can form donor-derived human

hepatic functional units in animal livers, while the plasma in humanized mice carrying a humanized liver contains human Alb and additional 21 human proteins<sup>[25,26,32,33]</sup>. However, the following significant drawbacks greatly limit the widespread use of such humanized mice derived from immunodeficient mice in study of human liver development, repair and regeneration, and pathogenesis of human hepatitis virus infection. On the one hand, transplanted foreign cells migrating into host liver from host spleen may not develop into all cell types in human liver required for normal liver function. On the other hand, immunodeficient mouse recipients have no normal immune system. In other words, after birth, this model is lack of a completely normal physiological environment, but the body immune system is involved in the pathogenesis of human hepatitis, and in control of human liver development, repair and regeneration.

Naturally occurring migration patterns of stem cells, availability of extending homing and engraftment sites, tissue- and organ-specific signals from niche in early embryo of animals greatly facilitate the widespread distribution of human donor cells in the recipient body, and promote them to home and engraft into various tissues and organs, in which human donor cells are actively influenced by the signals from niches to undergo reprogram, proliferation and differentiation in specific tissues and organs of recipients<sup>[19,20,34]</sup>. *In utero* or blastocyst transplantation of hSCs from multiple sources can form all possible donor-derived human cell types in the recipient liver, which in turn constitute human hepatic parenchymal and stromal components<sup>[2,6,18-20,22,24]</sup>. In contrast to the immunodeficient mice harboring a humanized liver, HAC in liver humanization possess and potential human immune system can be reconstituted with donor-derived human differentiated cells (data not shown). Besides the functional donor-derived human mature HLCs positive for human Alb, AFP, HNF-4, etc, throughout the host liver parenchyma, an “ideal human-animal chimeric liver” can also synthesize and secrete human Alb, ALT, AST and ALP into the circulation of host animals<sup>[2,6,18-20,22,24]</sup>. Our findings demonstrate that donor-derived human mature hepatocytes, endothelial and biliary duct cells in human-rat chimeric liver, constitute the “humanized hepatic functional units” in animal liver<sup>[2,19,20]</sup>. In addition, other donor-derived human cell types, such as liver stem/progenitor cells, stellate and Kupffer cells (K), fibroblasts and leukocytes in human liver, remain to be further identified in HRC liver.

It has been reported that after *in utero* transplantation of hMNCs, the transplanted human stem/progenitor cells can engraft into the recipient liver, and are actively influenced by niche signals to participate in organogenesis of recipients in the xenogeneic competitive settings<sup>[19,20]</sup>, indicating that HAC carrying humanized liver cells derived from *in utero* or blastocyst transplantation of hSCs is more superior to that in immunodeficient mice harboring humanized liver.

Since most of our present knowledge on liver regeneration is derived from laboratory mice and rats,

but not from human beings due to the lack of an ideal *in vivo* model<sup>[35]</sup>, HAC harboring a humanized liver will become an ideal *in vivo* system for investigating human liver regeneration and its mechanism.

Mature hepatocytes, liver stem cells or bone marrow (BM)-derived stem cells will be mobilized to participate in liver regeneration and repair, while various liver injury models, such as PHx, AAF/PHx, and AAF models, can induce different cell proliferative responses in liver<sup>[31]</sup>. In this study, the well-established 70% PHx model was used to induce acute chimeric liver injury and liver regeneration was induced with PHx. Donor-derived AFP<sup>+</sup>, CD34<sup>+</sup> and CD45<sup>+</sup> human cells were found in the chimeric liver on day 10 in PHx group but not in control group, suggesting that the donor-derived hSCs, engrafted into the BM of HAC, can be mobilized to migrate into the chimeric liver from the chimeric BM of HRC, thus participating in the regeneration and repair of injured chimeric liver during the acute liver damage. Additionally, the donor-derived human cells were engrafted in rat BM (Figure 1A). During chimeric liver regeneration, the donor-derived CD34<sup>+</sup> and CD45<sup>+</sup> human cells were detected in the portal area of chimeric liver, indicating that BM-derived hSCs participate in the regeneration of injured chimeric liver, which needs to be confirmed by further experiments. Generally, liver stem cells are not significantly proliferated after mild liver damage and BM-derived stem cells can directly enter liver from outside and subsequently develop into mature hepatic cells, but do not migrate into the portal area<sup>[31]</sup>. In this study, the markers of various donor-derived human cells in chimeric liver varied with time, demonstrating the changing process of surface markers of BM-derived hSCs, mature hepatocytes or liver stem cells during the regeneration of damaged chimeric liver. During liver regeneration after acute liver damage, the actively mobilized BM-derived hSCs in chimeric BM migrate into the chimeric liver, and further differentiate into hepatocytes to support the regeneration of injured chimeric liver, while donor-derived human mature hepatocytes and liver stem cells in chimeric liver participate in the regeneration and repair of injured chimeric liver<sup>[36]</sup>.

Anyway, HAC liver will become a powerful *in vivo* system for examining human-specific biological processes of damaged liver regeneration and repair.

Remaining constraints of such a “HAC” containing humanized liver cells include non-consistent engraftment of human cells in the same organ of different individuals, and inadequate human cells engrafted in HAC liver. For example, the percentage of donor-derived human cells in the chimeric liver of HRC is 0.1%-10.7%<sup>[20]</sup>.

The countermeasures for the elimination of the above constraints include definite cell components of human stem/progenitor cells used for transplantation, standard manipulation of transplantation, the need for genetic modifications to further humanize the host strain<sup>[37]</sup>, and loss of host hepatocytes, etc.

In summary, humanized liver can show insights into

*in vivo* human liver biology, and provide an *in vivo* powerful system for more precisely replicating the complex biological and pathological processes of human liver, and further allow us to investigate human liver-specific biological processes and diseases. However, efforts should be made to develop the optimal and pragmatic humanized animal models meeting the growing needs for animal models to carry out *in vivo* studies of human cells, tissues and organs.

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## COMMENTS

### Background

Data demonstrate that donor-derived human hepatocyte-like cells with different cellular phenotypes and of functional human hepatocytes are formed in chimeric liver of human-rat chimera (HRC) after *in utero* transplantation of human cells. Formation of human hepatic parenchymal and stromal components in rat liver was further examined, and humanized liver harboring HRC was used to investigate the phenotypic changes of donor-derived human cells engrafted into rat liver during 70% partial hepatectomy (PHx)-induced liver regeneration.

### Research frontiers

HRC carrying humanized liver generated by *in utero* transplantation of human cells has been developed by the authors. HRC harboring human-rat chimeric liver was used to study the human liver-specific biological processes and human liver-specific diseases.

### Innovations and breakthroughs

Humanized liver was employed to investigate the process of human hepatic ontogeny and to examine the phenotypic changes of donor-derived human cells engrafted into rat liver during PHx-induced liver regeneration in this study.

### Applications

Compared with the general laboratory animals including mice, rats, pigs, dogs and non-human primates, and immune-deficient mice (*in vivo* injury model) carrying humanized liver reconstructed with human hepatocytes or stem cells, HRC carrying growing humanized liver with a relatively large number of donor-derived human liver cells clustering to form functional human liver units in host liver, is the *in vivo* non-injury human-animal xenograft animal model with normal physiological conditions, and will become an ideal and suitable *in vivo* system for studies of the mechanisms underlying human liver development, repair and regeneration, the pathogenesis of human liver-related diseases including viral hepatitis, liver cirrhosis, hepatocellular carcinoma, and drug discovery and drug preclinical assessment of ADME-Tox.

### Terminology

Humanized animals generated by *in utero* transplantation or blastocyst transplantation of various human stem cells were defined in this study as normal animals engrafted with human cells, tissues or organs with normal physiological conditions.

### Peer review

The manuscript describes the successful development of HRC carrying humanized liver generated by *in utero* transplantation of human cells. The findings of this study indicate that donor-derived functional human hepatocytes generated in this model constitute human hepatic functional units with donor-derived human endothelial and biliary duct cells in host liver. More importantly, HRC harboring human-rat chimeric liver was used to preliminarily examine the phenotypic changes of donor-derived human cells engrafted into rat liver during PHx-induced liver regeneration.

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ORIGINAL ARTICLES

# Interleukin-6, desmosome and tight junction protein expression levels in reflux esophagitis-affected mucosa

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## Abstract

**AIM:** To investigate the correlation between the expression levels of interleukin (IL)-6 and proteins in tight junctions (TJs) in the esophageal mucosa of rats modeling different types of reflux esophagitis (RE), and the ability of aluminum phosphate to protect against RE-induced mucosal damage *via* these proteins.

**METHODS:** Male SPF Wistar rats aged 56 d were divided randomly into acid RE, alkaline RE, mixed RE, and control groups. Various surgical procedures were performed to establish rat models of acid RE. At 14 d after the procedure, some of the rats started aluminum phosphate treatment. Transmission electron microscopy (TEM) was used to observe the morphological features of TJs and desmosomes in the esophageal epithelium. Immunohistochemical methods and Western blotting were used to measure expression of claudin 1, occludin, ZO-1, JAM-1, DSG-1 and IL-6; reverse transcription polymerase chain reaction (RT-PCR) was used to measure expression of mRNA of claudin 1, occludin, ZO-1, JAM-1, DSG-1 and IL-6.

**RESULTS:** At day 14 after the procedures, an RE model was established in all subsequently sacrificed rats of

groups A, B and C. By both gross and microscopic observation, the mucosa was damaged and thickened as the disease progressed. With TEM observation, a widened intercellular space was noticed, with significantly fewer desmosomes. Immunohistochemistry showed significantly higher levels of all proteins in all RE models compared to control rats at 3 d after operation ( $65.5\% \pm 25.6\%$  vs  $20.5\% \pm 2.1\%$ ,  $P < 0.05$ , respectively). At 14 d after operation, along with continuing hyperplasia in the basal layer, the expression of TJ proteins in individual cells gradually decreased ( $12.4\% \pm 2.1\%$  vs  $20.5\% \pm 2.1\%$ ,  $P < 0.05$ , respectively). Western blottings and RT-PCR showed a directly proportional increase in IL-6 levels in relation to TJ proteins, as compared to controls ( $0.878 \pm 0.024$  vs  $0.205 \pm 0.021$  and  $0.898 \pm 0.022$  vs  $0.205 \pm 0.021$ ,  $P < 0.05$ , respectively). Upon treatment with aluminum phosphate, however, these protein levels were restored to normal gradually over 30-60 d in rats with acid RE ( $30.4\% \pm 2.1\%$  vs  $20.5\% \pm 2.1\%$ ,  $P > 0.05$ , treated vs untreated, respectively). These levels increased in the rat with alkaline RE, and this increase was accompanied by continued hyperplasia in comparison with controls ( $85.5\% \pm 25.6\%$  vs  $20.5\% \pm 2.1\%$ ,  $P < 0.05$ , respectively). Furthermore, the expression of TJ proteins was not correlated significantly with that of IL-6 in this group.

**CONCLUSION:** These findings indicate that TJ proteins are highly expressed as an early molecular event involved in RE development, and that IL-6 is an inflammatory factor in this process.

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**Key words:** Reflux esophagitis; Desmosome; Tight junction; Proteins; Mucosa

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## INTRODUCTION

Gastro-esophageal reflux disease (GERD) is a common condition that has an impact on many millions of people worldwide, and is gaining more clinical recognition as a significant gastrointestinal disease. Lifestyle factors, such as obesity, high-fat diet, and physical inactivity, boost the incidence of GERD. GERD occurs when stomach and duodenal contents reflux into the esophagus. Reflux-induced damage and inflammation in the esophageal mucosa are referred to as reflux esophagitis (RE).

The pathogenic process of RE is highly complex. However, the current consensus among scientists is that RE mainly results from damage induced by reflux of the defending esophageal mucosa system. An imbalance between offensive and defensive factors in the esophagus leads to the development of RE. The epithelial defense system consists of three parts: pre-epithelial defense, epithelial defense and post-epithelial defense. In 2002, the German cellular biologist Langbein *et al*<sup>[1]</sup> reported that, in addition to desmosomes, tight junctions (TJs) could be found in the esophageal epithelium; and both were involved in maintaining the cellular barrier. Since then, TJs and desmosomes are considered to be important contributors to the epithelial defense of the esophageal mucosa. Other contributors include intercellular lipids, mucin and the epithelial transportation system (such as  $\text{Na}^+ \text{-H}^+$  exchanger and  $\text{Na}^+$ -dependent  $\text{Cl}^- \text{-HCO}_3^-$  exchanger). Recent studies showed that TJs created a circular substance-blocking barrier between adjacent cells. TJs also function in cell adhesion, cell polarity and permeability, and in signal transduction resulting in cell proliferation and differentiation. Intercellular TJs consist mainly of claudins, occludins, JAM and ZOs. In 2003, Calabrese *et al*<sup>[2]</sup> discovered in their studies that the intercellular space of the lower esophageal epithelium in patients with GERD was three times as wide as that in normal controls, and suggested 0.74  $\mu\text{m}$  as the threshold between normal and abnormal intercellular space. In 2004-2005, Asaoka *et al*<sup>[3]</sup> reported for the first time that expression of TJ proteins was altered during esophagitis; the expression level of claudin-3 decreased while that of claudin-1 increased<sup>[4]</sup>. Such changes have a determinant effect on the permeability of the esophageal epithelium. Therefore, further investigation is warranted using experiments testing the hypothesis that altered expression of TJ proteins and desmosome proteins results in failure of TJs and desmosomes, and thus leads to widened intercellular space and contributes to RE. In the investigation into RE, the use of human samples has methodological limitations. Hence, we created animal models of RE in rats, and sought to analyze the changes in intercellular space, changes in the distribution and expression of TJ proteins and desmosome proteins, and their correlation with the expression level of interleukin (IL)-6 in the pathogenic process of RE. Moreover, some rats were treated with aluminum phosphate to determine its efficacy in treating RE.

To explore the role of TJ and desmosome proteins in the pathogenesis of RE, we established three animal models of RE on the Wistar rat background. We performed three types of surgical procedures on these rats, and measured the distribution and expression profiles of claudin 1, occludin, ZO-1, JAM-1, DSG-1 and IL-6 in the esophageal mucosa of the various RE rats, untreated and treated with aluminum phosphate.

## MATERIALS AND METHODS

### Establishment of animal models

Two-hundred and twenty male SPF Wistar rats aged 56 d were purchased as laboratory animals at Shengjing Hospital of China Medical University, acclimatized and fed with standard chow and water *ad libitum* for 14 d in an animal room with controlled room temperature (24  $\pm$  2°C). Following the surgical procedures, the rats were fasted for 24 h and were given water *ad libitum*. Before surgery, the rats were divided into four experimental groups. The control group was comprised of 10 rats, while the experimental groups A, B and C, respectively modeling acid RE, alkaline RE and mixed RE, were comprised of 70 rats each. Prior to surgery, the rats were anesthetized with chloral hydrate (Qingdao Yulong Algae Co., Ltd, Shangdong, China) 0.3% mL/100 mg *via* abdominal injection. The control rats underwent laparotomy, modified from the methods described by Yu *et al*<sup>[5]</sup>, without any further surgical procedures<sup>[3]</sup>. After laparotomy, a pyloric stenosis was created by wrapping and ligating the pylorus with 1-0 silk thread around a metal tube (diameter: 2 mm) which was retrieved slowly after ligation. To enhance gastric reflux, a 0.5 cm longitudinal incision was made by cardiomyotomy in the esophageal-gastric junction. The incision was deep enough to expose the mucosa.

Group A rats underwent partial pyloric ligation (which is also known as incomplete pyloric ligation) and cardiomyotomy, while those in group B were treated with total gastrostomy plus end-to-side esophago-duodenal anastomosis. In the group B rats, the gastric artery and vein were separated and ligated, and then the cardia and pylorus were ligated and incised. The stomach was removed, and the cardia was then connected to the pylorus by end-to-side anastomosis<sup>[6]</sup>. The group C rats underwent cardiomyotomy and cardia ligation plus end-to-side esophago-duodenal anastomosis. To initiate surgery, the group C rats were placed in a supine position and an incision was made in the medium of upper abdomen. The lower esophagus was separated and incised and the vagus nerve was preserved. The cardia end of the stomach was disclosed with purse-string sutures, and the stomach was excluded. A 0.4-0.5 cm longitudinal incision was made on the duodenal wall at 1.0 cm from the pyloric end. The lower esophagus was connected to the duodenum with end-to-side anastomosis<sup>[7]</sup>.

On post-procedure days 3, 6, 9 and 14, subgroups of the rats were sacrificed and the middle-low esophagi

were dissected for evaluating the experimental success rates. The severity of the damage in esophagi was examined according to the diagnostic criteria of RE as determined by the Chinese Medical Association Digestive Endoscopy Society (1999).

One week following the procedure, 35 rats in group A, seven rats in group B and 21 rats in group C died. Five rats from groups A, B and C were sacrificed at post-procedure days 3, 6 and 9, respectively. At day 14, five rats in group A, five rats in the control group, 21 rats in group B and 13 rats in group C were sacrificed, and the middle-lower esophagus was obtained for calculating the success rate of the model.

**Aluminum phosphate treatment:** The remaining rats were fed 0.5 g aluminum phosphate (Boryung Pharmaceutical Co., Ltd. Fusan, Korea) in water, twice daily for 15 successive days. Five rats in group A, five rats in control group, nine rats in group B and seven rats in group C were sacrificed at post-treatment days 30 or 60. The survival, incidence of esophagitis, and average change in body mass in these model groups and in the control group were calculated.

#### **Gross and microscopic observations for evaluating the damage severity in the esophagus**

At post-procedure days 3, 6, 9 and 14, the lower esophagi were dissected for mucosal damage evaluation based on the Chinese Medical Association Digestive of Erosive Esophagitis (1999), and epithelial thickness was measured and evaluated as an indication of damage. Esophageal tissues were then fixed in 10% buffered-formalin, shaped into 4 mm tissue masses, dehydrated with gradient ethanol, clarified with xylene, embedded in paraffin and sectioned into 4  $\mu\text{m}$  slices, which were then stained with HE solution. The stained slices were then observed under the optical microscope, and a diagnosis was made based on the pathological features according to diagnostic criteria of RE by the Chinese Medical Association Digestive Endoscopic Society (1999), revealing the incidence of RE in these animal models<sup>[8]</sup>.

#### **Transmission electron microscopy (TEM) examination**

At days 30 or 60 following aluminum phosphate treatment initiation, the rats were sacrificed and a TEM (TEM; 2600 J EME2000X, Hitachi) was used to observe the morphological features of TJs and desmosomes in the esophageal epithelium. Samples were obtained from the lower esophagus at 5 cm from the lower end, fixed in glutaraldehyde solution at 4°C, and then clarified, dehydrated, embedded and sectioned into ultra-thin slices. Ten slices, prepared with samples obtained from different sites of the same esophagus, were selected for each esophagus and then observed under TEM. The LEICA image analyzing system was used to measure the intercellular space and calculate the number of desmosomes. Ten pictures were taken for each sample. In each picture, an intact cell was selected

and an intercellular space was calculated by detecting the vertical distance between the selected cell and its adjacent cells in 10 randomly selected directions, with each direction at least 1.0  $\mu\text{m}$  apart. 100 intercellular spaces were measured in 10 pictures and average width of intercellular space was calculated. We counted the number of desmosomes in the intercellular spaces of the 10 selected cells, and totaled and averaged these values<sup>[9]</sup>.

#### **Immunohistochemical assay**

Immunohistochemical methods were used to measure the expression levels of claudin 1, occludin, ZO-1, JAM-1, DSG-1 and IL-6. For these assays, the middle-lower esophagus was obtained and fixed in 10% buffered-formalin, shaped into 4.0 mm tissue masses, dehydrated with gradient ethanol, clarified with xylene, embedded in paraffin and sectioned into 4.0  $\mu\text{m}$  slices, which were then dewaxed with xylene, debenzolized with anhydrous ethanol and dehydrated with gradient ethanol, and then treated with 3.0% hydrogen peroxide and rinsed with water. After being restored at high pressure and high temperature, blocking agent (10% normal goat serum) was added to dissolve the slices at room temperature. Thirty minutes later, rabbit anti-IL-6, claudin 1, ZO-1, JAM-1 and DSG-1 antibodies were added at a dilution of 1:80, and goat anti-occludin antibody added at a 1:100 dilution at 4°C. The slices were then incubated overnight and washed with phosphate-buffered saline (PBS) 3  $\times$ , 10 min each, the next day. At room temperature, general secondary antibody was added, and the slices were incubated at 37°C for 30 min. The slices were washed with PBS 3  $\times$ , for 10 min each time. Streptavidin-peroxidase was added at 37°C. Thirty minutes later, the slices were washed again with 3  $\times$  PBS for 10 min each time. The slices were then stained with 3,3-Diaminobenzidine for 5 min, rinsed with water, and then stained with hematoxylin for another 5 min and rinsed with water again. The slices were then treated with acidic ethanol, and washed with running water for 30 min, dehydrated in gradient ethanol, and clarified with xylene, and sealed with gum. These slices of mucosa of RE were observed under the microscope for the measurement of expression of IL-6, claudin 1, occludin, ZO-1, JAM-1 and DSG-1.

#### **Western blotting**

Western blotting was used to measure the expression levels of claudin 1, occludin, ZO-1, JAM-1, DSG-1 and IL-6 (all from Santa Cruz Biotechnology, Santa Cruz, CA). For these assays, the esophageal tissue (100 mg) was obtained and added to 1.0 ml of cell lysis buffer [Tris/HCl (100 mmol/L, pH 7.5), NaCl (100 mmol/L), 0.5% sodium deoxycholate, ethylene diamine tetra-acetate (EDTA, 1 mmol/L), 1% NP40, 0.1% sodium dodecyl sulfate (SDS) and protease inhibitor]. Total protein was extracted, and the concentration was measured by the Lowry method. The extracted protein (50  $\mu\text{g}$ ) was added to 15% SDS-polyacrylamide gel for electrophoresis

**Table 1** Primer sequences for RT-PCR

Primers	Upstream primers	Downstream primers	Amplification
JAM-1	5'-ACTGCCGTCCAGGTT-3'	5'-TTCGCCACTATCAAAGG-3'	524 bp
Claudin-1	5'-GGATGCCCTGCCTTC-3'	5'-CACAGCCAAGACCCCTC-3'	674 bp
Occludin	5'-CCACTATGAAACCGACTA-3'	5'-CCAGCAACCAGCATC-3'	399 bp
Zo1	5'-CTCGGGCATTATTCTG-3'	5'-GCTTCCTGGCACTTT-3'	205bp
Dsg1	5'-CCTGCTGCTTGCTT-3'	5'-GGTTATTGGGCTCGTC-3'	538bp
IL-6	5'-CTCCATCTGCCCTCA-3'	5'-CCAGGATAGAGCCACCAAT-3'	586bp
β-actin	5'-GGTCCTTAGCCACTCC-3'	5'-CCAGGATAGAGCCACCAAT-3'	701bp

(100 V for 1.5 h). When the bromophenol blue reached the bottom of the gel, the protein was blotted onto a nitrocellulose membrane. The blots were immersed in 5.0% milk (prepared with milk powder) for 2 h. Then at 4°C, rabbit anti-IL-6, claudin 1, ZO-1, JAM-1 and DSG-1 antibodies at dilutions of 1:80, and goat anti-octoccludin antibody at dilutions of 1:100 were added to the blots, which were then incubated overnight. The next day, the membrane was washed, and the corresponding alkaline phosphate-labeled secondary antibodies were added at room temperature. The membrane was then stained with O-Dianisidine Tetrazotized and β-Naphthyl acid phosphate, and analyzed with an electrophoresis gel imaging system.

Using the grey scale measurement of the electrophoresis gel imaging system, the expression levels of IL-6, claudin 1, occludin, ZO-1, JAM-1 and DSG-1 were calculated and expressed as percentages relative to the β-actin control (absorbance of these proteins/absorbance of β-actin).

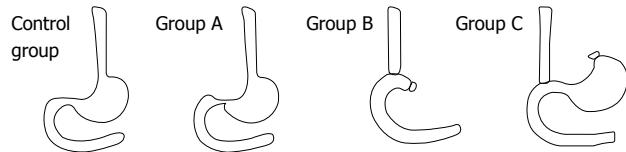
#### Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR was employed for cognate mRNA detection and quantitation. Total RNA was extracted with a Trizol Kit, (Invitrogen, Carlsbad, CA), measured, and verified with an ultra-violet spectrophotometer. Primers were designed by Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA) according to mRNA sequences (by GenBank) of IL-6, claudin 1, occludin, ZO-1, JAM-1, DSG-1 and β-actin (as control). The primer sequences are shown in Table 1. RT-PCR was performed using the Takara RNA LA PCR kit (Takara, Kyoto, Japan) according to the manufacturers' instructions. RT-PCR was catalyzed under the following temperatures: 5 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 59°C and 1 min at 72°C; finally 5 min at 72°C. 5 μg of the PCR product was added to 2 μL loading buffer, and electrophoresed in a 1.5% agarose gel at 100 V for 1.5 h. Analysis was carried out using a gel imaging system (Glyko, Novato, CA, USA).

#### Statistical analysis

Data were processed with a statistical package SPSS version 10.0 (SPSS Inc, USA).

The means between two groups were compared for differences using the student's *t*-test; comparison of the ratio between two samples was tested with the chi-square test. The data were reported as mean ± SD. A 95% confidence interval for the average weight of rats



**Figure 1** Procedure for the establishment of animal models in each group. Control: Without treatment; A: Acid RE; B: Alkaline RE; C: Mixed RE.

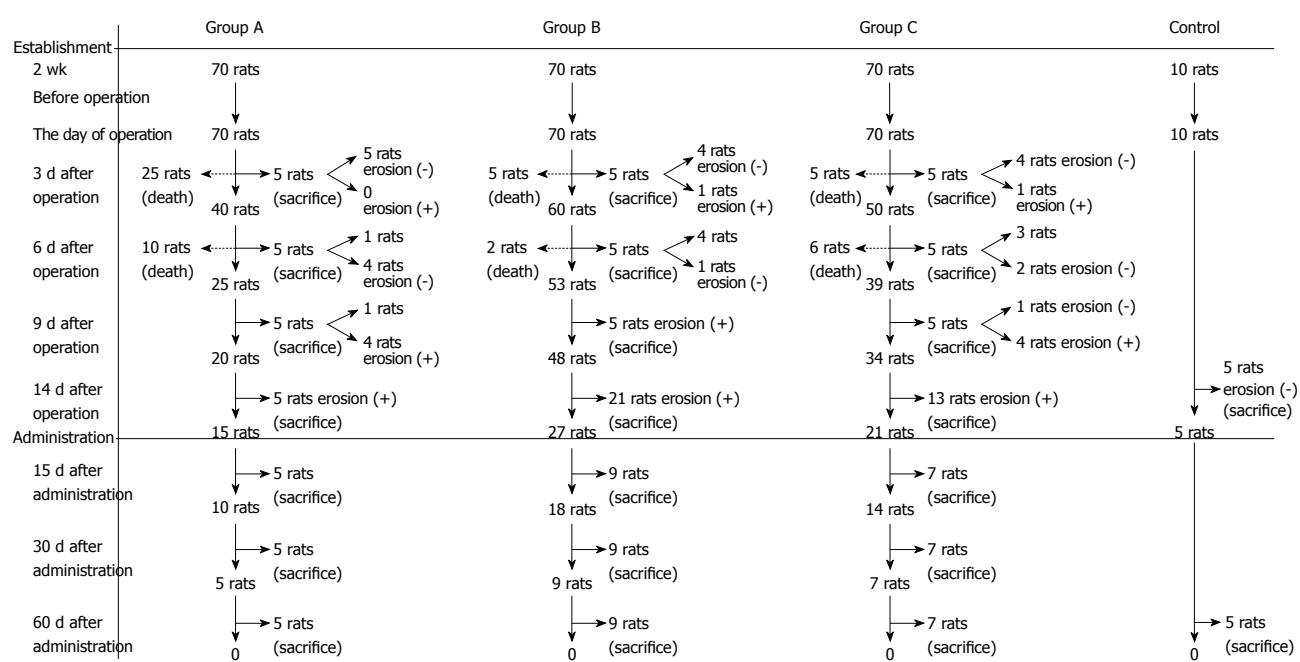
was calculated. In the analysis of correlation between expression of proteins, differences were considered significant at the  $P < 0.05$  level.

## RESULTS

At post-procedure day 14, the RE model was established in all group A-, B- and C-sacrificed rats. By gross and microscopic observation, we ascertained that the mucosa was damaged and thickened as the disease progressed. Figure 1 helps illustrate the establishment of the animal models with the three modified procedures. In the modified RE models, the survival of group A was 50% at day 14 after procedures ( $n = 35/70$ ); 90% ( $n = 63/70$ ) for group B, and 70% ( $n = 49/70$ ) for group C. At 14 d after the procedures, the RE models were established in all the rats ( $n = 39/39$ ). Survival and incidence of RE are shown in Figure 2. At days 3, 6 and 9 after the procedures, the average weight of rats in the model groups continuously decreased as compared to the control group. In aluminum phosphate-treated rats, the average weight improved as treatment progressed and was comparable to the control group at 1 mo afterward.

#### Gross observation and histological assessment

In RE models, erosions of various sizes could be seen in the mucosa, while no erosion was detected in the control group. In most cases, esophagitis occurred in the middle-low part of esophagus. Histologically, the normal esophagus revealed a thin epithelial layer with squamous cells (Figure 3A). In experimental RE models, the epithelium was markedly thickened and the lamina propria papillae were elongated into the epithelium. Basal cell hyperplasia and inflammatory cell infiltration were prominent in the lamina propria and the thickness of the esophageal epithelium increased with time in RE models (Figure 3B-E). In the control group, no expression of IL-6 was detected; claudin-1, occludin, ZO-1 and JAM-1 could be seen on the cellular



**Figure 2** Survival and incidence of RE. The modeling details are described. RE incidence rate was 100% 2 wk after treatment and rat survival rate was 50% ( $n = 35/70$ ) in group A, 90% ( $n = 63/70$ ) in group B and 70% ( $n = 49/70$ ) in group C.

membrane of the esophageal epithelial cells, mostly in the spinous and granular layers, while DSA-1 and JAM-1 could be seen on the cellular membrane of all mucosal layers. Amongst these proteins, DSG-1 had the highest expression level. In RE model rats, expression of IL-6 was only seen in inflammatory cells; the expression levels of claudin-1, occludin, ZO-1, JAM-1 and DSG-1 increased in both the cellular membrane and cytoplasm of spinous and granular layers in the mucosa around erosions, and these proteins were seen in cytoplasm as well as in membrane. However, as hyperplasia continued in the basal cells, expression of claudin-1, occludin, ZO-1, JAM-1 and DSG-1 decreased in individual cells of the spinous and granular layers (Figure 4A-C). One month after treatment started, the histological changes of RE disappeared in acid RE rats, with comparable expression levels of the proteins to the control group. Treatment was not effective for alkaline RE rats, in which hyperplasia and elevated expression of IL-6 continued and vesicles could be seen. But expression of these proteins was significantly lower in individual cells as compared to control group (Figure 5).

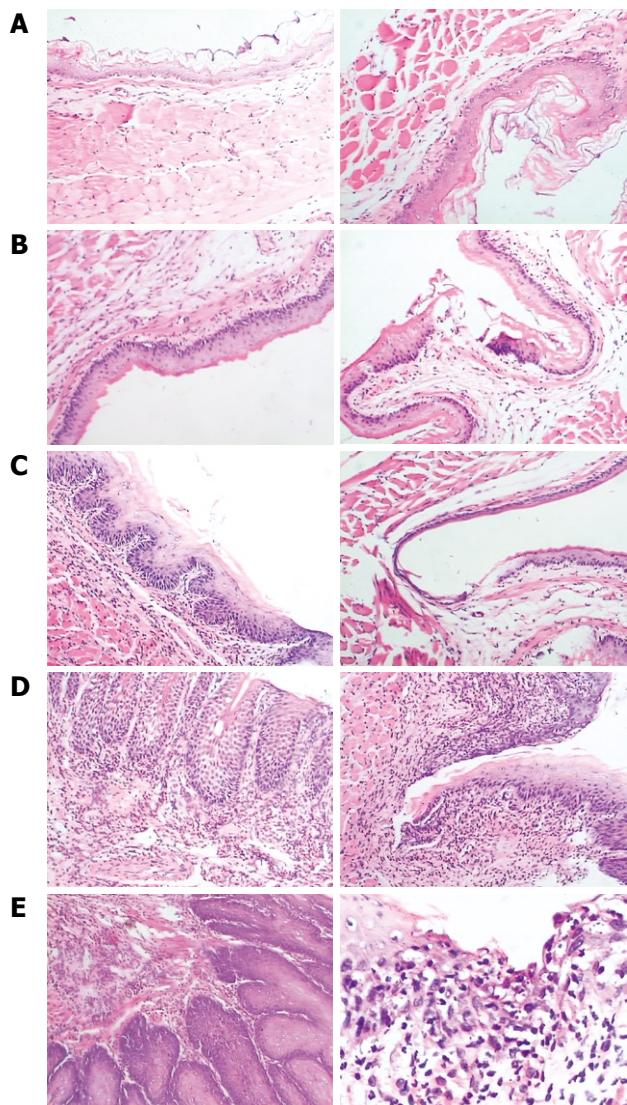
#### Changes in ultra-micro structures under TEM

As shown with TEM, the nucleus and cellular membrane were generally intact. However, a swollen cellular membrane within the intercellular space was noted; intercellular space was significantly wider in some cells, with fewer or even no desmosomes in these widened spaces. The number of desmosomes per unit area decreased as well. In RE rats, the width of the intercellular space was  $2.39 \pm 0.42 \mu\text{m}$ . As for the control rats, the width was  $0.63 \pm 0.21 \mu\text{m}$ , which was significantly smaller as compared to that in the RE rats ( $P < 0.05$ ). The average number of desmosomes was  $0.124$

$\pm 0.044/\mu\text{m}^2$ , which was also significantly smaller than that observed in the control group ( $0.221 \pm 0.031/\mu\text{m}^2$ ,  $P < 0.05$ ) (Figure 6).

#### Western blotting and RT-PCR

Compared to the control group, the Western blottings revealed that the expression levels of IL-6, claudin-1, occludin, ZO-1, JAM-1 and DSG-1 increased with time in the RE groups. In the aluminum phosphate-treated acid RE rats, the expression levels of claudin-1, occludin, ZO-1, JAM-1 and DSG-1 gradually decreased as treatment progressed and reverted to normal levels 30 d after treatment initiation. As for the mixed RE rats, the expression levels of IL-6, claudin-1, occludin, ZO-1, JAM-1 and DSG-1 returned to normal within 60 d after treatment initiation, while the expression levels of these proteins in the alkaline RE rats were markedly high at all time points during the study (Figures 7 and 8). The results of the Western blotting and RT-PCRs suggested that as the expression level of IL-6 increased, those of claudin-1, occludin, ZO-1, JAM-1 and DSG-1 increased as well and became more prominent as the disease progressed. Within 30 d after initiation of the aluminum phosphate treatment, the Western blotting and RT-PCR results indicated that the expression levels of IL-6, claudin-1, occludin, ZO-1, JAM-1 and DSG-1 in the acid RE rats (group A) decreased gradually and became comparable to that of the normal control group. Hyperplasia and widening of the intercellular spaces were restored as observed by microscopy. At day 60, the Western blotting and RT-PCR results suggested that the expression levels of these proteins decreased gradually in the mixed RE rats (group C), but increased dramatically in the alkaline reflux group (group B).



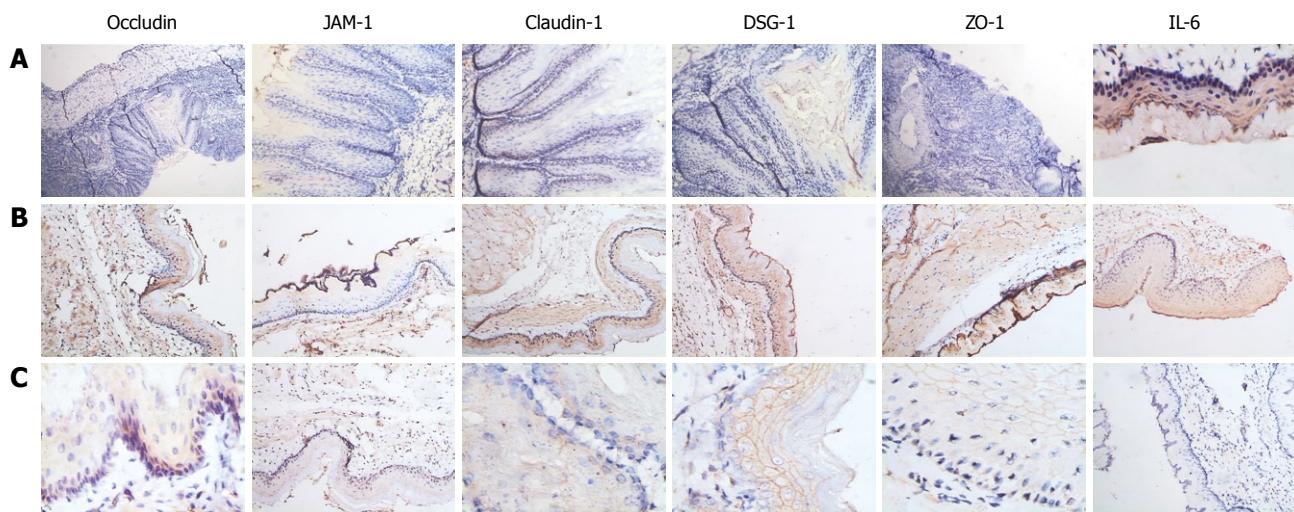
**Figure 3** Histological findings of control esophagus and chronic reflux esophagitis ( $\times 100$ ). A: Normal mucosa; B: At day 3 after operation; C: At day 6 after operation; D: At day 9 after operation; E: at day 14 after operation. B-E (left): Basal cell hyperplasia became severe along with the reflux time; B-E (right): Epithelial mucosa ulcer became severe along with the reflux time.

## DISCUSSION

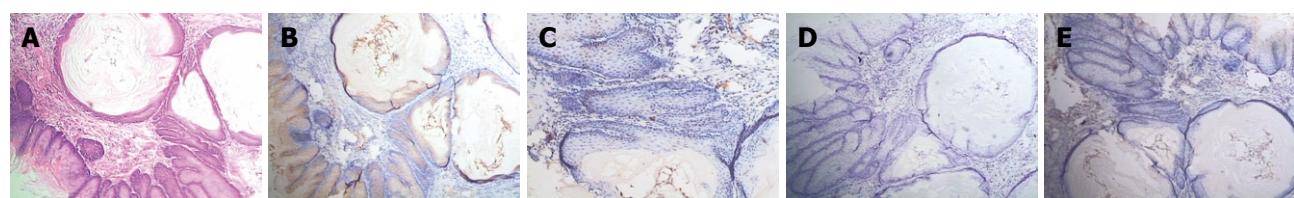
Numerous studies have described the establishment of animal models of RE. The success rate was about 10% when the rat model was created by using the method described by Yu *et al*<sup>[5]</sup>. Most rats died of reflux-related complications, such as asphyxia, pulmonary edema and pulmonary hemorrhage. Therefore, we modified their method by using a 2.0 mm metal tube in the partial pyloric ligation instead of a 1.5 mm tube. Furthermore, in our study, the tube was not inserted into the stomach as it was in Qiang Yu's method, which helped avoid artificial gastric perforation. These modifications increased the success rate to 50% ( $n = 35/70$ ). Two weeks after the procedures, the success rate was 100% ( $n = 5/5$ ) as confirmed by histological examination. RE models created with the other procedures were even more stable, with higher survival levels of 70% and

90%. Prominent hyperplasia and erosion appeared at post-procedure day 6. At post-procedure day 14, the RE model was established in all examined rats<sup>[8]</sup>.

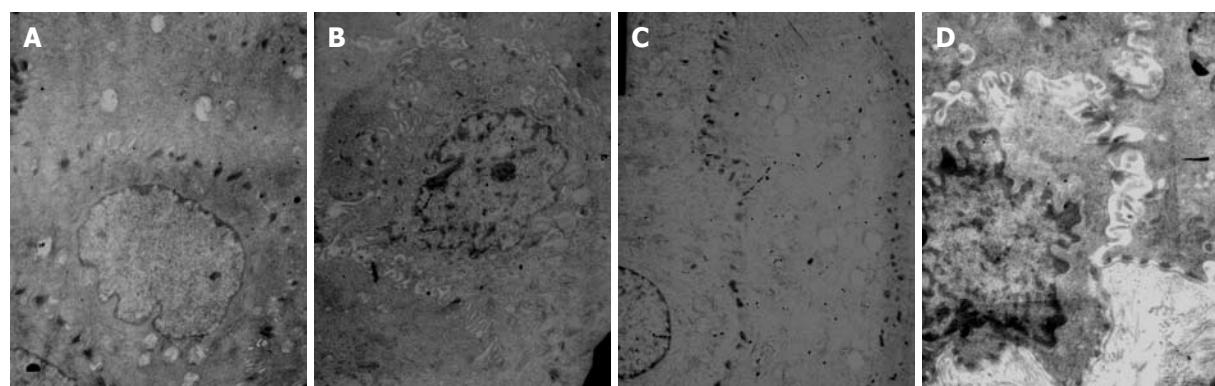
Histological assessment of the esophagitis models showed that the epithelium was significantly thickened; the lamina propria mucosa was markedly elongated into the epithelium. Inflammatory cell infiltration was noted in lamina propria. Such histological findings were extremely similar to those seen in examples of human RE. Thus, these models can help us deduce the development of the process of erosion. In our study, no prominent erosions were noticed within 6 d after the procedure. However, microscopic observation revealed that the thickness of the esophageal mucosa had already increased at this time point, and continued to increase with time. This finding suggested that irritants such as inflammation had promoted cellular proliferation and epithelial hyperplasia before the erosions occurred<sup>[10,11]</sup>. Moreover, we found that expression levels of claudin-1, occludin, ZO-1, JAM-1 and DSG-1 in the model rats were significantly higher than that of the control group; however, as hyperplasia of basal cells occurred, expression of these proteins in the individual cells decreased. These findings suggested elevated expression of these proteins was an early molecular event in the pathogenesis of RE, which occurred significantly earlier than hyperplasia. In the immunohistochemical staining, we noted that DSG-1 could be found on the membrane in all layers of the mucosa and had the highest expression level among these proteins, which suggests that the desmosome is the main cell junction in the esophageal epithelium. With TEM, we found that as RE progressed, the swollen cellular membrane within the intercellular space was noted in model rats in comparison with control rats; the intercellular space was significantly widened in some cells, with fewer or even no desmosomes in these spaces, which was consistent with the findings of Calabrese *et al*<sup>[2]</sup> and Tobey *et al*<sup>[12]</sup> in 2003. Western blottings and RT-PCR revealed that, as the expression of the inflammatory factor IL-6 increased in the model rats, the expression levels of claudin-1, occludin, ZO-1, JAM-1 and DSG-1 in esophageal mucosa increased as well. With the information from immunohistochemical assays, we found that the expression level of these TJ proteins in the model rats was significantly higher than that in control rats as early as 3 d after the procedures, but the Western blotting and RT-PCR suggested that the total amount of these proteins remained the same at this time point. When elevated expression levels of these proteins were detected by these assays, we could see hyperplasia in the basal cells under the microscope, while expression of these proteins decreased in the individual cells. These findings suggest that hyperplastic cell populations require more cell junctions and more cell junction proteins, but production of these proteins has started to decrease in individual cells, which leads to failure of cell junctions in these cells. A possible explanation is: when reflux occurs and TJs and desmosomes in



**Figure 4** Immunostaining for occludin, JAM-1, claudin-1, DSG-1, ZO-1, IL-6 in controls and reflux esophagitis model ( $\times 400$ ). A: Protein expression on day 14 in chronic RE model; B: Protein expression on day 3 in chronic RE model; C: Protein expression on day 14 in control esophagus.



**Figure 5** HE and immunostaining ( $\times 40$ ). A: HE; B: DSG-1; C: Claudin-1; D: ZO-1; E: JAM-1. Hyperplasia and vesicles could be seen, but expression of these proteins was significantly lower in individual cells as compared to control group.

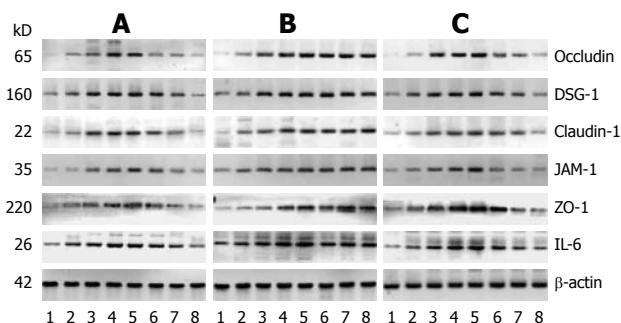


**Figure 6** Transmission electron microscopy. A: Normal; B: 14 d after operation; C: A model of 60 d after aluminium phosphate administration; D: B model of 60 d after aluminium phosphate administration.

the intercellular space are damaged, the cells begin to express more TJ and desmosome proteins in response in an effort to repair the damaged TJs and desmosomes; if damage persists and reflux starts to provoke hyperplasia, even more proteins are needed in the formation of cell junctions in these new cells. At this point, the production of TJ proteins and desmosome proteins falls short of the need, which leads to the failure of this protein compensating mechanism. Subsequent decreased expression of these proteins in individual cells leads to failure of cell junctions and further widening of the intercellular space and elevated permeability in the esophageal epithelium, resulting in damaged epithelial

defense and the pathological changes of RE, such as swollen mucosa, erosion and ulcer. These findings also suggest that elevated expression of claudin-1, occludin, ZO-1, JAM-1 and DSG-1 is a part of the pathogenesis of RE and an early molecular event in the development and progression of RE, and also one protective mechanism against the damage in RE.

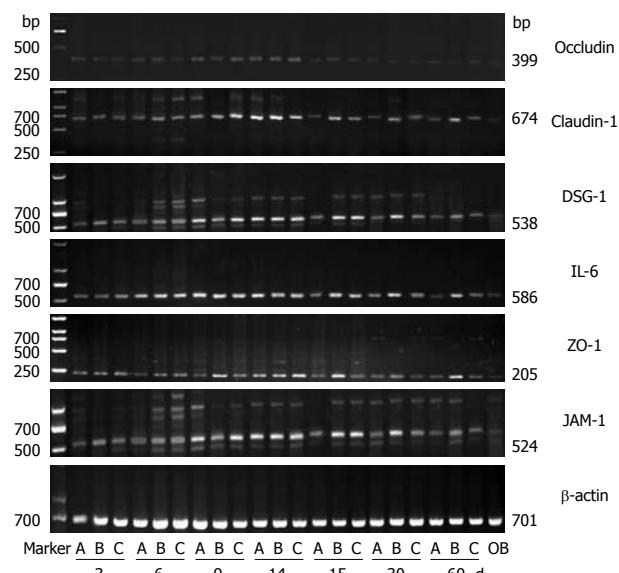
In 2005, Calabrese *et al*<sup>[2]</sup> found that widening of the intercellular spaces in squamous cells of the lower esophagus in patients with GERD could be restored after being treated with omeprazole for 3-6 mo. In our study, we treated RE rats with aluminum phosphate and it emerged that the three groups of rats responded



**Figure 7 Western blotting.** A: Model GER (acid reflux esophagitis); B: Model DER (alkaline reflux esophagitis); C: Model DG-ER (mixed reflux esophagitis). 1: Samples from control rats ( $n = 5$ ); 2-8: Samples from rats with 3, 6, 9, 14, 15, 30 and 60 d after operation ( $n = 5$ ).

differently to the treatment, with the most significant efficacy noticed in acid RE rats. Western blotting and RT-PCR assays suggested the expression of claudin-1, occludin, ZO-1, JAM-1 and DSG-1 decreased in these rats accordingly and gradually. At 1 mo after treatment started, expression of these proteins reverted to normal levels. Microscopic observation revealed that swelling and hyperplasia of the basal cells were restored, and the intercellular space was comparable to the normal controls. Within 60 d after treatment started, expression of these proteins gradually decreased in mixed RE rats, with milder hyperplasia under the microscope as compared to alkaline RE rats; swollen mucosa, erosion and ulcer disappeared as well. However, no significant treatment benefit was seen in alkaline RE rats. Western blotting and RT-PCR assays revealed that with persistent elevated expression of IL-6, claudin-1, occludin, ZO-1, JAM-1 and DSG-1, hyperplasia continued and vesicles developed. Microscopically, we found that expression of cell junction proteins was significantly lower in individual cells compared to those of control group. Aluminum phosphate is a weak acidic insoluble gel with a high level of absorbance. It creates a colloid membrane that lasts for 1.5 h on the surface of the esophageal and gastric mucosa. It can also repair damaged digestive mucosa by preventing erosions caused by esophageal reflux and gastric acid, and by preventing self-digestion induced by digestive juice. Therefore, it is more effective in treating acid RE.

Result of the four TJ proteins and one desmosome protein in our study revealed positive correlation between two of these proteins, which suggests that positive regulation and synergism might occur in the pathogenesis of esophagitis and that these proteins function as a group. Furuse *et al*<sup>[13]</sup> found TJs in occludin gene-deleted rats, indicating that these four TJ proteins work in synergism in maintaining cell junctions. Claudins, the main components of TJs, are potent adhesive proteins that create a cell junction and seal the intercellular space<sup>[14]</sup>. They also play an important role in specific functions of TJs, such as fence and barrier functions. Altered expression of claudins might lead to malformation of TJs. Occludins are mainly seen in TJ fibrils and are involved in signal transduction in the formation of TJs<sup>[15-17]</sup>. ZO-1



**Figure 8 RT-PCR.** Samples from rats with 3, 6, 9, 14, 15, 30 and 60 d after operation. A: GER model; B: DER model; C: DG-ER model; OB: Samples from control rats ( $n = 5$ ).

is a bridge that connects the TJs to intracellular structures<sup>[18-20]</sup>, while JAM has a role in regulating permeability of TJs<sup>[21,22]</sup>. Desmosomes, a kind of cell junction that specifically localizes in epithelium and mainly consists of DSG, is a structure that facilitates connection and signal transduction between cells. Inspired by the pathogenesis of inflammatory bowel disease<sup>[23-25]</sup> as described by Gassler *et al*<sup>[26]</sup>, we deduce that from the outset, esophageal reflux might destroy the structure of claudin-1 and occludin, and then ZO-1 fails to connect claudin-1 to the cellular membrane resulting in the re-distribution of ZO-1 and damage to the TJs and a subsequent increase in the permeability of esophageal epithelium; in response, expression of JAM and DSG increases, leading to more damage to the desmosomes, and eventually RE. These findings have certain implications in explaining the molecular mechanism of RE.

Statistical analysis was done to investigate the correlation between IL-6 and TJ proteins, and desmosome proteins. The results revealed no correlation. IL-6, an inflammatory factor in the development and progression of RE, is a pleiotropic cytokine produced by monocytes/macrophages. Jia *et al*<sup>[27]</sup> indicated that the IL-6 level in patients with active ulcerative colitis (UC) was markedly higher than that in patients with inactive disease and normal controls, but was not related to site and scale of the lesion. Martinez de Haro *et al*<sup>[11]</sup> found that the IL-6 level in normal mucosa of patients was higher than in normal controls but levels were not significantly different between normal mucosa and affected mucosa obtained from the same patient. These data suggest that the IL-6 level might reflect the activity of UC and RE to a certain extent.

Hamaguchi *et al*<sup>[28]</sup> found that in chronic esophagitis rats, expression of cytokines and adhesive molecules increased. Although currently, we don't see any connection between cytokines and TJ proteins, we might assume

that inflammatory cytokines and subsequent damage to the tissue might have regulated the permeability of esophageal epithelium in esophagitis. Cytokines might damage the barrier function and increase the permeability of the epithelium.

In summary, in molecular cell biology and microscopic technology which accelerate research in this field<sup>[29,30]</sup>, we now have an increasingly profound understanding of the function and structure of TJ, TJ proteins' regulation of the TJ formation and structure, as well as the roles of epithelial permeability and TJs in epithelial defense. Simonovic *et al*<sup>[31]</sup> and Jin *et al*<sup>[32]</sup> proposed that phosphorylation of occludins was the determinant factor in the distribution of these proteins, but its regulating mechanism, as well as the biological mechanism underlying the feedback regulation of these proteins, are still unclear and need to be further explored in more studies.

## COMMENTS

### Background

Tight junction (TJ) and desmosome proteins in esophageal epithelium create a circular substance-blocking barrier between adjacent cells. These proteins function in cell adhesion, contribute to cell polarity and permeability and facilitate signal transduction in cell proliferation and differentiation. We investigated the correlation between the expression levels of IL-6 and the proteins in TJs, including claudin 1, occludin, ZO-1, JAM-1 and DSG-1, in the esophageal mucosa of rats modeling different types of reflux esophagitis (RE), and the ability of aluminum phosphate to protect against RE-induced mucosal damage via these proteins.

### Innovations and breakthroughs

Further investigations are warranted using experiments testing the hypothesis that altered expression of TJ proteins (claudin 1, occludin, ZO-1, JAM-1) and desmosome proteins (DSG-1) results in failure of TJs and desmosomes, which thus leads to widened intercellular space and contributes to RE. Moreover, some rats were treated with aluminum phosphate to determine its efficacy in treating RE.

### Applications

These findings indicate that increased expression of TJ proteins, as an early molecular event, is involved in RE development and that IL-6 is an inflammatory factor in this process. Aluminum phosphate significantly protects esophageal mucosa by enhancing its defense against damage. However, efficacy of the drug is related to pH value of the reflux: it is effective in treating acid RE rather than alkaline RE.

### Terminology

GERD occurs when stomach and duodenal contents reflux into the esophagus. Reflux-induced damage to, and inflammation in, the esophageal mucosa are referred to as RE. TJs create a circular substance-blocking barrier between adjacent cells. TJs also function in cell adhesion, cell polarity and permeability, and in signal transduction resulting in cell proliferation and differentiation. Intercellular TJs consist mainly of claudins, occludins, JAM and ZOs.

### Peer review

This interesting study was to determine the changes of IL-6 and various TJ proteins in the esophageal mucosa induced by RE. Authors conducted these studies in rats and nicely correlated between the expression pattern of claudin, occludin, ZO-1 and JAM-1 proteins in different types of RE and the ability of aluminum phosphate to protect against RE. Results presented in this manuscript showed that there is a significant difference between RE induced and sham-control groups.

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## Spot urinary sodium for assessing dietary sodium restriction in cirrhotic ascites

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### Abstract

**AIM:** To evaluate the accuracy of spot urinary Na/K and Na/creatinine (Cr) ratios as an alternative to 24-h urinary sodium in monitoring dietary compliance in patients with liver cirrhosis and ascites treated with diuretics.

**METHODS:** The study was carried on 40 patients with liver cirrhosis and ascites treated with diuretic therapy. Patients were divided into two groups according to 24-h urinary sodium. We measured spot urine Na/K ratio, Na/Cr ratio and 24-h urinary sodium. Student's *t* test was used to compare the interval variables and  $\chi^2$  test to compare the nominal variables between the two groups. Receiver operator characteristic curve was used to identify the best cutoff point for Na/K and Na/Cr ratio.

**RESULTS:** The best cutoff point for Na/K ratio was 2.5 ( $P < 0.001$ ) and area under the curve (AUC) was 0.9, and for Na/Cr ratio, the best cutoff point was 35 ( $P < 0.001$ ) and AUC was 0.885. Na/K ratio showed higher sensitivity and accuracy compared to Na/Cr ratio (87.5% and 87% for Na/K ratio; 81% and 85% for Na/Cr ratio, respectively).

**CONCLUSION:** Spot urine Na/K ratio has adequate accuracy for assessment of dietary sodium restriction

compared with 24-h urinary sodium in patients with liver cirrhosis and ascites.

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**Key words:** Ascites; Liver cirrhosis; Portal hypertension; Urinary sodium

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### INTRODUCTION

Ascites is considered to be the most common of the three major complications of cirrhosis; other complications are hepatic encephalopathy and variceal bleeding. It is estimated that about 50% of patients with compensated cirrhosis will develop ascites within 10 years of observation<sup>[1]</sup>. The development of ascites in patients with liver cirrhosis is associated with poor prognosis and an increased risk of mortality, as approximately 50% of patients with ascites are expected to succumb within 2 years<sup>[2]</sup>.

The development of ascites is secondary to renal retention of sodium and water because of underlying activation of neurohormonal mechanisms<sup>[3]</sup>. Therefore, patients who accumulate ascites have urinary excretion of sodium that is significantly lower than their dietary salt intake. This means that, in order to achieve successful ascites mobilization, patients should have a negative sodium balance. This can be achieved through education regarding dietary sodium restriction (2 g/d) in addition to oral diuretic therapy<sup>[4]</sup>. The usual diuretic regimen starts with single morning doses of oral spironolactone (100 mg) and furosemide (40 mg). Efficacy of therapy is monitored through the goal of average weight loss of 300-500 g/d in patients without peripheral edema and 800-1000 g/d in those with peripheral edema. Doses can be increased gradually in patients who show inadequate response,

to reach a maximum of 400 mg/d spironolactone and 160 mg/d furosemide<sup>[5]</sup>.

This approach is effective in approximately 90% of patients and 10% are considered diuretic-resistant and second-line therapy is indicated for ascites mobilization<sup>[6]</sup>. However, patients who are not compliant with diet may also show inadequate response to maximum diuretic doses. Assessment of dietary compliance is important in order to avoid mislabeling patients with refractory ascites, while their problem is inadequate dietary salt restriction<sup>[7]</sup>. Several methods have been suggested for assessment of dietary compliance: (1) The usual method is measuring 24-h urinary sodium excretion. Patients who gain weight despite excreting more than 78 mEq Na/d are not compliant with the diet<sup>[8]</sup>. However, the main problem here is that it may be difficult for the patient to accurately collect 24 h urine. At the same time, using spot urinary sodium is easier for the patient but it is not accurate because of the lack of uniform excretion of sodium throughout the day. (2) Furosemide-induced natriuresis can be used. A single intravenous 80-mg dose of furosemide is given and urinary sodium is measured in the next 8 h. Patients with diuretic resistance have sodium excretion < 50 mEq/8 h<sup>[9,10]</sup>. (3) There is some evidence that spot urinary Na/K ratio may be as helpful as 24-h urinary sodium collection, with adequate accuracy, when the ratio more than a given cut value (one in some studies) is equivalent to 24 h sodium more than 78 mmol Na/d<sup>[11,12]</sup>.

The aim of this study was to assess the accuracy of spot urinary Na/K and Na/creatinine (Cr) ratio for assessment of dietary sodium restriction in patients with liver cirrhosis and ascites treated with diuretic therapy.

## MATERIALS AND METHODS

The study was conducted on 40 patients (28 male and 12 female; mean age 50 ± 4.5 years) with liver cirrhosis and ascites treated with diuretics, who were admitted to the Department of Internal Medicine in Ain Shams University Hospitals. The study was performed according to the ethical standards for human experimentation and was approved by the scientific committee of Ain Shams University. Informed consent was obtained from the selected patients after explaining the aim of the study and the nature of the investigations required. Liver cirrhosis was documented by clinical assessment, laboratory findings and evidence of liver cirrhosis upon abdominal ultrasound. Ascitic fluid analysis was done to exclude malignancy and spontaneous bacterial peritonitis. Patients with evidence of portosystemic encephalopathy or intrinsic renal disease were excluded from the study. All patients had normal kidneys upon ultrasound and no proteinuria or active urinary sediment upon urine analysis. Daily monitoring of patient weight and lack of response to diuretics was defined according to the consensus conference of the International Ascites Club, which is mean weight loss of < 0.8 kg over 4 d<sup>[5]</sup>.

Collection of 24-h urine sample for calculation of sodium was done in sterile plastic containers by recording the volume in 24 h, starting at 08:00. Verbal instructions

were given to assure completeness of collection. Samples were centrifuged and sodium concentration was measured in mEq/L using a Beckman Synchron CX5 ISE (NJ, USA) chemistry analyzer. All samples were processed on the day of collection. In order to obtain the whole 24-h urinary sodium, we multiplied sodium concentration by the volume in liters. Spot urine samples were collected for measurement of sodium, potassium and creatinine. Samples were handled as previously described for 24-h samples, except that volume was not recorded, as it has no significance in this setting. Patients were divided into two groups according to 24-h urinary sodium.

## Statistical analysis

Data were collected, revised, verified and edited on a PC. Data were then analyzed statistically using SPSS statistical package version 25 (SPSS, Chicago, IL, USA). Patients were divided according to 24-h urinary sodium excretion into diuretic-resistant (16 patients with 24-h urinary sodium < 78 mEq) and diuretic-sensitive (24 patients with 24-h urinary sodium ≥ 78 mEq). Data were reported in the form of mean ± SD. Student's *t* test was used to compare the interval variables and the  $\chi^2$  test was used to compare the nominal variables between the two groups. Correlation between 24-h urinary sodium and other variables was done using Pearson correlation. In order to identify the best cutoff point for Na/K ratio and Na/Cr ratio, a receiver operator characteristic (ROC) curve was used.  $P < 0.05$  was considered statistically significant.

## RESULTS

According to the type of diuretic used, 28 patients were taking furosemide and spironolactone in combination; four, furosemide alone; three, spironolactone alone; and five, spironolactone in combination with bumetanide. There was no significant difference between the two groups with regard to type of diuretics used.

As shown in Table 1, there was no significant difference between the groups as regards age, sex, serum creatinine, serum potassium, liver enzymes, serum bilirubin, total proteins, international normalized ratio or partial thromboplastin time. The following parameters were significantly lower in the diuretic-resistant group: serum sodium, serum albumin, white blood cell (WBC) count and platelet count. However, the diuretic-resistant patients had higher blood urea nitrogen (BUN) and higher Child score. Table 2 shows that 24-h urinary sodium, spot urine Na/K ratio and spot urine Na/Cr ratio were significantly lower in the diuretic-resistant patients.

Significant correlation was noted between 24-h urinary sodium and Na/K ratio ( $r = 0.76, P = 0.001$ ), Na/Cr ratio ( $r = 0.56, P = 0.001$ ), serum sodium ( $r = 0.59, P = 0.001$ ), serum BUN ( $r = -0.31, P = 0.046$ ) and Child score ( $r = -0.31, P = 0.05$ ).

## Determination of best cutoff point for Na/K ratio

Figure 1A represents the ROC curve for the best cutoff point to differentiate between diuretic sensitivity and resistance using spot urine Na/K ratio. Area under

**Table 1** Patients characteristics (mean  $\pm$  SD)

	Diuretic-resistant group (n = 24)	Diuretic-sensitive group (n = 16)	P
Age (yr)	50 $\pm$ 4	49 $\pm$ 4	NS
Sex ratio (Male/female)	17/7	11/5	NS
Weight loss (yes/no)	0/24	9/7	0.001
Serum creatinine (mg/dL)	1.1 $\pm$ 0.2	0.9 $\pm$ 0.2	NS
BUN (mg/dL)	23.5 $\pm$ 8.3	18.5 $\pm$ 8.3	0.033
Serum sodium (mEq/L)	128 $\pm$ 5	136 $\pm$ 5	0.001
Serum potassium (mEq/L)	4.3 $\pm$ 0.6	4.3 $\pm$ 0.5	NS
AST (U/L)	68 $\pm$ 34	69 $\pm$ 40	NS
ALT (U/L)	56 $\pm$ 27	57 $\pm$ 27	NS
ALP (U/L)	283 $\pm$ 56	246 $\pm$ 84	NS
Total bilirubin (mg/dL)	3.8 $\pm$ 1.7	3.2 $\pm$ 1.8	NS
Direct bilirubin (mg/dL)	1.9 $\pm$ 1.1	1.6 $\pm$ 0.9	NS
Total proteins (g/dL)	7.1 $\pm$ 0.4	7.2 $\pm$ 0.5	NS
Serum albumin (g/dL)	2.4 $\pm$ 0.4	2.8 $\pm$ 0.4	0.012
WBC count ( $\times 10^9$ /L)	6.6 $\pm$ 2.4	8.8 $\pm$ 4.3	0.045
Hemoglobin (g/dL)	9.7 $\pm$ 1.7	9.5 $\pm$ 2.6	NS
Platelet count ( $\times 10^9$ /L)	73 $\pm$ 24	108 $\pm$ 58	0.014
INR	2.9 $\pm$ 1.3	1.5 $\pm$ 0.2	NS
PTT (s)	36 $\pm$ 9	32 $\pm$ 7	NS
Child classification (B/C)	1/23	6/10	0.011

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; NS: Not significant.

**Table 3** Sensitivity and specificity of different Na/K and Na/Cr ratios in determination of diuretic resistance or sensitivity

Ratio	Sens (%)	Spec (%)	PPV (%)	NPV (%)	Acc (%)
Na/K ratio	1	93.8	58.3	76.7	90.0
	2.5	87.5	87.5	91.3	82.4
	3.5	50	95.8	92.3	55.6
Na/Cr ratio	20	93.8	66.7	82.1	91.7
	35	81	87.5	91	77.8
	45	62.5	87.5	88.2	60.9

PPV: Positive predictive value; NPV: Negative predictive value; Sens: Sensitivity; Spec: Specificity; Acc: Accuracy.

the curve (AUC) was 0.9 and  $P < 0.001$ . According to the curve, the best cutoff point was 2.5, with 87.5% sensitivity and specificity and 85% accuracy. Using higher or lower cutoff points shows lower accuracy although sensitivity or specificity may be higher (Table 3).

#### Determination of best cutoff point for Na/Cr ratio

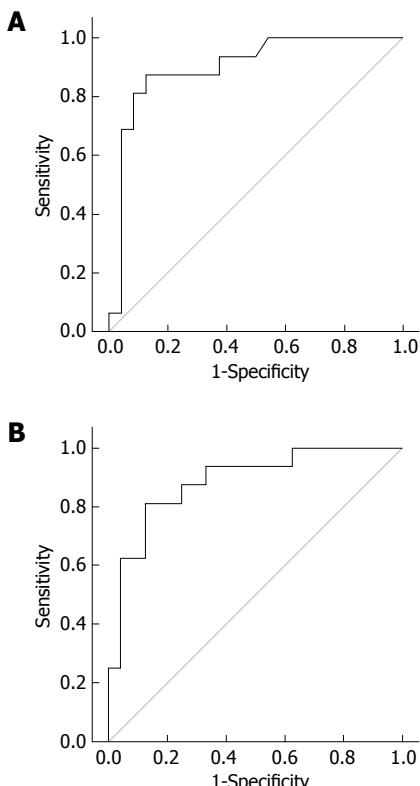
For Na/Cr ratio, AUC was 0.885 and  $P$  was  $< 0.001$ . The best cutoff point according to the curve (Figure 1B) was 35, with 81.3% sensitivity, 87.5% specificity and 87% accuracy. Other cutoff values showed lower accuracy (Table 3).

## DISCUSSION

During treatment of ascites in patients with liver cirrhosis, excess sodium intake can be misinterpreted as diuretic unresponsiveness, as in the present study, seven patients showed no weight loss in response to diuretics, while their 24-h sodium excretion was  $> 78$  mEq. Appropriate identification of such patients is also important to avoid complications of unnecessary increase in diuretic dosage

**Table 2** Difference between groups as regards 24-h urinary sodium and spot urine ratios (mean  $\pm$  SD)

	Diuretic-resistant group (n = 24)	Diuretic-sensitive group (n = 16)	P
24 h urinary sodium (mEq)	33 $\pm$ 19	126 $\pm$ 46	0.001
Spot urine Na/K ratio	1.3 $\pm$ 1.3	3.7 $\pm$ 1.6	0.001
Spot urine Na/Cr ratio	21 $\pm$ 18	100 $\pm$ 87	0.001



**Figure 1** ROC curve for the best cutoff point to differentiate between diuretic sensitivity and resistance using spot urine Na/K ratio (A) and Na/Cr ratio (B).

(mainly encephalopathy and electrolyte disturbance) and complications of unnecessary large volume paracentesis (mainly mechanical trauma, post-paracentesis circulatory dysfunction and possible increased incidence of spontaneous bacterial peritonitis caused by ascitic fluid protein depletion)<sup>[13]</sup>.

Measuring 24-h urinary sodium is used currently to identify such patients; however, it may be difficult for patients to collect urine appropriately and lack of appropriate collection can lead to false low results, and again, false-labeling of the patient as unresponsive to diuretics. Using 24-h urinary creatinine has been proposed as a method to ensure adequate collection of urine<sup>[14]</sup>, but even this may not be accurate, because patients with advanced liver cirrhosis can have muscle wasting, and therefore, lower creatinine excretion in urine, even with complete collection<sup>[15]</sup>.

The present study shows that spot urine Na/K ratio can be used as an easier alternative to 24-h urinary sodium excretion, with adequate accuracy. At the best cutoff point, Na/K ratio was shown to be more accurate than

Na/Cr ratio, with better sensitivity and specificity. Similar observations as regards spot urine Na/K ratio have been made by others<sup>[11,12]</sup>. However, according to these other studies, the best cutoff point is 1, while in the present study, the best sensitivity, specificity and accuracy was noted at a cutoff point of 2.5. This difference may have been caused by a difference in types of diuretics used, which may affect urinary sodium and potassium excretion. Further studies are needed with classification of patients into groups according to types of diuretics used in order to document any significant effect on Na/K ratio.

Another test used is furosemide-induced natriuresis, which can identify diuretic unresponsiveness with adequate accuracy<sup>[9,10]</sup>. However, it still needs urine collection for 8 h after furosemide administration. At the same time, it can identify patients with refractory ascites (those who do not respond to maximum doses of diuretics), even before reaching the maximum dose, which is an advantage over spot urine Na/K ratio. Probably we should use Na/K ratio for routine assessment of sodium restriction during diuretic therapy, and keep the furosemide-induced natriuresis test for patients that we suspect are completely resistant to medical treatment, therefore, we can make early referral for liver transplantation or procedures like transjugular intrahepatic portosystemic shunt.

Patients with more advanced liver disease have more deterioration in liver function and marked degrees of circulatory dysfunction and neurohumoral activation [including antidiuretic hormone (ADH)], which results in enhanced sodium renal tubular reabsorption, and therefore, more diuretic resistance<sup>[16]</sup>. It has been shown also by another study that lower urinary sodium in cirrhotic patients with ascites is associated with reduced survival<sup>[17]</sup>. This was noted in the present study, as patients with lower urinary sodium had more advanced liver disease in the form of lower serum albumin and higher Child-Pugh score. Also, lower serum sodium in the resistant group represents more impairment of free water excretion because of a high level of ADH that leads to dilutional hyponatremia in patients with more resistant ascites<sup>[7,18]</sup>.

According to the main theory of ascites formation, the initial step in pathogenesis is the development of portal hypertension<sup>[16]</sup>. Platelet and WBC counts are already suggested as noninvasive tests for prediction of esophageal varices<sup>[19,20]</sup>, another complication of portal hypertension. The lower platelet and WBC counts associated with diuretic resistance in the present study may also represent more advanced liver disease and more severe portal hypertension in the unresponsive patients.

One of the results of neurohormonal activation in advanced liver disease is renal vasoconstriction, which may reduce the glomerular filtration rate (GFR) and present later as hepatorenal syndrome<sup>[6,21]</sup>. The reduction in GFR in these patients may be masked by serum creatinine concentration that appears to be within the normal range, with a GFR as low as 20 mL/min<sup>[15,22]</sup>. In the present study, patients in the resistant group had higher BUN, which was similar to that in a previous study<sup>[12]</sup>. This may represent impairment in renal function masked by normal serum creatinine.

In conclusion, dietary sodium restriction is essential in mobilization of ascites caused by liver cirrhosis in addition to diuretics. Monitoring of dietary compliance is essential and exclusion of excess sodium intake is important in patients who appear unresponsive to diuretics. However, this is often missed, perhaps because of the difficulty of 24-h urine collection. Using spot urine Na/K ratio may be as accurate as 24-h urinary sodium measurement, with the advantage of being more applicable for the patient. However, further studies are still needed before accepting this test in practice, mainly testing the effect of the type of diuretic used on the best cutoff value. Another point we recommend testing is whether there is diurnal variation for this ratio, and if there is a preference for a first morning voiding sample.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Monitoring dietary compliance is an important aspect in treatment of ascites caused by liver cirrhosis. The standard test used is 24-h urinary sodium, however, its use is limited by difficulty of urine collection over 24 h. Few studies have evaluated spot urine Na/K ratio as an alternative.

### Research frontiers

In the present study, the standard 24-h urine test was compared to spot urine Na/K and Na/creatinine (Cr) ratio in patients with ascites caused by cirrhosis, treated with diuretics.

### Innovations and breakthroughs

The present study showed adequate accuracy for spot urine tests when compared to 24-h urinary sodium assessment. The accuracy, sensitivity and specificity of Na/K ratio are higher than those for Na/Cr ratio. Also, the study showed greater deterioration in liver function in patients who had lower urinary sodium excretion.

### Applications

Na/K ratio may be used in clinical practice to monitor dietary sodium compliance in patients with ascites and liver cirrhosis. It should be more convenient for the patient compared to the 24-h urinary sodium test.

### Peer review

Spot urinary Na/K ratio was previously proposed a few years ago as a substitute for 24-h urinary sodium. Nevertheless the first communications were only published as abstracts. As far as we know, this is the first full publication regarding this issue. The statistical procedures are adequate and the methods of the research are good. The only criticism is the low sample size. This paper should be useful for readers of the journal.

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BRIEF ARTICLES

## A randomized controlled trial of imipramine in patients with irritable bowel syndrome

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### Abstract

**AIM:** To study the efficacy of low-dose imipramine in relieving symptoms associated with the irritable bowel syndrome (IBS).

**METHODS:** A randomized, double-blind trial of 25 mg imipramine vs matched placebo for 12 wk was performed. Doubling the dose was allowed once at week 2 in case of an unsatisfactory early response. Primary efficacy variables were subjective global symptom relief and quality of life (QoL) using SF-36 at week 12.

**RESULTS:** One hundred and seven patients were enrolled by advertisement or referral by general practitioners and 56 (31 imipramine: 25 placebo) completed the 16-wk study. Baseline characteristics were comparable. A high overall dropout rate was noted in the imipramine and placebo arms (47.5% vs 47.9%,  $P > 0.05$ ), a mean of 25.0 and 37.4 d from enrollment, respectively ( $P < 0.05$ ). At the end of 12 wk, there was a significant difference in global symptom relief with imipramine over placebo (per-protocol: 80.6% vs

48.0%,  $P = 0.01$ ) and a trend on intent-to-treat (ITT) analysis (42.4% vs 25.0%,  $P = 0.06$ ). This improvement was evident early and persisted to week 16 ( $P = 0.024$  and 0.053 by per-protocol and ITT analyses, respectively). Mean cumulative and component-specific SF-36 scores improved in the imipramine group only (per-protocol,  $P < 0.01$ ). Drug-related adverse events leading to patient dropout were more common in the imipramine group (25.4% vs 12.5%,  $P > 0.05$ ).

**CONCLUSION:** Imipramine may be effective in the treatment of IBS patients and is associated with improved QoL. Careful patient selection, initiation of a low dose with gradual escalation and monitoring for side effects may result in an improved therapeutic response.

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**Key words:** Tricyclic antidepressants; Quality of life; Functional gastrointestinal disorders; Irritable bowel syndrome; Pain

**Peer reviewer:** Dr. Giuseppe Chiarioni, Gastroenterological Rehabilitation Division of the University of Verona, Valeggio sul Mincio Hospital, Azienda Ospedale di Valeggio s/M, Valeggio s/M 37067, Italy

Abdul-Baki H, El Hajj II, ElZahabi L, Azar C, Aoun E, Skoury A, Chaar H, Sharara AI. A randomized controlled trial of imipramine in patients with irritable bowel syndrome. *World J Gastroenterol* 2009; 15(29): 3636-3642 Available from: URL: <http://www.wjgnet.com/1007-9327/15/3636.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.3636>

### INTRODUCTION

Irritable bowel syndrome (IBS) is a common disorder of the gastrointestinal (GI) tract characterized by abdominal pain or discomfort and altered bowel habits. The exact pathophysiology of IBS remains unclear but is thought to involve altered intestinal motility and increased visceral sensitivity as a result of a dysregulated bidirectional communication between the enteric nervous system and the brain, the so-called brain-gut axis. The role of tricyclic antidepressants (TCAs) in the

treatment of IBS has been systematically reviewed by the American Gastroenterology Association and the American College of Gastroenterology<sup>[1,2]</sup>. TCAs have been found to improve abdominal pain in IBS patients; however there was inadequate evidence to support an effect on improvement of global IBS symptoms. Notably, most randomized, controlled trials of TCAs in IBS were completed before the publication of the ROME II committee recommendations for the study design of treatment trials for IBS and suffered from suboptimal study design, small sample size, and short treatment duration<sup>[3,4]</sup>.

The exact mechanism of action of TCAs is not known, but may be mediated *via* potentiation of adrenergic synapses by blocking uptake of the neurotransmitters, norepinephrine and serotonin, at nerve endings<sup>[5,6]</sup>. Their reported benefit in IBS appears, however, to be unrelated to an antidepressant effect given that a response is commonly noted early and with doses generally well below the effective antidepressant doses. Potential beneficial mechanisms of TCAs in IBS include a reduction in visceral pain sensitivity and, to a lesser extent, their anticholinergic effects.

The aim of this study was to evaluate the efficacy and safety of imipramine hydrochloride, a tertiary amine TCA, in patients with IBS who have failed to respond satisfactorily to antispasmodics. The primary study endpoint was global symptom relief as assessed by the patient at the end of the 12-wk treatment. Secondary endpoints were symptom relief at week 16 and changes in quality of life (QoL) as measured using the short form SF-36 questionnaire.

## MATERIALS AND METHODS

Figure 1 shows the general design of the study. Study subjects were recruited by either advertisements posted in clinics and pharmacies or by referral from primary care or specialty clinics at the American University of Beirut Medical Center (AUBMC). A preliminary telephone interview was conducted for patients who answered to posted advertisements. Those who satisfied inclusion criteria were asked to present for interview and examination by a general practitioner to confirm adherence to study inclusion criteria: (1) fulfillment of the Rome II criteria for the diagnosis of IBS and (2) history of an unsatisfactory response to one or more prescription antispasmodics available on the Lebanese market (trimebutine, mebeverine hydrochloride, otilonium bromide, or alverine citrate). Exclusion criteria were age below 18 years, allergy to imipramine, history of hematochezia or melena, constitutive symptoms (such as fever and weight loss), severe constipation (less than one bowel movement per 3 d), pregnancy, history of cardiac arrhythmias, use of any drug that could influence bowel function within 1 mo of entering the study (such as tegaserod, laxatives, antibiotics, or probiotics), known lactose intolerance, use of antidepressants or presence of signs and symptoms suggestive of clinical depression, or any evidence of advanced organic or psychiatric

disease that may impact compliance or adherence to the study protocol. Similarly, patients who were referred from primary care or specialty clinics were screened for inclusion and exclusion criteria and records of previous medical investigations relating to the patient's complaint were carefully reviewed to exclude organic disease.

After initial evaluation and assessment, written informed consent was obtained, and patients were asked to complete a pre-treatment QoL questionnaire, the SF-36, a reliable and valid measure of QoL<sup>[7-9]</sup>. A word-for-word Arabic language translation of the SF-36 was available for non-English speaking patients; however the translated version was not subjected to validation studies.

### Assignment

All subjects were randomized by an independent investigator using a computer-generated random numbers table with a 1.2 to 1 stratification in favor of imipramine. The randomization key was kept under lock until the completion of the study.

### Masking

Study drugs were provided in opaque envelopes as imipramine (Tofranil, Novartis Pharma AG, Basle, Switzerland) 25 mg tablets, one tablet daily before bedtime for 84 d (12 wk) or matching placebo tablets.

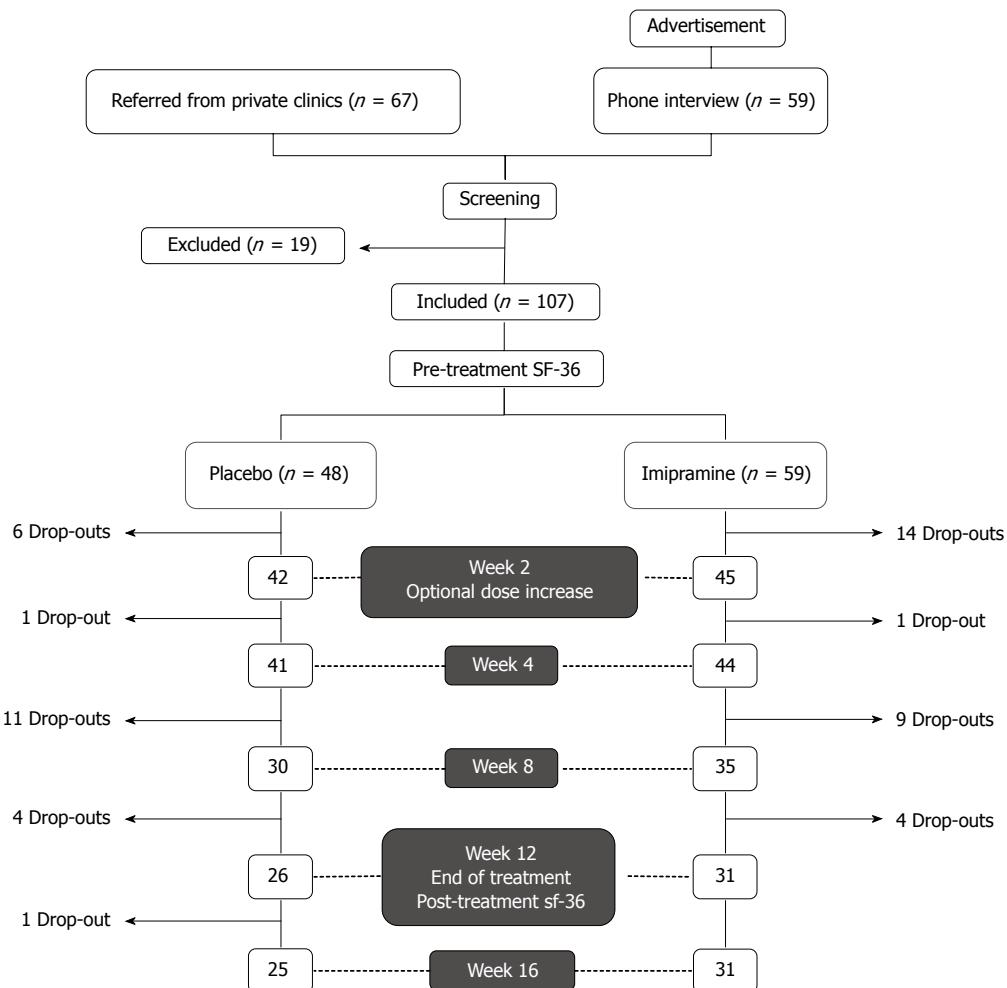
### Protocol and patient follow-up

Patients were contacted by phone at day 7 and day 14 of treatment to report any side effects. At day 14, patients who reported unsatisfactory global improvement of symptoms were given the choice of either continuing the treatment as before or doubling the daily dose (one tablet twice daily). The decision was left to the patients based on their level of tolerance to side effects, if any (the change, however, had to be effected once and on, or starting at, day 14).

The study's main variable was the subjective feeling of global symptom relief as reported by the subjects in response to the following question: "Have your symptoms improved satisfactorily since starting the study drug?" Patients were contacted on weeks 4, 8 and 12 of treatment to answer this question. At week 12, patients were requested to complete a post-treatment SF-36 questionnaire. An off-treatment follow-up was done at week 16 to answer the same global relief question mentioned above. Compliance was checked by pill count. The trial was approved by the Institutional Research Board of the AUBMC.

### Statistical analysis

Sample size calculation was estimated based on the assumption of a 60% response to imipramine *vs* a 30% response to placebo. The estimated sample size was 56 patients per arm. Projecting a 20%-30% dropout rate, the sample size was calculated at 70 patients per arm. The data were entered and analyzed using SPSS version 11.5. Frequency tables and cross-tabulations were derived in order to depict any associations between the different variables. Analysis of the primary end-point (global symptom relief) was done according to an intent-



**Figure 1** Study design.

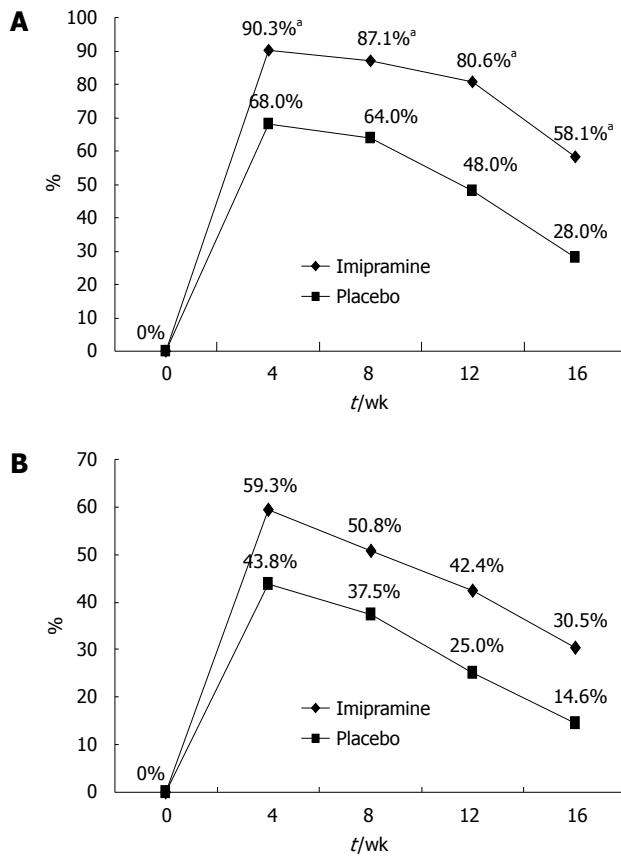
to-treat (ITT) basis. The paired samples *t*-test and the independent-samples *t*-test were used to compare the QoL scores before and after treatment. The change in SF-36 scores between week 12 (end of treatment) and baseline was calculated only in patients who completed the study and had paired SF-36 scores (PP analysis). A *P*-value at or below 0.05 was considered as the cut-off point for statistical significance.

## RESULTS

From December 2004 to May 2006, 67 patients were referred from private clinics and 59 patients answered to posted advertisements. Because of significant thinning of patient recruitment late in the study period as well as extenuating political circumstances in Lebanon, the study was closed in June 2006 before the preset sample size could be reached. Of the 136 screened subjects, 107 met the criteria for enrollment; 59 were randomized to imipramine and 48 to placebo. Since enrollment was stopped prematurely, the power calculation was performed *ad hoc*. Assuming a 60% response rate to imipramine *vs* a 30% response rate to placebo, with  $\alpha = 0.05$  (two-tailed), the above sample size allowed us to detect a significant difference between the two groups with a calculated power of 88.4%.

Both groups were comparable with regard to age, sex, and symptoms (Table 1). The mean age in the imipramine arm was  $42.6 \pm 12.4$  years *vs*  $45.3 \pm 13.8$  years for the placebo arm, with a slight male predominance. Fifty-seven individuals of the total patient sample (52.5% *vs* 54.2%) had undergone endoscopic procedures with no abnormal findings. The remaining patients had undergone other diagnostic testing based on their primary physician's recommendations (blood count, inflammatory markers, stool studies, and imaging) which were non-revealing.

All patients fulfilled the Rome II criteria for IBS and had a history of at least one previous treatment using off-the-counter or prescribed antispasmodic medications with unsatisfactory results. The patients' global relief of symptoms during the different intervals of the trial is shown in Figure 2. The ITT population included all 107 patients while the per-protocol analysis was based on results from the 56 patients (31 on imipramine and 25 on placebo) who completed the 12-wk active treatment period and the 4-wk off-treatment follow-up. The imipramine group reported relief of baseline symptoms in 80.6% of patients at the end of the 12-wk treatment period as compared to 48.0% in the placebo arm (*P* = 0.01). The highest rate of relief was achieved at week 4 in both groups: 90.3% for imipramine and 68.0% for



**Figure 2** Rate of global symptom relief (A) per-protocol and (B) per intent-to-treat analysis (<sup>a</sup> $P < 0.05$ ).

placebo ( $P = 0.037$ ). Per-protocol analysis revealed a significantly greater improvement of baseline symptoms in the imipramine arm *vs* placebo group at all checkpoints of the 12-wk trial including 4 wk after stopping treatment ( $P = 0.024$ ). ITT analysis of all 107 patients who received at least one tablet of the assigned treatment showed higher rates of global symptom relief in favor of imipramine at all study points; however, the difference was not statistically significant (42.4% *vs* 25% at week 12,  $P = 0.06$ , Figure 2).

The dropout rate was similar in both study groups: 28/59 (47.5%) for imipramine and 23/48 (47.9%) for placebo. Subjects receiving imipramine dropped out of the trial earlier than in the placebo arm with a mean dropout time of  $25.0 \pm 17.9$  d *vs*  $37.4 \pm 20.8$  d, respectively ( $P = 0.026$ ). The reasons for dropout were loss to follow-up, premature withdrawal of treatment without side effects (at or before 2 wk of treatment), protocol violation, and side effects (Table 2). Premature withdrawal accounted for 60.9% of dropouts in the placebo group. Fourteen of the 28 dropouts (50%) on imipramine reported that side effects were the main reason for withdrawal with a predominance of anticholinergic side effects (Table 3). Of those 14 patients, only two had doubled the dose of medication (to 50 mg imipramine) after a sub-optimal response at 2 wk. In general, the rate of side effects was higher in the imipramine group (64.4% *vs* 39.6%, respectively,  $P = 0.01$ ). However, when comparing the rates of side effects which led to subject

**Table 1** Baseline patient characteristics *n* (%)

	Imipramine ( <i>n</i> = 59)	Placebo ( <i>n</i> = 48)
Mean age (yr)	$42.6 \pm 12.4$	$45.3 \pm 13.8$
Male sex	33 (55.9)	29 (60.4)
Type of recruitment	38 (64.4) referrals	29 (60.4) referrals
Bloating/distention	57 (96.6)	46 (95.8)
Abdominal pain	58 (98.3)	47 (97.9)
Flatulence	45 (76.3)	40 (83.3)
Constipation	17 (28.8)	15 (31.3)
Diarrhea	11 (18.6)	7 (14.6)
Mixed pattern	14 (23.7)	15 (31.3)
Mean baseline SF-36 score	$98.6 \pm 21.3$	$102.8 \pm 16.6$

**Table 2** Reason for dropout in both study groups *n* (%)

	Imipramine ( <i>n</i> = 59)	Placebo ( <i>n</i> = 48)	P-value
Total dropouts	28 (47.5)	23 (47.9)	NS
Premature withdrawal	8 (13.6)	14 (29.2)	< 0.05
Lost to follow-up	3 (5.1)	3 (6.3)	NS
Protocol violation	3	0	NS
Side effects	14 (23.7)	6 (12.5)	0.094

NS: Not significant.

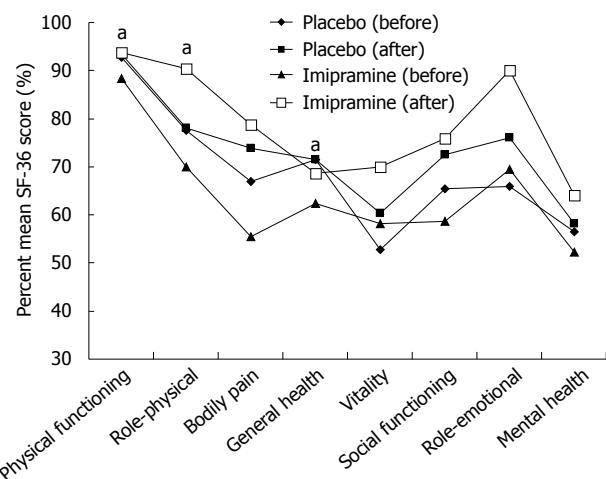
**Table 3** Side effect leading to dropout in the imipramine treatment group

Side effect	Number of patients ( <i>n</i> = 14)
Sleep disturbance	3
Urologic symptoms	2
Palpitations	2
Anxiety	1
Dry mouth	1
Dizziness	3
Flushing & sweating	1
Constipation	1

dropout, the difference was not statistically significant (25.4% *vs* 12.5%,  $P = 0.094$ ).

Sixteen patients (27.1%) from the imipramine group compared to 19 (39.6%) patients from the placebo arm opted for doubling of the dose at day 14 of treatment ( $P = 0.188$ ). There was no association between increasing the dose in the imipramine group and global symptomatic improvement, development of side effects, or adverse events leading to dropout; however, it was observed that 8/16 (50%) of patients whose treatment dose was doubled dropped out of the trial as compared to 15/38 (39.5%) patients maintained on the original dosage ( $P = 0.038$ ). Furthermore, 41.8% (28/67) of patients recruited from clinics dropped out compared to 57.5% (23/40) of self-referred patients ( $P = 0.12$ ), independent of assigned treatment ( $P = 0.10$  for imipramine;  $P = 0.60$  for placebo).

The change in QoL was assessed by asking patients to complete the SF-36 questionnaire upon onset of treatment and at completion of therapy (week 12). Only patients with paired completed SF-36 questionnaires



**Figure 3** Percent mean SF-36 before and after treatment ( $^aP < 0.05$ ).

were included in this analysis (PP group,  $n = 56$ ). Before beginning treatment, the mean SF-36 scores were similar for imipramine and placebo groups ( $96.1 \pm 25.0$  vs  $102.2 \pm 17.0$ ,  $P = 0.307$ ). After treatment, the mean SF-36 scores for the imipramine and placebo groups increased to  $113.7 \pm 19.4$  and  $108.6 \pm 15.9$ , respectively ( $P = 0.3$ ). The mean percent difference in SF-36 scores before and after treatment for imipramine was  $11.8\% \pm 13.2\%$  compared to  $4.3\% \pm 9.0\%$  for placebo ( $P = 0.02$ ). Further analysis of SF-36 results showed a trend for greater improvement in all components of the questionnaire in the imipramine arm compared to placebo (Figure 3).

## DISCUSSION

The current ROME II committee recommendations for conducting randomized, controlled trials in IBS have stressed the use of the Rome criteria to identify patients with IBS, and a randomized, parallel-trial, double-blinded design with no placebo run-in, with minimum treatment duration of 8–12 wk, follow-up of symptoms after treatment is stopped, and assessment of compliance with therapy. Moreover, trials should include baseline assessment of symptoms, account for patient disposition (discontinuations, withdrawals, etc), provide sample size calculation and enroll an adequate number of patients, with the primary outcome being improvement of global IBS symptoms based on patient assessment and/or use of a validated scale to assess IBS symptoms<sup>[3]</sup>. To date, 10 randomized, controlled trials and two crossover studies have evaluated the effectiveness of TCAs in the treatment of IBS<sup>[10–18]</sup>. The largest and best study to date on TCAs was done by Drossman *et al*<sup>[19]</sup>, investigating the role of desipramine in patients with functional disorders. However, the study involved patients with functional abdominal disorders (IBS, functional abdominal pain, painful constipation, and unspecified functional bowel disorders) and was therefore not restricted to IBS. Although the findings of that study supported a role of TCAs in functional bowel disorders, it is difficult to draw a firm conclusion regarding IBS

given the inhomogeneous study population. On the other hand, few of the other trials used the Rome criteria to identify patients with IBS, measured compliance, or presented sample size calculations. Most had small sample sizes, and only one trial was more than 8 wk in duration. A most recent study by Rajagopalan *et al*<sup>[11]</sup> met most recommendations for optimal study design but again suffered from a small sample size (20 patients per arm of whom nine dropped out from each group), and the lack of patient follow-up after the treatment was stopped. Our study meets almost all of the above requirements and is the largest randomized trial to date investigating the role of TCAs in patients with IBS. The study findings provide support for the efficacy of TCAs in the treatment of IBS, particularly after failure of antispasmodics, commonly used as first-line agents in IBS.

The fact that antidepressants are more consistent in improving global measures than specific GI symptoms has raised concerns about their true effect in functional GI disorders including IBS<sup>[20]</sup> where changes in symptoms are weak predictors of changes in well-being following treatment with TCAs<sup>[21]</sup>. Although controversial<sup>[22]</sup>, the use of global symptom improvement as the primary end point may arguably be more important because of the wide and varied symptomatology of IBS and functional abdominal symptoms, and the varying importance that patients place on particular symptoms. This helps overcome inherent disadvantages of symptom score systems, which measure physiologic epiphénoména such as stool characteristics and subtypes of IBS<sup>[23]</sup>, but do not address the impact of this on global well-being.

The mechanism of action of TCAs in IBS remains unclear but appears to primarily involve a modulation of the brain-gut neurologic axis. Using functional magnetic resonance imaging, Morgan *et al*<sup>[24]</sup> have shown that rectal pain following balloon distension induced significant activation of the perigenual anterior cingulate cortex, right insula and right prefrontal cortex, and that amitriptyline use was associated with reduced pain-related cerebral activation in some centers, but only during stress. This effect was thought to occur in the central nervous system, rather than peripherally, to blunt pain and other symptoms exacerbated by stress in IBS. Another putative mechanism of action of TCAs is by modulation of gut motility *via* action on peripheral muscarinic receptors and/or on ATP-sensitive K<sup>+</sup> channels in interstitial cells of Cajal<sup>[25,26]</sup>. Identifying an anxiety or affective disorder is not necessary for initiating TCAs since they appear to have an analgesic advantage on somatic pain independent of psychiatric effects. Response to TCAs may in fact be attenuated in the presence of active psychiatric illness<sup>[27,28]</sup>. Because of their side effects, particular effort must be taken to initiate treatment with TCAs. In our study, major side effects leading to premature dropout were more common in the imipramine group and were primarily anticholinergic side effects. The incidence of adverse events was similar to previously published trials of TCAs in patients with functional GI disorders or neuropathic pain<sup>[29]</sup>. This emphasizes the need to warn

patients about potential side effects of TCAs and weigh these against any expected or perceived benefit.

Health-related QoL is affected significantly in patients with IBS compared to the general population. The health-related QoL is associated with perceived IBS severity defined by the overall disease limitations rather than symptoms and appears to improve in treatment responders and correlates with symptom improvement<sup>[30,31]</sup>. For that reason, the SF-36 model, a general health status instrument, was used in our study to assess QoL. We found that the post-treatment SF-36 mean total score was significantly higher than the pre-treatment score in the imipramine group only with a notable improvement in all components of the health-related QoL instrument used.

The limitations of this study include the single center nature of the study, the lack of formal assessment of baseline co-morbid psychiatric distress and the possibility of unblinding given the untoward, primarily anticholinergic adverse events of TCA in some patients. This potential unblinding is unlikely, however, to have biased the measured patient-specific endpoints of global symptom relief or the SF-36 components. Other limitations include the fact that dose escalation was allowed at week 2 and the high dropout rate noted in both the imipramine and placebo arms (47.5% vs 47.9%, respectively) as well as the inability to meet the preset sample size for the study. The restriction of dose escalation to a single early time point (at 2 wk) was felt to best reflect the real-life clinical situation where dose adjustments are often made in the dose of TCAs according to patient response and possible drug-related adverse events. The high dropout rate in our trial can be explained by several factors. First, the IBS patient population is hard to study because of an existing high degree of psychosomatization. Second, and perhaps more importantly, these patients have an often unrealistic expectation of rapid symptomatic relief or even cure, and a 12-wk period may therefore constitute an unduly long period of time to "experiment" with a placebo or a drug that they may perceive as investigational and possibly ineffective. It is for this reason that most large studies on functional GI disorders have included a 2-4 wk run-in phase wherein patients who are more likely to continue the trial period are consequently selected. However, and despite these run-in or screening periods, dropout rates in these large trials remain in the order of 20%-25%<sup>[32,34]</sup>. Lastly, and perhaps most importantly, adherence may have been further reduced in our patient population for cultural reasons such as the unfamiliar concept of a "placebo" control arm and the fact that the use of the active drug in this particular study (a medication approved for the use of depression) may suggest or connote a "psychiatric" label to the patients' condition. These factors, as well as the single center nature of the study, may have contributed to our inability to reach the preset sample size and to the premature closure of the study. The above notwithstanding, this study remains the largest placebo-controlled trial of TCAs in patients with IBS as defined by strict clinical criteria.

In conclusion, this randomized, double-blind, place-

bo-controlled trial provides evidence in support of the efficacy of imipramine in reducing symptoms of IBS and providing global relief. Symptom improvement is associated with improved QoL. Careful patient selection and education about the role of TCAs, the rationale for their use, and the recognition of potential side effects are important considerations. Gradual dose escalation, perhaps in small increments of 10 mg, and close monitoring are likely to result in a better therapeutic response.

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## COMMENTS

### Background

Tricyclic antidepressants (TCAs) were found to improve abdominal pain in irritable bowel syndrome (IBS) patients. This trial aimed to study the effect of imipramine, a TCA, on global symptom relief in patients with IBS.

### Research frontiers

TCAs have been shown in a few trials to improve abdominal pain in patients with IBS; however there was inadequate evidence to support an effect on improvement of global IBS symptoms. Notably, most randomized, controlled trials of TCAs in IBS were completed before the publication of the ROME II committee recommendations for the study design of treatment trials for IBS and suffered from suboptimal study design, small sample size, and short treatment duration.

### Innovations and breakthroughs

This trial did not include a run-in-phase in order to simulate the real-life scenario in the clinic, and to obtain a valid assessment of the utility and compliance with TCAs. It was rigorously designed and included a formal assessment of quality of life (QoL) indicators and followed all the recommendations of the Rome committee on the optimal design of IBS trials. Only a few published trials on TCAs have done so and all included less than 20 patients per arm, making this trial the largest ever conducted on the use of TCAs in patients with IBS.

### Applications

IBS remains a common intestinal disorder causing significant discomfort and poor QoL to patients who have the diagnosis. TCAs have been shown to improve abdominal pain in patients with IBS; however, there is insufficient evidence of global symptom relief. The search for an optimal treatment to improve symptoms and QoL in IBS remains ongoing.

### Terminology

IBS is a common disorder of the gastrointestinal tract characterized by abdominal pain or discomfort and altered bowel habits. The exact pathophysiology of IBS remains unclear; however, it is thought to involve altered intestinal motility as a result of dysregulated communication between the brain and enteric nervous system. The mechanism by which TCAs affect this neural communication is yet unknown.

### Peer review

Few trials have adequately studied the effect of TCAs on global relief of symptoms in patients with IBS. The aim of this study was to evaluate the efficacy and safety of imipramine in patients with IBS who have failed to respond satisfactorily to antispasmodics.

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## Association of Fas/Apo1 gene promoter (-670 A/G) polymorphism in Tunisian patients with IBD

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### Abstract

**AIM:** To detect a possible association between the polymorphism of the (-670 A/G) Fas/Apo1 gene promoter and susceptibility to Crohn's disease (CD) and ulcerative colitis (UC) in the Tunisian population.

**METHODS:** The (-670 A/G) Fas polymorphism was analyzed in 105 patients with CD, 59 patients with UC, and 100 controls using the polymerase chain reaction restriction fragment length polymorphism method.

**RESULTS:** Significantly lower frequencies of the Fas -670 A allele and A/A homozygous individuals were observed in CD and UC patients when compared with controls. Analysis of (-670 A/G) Fas polymorphism with respect to sex in CD and UC showed a significant difference in A/A genotypes between female patients and controls ( $P$  corrected = 0.004 in CD patients and  $P$  corrected = 0.02 in UC patients, respectively). Analysis also showed a statistically significant association between genotype AA of the (-670 A/G) polymorphism and the ileum localization of the lesions ( $P$  corrected = 0.048) and between genotype GG and the colon localization ( $P$

corrected = 0.009). The analysis of inflammatory bowel disease patients according to clinical behavior revealed no difference.

**CONCLUSION:** Fas-670 polymorphism was associated with the development of CD and UC in the Tunisian population.

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**Key words:** Fas/Apo1; Gene polymorphisms; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis

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### INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic disorder of the gastrointestinal tract characterized by immune dysregulation and leukocyte recruitment<sup>[1]</sup>. IBD may manifest as either Crohn's disease (CD) or ulcerative colitis (UC), which are two distinct forms of IBD with some common clinical, epidemiological, and immunological features, but they can be distinguished by anatomical and histological features as well as by serologic markers<sup>[2,3]</sup>.

The etiology of IBD is unknown, but the condition seems to be the result of a combination of environmental, genetic, and immunologic factors in which an uncontrolled immune response within the intestinal lumen leads to inflammation in genetically predisposed individuals<sup>[4]</sup>.

Genetic factors are known to play an important role in determining an individual's susceptibility to IBD. Significant linkages in chromosomes 1, 3, 6, 7, 12, 14, 16 and 19 have been reported<sup>[5-8]</sup>. However, IBD-1, a susceptibility locus in chromosome 16, was the first gene to be clearly associated with CD<sup>[9,10]</sup>.

Although the underlying genetic and environmental causes remain to be elucidated, CD4+ T helper 1 (Th1) lymphocytes play a pivotal role in the pathogenesis of these diseases. A Th1-like phenotype, with its signature cytokines interferon (IFN)- $\gamma$  and tumor necrosis factor (TNF)- $\alpha$ , is shared among many colitis models and is found in patients with active CD<sup>[1,11,12]</sup>. Moreover, IFN- $\gamma$  not only cooperates with TNF- $\alpha$  to cause colonic cell death, it also modulates epithelial Fas expression and sensitizes colonic epithelial cells to Fas-induced apoptosis<sup>[1]</sup>.

Programmed cell death, or apoptosis, is regulated by tightly controlled intracellular signaling events in response to pathological cytotoxic stimuli including TNF- $\alpha$ , TNF-related apoptosis-inducing ligand (TRAIL), and Fas<sup>[13-15]</sup>. Fas, a member of the tumor necrosis factor superfamily, is constitutively expressed by the basolateral membrane of normal colon and small intestinal epithelium<sup>[11,12,16-19]</sup>. Fas ligation induces apoptosis in colonic epithelial cells and is implicated in the epithelial damage seen in ulcerative colitis<sup>[11,12,16-19]</sup>. The ligand for Fas (FasL) is expressed by intraepithelial and lamina propria lymphocytes, and its expression is increased in the lamina propria of UC patients, suggesting that Fas-FasL-induced apoptosis participates in the mucosal damage of UC<sup>[11,12]</sup>. Inhibition of cytokine-regulated apoptosis may therefore be useful in preventing or treating intestinal lesions in patients with IBD.

The Fas/Apo-1 gene has been mapped to the chromosome 10q24.1 region<sup>[20]</sup>. The gene consists of nine exons and eight introns. Two polymorphisms located in the promoter region of the Fas gene have been reported<sup>[21]</sup>. One of these polymorphisms is a single nucleotide substitution (A/G) at the -670 position. Several studies have shown an association between this A/G single nucleotide polymorphism (SNP) and autoimmune diseases such as type 1 diabetes<sup>[22]</sup>, multiple sclerosis<sup>[6]</sup>, Sjögren's syndrome<sup>[23]</sup>, rheumatoid arthritis<sup>[24]</sup>, and systemic lupus erythematosus<sup>[6,24]</sup>.

In this study, we have analyzed the Fas/Apo1 gene promoter (-670 A/G) polymorphism in unrelated Tunisian patients with CD and UC to evaluate the contribution of the Fas/Apo1 (CD95) gene to genetic susceptibility to IBD.

## MATERIALS AND METHODS

### Patients

Blood samples were obtained from 164 subjects with IBD, composed of 13 cases of familial forms and 151 cases of sporadic forms. These patients were classified into 105 patients with CD (50 men, 55 women) with a mean age of 36.07 years (range, 23-60 years), and 59 patients with UC (17 men, 42 women) with a mean age of 37.89 years (range, 25-74 years). A total of 100 healthy individuals (52 men and 48 women) matched for age, sex and ethnicity were included as controls. None of the healthy controls had any evidence of autoimmune diseases such as inflammatory bowel disease, diabetes, or

other autoimmune diseases. All subjects were unrelated Tunisians treated at the Department of Gastroenterology of Charles Nicolle and La Rabta Hospitals in Tunis. The diagnoses of CD and UC were determined according to conventional endoscopic, radiological, and histological criteria<sup>[25]</sup>. Data obtained from each patient included age at diagnosis, disease location, disease characteristics, and extraintestinal location, which were used to group the patients according to the Vienna classification<sup>[26]</sup>. All patients and controls gave informed consent to participate in this study which was approved by the Ethics Committee of Charles Nicolle Hospital in Tunis.

### Methods

Transition A/G, in position -670 of the promoter of the Apo1/Fas gene, was analyzed by the polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) method. The Apo1/Fas marker frames an area of 232 Pb in the area of the promoter and was able to contain a site of cut for the enzyme Bst I (Promega). The digestion of the amplified fragment gave rise to two types of allele: the allele G marked by the presence of two fragments 188 Pb and 232 Pb and the allele A characterized by the absence of cut (A), therefore a fragment of 232 Pb. The PCR was carried out in a final volume of 20  $\mu$ L containing 5  $\mu$ L of genomic DNA at 40 ng/ $\mu$ L, 1 U of *Taq* polymerase, 1.5 mmol/L of MgCl<sub>2</sub>, 0.2 mmol/L of dNTP and 10 pmol of each primer.

The primers used were: Fas (1); 5'-CTACCTAAGA GCTATCTACCGITTC-3' and Fas (2); 5'-GGCTGTCC ATGTTGTGGCTGC-3'. The amplification of product was carried out on 2% agarose gel and visualized under UV. Ten microliter of the amplified fragments were then digested by 5 U of Bst I in a final volume of 20  $\mu$ L and the mixture was left 2.5 h at 60°C. The separation of the products of digestion was performed on 3% agarose gel and visualized under UV.

### Statistical analysis

Frequencies of the genotypes, alleles, and phenotypes were analyzed by using the  $\chi^2$  test. The odds ratio (OR) and 95% confidence interval were calculated to measure the strength of the association observed. Hardy Weinberg equilibrium was tested by calculating the  $\chi^2$  for reliability of fit. Calculations were made by using Internet programs from [www.myatt.demon.co.uk/epicalc.htm](http://www.myatt.demon.co.uk/epicalc.htm). Statistical significance was defined as  $P < 0.05$ .  $P$  values were corrected by Bonferroni correction for multiple comparisons, taking into account the number of alleles studied.

## RESULTS

As shown in Table 1, Crohn's disease (64%) was more frequent than ulcerative colitis (36%) in the study. The peak of the disease is between 20 and 40 years. The familial forms represent 8.57% of the CD cases and 6.77% of the UC cases. The principal clinical manifestations observed during evolutionary flares of

**Table 1** Clinical characteristics of study subjects *n* (%)

	<b>CD (<i>n</i> = 105)</b>	<b>UC (<i>n</i> = 59)</b>
Sex (Male/female)	50/55	17/42
Age average	36.07	37.89
Age (Min/max)	23/60	25/74
Familial forms	9 (8.57)	4 (6.77)
Surgical history	11 (10.47)	4 (6.77)
Activity of the disease ( <i>n</i> )	In remission (75) Moderated (18) Severe (12)	In remission (46) Moderated (13) Severe (0)
Location of lesion, ( <i>n</i> )	Ileum (27) Colon (24) Ileocolon (54)	Distal (21) Left-sided colitis (26) Pancolitis (12)
Forms, <i>n</i> (%)	Stenosing, 43 (40.95) Fistula, 37 (35.23)	
Extra-intestinal manifestation	31 (29.53)	5 (8.47)
Evolution, <i>n</i> (%)	Favorable, 89 (84.16) Cortico dependency, 12 (11.42) Cortico-resistance, 2 (1.92) Unfavourable, 2 (1.92)	Cortico dependency, 1 (1.69) Cortico-resistance, 0 Unfavourable, 0
Production of pANCA	43	28
Production of ASCA	22	9
Treatments	5-ASA, 32 (30.47)	5-ASA, 58 (98.30)

**Table 2** Allelic and genotypic frequency of (-670 A/G) Apo1/Fas gene polymorphisms in patients with inflammatory bowel disease and controls *n* (%)

<b>Apol/Fas (-670)</b>	<b>Controls (<i>n</i> = 100)</b>	<b>IBD (<i>n</i> = 164)</b>	<b>UC (<i>n</i> = 59)</b>	<b>CD (<i>n</i> = 105)</b>
Genotypic frequencies				
A/A	49 (49)	46 (28.04) <sup>c</sup>	15 (25.42) <sup>b</sup>	31 (29.52) <sup>a</sup>
A/G	32 (32)	70 (42.68)	30 (50.85)	40 (38.10)
G/G	19 (19)	48 (29.26)	14 (23.73)	34 (32.38)
Allelic frequencies				
A	130 (70)	162 (49.39) <sup>d</sup>	60 (50.84) <sup>f</sup>	102 (48.57) <sup>e</sup>
G	70 (35)	166 (50.61)	58 (49.16)	108 (51.43)

<sup>a</sup>Comparing AA genotype frequencies in CD patients with controls: *P* corrected = 0.004, OR: 0.44, 95% CI, 0.24 < OR < 0.81; <sup>b</sup>Comparing AA genotype frequencies in UC patients with controls: *P* corrected = 0.0034, OR: 0.35, 95% CI, 0.16 < OR < 0.76; <sup>c</sup>Comparing AA genotype frequencies in IBD patients with controls: *P* corrected = 0.0005, OR: 0.41, 95% CI, 0.23 < OR < 0.70; <sup>d</sup>Comparing A/Allele frequencies in IBD patients with controls: *P* corrected = 0.0004, OR: 0.53, 95% CI, 0.36 < OR < 0.77; <sup>e</sup>Comparing A/Allele frequencies in CD patients with controls: *P* corrected = 0.0007, OR: 0.57, 95% CI, 0.34 < OR < 0.77; <sup>f</sup>Comparing A/Allele frequencies in UC patients with controls: *P* corrected = 0.01, OR: 0.56, 95% CI, 0.34 < OR < 0.91. IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease; OR: Odds ratio; CI: Confidence interval.

the disease depended on the site: Ileum (25.71%), Colon (22.85%) and Ileocolon (51.42%) for CD patients and Pancolitis (20.33%), Distal (35.59%) and Left-sided colitis (44.06%) for UC patients. Results for the genotyping of the Fas/Apo1 gene promoter (-670 A/G) SNP in 105 patients with CD, 59 subjects with UC, and 100 healthy controls are summarized in Table 2. The allele frequencies were in Hardy-Weinberg equilibrium both in the patients and in controls. We have compared the A/A genotype frequencies found in various subgroups of IBD patients with those in the entire control population.

When comparing CD patients with the control group, the frequency of the -670 A allele was found to be significantly lower in CD than in controls. Also, the distribution of the -670 A/A genotype was significantly lesser in CD patients than in controls. When comparing UC patients with the control group, the frequencies of the -670 A allele and the homozygous -670 A/A genotype were lower in UC patients than in controls and these differences were statistically significant. When we analyzed the Fas/Apo1 gene promoter (-670 A/G) polymorphism with respect to gender (Table 3), we found that Fas/Apo1 genotype frequencies were similar in controls of both sexes (48 females and 52 males). However, the frequency of CD female patients carrying the -670 A/A genotype was significantly less when compared with the control group. The same result was obtained in women with UC when compared with those in the control group. Thus, the homozygosity for the A allele could be a protective factor for development of IBD in female patients. An analogous analysis has been done on male patients with CD and UC. The results did not show a statistically significant association for either CD or UC patients compared with the control group. Subsequently, we sought to investigate whether this polymorphism could be linked to a particular clinical phenotype. When stratifying CD patients according to the Vienna classification, as shown in Table 4, we found a statistically significant association ( $\chi^2 = 3.89$ , two degrees of freedom, *P* corrected = 0.048) odds ratio of 2.48 (0.90 < OR < 6.87, CI = 95%) between genotype AA of the Fas/Apo1 gene promoter (-670 A/G) polymorphism and the ileum localization of the lesions. In addition, we found a statistically significant association ( $\chi^2 = 6.74$ , two degrees of freedom, *P* corrected = 0.009) odds ratio of 3.38 (1.19 < OR < 9.67, CI = 95%) between genotype GG of the polymorphism and the colon localization of the lesions. Analysis of IBD patients according to clinical behavior revealed no difference between those carrying

**Table 3** Incidence of (-670 A/G) Apo1/Fas gene polymorphism analyzed with respect to sex in IBD patients *n* (%)

Apol/Fas	Controls (n = 100)	CD females (n = 55)	CD males (n = 50)	UC females (n = 42)	UC males (n = 17)
Genotypic frequencies					
A/A	49 (49)	14 (25.45) <sup>a</sup>	17 (34)	12 (28.57) <sup>b</sup>	5 (29.41)
A/G	32 (32)	24 (43.64)	16 (32)	23 (54.76) <sup>c</sup>	7 (41.18)
G/G	19 (19)	17 (30.91)	17 (34)	7 (16.67)	5 (29.41)
Allelic frequencies					
A	130 (70)	52 (47.27)	50 (50)	47 (55.95)	17 (50)
G	70 (35)	58 (52.73)	50 (50)	37 (44.05)	17 (50)

Fas genotypes were similar in controls of both sexes: 52 males and 48 females;  $\chi^2 = 0.06$ ,  $P = 0.8$ , two degrees of freedom; <sup>a</sup> $\chi^2$  test of heterogeneity between CD females and controls ( $\chi^2 = 8.15$ , two degrees of freedom,  $P$  corrected = 0.004) odds ratio for AA genotype is 0.36 (0.16 < OR < 0.77, CI = 95%); <sup>b</sup> $\chi^2$  test of heterogeneity between UC females and controls ( $\chi^2 = 5.04$ , two degrees of freedom,  $P$  corrected = 0.02) odds ratio for AA genotype is 0.42 (0.18 < OR < 0.96, CI = 95%); <sup>c</sup> $\chi^2$  test of heterogeneity between UC females and controls ( $\chi^2 = 6.46$ , two degrees of freedom,  $P$  corrected = 0.01) odds ratio for AG genotype is 2.57 (1.15 < OR < 5.76, CI = 95%).

or not carrying the (-670 A/G) allele (data not shown). We did not find an association between (-670 A/G) polymorphism and the severity of disease in any of the IBD patients, as defined by the need for surgery (data not shown).

## DISCUSSION

There are several candidate genes potentially involved in the pathogenesis of IBD because of chromosomal location or function within inflammatory processes or both<sup>[27]</sup>. Much investigation has surrounded the FasL system, a major pathway responsible for inducing apoptosis of T cells and enterocytes in the colonic mucosa<sup>[28]</sup>. Fas is a type I transmembrane protein and a member of the TNF receptor superfamily that may be expressed constitutively by gut lamina propria T (LPT) cells<sup>[29]</sup>. FasL is a type II transmembrane protein that is expressed on cytotoxic T cells and that induces apoptosis of cells expressing Fas<sup>[30]</sup>. Defective apoptosis of LPT cells may be a factor in mucosal immune dysregulation and tissue inflammation. Rioux *et al*<sup>[31]</sup> found that 15% of LPT cells underwent apoptosis in normal individuals. There was a marked reduction in apoptosis of LPT cells in patients with UC and CD and those with specific colitis. In the present study, we genotyped Fas-670 polymorphisms in Tunisian patients with IBD and healthy controls, and found that the frequencies of the A allele and the AA genotype were significantly lower in patients with IBD compared with those in the control group, and in UC patients compared to CD patients, but here the difference was not significant. Thus, we have demonstrated an association between a promoter polymorphism of the (-670 A/G) Fas/Apo1 gene and IBD in this study. The significance of this association may differ according to the population studied and the type of inflammatory bowel disease. In fact, no association of (-670 A/G)

**Table 4** Association between (-670 A/G) Apo1/Fas gene polymorphism and location of lesion

Apol/Fas	UC (n = 59)			CD (n = 105)			
	G/G	G/A	A/A	G/G	G/A	A/A	
Distal	4	9	8	Ileum	5	10	12 <sup>a</sup>
Left-sided colitis	5	15	6	Colon	13 <sup>b</sup>	8	3
Pancolitis	3	8	1	Ileocolon	16	22	16

<sup>a</sup> $\chi^2$  test between AA genotype and ileum location of lesion in CD patients ( $\chi^2 = 3.89$ , two degrees of freedom,  $P$  corrected = 0.048) odds ratio is 2.48 (0.90 < OR < 6.87, CI = 95%); <sup>b</sup> $\chi^2$  test between GG genotype and colon location of lesion in CD patients ( $\chi^2 = 6.74$ , two degrees of freedom,  $P$  corrected = 0.009) odds ratio is 3.38 (1.19 < OR < 9.67, CI = 95%).

Fas/Apo1 SNP with IBD in Chinese, Dutch, Australian and Korean patients exists<sup>[32-35]</sup>. Confusingly, in some studies the A variant was found as a susceptibility factor for rheumatoid and multiple sclerosis disease. Stratification analyses revealed that the association was stronger in males than in females. Sex effects in IBD have already been reported by Huang *et al*<sup>[36]</sup> who have identified several putative regions of sex-specific linkage. Regions on chromosomes 6, 11, 14 and 18 showed strong evidence of linkage in male-affected families but not in female-affected families. Moreover, oral contraceptives have been shown to be associated with increased risk for CD<sup>[37]</sup>. It has been proposed that epigenetic factors play an important role in the pathogenesis of IBD<sup>[38]</sup>, and that sex effects are mediated by sexual hormones, which have an effect on gene expression and consequently could lead to differential expression of disease susceptibility genes in males and females. According to our findings, it appears that Fas/Apo1 gene polymorphisms decrease susceptibility to CD and UC in females in the Tunisian population. However, because there was no evidence of linkage of the Fas/Apo1 gene with the X chromosome, it is possible that this difference could be a random variation. On the other hand, it appears that CTLA-4 +49 gene polymorphisms increase susceptibility to CD in females in the Tunisian population and in the same patients as have Fas/Apo1 polymorphisms. No explanations have been presented for the discrepancy between positive findings, like ours, and the negative findings of others. The most plausible is the known genetic diversity of the different populations at the haplotype level. Our present results and the lack of previous studies on the sex distribution of Fas/Apo1 polymorphisms in patients with IBD warrant further investigation. The reason for this divergence is not clear but might reflect an ethnic difference in the contribution of genetic factor(s).

Fas-670 polymorphism within the promoter region is situated at a transcriptional binding site (the  $\gamma$  interferon activation site) and may potentially have a functional effect on gene regulation of factors such as STATs as well as on expression of proapoptotic proteins (Fas, FasL). In fact, in patients with UC, concentration of a systemic soluble form of Fas was found to be significantly lower in active UC than in controls<sup>[35]</sup>. Garcia Rodriguez *et al*<sup>[39]</sup> have studied Fas/FasL expression on activated colonic T

cells of UC patients, as well as the susceptibility of such T cells to activation-induced cell death, and have reported that CD4+ and CD8+ T cells in UC mucosa expressing FasL were significantly enhanced, suggesting that T cells in UC are less sensitive to apoptotic signals mediated by Fas. A few studies reported the association between the Fas/Apo1 gene promoter (-670 A/G) polymorphism and the localization of the lesions in IBD. Xia *et al*<sup>[40]</sup> have not found a difference between left-sided and total colitis ( $P = 0.8242$ ). Moreover, in our study we found an association between ileum and colon localization of the lesions in CD patients and (-670 A/G) polymorphism.

Regarding the Fas-670 polymorphism, it has been hypothesized that either increased apoptosis of intestinal epithelium or decreased apoptosis of lamina propria lymphocytes may induce inflammation of the gut and, although expression and functional effects of the Fas antigen have been found to be associated with IBD, the relationship between Fas-670 polymorphism and IBD has not been reported yet. However, in this study, a Fas-670 polymorphism was associated with the development of CD and UC, providing strong support for an IBD susceptibility gene in the region surrounding Apo1/Fas. It remains to be determined precisely how the Fas alleles influence the pathogenesis of IBD. Finally, it is possible that haplotypes exist in our population which we may have missed. We conclude that our results must await confirmation by other investigators.

## COMMENTS

### Background

In recent years, a few studies have been published addressing the question of where and under what conditions ligand for Fas (FasL) is produced in the gut in the normal and neoplastic situation. Some of these studies have considerably influenced our view of the role of the CD95/CD95L system. That is why it is necessary to analyze the Fas/Apo1 polymorphism in unrelated Tunisian patients with Crohn's disease (CD) and ulcerative colitis (UC) to evaluate the contribution of the CD95 gene to genetic susceptibility to inflammatory bowel disease (IBD).

### Research frontiers

Inhibition of cytokine-regulated apoptosis may be useful in preventing or treating intestinal lesions in patients with IBD.

### Innovations and breakthroughs

The relationship between Fas-670 polymorphism and IBD has not been reported yet. This is probably the first report on the association of Fas-670 polymorphisms in Tunisian IBD patients. However, this polymorphism was associated with the development of CD and UC, providing strong support for an IBD susceptibility gene in the region surrounding Apo1/Fas.

### Applications

By understanding how the Fas-670 polymorphism is associated with the development of CD and UC, this study may indicate a future strategy for therapeutic intervention in patients with IBD.

### Peer review

The manuscript by Ben Aleya *et al* reports on the association of a Fas/Apo1 promoter polymorphism with IBD in Tunisian patients. The experiments are performed well and the results are interesting.

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# Endoscopic retrograde cholangiopancreatography during pregnancy without radiation

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## Abstract

**AIM:** To present our experience with pregnant patients who underwent endoscopic retrograde cholangiopancreatography (ERCP) without using radiation, and to evaluate the acceptability of this alternative therapeutic pathway for ERCP during pregnancy.

**METHODS:** Between 2000 and 2008, six pregnant women underwent seven ERCP procedures. ERCP was performed under mild sedoanalgesia induced with pethidine HCl and midazolam. The bile duct was cannulated with a guidewire through the papilla. A catheter was slid over the guidewire and bile aspiration and/or visualization of the bile oozing around the guidewire was used to confirm correct cannulation. Following sphincterotomy, the bile duct was cleared by balloon sweeping. When indicated, stents were placed. Confirmation of successful biliary cannulation and stone extraction was made by laboratory, radiological and clinical improvement. Neither fluoroscopy nor spot radiography was used during the procedure.

**RESULTS:** The mean age of the patients was 28 years (range, 21-33 years). The mean gestational age for the fetus was 23 wk (range, 14-34 wk). Five patients underwent ERCP because of choledocholithiasis and/or

choledocholithiasis-induced acute cholangitis. In one case, a stone was extracted after precut papillotomy with a needle-knife, since the stone was impacted. One patient had ERCP because of persistent biliary fistula after hepatic hydatid disease surgery. Following sphincterotomy, scoleces were removed from the common bile duct. Two weeks later, because of the absence of fistula closure, repeat ERCP was performed and a stent was placed. The fistula was closed after stent placement. Neither post-ERCP complications nor premature birth or abortion was seen.

**CONCLUSION:** Non-radiation ERCP in experienced hands can be performed during pregnancy. Stent placement should be considered in cases for which complete common bile duct clearance is dubious because of a lack of visualization of the biliary tree.

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**Key words:** Cholangitis; Choledocholithiasis; Endoscopic retrograde cholangiopancreatography; Jaundice; Pregnancy

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## INTRODUCTION

Hormonal changes during pregnancy increase the lithogenicity of bile and impair gallbladder emptying, which create a favorable environment for gallstone formation. The prevalence of gallstones in pregnancy has been reported as 3.3%-12.2%<sup>[1-3]</sup>. Acute cholecystitis is the second most common non-obstetric emergency in pregnant women. Choledocholithiasis and consequent

**Table 1** Demographic and laboratory results of the patients

Patient number	Age (yr)	WBC/ $\mu$ L	AST (IU/L)	ALT (IU/L)	GGT (IU/L)	Bilirubin (mg/dL)	Ultrasonography	MRCP
1	32	10.800	61	58	144	1.09	Normal	Fistula presence
1 <sup>1</sup>	32	6.690	52	54	136	0.75	Normal	Fistula persistence
2	33	12.000	244	186	324	16.5	Stone in CBD	-
3	27	11.500	126	92	212	10.8	Stone in CBD	-
4	21	14.000	204	144	289	4.85	Stone in CBD	-
5	28	15.200	195	127	326	15.3	Stone in CBD	-
6	29	14.300	262	210	188	8.22	Dilated CBD	Stone in CBD

<sup>1</sup>Same patient with repeat ERCP. WBC: White blood cells; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CBD: Common bile duct.

complications such as pancreatitis and cholangitis are potentially lethal diseases for the mother and fetus. Acute pancreatitis in pregnancy has been shown to be closely related to gallstone disease<sup>[4]</sup>. Biliary pancreatitis has been found to be associated with 15% and 60% of maternal and fetal mortality rates, respectively<sup>[5]</sup>.

During pregnancy, the treatment is usually conservative since surgery is associated with an increased rate of complications such as preterm labor and spontaneous abortion. In choledocholithiasis, endoscopic retrograde cholangiopancreatography (ERCP) is the first-line treatment of choice. One should try to avoid the use of fluoroscopy during pregnancy, especially in the first trimester. ERCP with 3.2 min mean fluoroscopy time and 310 mrad estimated mean fetal radiation exposure has been found to be safe for the mother and fetus during pregnancy<sup>[6]</sup>. However, a clear-cut safe radiation dose for ERCP in pregnancy is still unknown. Here, we present our experience with pregnant women who underwent ERCP without using radiation.

## MATERIALS AND METHODS

Seven hundred and fifty ERCP procedures for choledocholithiasis and/or choledocholithiasis-induced acute cholangitis were performed on 597 patients in our Endoscopy Unit at Vakif Gureba Research and Training Hospital from 2000 to 2008. Six out of 598 ERCP patients were pregnant. One patient with biliary fistula caused by surgery for hepatic hydatid disease was also included in the study. The data regarding laboratory, ultrasonographic and endoscopic findings, and clinical course of the patients were analyzed. Magnetic resonance cholangiopancreatography (MRCP) was used for diagnosis of fistula after hydatid surgery, and for control of fistula closure after the first ERCP procedure in the patient with hepatic hydatid disease. MRCP was also used for diagnosis of choledocholithiasis in the patient with elevated bilirubin levels and a dilated common bile duct shown by ultrasonography. Patients with fever, jaundice, abdominal pain and radiological findings were diagnosed with choledocholithiasis-induced acute cholangitis.

All patients included in the study signed an informed consent for the procedures, after receiving an explanation on the risks, benefits and alternatives of ERCP and associated therapeutic procedures.

The patients were counseled by the Gynecology and Obstetrics Department before the procedure. Pre-procedural complete blood count, bilirubin, transaminase and  $\gamma$ -glutamyl transferase levels were noted. The fetal heart sounds were controlled before the ERCP procedure. Pethidine HCl ( $\leq 100$  mg) and midazolam ( $\leq 3$  mg) were used for analgesia and sedation. The patients were positioned in the left lateral position to avoid vena caval or aortic compression. After introducing the duodenoscope into the duodenum, the bile duct was cannulated with a guidewire through the papilla, and bile aspiration and/or visualization of the bile oozing around the guidewire was used to confirm correct cannulation. Following sphincterotomy, the stones were extracted by balloon sweeping. When indicated, stents were placed.

Confirmation of successful therapeutic ERCP was made by laboratory and clinical improvement of the patients. After all ERCP procedures, one experienced and the same radiologist performed ultrasonographic evaluation of the biliary system in order to eliminate inter-observer variations. Neither fluoroscopy nor spot radiography were used during the procedure. After the ERCP procedure, laboratory findings and clinical progression of the patients were recorded and followed until discharge.

## RESULTS

The mean age of the patients was 28 years (range, 21-33 years). The mean gestational age of the fetus was 23 wk (range, 14-34 wk). The demographic and laboratory data of the patients are shown in Table 1. The treatment results and obstetric findings of the patients are summarized in Table 2. There were no complications related to sedoanalgesia in the mother and fetus after the procedure.

In one case, stone extraction after precut papillotomy with a needle-knife was performed since the stone was impacted. One patient had ERCP because of persistent biliary fistula after hepatic hydatid disease surgery. Following sphincterotomy performed by bipolar current, scoleces were extracted from the common bile duct. Two weeks later, since the fistula persisted, repeat ERCP was performed and a polyethylene stent was placed. The fistula was closed after the procedure. The laboratory abnormalities of the other patients with choledocholithiasis and/or choledocholithiasis-

**Table 2** Treatment results and obstetric findings

Patient number	Diagnosis	Procedure	CBD content	Gestational time (wk)	Delivery time (wk)
1	Biliary fistula <sup>1</sup>	Sphincterotomy and scolices extraction	Scolices	19	
1	Persistent fistula <sup>2</sup>	Stent placement	-	21	Term
2	Choledocholithiasis	Sphincterotomy and stone extraction	Stone	14	34
3	Choledocholithiasis	Sphincterotomy and stone extraction	Stone	20	36
4	Choledocholithiasis	Sphincterotomy and stone extraction	Multiple stones	34	Term
5	Cholangitis	Sphincterotomy and stone extraction	Impacted stone	28	36
6	Choledocholithiasis	Sphincterotomy and stone extraction	Multiple stones	23	Term

<sup>1</sup>After hydatid disease surgery; <sup>2</sup>Same patient with repeat ERCP.

induced acute cholangitis were normalized after ERCP. Ultrasonographic confirmation of stone extraction was made in all patients. Post-ERCP complications, premature birth, abortion or intrauterine growth retardation were not observed. The clinical follow-up of the patients until discharge was uneventful.

## DISCUSSION

Cholangitis in pregnancy is a serious condition for the mother and fetus, and results in high mortality and morbidity. ERCP is the first-line treatment of choice for cholangitis and pancreatitis caused by choledocholithiasis. However, the use of ERCP in pregnancy is limited because of the use of radiation. In previously described ERCP procedures performed during pregnancy, abdominal shielding was used to minimize radiation exposure<sup>[7]</sup>. Fetal damage by radiation is related to the time of pregnancy and the radiation dose. Several studies have demonstrated that ERCP can be successfully and safely performed during pregnancy<sup>[2,6,8-10]</sup>. The role of ERCP during pregnancy for complicated biliary tract diseases is well established. However, there remains the primary concern of fetal safety in relation to exposure to ionizing radiation. The putative risks may include fetal death, intrauterine growth retardation, malformations, and childhood cancer. It has been recommended that imaging modalities without ionizing radiation be used during pregnancy<sup>[9]</sup>. Therefore, attempts for complete exclusion of radiation are of paramount importance in ERCP. The literature has shown that ERCP can be performed safely without necessitating fluoroscopic use<sup>[11,12]</sup>.

Therapeutic ERCP is used routinely in pregnancy, with strict indications in clinical practice. Although the literature related to radiation exposure in ERCP has shown that ERCP is safe and effective in the early period after the procedure, the long-term negative effects of radiation exposure of children are unknown. Late adverse effects, such as cancer, may take years to develop after delivery, therefore, it is very difficult to determine the safe dose of radiation in pregnancy for ERCP. In order to overcome this problem, ERCP without radiation in pregnancy should be the first-choice treatment modality for therapeutic ERCP procedures.

In our study group, we cannulated the bile duct with the assistance of a guidewire. Cannulation was

confirmed by bile aspiration and/or visualization of the bile oozing around the guidewire after cannulation. Afterwards, bipolar biliary sphincterotomy with balloon sweeping of the biliary duct was performed. At least three times, the stone extraction balloon catheter had to be passed inside the bile duct and pulled out to achieve complete ductal clearance in patients with multiple stones. Improvements in laboratory, clinical and ultrasonographic findings were used as simple and accessible reference points for tracking the patients after ERCP. Maintenance of normal findings during follow-up of the patients after ERCP was thought to be related closely to the success of the procedure with regard to stone extraction. As wire-guided cannulation in non-radiation ERCP has the limitations of incorrect placement of the wire in the cystic duct and retained proximal stones after the procedure, choledochoscopy, real-time percutaneous ultrasonography, linear echoendoscopy, and intraductal ultrasonography are recommended for improving the performance of non-radiation ERCP<sup>[12]</sup>. Linear echoendoscopy, intraductal ultrasonography, and choledochoscopy may necessitate additional sedoanalgesia, which has a potentially jeopardizing effect on the fetus. As a result of this concern, we recommend simple monitoring of the patient with ultrasonography and biochemical and clinical parameters after the procedure. In our study, no complication was observed using these monitoring procedures.

Here, we presented six pregnant women with different etiologies, who underwent successful ERCP without the use of fluoroscopy. One case that makes our series interesting is the patient with biliary fistula after hepatic hydatid surgery. The patient was operated on because of liver hydatid disease that imitated acute abdominal findings. As a result of persistent biliary fistula after the operation, she underwent ERCP and sphincterotomy. Since the fistula lasted for an additional 2 wk after the procedure, which was documented by continuous drainage of the bile from the catheter and by MRCP, repeat ERCP with stenting was performed. In the following period, the clinical course was uneventful with closure of the fistula, and delivery occurred without any complication. The stent was removed after delivery.

In conclusion, there have been only a few studies of non-radiation ERCP during pregnancy. Therapeutic non-radiation ERCP with wire-guided cannulation, in

pregnant women with strong indications for ERCP, may be an effective treatment strategy in experienced centers. Laboratory and ultrasonographic follow-up of the patients after the procedure may be a safe, simple and cost-effective strategy for monitoring the effectiveness of the procedure. Stent placement should be considered in cases in which clearance of the common bile duct has failed. Larger studies are needed to draw strong conclusions about non-radiation ERCP.

## COMMENTS

### Background

Acute cholecystitis is the second most common non-obstetric emergency in pregnant women. Choledocholithiasis and consequent complications such as pancreatitis and cholangitis are potentially fatal diseases for the mother and fetus. During pregnancy, treatment is usually conservative since surgery is associated with an increased rate of complications such as preterm labor and spontaneous abortion. In choledocholithiasis and/or choledocholithiasis-induced acute cholangitis, endoscopic retrograde cholangiopancreatography (ERCP) is the first-line treatment of choice. Already, it has been reported that non-radiation ERCP might be an option for pregnant women with choledocholithiasis and/or choledocholithiasis-induced acute cholangitis.

### Research frontiers

Non-radiation ERCP has been used in some studies for the treatment of choledocholithiasis in pregnant women and strict guidelines have not been established.

### Innovations and breakthroughs

The authors reported that therapeutic non-radiation ERCP with wire-guided cannulation in pregnant women with strong indications for ERCP may be an effective treatment strategy in experienced centers. Laboratory and ultrasonographic follow-up of the patients after the procedure may be a safe, simple and cost-effective strategy for monitoring the effectiveness of the procedure.

### Applications

This study may be useful for showing the safety and effectiveness of non-radiation ERCP in pregnant women with choledocholithiasis and/or choledocholithiasis-induced acute cholangitis or biliary fistula.

### Peer review

This study examined the acceptability of ERCP without radiation during pregnancy. The authors showed their experience with six pregnant women who underwent ERCP without using radiation. The results are interesting and may form a reference for the treatment of pregnant women with strong indications for ERCP.

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## Factors associated with use of gastric cancer screening services in Korea

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[adjusted odds ratio (aOR), 1.66; 95% CI: 1.21-2.26], middle/high school (aOR, 1.38; 95% CI: 1.01-1.89), and university or higher (aOR, 1.64; 95% CI: 1.13-2.37) were more likely to undergo gastric cancer screening than those who received no formal education at all. The population with the highest income tertile had more attendance at gastric screening compared to those with the lowest income tertile (aOR, 1.36; 95% CI: 1.06-1.73). Gastric screening was also negatively associated with excessive alcohol consumption (aOR, 0.71; 95% CI: 0.53-0.96). A positive attitude to preventive medical evaluation was significantly associated with better participation in gastric cancer screening programs (aOR, 5.26; 95% CI: 4.35-6.35).

**CONCLUSION:** Targeted interventions for vulnerable populations and public campaigns about preventive medical evaluation are needed to increase gastric cancer screening participation and reduce gastric cancer mortality.

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**Key words:** Socioeconomic factors; Health behavior; Health status disparity; Mental health; Early detection of cancer

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Kwon YM, Lim HT, Lee K, Cho BL, Park MS, Son KY, Park SM. Factors associated with use of gastric cancer screening services in Korea. *World J Gastroenterol* 2009; 15(29): 3653-3659 Available from: URL: <http://www.wjgnet.com/1007-9327/15/3653.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.3653>

### Abstract

**AIM:** To identify the factors associated with participation in gastric cancer screening programs.

**METHODS:** Using data from the Korea National Health and Nutrition Examination Survey 2005 (KNHANES III), a nationwide health-related survey in Korea, a cross-sectional study was performed to investigate the multiple factors associated with gastric cancer screening attendance among persons aged at least 40 years. The study population included 4593 individuals who completed a gastric cancer screening questionnaire and had no previous cancer history. Four groups of individual-level or environmental level covariates were considered as potential associated factors.

**RESULTS:** Using KNHANES III data, an estimated 31.71% of Korean individuals aged at least 40 years adhered to gastric cancer screening recommendations. Subjects who graduated from elementary school

### INTRODUCTION

Gastric cancer is a major public health burden. Although the worldwide incidence of gastric cancer and associated mortality is decreasing, gastric cancer is still the fourth most common cancer and the second leading cause of cancer-related death, causing 700 000 deaths annually<sup>[1]</sup>. In contrast to the worldwide trend, in Korea the incidence of gastric cancer has been reported to be stable or increasing, and is still one of the most common forms

of cancer and one of the leading causes of death from cancer among both sexes<sup>[2,3]</sup>. According to the Korea Central Cancer Registry Data, gastric cancers comprised 18% of all new cancers and caused 15.3% of cancer deaths in 2005.

Despite the increasing incidence of gastric cancer in Korea, mortality due to gastric cancer is decreasing. This is due to advances in surgical techniques, chemotherapy and radiological therapy, and to early detection by gastric cancer screening programs<sup>[4]</sup>. Because patients with early-stage gastric cancer often show no clinical symptoms, a significant proportion of patients are diagnosed when the disease is at an advanced stage, which is associated with poor prognosis. If screening for gastric cancer were universal, beginning at the age of 40 years, and combined with timely treatment of surgical or endoscopic mucosal removal of early cancers, the gastric cancer mortality rate could be markedly reduced<sup>[5,6]</sup>. A recent study in Korea also showed that repeated endoscopic screening within 2 years decreased the incidence of gastric cancer and endoscopic resection could be applied to more patients who underwent EGD screening within 2 years<sup>[7]</sup>.

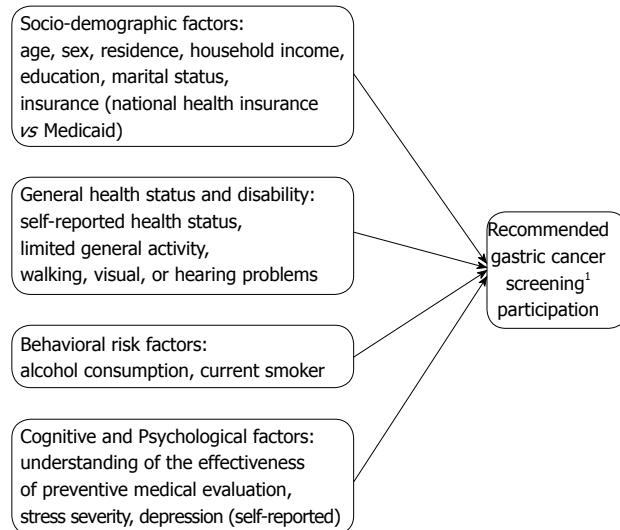
In the past decade, the Korean Ministry of Health and Welfare (MOHW) agency has established multiple cancer control programs. In 1996, the Korean government initiated a comprehensive “10-year plan for cancer control”. As part of this program, the Korean Government initiated the National Cancer Screening Program (NCSP) in 1999. Since then, the NCSP has provided free cancer screening for common cancers to low-income individuals receiving medical aid<sup>[8]</sup>. However, the rate of participation in gastric cancer screening programs is not optimal<sup>[9,10]</sup>. Therefore, to increase the participation rate and improve the survival rate of gastric cancer patients, identification and removal of potential barriers to cancer screening participation might be of great importance. However, few studies have investigated the individual and environmental predictors of gastric cancer screening participation in the Korean population.

In the present study, we analyzed the relationship among four dimensions of individual-level factors, including socio-demographic characteristics, general health status, gastric cancer risk factors and cognitive factors, and gastric cancer screening participation (Figure 1), among individuals included in the Third Korea National Health and Nutrition Examination Survey 2005 (KNHANES III).

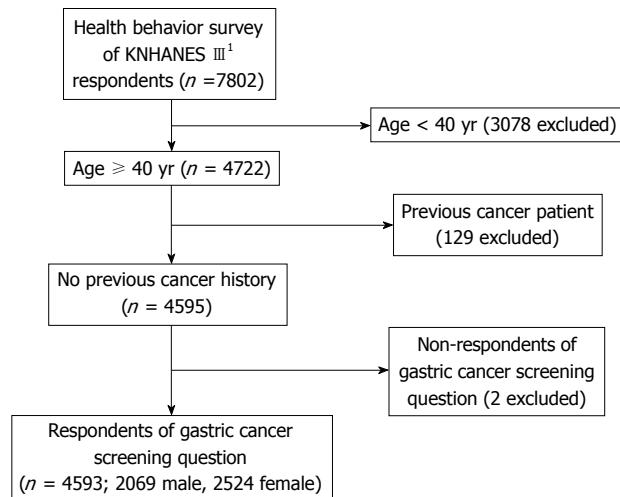
## MATERIALS AND METHODS

### **Study population**

This study was based on data obtained from the KNHANES III. The KNHANES is a national household survey that provides comprehensive information on health status, health care utilization, and socio-demographics of a nationally representative sample of 34145 individuals from 13800 households. The KNHANES III is composed of four parts: health interview survey, health behavior survey, health examination survey and nutrition survey.



**Figure 1** Framework of study investigating factors associated with gastric cancer screening participation. <sup>1</sup>Endoscope or upper gastrointestinal series; every 2 years, starting at the age of 40 years.



**Figure 2** The study population framework. <sup>1</sup>2005 Korean National Health and Nutrition Examination Survey.

We performed a cross-sectional analysis of data from the 7802 individuals who completed the health behavior survey. We then selected individuals aged at least 40 years who answered the gastric cancer screening behavior question, yielding 4722 individuals. After excluding 129 individuals who had previously been diagnosed with cancer, 4593 people (2069 men and 2524 women) were eligible for our analyses (Figure 2).

### **Gastric cancer screening behavior outcome**

According to the KNCSP guidelines, persons aged at least 40 years should undergo gastroscopy or upper gastrointestinal series (UGIS) examinations every 2 years. Subjects were asked the question “when was the last time you had a gastric cancer screening examination (endoscope or UGIS)?” The possible responses were “never”, “less than 1 year”, “1-2 years”, “more than 2 years”.

In the present study, the outcome variable is whether

the individuals adhere to KNCSP guidelines or not. We recognized that individuals who reported never taking a gastric cancer screening examination or having undergone examinations more than 2 years prior to completing the questionnaire would be classified as not adhering to KNCSP guidelines.

### **Independent variables**

Data about variables were also obtained from the KNHANES III and their associations with gastric cancer screening were investigated. We considered 17 variables as potential factors that could be associated with gastric cancer screening (Figure 1); these 17 variables were classified into one of four groups: (1) socio-demographic factors [age, gender, residential area (metropolitan area or not), household income, education level, marital status, insurance (national health insurance or Medicaid)]; (2) general health status and disability (self-reported health status, limitation of general activity, walking problem, visual problem, hearing problem; (3) gastric cancer risk factors (alcohol consumption, current smoker); (4) cognitive and psychological factors (attitude to routine health checks, stress severity, depression).

Patients were stratified by age; age 40-49, 50-59, 60-69 and  $\geq 70$  years. Residential area was classed as metropolitan or non-metropolitan area. Income was calculated by dividing the household monthly income by the square root of the household size (equivalized income), and was categorized into three groups<sup>[11,12]</sup>. Education level was categorized as uneducated, elementary school graduate, high school graduate, and college or higher education graduate. Marital status was recorded as married, unmarried, widowed, or divorced, and was dichotomized as living with or without spouse. South Korea has a universal health insurance system; hence we compared individuals with national health insurance (NHI) and those receiving Medicaid (the Korean government program for low-income or medically needy individuals).

Alcohol consumption was assessed using the question "how often do you binge drink?" (binge drinking was defined as seven or more drinks for men and five or more drinks for women), and was categorized into three groups: (1) non-binge drinker, nondrinker or social drinker who reported binge drinking no more than once per month; (2) binge drinker, reported binge drinking 1-4 times per month; and (3) frequent binge drinker, reported binge drinking more than twice per week<sup>[13]</sup>. Individuals were asked "Do you now smoke cigarettes?" and, based on responses, were categorized by smoking status into three groups: (1) never smoker, have never smoked; (2) ex-smoker, have quit smoking; and (3) current smoker, smoke daily or intermittently smoke.

For cognitive and psychological factors, we considered attitudes to preventive medical evaluation, stress severity and self-reported depression.

### **Statistical analysis**

The dependent variable of interest was whether the

patient had undergone gastric cancer screening within the previous 2 years; independent variables were the 17 factors described above.

Descriptive statistical methods were used to describe the basic characteristics of the study population; numbers and percentages are reported for each variable. First, to identify the factors associated with undergoing gastric cancer screening, we used univariate logistic regression analysis. Odds ratios and confidence intervals for the associations between gastric cancer screening attendance and each factor were calculated. A *P*-value less than 0.05 was considered significant. Next, the factors identified as significantly associated with gastric cancer screening by univariate analysis (*P* < 0.05) were analyzed by multivariate logistic regression analysis. All statistical tests were performed using STATA, version 10.0.

## **RESULTS**

### **General characteristics of the study population**

Characteristics of the study population are summarized in Table 1. In the study population, 1457 individuals (31.71%) had undergone a gastric cancer screening examination within the previous 2 years. The mean age of our study population was 55.12 years; 54.95% were women. Three-quarters of the population were living with a spouse and most were enrolled in the national health insurance program. Of the study population, 8.69% were frequent binge drinkers, consuming large quantities of alcohol almost daily. A total of 6.30% of women were current smokers, compared with 48.77% of men; this result is similar to that reported for adult men in Korea in 2006 (44.7%)<sup>[8]</sup>.

### **Factors associated with gastric cancer screening attendance**

Table 2 shows the univariate analysis results; crude odds ratios (ORs) and 95% confidence intervals (95% CIs) were reported. The factors associated with gastric cancer screening participation were age (more than 70 years), income, education level, marital status, limited general activity, visual problems, smoking status, attitude to preventive medical health assessments, stress and alcohol consumption.

By multivariate logistic regression analysis, only four of these factors were significantly and independently associated with gastric cancer screening (Table 3): household monthly income, education level, alcohol consumption and attitude to preventive medical evaluation.

Of the socio-demographic factors considered, higher household income was found to be associated with a higher OR. Compared with the lowest income tertile, the adjusted OR (aOR) of the highest income tertile was 1.36 (95% CI: 1.06-1.73). There was also a clear trend to increased rates of gastric cancer screening with greater education level (elementary school graduate: aOR, 1.66; 95% CI: 1.21-2.26; middle or high school graduate: aOR, 1.38; 95% CI: 1.01-1.89; university or higher education graduate: aOR, 1.64; 95% CI: 1.13-2.37).

**Table 1 Basic characteristics of the study population (*n* = 4593)**

<b><i>n</i> (%)</b>		
Socio-demographic factor		
Age (yr)	40-49	1854 (40.37)
	50-59	1204 (26.21)
	60-69	924 (20.12)
	≥ 70	611 (13.3)
Sex	Male	2069 (45.05)
	Female	2524 (54.95)
Residence <sup>1</sup>	Metropolis	2058 (44.81)
	Town or country	2535 (55.22)
Household income <sup>2</sup>	Lowest tertile	1446 (31.88)
	Middle tertile	1550 (34.17)
	Highest tertile	1540 (33.95)
Education	Uneducated	497 (10.83)
	Elementary school	1117 (24.32)
	Middle-high school	2206 (48.03)
	University or higher	773 (16.83)
Marital status <sup>3</sup>	Without spouse	1053 (22.94)
	With spouse	3537 (77.06)
National health insurance (NHI) vs Medicaid <sup>4</sup>	NHI	4355 (95.17)
	Medicaid	221 (4.83)
General health status		
Self-reported health status	Healthy	1522 (33.14)
	Middle	1682 (36.62)
	Unhealthy	1389 (30.24)
Limitation of general activity	Limited	671 (14.61)
	Unlimited	3922 (85.39)
Walking problem	Limited	881 (19.19)
	Unlimited	3711 (80.81)
Visual problem	Limited	1600 (34.84)
	Unlimited	2993 (65.16)
Hearing problem	Limited	635 (13.83)
	Unlimited	3958 (86.17)
Cancer risk factor		
Smoking	Never	2536 (55.21)
	Ex-smoker	889 (19.36)
	Current smoker	1168 (25.43)
Alcohol consumption (number of binge drinking sessions per month) <sup>5</sup>	Non-binge drinker	2781 (60.55)
	Binge drinker	1413 (30.76)
	Frequent binge drinker	399 (8.69)
Psychological factors		
Gastric cancer screening <sup>6</sup>	Participated	1457 (31.72)
	Did not participate	3136 (68.28)
Attitude to health check-up	Not effective <sup>7</sup>	1730 (15.46)
	Effective	2862 (84.54)
Stress severity	None	845 (18.4)
	Mild	2126 (46.29)
	Moderate	1311 (28.54)
	Severe	311 (6.77)
Depression (self-reported)	No	3788 (82.49)
	Yes	804 (17.51)

<sup>1</sup>There are seven metropolitan areas in Korea, the other areas were defined as town or country; <sup>2</sup>Defined as "household income/square root of number of persons in household"; <sup>3</sup>State of "without spouse" includes never married, divorced, and separation by death; <sup>4</sup>National health insurance includes regional health insurance and workplace health insurance; <sup>5</sup>Binge drinking means alcohol consumption of more than 70 g of alcohol per episode for men or 50 g per episode for women in this study. One glass of Soju contains 10 g of alcohol; <sup>6</sup>Experience or not of EGD (Esophago-Gastro-Duodenoscopy) or UGIS (Upper Gastrointestinal Series) within the previous 2 years; <sup>7</sup>Includes people with negative thoughts about medical check-ups and those who have not previously undergone a medical check-up.

Analysis of gastric cancer risk factors revealed that frequent binge drinkers who consumed more than seven glasses of Soju (70 g alcohol in total), the most widely consumed traditional beverage in Korea, per day more

**Table 2 Factors associated with gastric cancer screening<sup>1</sup> by univariate analysis (*n* = 4593)**

		<b>OR (crude)</b>	<b>95% CI</b>
Socio-demographic factor			
Age (yr)	40-49	1 (ref)	
	50-59	1.07	0.89-1.29
	60-69	0.99	0.80-1.22
	≥ 70	0.61	0.47-0.79
Sex	Male	1 (ref)	
	Female	0.95	0.84-1.07
Residence	Metropolis	1 (ref)	
	Town or country	1.15	0.96-1.36
Household income	Lowest tertile (≤ 1.00 × 10 <sup>6</sup> kW)	1 (ref)	
	Middle tertile (1.00-2.42 × 10 <sup>6</sup> kW)	1.25	1.03-1.53
	Highest tertile (≥ 2.45 × 10 <sup>6</sup> kW)	1.80	1.47-2.20
Education	Uneducated	1 (ref)	
	Elementary school	1.83	1.38-2.43
	Middle or high school	1.71	1.31-2.26
	University or higher	2.51	1.84-3.46
Marital status	Without spouse	1 (ref)	
	With spouse	1.42	1.20-1.68
NHI vs Medicaid	NHI	1 (ref)	
	Medicaid	0.98	0.66-1.45
General health status			
Self-reported health status	Healthy	1 (ref)	
	Middle	1.07	0.91-1.27
	Unhealthy	1.09	0.92-1.29
Limitation of general activity	Limited	1 (ref)	
	Unlimited	1.20	1.00-1.45
Walking problem	Limited	1 (ref)	
	Unlimited	1.15	0.96-1.39
Visual problem	Limited	1 (ref)	
	Unlimited	1.15	1.00-1.32
Hearing problem	Limited	1 (ref)	
	Unlimited	1.16	0.97-1.39
Cancer risk factor			
Alcohol consumption	Non-binge drinker	1 (ref)	
	Binge drinker	1.03	0.89-1.20
	Frequent binge drinker	0.76	0.59-0.98
Smoking	Never	1 (ref)	
	Ex-smoker	1.05	0.88-1.24
	Current smoker	0.80	0.69-0.94
Psychological factor			
Attitude to health check-up	No	1 (ref)	
	Yes (cancer screening is effective)	5.61	4.66-6.75
Stress severity	None	1 (ref)	
	Mild	1.18	0.99-1.41
	Moderate	1.23	1.01-1.49
	Severe	0.95	0.72-1.24
Depression (self-reported)	No	1 (ref)	
	Yes	1.03	0.87-1.23

OR: Odds ratio; CI: Confidence interval. <sup>1</sup>Endoscope or Upper Gastrointestinal Series (UGIS) every 2 years.

than twice per week were significantly less likely to undergo gastric cancer screening compared with non-binge drinkers or binge drinkers (aOR, 0.71; 95% CI: 0.53-0.96).

A positive attitude to preventive medical evaluation showed the greatest association with gastric cancer screening participation. Individuals who recognized

**Table 3** Factors associated with gastric cancer screening<sup>1</sup> by multivariate analysis (*n* = 4593)

		Multivariate	95% CI
		OR	
Socio-demographic factor			
Age (yr)	40-49	1 (ref)	
	50-59	1.02	0.83-1.25
	60-69	0.95	0.74-1.23
	≥ 70	0.82	0.59-1.16
Household income	Lowest tertile (≤ 1.00 × 10 <sup>6</sup> kW)	1 (ref)	
	Middle tertile (1.00-2.42 × 10 <sup>6</sup> kW)	1.08	0.86-1.36
	Highest tertile (≥ 2.45 × 10 <sup>6</sup> kW)	1.36	1.06-1.73
Education	Uneducated	1 (ref)	
	Elementary school	1.66	1.21-2.26
	Middle or high school	1.38	1.01-1.89
	University or higher	1.64	1.13-2.37
Marital status	Without spouse	1 (ref)	
	With spouse	1.05	0.87-1.26
General health status			
Limitation of general activity	Limited	1 (ref)	
	Unlimited	0.95	0.76-1.19
Visual problem	Limited	1 (ref)	
	Unlimited	1.03	0.88-1.21
Cancer risk factor			
Alcohol consumption	Non-binge drinker	1 (ref)	
	Binge drinker	0.93	0.77-1.13
	Frequent binge drinker	0.71	0.53-0.96
Currently smoking	Never	1 (ref)	
	Ex-smoker	1.08	0.87-1.33
	Current smoker	0.86	0.70-1.06
Psychological factor			
Attitude to health check-up	No	1 (ref)	
	Yes (cancer screening is effective)	5.26	4.35-6.35
Stress severity	None	1 (ref)	
	Mild	1.06	0.87-1.30
	Moderate	1.20	0.98-1.48
	Severe	1.06	0.80-1.41

<sup>1</sup>Endoscope or UGIS every 2 years.

the effectiveness of preventive medical evaluation were more likely to undergo recommended gastric cancer screening compared with those who did not (aOR, 5.26; 95% CI: 4.35-6.35).

## DISCUSSION

In this national representative household survey, we found that the participation rate of gastric cancer screening in Korea amongst individuals aged 40 years or more, is only 31.71%, even though gastric cancer is largely treatable when detected at an early stage. Our study also found that low household income, low education level, frequent binge drinking and a negative attitude to preventive medical evaluation were significantly associated with poor participation in gastric cancer screening programs. Our study is the first to use multivariate analysis to identify the factors associated with gastric cancer screening in clinical practice, including demographic factors, general health status, behavioral risk factors and psychological factors.

Factors associated with unhealthy behavior, such as alcohol consumption and smoking, are considered

gastric cancer risk factors<sup>[14-17]</sup>, and high levels of tobacco smoking and alcohol consumption have been shown to substantially increase the relative risk of this cancer<sup>[15]</sup>. Therefore, preventive measures, such as gastroscopy or UGIS, are likely to be of particular benefit in frequent binge drinkers and smokers. However, in this study, we found that frequent binge drinkers were less likely to undergo gastric cancer screening tests compared with others. Current smokers also showed a low participation rate in screening programs, although the difference compared with non-smokers was not significant. These findings indicate that more interventions are needed for individuals with unhealthy behavioral risk factors, and suggest that successful preventive measures could markedly reduce the number of gastric cancer deaths.

Our study also showed that a positive attitude to the effectiveness of preventive medical evaluation is strongly associated with gastric cancer screening participation. Previously, several studies have demonstrated that cognitive elements can directly affect the decision to undergo gastric cancer screening, and personal background and other socio-demographic factors might have indirect effects through their effects on cognitive variables<sup>[9,18]</sup>. These findings indicate that improved public education about the benefits of preventive cancer screening and routine health checks could have important influences on individual patient decisions to undergo gastric cancer screening.

Household income and education level were shown to be significant predictors of participation in gastric cancer screening in the present study. Previous studies have reported inconsistencies in the relative strength and significance of the correlations between cancer screening and socio-demographic factors<sup>[1,9,18-21]</sup>. There are several possible reasons for this inconsistency. One possibility is that physiologic medical accessibility may vary with health care policy and location; for example, developed *vs* non-developed countries or urban *vs* rural area. Therefore, the study location and health policy status should be considered when different studies are compared. Another possible reason is the differences in study design. Several recent reports in Korea have highlighted the factors associated with cancer screening participation, but showed no associations between gastric cancer screening and socio-economic factors<sup>[9,18]</sup>. This can be explained by the differences in study design, such as variable categories and outcome variable. In the present study, we used equivalized household income which was calculated by dividing the household monthly income by the square root of the household size, while other previous studies usually did not consider the household size and simply used the crude household income<sup>[9]</sup>. In addition, a major focus of one study was the intention to receive gastric cancer screening, not receipt of gastric cancer screening, which could lead to different results from our study<sup>[18]</sup>.

The Korean Government initiated the National Cancer Screening Program (NCSP) in 1999, and it has since expanded its target population. Currently, NCSP provides Medical Aid recipients and NHI beneficiaries within the lower 50% income bracket with free gastric cancer screening services<sup>[8]</sup>.

In addition, the costs of gastric cancer screening in Korea are low compared with other countries, which could improve the accessibility of these services to low-income individuals<sup>[22]</sup>. However, our results indicate that socio-economic inequalities in gastric cancer screening participation have not yet been sufficiently overcome. To increase gastric cancer screening participation, improved intervention programs for individuals with low income and education level are needed.

No general health status or disability-related factor was significantly associated with gastric cancer screening participation, which is in contrast to our original hypothesis that there might be disability-related barriers to participation in screening programs. A recent cross-sectional study reported that individuals with a disability showed reduced levels of participation in mass screening programs compared with those without a disability<sup>[23]</sup>. However, few studies have assessed the association between physical disability and gastric cancer screening participation. Further research about gastric cancer screening patterns among individuals with a disability is required to determine these associations.

Our study has several limitations. First, as the findings were based on patient self-reported health status data, respondents may under-report, over-report, or choose not to respond, leading to possible inaccuracy. Second, information about gastric cancer screening was obtained from the responses to a single question, and any symptoms at the time of the examination were not reported. Cancer screening prevention programs are designed for individuals with no associated symptoms; therefore, there could be some misclassification of gastric cancer screening participation by including individuals with symptoms indicative of gastric cancer. However, previous studies have also not considered accompanying symptoms, and used a similar definition of cancer screening participation<sup>[21,24,25]</sup>.

In conclusion, we found that more than two-thirds of the Korean population did not comply with the gastric cancer screening recommendations. These findings indicate that there is scope for further improvement. In particular, targeted interventions are needed for vulnerable populations such as those with low income, low education level and unhealthy behaviors. In addition, public campaigns to improve attitudes to preventive medical evaluation could be powerful methods to increase gastric cancer screening participation.

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## COMMENTS

### Background

The mortality of gastric cancer is decreasing despite the increasing incidence in Korea. This can be explained by surgical technique development and early detection by endoscopic screening or upper gastrointestinal study.

### Research frontiers

Despite the development in national cancer control programs, the rate of participation in gastric cancer screening programs is still not optimal. However, few studies have investigated the individual and environmental predictors of gastric cancer screening participation in the Korean population. In this study, the authors identified the factors associated with participation in gastric cancer screening programs.

### Innovations and breakthroughs

Recent reports in Korea have highlighted the factors associated with cancer screening participation, but reported inconsistencies in the results. It can mainly be explained by the differences in study design, that is, different variable categories and outcome variables. Our study shows predictors associated with gastric cancer screening in multiple dimensions, both at the individual level and at the environmental level.

### Applications

The authors' findings indicate that targeted interventions and public campaigns for vulnerable populations could be powerful methods to increase gastric cancer screening participation.

### Peer review

In the present study, authors have performed a cross-sectional study investigating the factors associated with participation in a gastric cancer screening program in Korea, and multivariate analyses have revealed independent predictive factors to be household income, education, alcohol consumption and attitude to health check-up. The manuscript is relatively well written, and the results are moderately interesting.

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BRIEF ARTICLES

## Bilateral liver resection for bilateral intrahepatic stones

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## INTRODUCTION

Intrahepatic stones is a common disease in Eastern Asia. Hepatectomy that can remove stones, strictured bile duct and atrophic liver tissue seems to be the optimal treatment for intrahepatic stones in selected patients. It has been accepted increasingly as the definitive treatment for intrahepatic stones in Eastern Asian<sup>[1-5]</sup> and some European countries<sup>[6,7]</sup>. Most of the previous studies have demonstrated that liver resection for intrahepatic stones has been confined to unilateral resection. The outcomes of bilateral liver resection for bilateral intrahepatic stones have not been clarified.

The aims of this study were to evaluate the short- and long-term outcomes of bilateral liver resection for bilateral intrahepatic stones.

## Abstract

**AIM:** To evaluate the short- and long-term outcomes of bilateral liver resection for bilateral intrahepatic stones.

**METHODS:** We reviewed retrospectively 101 consecutive patients with bilateral intrahepatic stones who underwent bilateral liver resection in the past 10 years. The short- and long-term outcomes of the patients were analyzed. The Cox proportional hazards model was used to identify the risk factors related to stone recurrence.

**RESULTS:** There was no surgical mortality in this group of patients. The surgical morbidity was 28.7%. Stone clearance rate after hepatectomy was 84.2% and final clearance rate was 95.0% following postoperative choledochoscopic lithotripsy. The stone recurrence rate was 7.9% and the occurrence of postoperative cholangitis was 6.5% in a median follow-up period of 54 mo. The Cox proportional hazards model indicated that liver resection range, less than the range of stone distribution ( $P = 0.015$ , OR = 2.152) was an independent risk factor linked to stone recurrence.

**CONCLUSION:** Bilateral liver resection is safe and its short- and long-term outcomes are satisfactory for bilateral intrahepatic stones.

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**Key words:** Intrahepatic stone; Hepatectomy; Risk factor; Outcome; Bilateral

## MATERIALS AND METHODS

### Patients

The study included 101 consecutive patients with bilateral intrahepatic stones who underwent elected bilateral liver resection between January 1997 and December 2007. There were 45 men and 56 women with an average age of 47.2 years (range, 20-72 years). Forty-nine (48.6%) patients had undergone previous biliary surgery. Fifty-two (51.5%) patients also had extrahepatic stones. Twenty (19.8%) patients also had common bile duct cysts, and nine (8.9%) had hilar stricture.

### Indications of hepatectomy for intrahepatic stone

The indications of hepatectomy for bilateral intrahepatic stones were: (1) bilateral segmental liver parenchymal atrophy caused by stones; (2) stricture of stone-bearing bile ducts; (3) estimated liver remnant is sufficient after hepatectomy; and (4) the general condition of patient

was good and liver function was Child-Pugh A class, and he/she could tolerate the hepatectomy.

### Preoperative preparation

Preoperative preparation included liver biochemistry, coagulation profile, ultrasound and computed tomography (CT). If the liver resection range was larger than four liver segments, volumetric CT was done to estimate the volume of the liver remnant. Percutaneous transhepatic cholangiography or endoscopic retrograde cholangiopancreatography was performed selectively for patients with intrahepatic bile duct dilatation, which aimed at delineating the site of the bile duct stricture. For patients with acute cholangitis, percutaneous transhepatic cholangiodrainage (PTCD) guided by ultrasound or endoscopic nasobiliary drainage (ENBD) was performed preoperatively, and definitive hepatectomy was performed 1-3 mo later<sup>[8]</sup>. There were 17 patients with acute cholangitis who received PTCD and ENBD before definitive hepatectomy.

### Definitions

Bile leakage, defined as bile fluid draining from the peritoneal cavity or oozing from the wound, was demonstrated by cholangiography through a T tube or transanastomotic tube<sup>[8]</sup>. Postoperative liver failure was defined as serum total bilirubin > 85 μmol/L and coagulopathy (international normalized ratio > 1.5) that lasted for > 2 wk after hepatectomy. Patients presented with ascites and/or encephalopathy. Surgical mortality was defined as death within 30 d after hepatectomy. Major hepatectomy was defined as resection of more than three liver segments.

### Follow-up

Patients after surgery were followed up twice a year. Liver biochemistry and ultrasound were performed routinely. If stone recurrence was highly suspected, CT was performed. Stone recurrence was defined as stone recurrence intra- or extrahepatically after complete initial clearance. Patients who presented with right upper quadrant pain, chill and fever, with or without jaundice, were considered to have an acute attack of cholangitis.

At the end of the study (December 2008), 92 of 101 patients completed a median follow-up period of 54 mo (12-120 mo).

### Statistical analysis

Patient data were analyzed by SPSS version 13.0 software (Chicago, IL, USA). The Cox proportional hazards model was used to identify the risk factors associated with stone recurrence.  $P < 0.05$  was considered statistically significant.

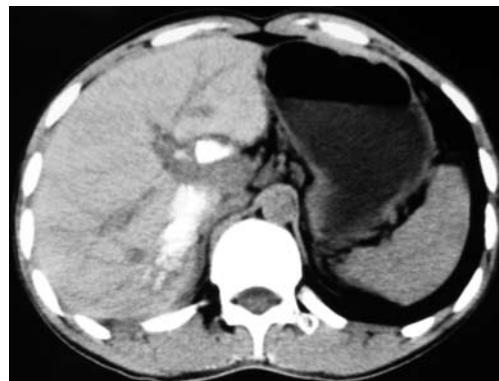
## RESULTS

### Procedures of bilateral liver resection

The detailed procedures of bilateral liver resection of the 101 patients are listed in Table 1. The most common

**Table 1 Liver resection procedures of 101 patients**

Procedures	n (%)
Left lateral sectionectomy plus right posterior sectionectomy	38 (37.6)
Left lateral sectionectomy plus segmentectomy of segments 5-7	18 (17.8)
Hemihepatectomy plus ipsilateral segmentectomy	21 (20.8)
Left hepatectomy plus right posterior sectionectomy	11 (10.9)
Left hepatectomy plus left caudate sectionectomy	9 (9.0)
Left trisegmentectomy	2 (2.0)
Left and right hepatectomy (leaving hypertrophic caudate lobe)	1 (1.0)
Segmentectomy of segments 2-5, 7, 8 (leaving hypertrophic segment 6)	1 (1.0)



**Figure 1** A patient with left lateral section and right posterior section stones underwent left lateral sectionectomy and right posterior sectionectomy.

procedure performed in this group of patients was left lateral sectionectomy plus right posterior sectionectomy (Figure 1), which accounted for 37.6% of patients. Four patients underwent more aggressive liver resection, which included two left trisegmentectomies; one left and right hepatectomy that left only the hypertrophic caudate lobe (970 g estimated by volumetric CT); and one segmentectomy of segments 2, 3, 4, 5, 7 and 8, which left only the hypertrophic segment 6 (670 g estimated by volumetric CT).

Twenty-nine of 101 (28.7%) patients had concomitant hepaticojejunostomy following resection of common bile duct cysts and bile duct strictureplasty.

The consistency between liver resection range and the range of stone distribution was classified into two categories: liver resection range equal to stone distribution, and liver resection range less than stone distribution. Fifty-eight of 101 (57.4%) patients had a liver resection range that was equal to the range of stone distribution, and the others had a resection range that was less than the range of stone distribution.

### Surgical mortality and morbidity

There was no surgical mortality in our group of patients. Twenty-nine (28.7%) patients developed one kind of complication (Table 2). One patient suffered from postoperative hemobilia at postoperative day 5, and he underwent emergency surgical exploration. Bleeding

**Table 2 Surgical complications**

Complications	n (%)
Wound infection	18 (17.8)
Bile leak	8 (8.0)
Peritoneal infection	7 (7.0)
Pleural effusion	10 (10.0)
Liver failure	2 (2.0)
Pulmonary infection	2 (2.0)
Hemobilia	1 (2.0)
Sepsis	1 (1.0)

of the anastomotic mouth was found and hemostasis was achieved by fine suturing the bleeding vessel at operation. He recovered well. The other complications were cured by conservative treatment.

### Stone clearance

Residual stone was confirmed by postoperative cholangiography through a T tube and transanastomotic tube, or ultrasound findings. Sixteen patients had residual stones after hepatectomy. The stone clearance rate was 84.2% (85/101). The final stone clearance rate was 95.0% (95/101) following 1-5 sessions of postoperative choledochoscopic lithotripsy through the T-tube or transanastomotic tube route. Residual stones could not be completely removed in five patients. This was because of tiny stones located at the peripheral bile duct or segregated bile duct that could not be reached by choledochoscopy ( $n = 3$ ), or because the sharp angle formation of the jejunal loop of hepaticojjunostomy hindered entry of the choledochoscope ( $n = 2$ ).

### Stone recurrence

At the end of this study, 92 patients had completed the follow-up, including 89 whose stones were completely removed at initial operation and three patients with residual stones. In a median follow-up period of 54 mo, seven (7.9%) patients developed stone recurrence. Six patients suffered from at least one attack of acute cholangitis. Four of six patients had stone recurrence and the other two had residual stones. The occurrence of acute cholangitis was 6.5% (6/92). Stone recurrence and residual stones were the major causes of postoperative acute cholangitis.

To identify the risk factors related to stone recurrence, six clinical factors including age ( $\geq 50$ ,  $< 50$  years), sex, previous history of biliary surgery, complicated with extrahepatic stones, concomitant hepaticojjunostomy, and consistency between liver resection range and the range of stone distribution were analyzed by the Cox proportional hazards model. It indicated that liver resection range less than the range of stone distribution ( $P = 0.015$ , OR = 2.152, 95% CI: 1.624-4.721) was the only independent risk factor associated with stone recurrence.

## DISCUSSION

The treatment principle of intrahepatic stones consists

of complete removal of the stones, strictured bile duct and atrophic liver parenchyma, and establishment of satisfactory biliary drainage. Bilateral intrahepatic stones is a complex condition, and treatment of this disease remains a challenge. Hepatectomy that can remove stones and resect strictured bile ducts seems to be the optimal treatment for intrahepatic stones. Whether bilateral liver resection is feasible for bilateral intrahepatic stones and its outcome have not been evaluated.

Our results demonstrated that stone clearance rate after hepatectomy was 84.2%, and the final clearance rate was 95% following choledochoscopic lithotripsy. There was no surgical mortality. The stone recurrence rate was 7.9% and the occurrence of acute cholangitis episodes was 6.5%, after a median follow-up period of 54 mo. This indicated that bilateral liver resection for bilateral intrahepatic stones was safe, and its short- and long-term outcomes were satisfactory.

The locations of intrahepatic stones are strictly within segments. Most commonly, the involved segments are destroyed and atrophic, and the stone-bearing bile ducts show fibrotic thickening or stricture because of repeated attacks of acute cholangitis, whereas, the non-involved segments are hypertrophic (especially in patients with bilateral stones). Therefore, anatomical hepatectomy for the affected segments is feasible, with the prerequisite of there being sufficient remnant liver. The Cox proportional hazards model indicated that a liver resection range less than the range of stone distribution was the only independent risk factor associated with stone recurrence in this study. A liver resection range less than the range of stone distribution may leave bile duct stricture, which is a key predisposing factor for stone recurrence<sup>[9-11]</sup>.

There is reluctance among some surgeons to perform bilateral liver resection for bilobar stones because of the associated surgical risks. It has been reported that hepatectomy for the more severely affected side, combined with hepaticojjunostomy for removal of stones on the other side, is an effective approach<sup>[2]</sup>. We do not advocate that the liver resection range should be consistent with the range of stone distribution for every patient with bilateral intrahepatic stones. However, we suggest strongly that the strictured stone-bearing bile ducts and atrophic liver tissue should be resected, with the prerequisite of leaving sufficient remnant liver. This is because the strictured inflammatory bile ducts and atrophic tissue are predisposing risk factors for stone recurrence<sup>[9,10]</sup> and the occurrence of cholangiocarcinoma<sup>[12-14]</sup>. If the estimated future liver remnant was not sufficient, hepatectomy was performed for the severely affected side and the ipsilateral stones were removed as cleanly as possible via intraoperative choledochoscopic lithotripsy. Whenever possible, we do not perform hepaticojjunostomy for intrahepatic stones, but attempt to preserve the normal anatomy of the common bile duct because hepaticojjunostomy cannot drain residual stones effectively<sup>[15]</sup>, and it has a high incidence of cholangitis after hepaticojjunostomy<sup>[16,17]</sup>. Twenty-nine patients in our group had concomitant

hepaticojejunotomy following resection of common bile duct cysts and bile duct strictureplasty.

All liver resections in our 101 patients were major hepatectomies (resection of more than three segments). There was no surgical mortality and the postoperative morbidity was 28.7%. We concluded that the zero surgical mortality and acceptable morbidity of this study were the result of deliberate preoperative preparations. Preoperative volumetric CT estimation is of critical importance for patients who undergo resection of more than four liver segments. Sufficient future remnant liver is mandatory for preventing postoperative liver failure. For patients with acute cholangitis, preoperative PTCD or ENBD is indicated for relieving biliary sepsis. Definitive hepatectomy is performed 1 mo later after sepsis subsides<sup>[8]</sup>. This management may decrease the surgical morbidity.

In conclusion, this study demonstrated that bilateral liver resection is safe and its short- and long-term outcomes are satisfactory. A liver resection range less than the range of stone distribution is an independent risk factor associated with stone recurrence. Complete resection of strictured stone-bearing bile ducts and atrophic liver tissue, with the prerequisite of sufficient future remnant liver may decrease stone recurrence.

## COMMENTS

### Background

Intrahepatic stones is a common disease in Eastern Asia. Hepatectomy that can remove stones, strictured bile duct and atrophic liver tissue seems to be the optimal treatment for intrahepatic stones, and has been accepted increasingly as the definitive treatment. Most of the previous studies have demonstrated that liver resection for intrahepatic stones is confined to unilateral resection. The outcomes of bilateral liver resection for bilateral intrahepatic stones have not been clarified. Therefore, this study was conducted to evaluate the short- and long-term outcomes of bilateral liver resection for bilateral intrahepatic stones.

### Research frontiers

This study evaluated the feasibility and outcomes of bilateral liver resection for bilateral intrahepatic stones.

### Innovations and breakthroughs

There have been an increasing number of studies on hepatectomy for intrahepatic stones in recent years. For the subgroup of patients with bilateral intrahepatic stones, the feasibility and outcome of bilateral liver resection have not been clarified. This study is believed to be the first to demonstrate that bilateral liver resection for bilateral intrahepatic stones is safe and its long-term outcome is satisfactory, with a stone recurrence rate of 7.9% and an acute cholangitis rate of 6.5%.

### Applications

The most important clinical application of this study is that bilateral liver resection is indicated in a subgroup of patients with bilateral intrahepatic stones. The results of this study expand the surgical indications of hepatectomy for intrahepatic stones.

### Terminology

Bilateral liver resection is defined as bilateral resection of stone-bearing segments, with the prerequisite of leaving a sufficient liver remnant, for example, left lateral sectionectomy plus right posterior sectionectomy.

### Peer review

This paper is very interesting and well written. The study is provocative and the findings might be helpful for understanding the indications for bilateral liver resection of bilateral intrahepatic stones.

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BRIEF ARTICLES

## Sequential use of transarterial chemoembolization and percutaneous cryosurgery for hepatocellular carcinoma

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**Author contributions:** Xu KC made the study plan, managed patient data and wrote the paper; Niu LZ and Hu YZ performed the cryosurgery procedures; Zhou Q performed transarterial chemoembolization; Guo DH, Liu ZP, Lan B and Mu F collected and provided the materials of patients; Li YF performed the statistical analysis; Zuo JS supervised the trial process.

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at the ablated area was 17% for all patients, 11% and 23% for patients in sequential group and cryo-alone groups, respectively ( $P = 0.001$ ). The overall 1-, 2-, 3-, 4- and 5-year survival rate was 72%, 57%, 47%, 39% and 31%, respectively. The 1- and 2-year survival rates (71% and 61%) in sequential group were similar to those (73% and 54%) in cryo-alone group ( $P = 0.69$  and 0.147), while the 4- and 5-year survival rates were 49% and 39% in sequential group, higher than those (29% and 23%) in cryo-alone group ( $P = 0.001$ ). Eighteen patients with large HCC (> 5 cm in diameter) survived for more than 5 years after sequential TACE while no patient with large HCC (> 5 cm in diameter) survived more than 5 years after cryosurgery. The overall complication rate was 24%, and the complication rates were 21% and 26% for the sequential and cryo-alone groups, respectively ( $P = 0.06$ ). The incidence of hepatic bleeding was higher in cryo-alone group than in sequential group ( $P = 0.02$ ). Liver crack only occurred in two patients of the cryo-alone group.

**CONCLUSION:** Pre-cryosurgical TACE can increase the cryoablation efficacy and decrease its adverse effects, especially bleeding. Sequential TACE and cryosurgery may be the better procedure for unresectable HCC, especially for large HCC.

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**Key words:** Hepatocellular carcinoma; Cryosurgery; Transarterial chemoembolization; Cryoablation; Treatment

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Xu KC, Niu LZ, Zhou Q, Hu YZ, Guo DH, Liu ZP, Lan B, Mu F, Li YF, Zuo JS. Sequential use of transarterial chemoembolization and percutaneous cryosurgery for hepatocellular carcinoma. *World J Gastroenterol* 2009; 15(29): 3664-3669 Available from: URL: <http://www.wjgnet.com/1007-9327/15/3664.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.3664>

## INTRODUCTION

Cryosurgery has been used for two decades in treatment of many benign, malignant, and metastatic tumors<sup>[1-3]</sup>.

**RESULTS:** During a mean follow-up period of 42 ± 17 mo (range, 24-70 mo), the local recurrence rate

Furthermore, hepatocellular carcinoma (HCC) has been successfully treated either with cryosurgery alone or in combination with resection<sup>[4-6]</sup>.

Transarterial chemoembolization (TACE) itself is not associated with improved survival for patients with HCC<sup>[7]</sup>. However, it can shrink the HCC mass. It has been shown that sequential use of TACE and hepatectomy improves the outcome of patients with large HCC<sup>[8]</sup>. Cryosurgery is similar to the surgical resection of HCC in terms of total destruction of the tumor tissue. Therefore, TACE in combination with cryosurgery is expected to yield a better therapeutic effect on HCC, especially on large HCC.

From March 2001 to December 2006, 510 patients with HCC underwent percutaneous cryosurgery with or without TACE. In this study, we retrospectively investigated the results of 420 patients with HCC. Of them, 290 were treated with sequential use of TACE and percutaneous cryosurgery, and 130 received percutaneous cryosurgery alone.

## MATERIALS AND METHODS

### **Patients**

A total of 420 patients (310 males and 110 females) at a median age of 43 years (range, 21-81 years) were enrolled in this study. The diagnosis of HCC was made by abdominal computed tomography (CT), liver ultrasonography, and/or whole positron emission tomography-CT (PET-CT). Two hundred and ninety-five patients had an elevated serum  $\alpha$ -fetoprotein (AFP) level. HCC was proven by biopsy in 286 patients. All the patients were considered to have unresectable tumors due to their location or size or comorbidity. Patients with Child-Pugh C tumors and cirrhotic ascites were excluded from this study. The patients were divided into sequential TACE-cryosurgery (sequential) group ( $n = 290$ ) and cryosurgery alone (cryo-alone) group ( $n = 130$ ) (Table 1). There were no differences in age, sex and Child-Pugh classification of the patients between the two groups. However, patients in the sequential group tended to have larger tumors and a greater number of tumors compared to those in the cryo-alone group ( $P < 0.05$ ). Tumors larger than 10 cm in diameter were only found in the sequential group. All patients gave their informed consent. The study was approved by the Ethical Committee of Fuda Cancer Hospital (Guangzhou, China).

### **Methods**

TACE was performed after cross-sectional images were reviewed as previously described<sup>[9]</sup>. A French vascular sheath was placed into the femoral artery, and a 0.035 inch diameter Mickaelson catheter was advanced into the celiac and superior mesenteric arteries. Contrast was injected into the arteries during rapid-sequence radiographic imaging. Arterial branches supplying the tumors were then located. The venous phase was carefully examined for patency of the portal veins. A 0.018 inch diameter Tracker catheter was advanced through the Mickaelson catheter to the arterial branches supplying the tumors. The mixture of

**Table 1 Clinical features of patients undergoing sequential TACE-cryosurgery or cryosurgery-alone  $n$  (%)**

Clinical features	Sequential group ( $n = 290$ )	Cryo-alone group ( $n = 130$ )	P
Age (median)	46	41	
Sex (M/F)	212/78	98/32	0.623
Child-Pugh A	91 (31.4)	42 (32.3)	0.850
Child-Pugh B	199 (68.6)	88 (67.7)	0.850
Tumors			
1	132 (45.5)	85 (57.7)	0.001
2	84 (28.9)	38 (25.4)	
3	32 (11.0)	6 (10.0)	
> 3	42 (14.5)	1 (6.9)	
Largest diameter of tumors			
Range (cm)	4.5-15.0	3.1-7.0	
mean $\pm$ SD (cm)	6.5 $\pm$ 3.1	4.6 $\pm$ 3.2	0.04
Tumor > 10 cm	69 (23.8)	0	

doxorubicin (50 mg), mitomycin (10 mg), and lipiodol (4-15 mL) was injected into the arterial branches until hemostasis was achieved. If the tumors had no shrinkage 2 wk after the procedure, a second TACE was performed.

Percutaneous cryosurgery was performed under the guidance of ultrasound. Right lateral intercostal access was used to place cryoprobes in most patients. The size of cryoprobes used depended upon the tumor size and number. Small lesions were ablated with one or two 2-mm cryoprobes. Larger caliber cryoprobes (5-mm) or multiple probes were used according to the size of lesions. The cryoprobes were placed in the centre of tumor using a modified Seldinger technique under real-time ultrasound guidance. Multiple cryoprobes were placed 1.5-2 cm apart in the tumor periphery. Once the cryoprobe was positioned, freezing was initiated using an argon/helium-based cryosurgery system (EndoCare, Inc., Irvine, Calif. USA). Two freeze cycles (15 min each) were applied for 10-15 min of helium thaw following each freeze. The size of the ice ball formed by the cryoprobes was evaluated by ultrasound and made at least 1 cm larger than the apparent ultrasonic size of the tumor. Generally, separate lesions were frozen sequentially rather than simultaneously. The probe was removed after the second freeze/thaw cycle and the tract was filled with Gelfoam strips soaked in thrombin to facilitate hemostasis<sup>[10,11]</sup>.

### **Follow-up**

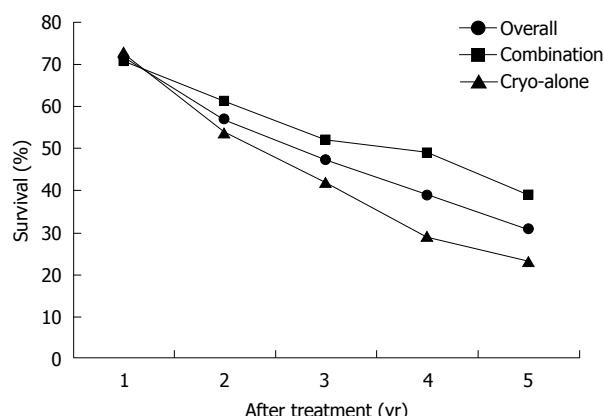
Follow-up consisted of ultrasonography and/or helical CT of the liver and serial check of AFP levels 1 mo after surgery and then every 2-3 mo for 1-2 years. Eighty-two patients underwent PET-CT during the follow-up and were subsequently reassessed every 6 mo with PET-CT. Successful tumor ablation (disease-free) was ideally defined by disappearance or no expansion of the ablated lesion on CT follow-up or no metabolic activity on PET-CT. Tumor recurrence or persistence was also defined using CT. Percutaneous biopsy was used in 79 patients to verify the eradication or recurrence of tumors.

### **Statistical analysis**

Survival rate of the patients was calculated from the

**Table 2 Survival rate of patients after sequential TACE-cryoablation or cryo-alone groups (%)**

	Patients (n = 420)	Sequential group (n = 290)	Cryo-alone group (n = 130)	P
Local recurrence	17	11	24	0.001
Survival rate				
1-yr	72	71	73	0.668
2-yr	57	61	54	0.147
3-yr	47	52	42	0.064
4-yr	39	49	29	0.001
5-yr	31	39	23	0.001

**Figure 1 Survival rate of HCC patients.****Table 3 Complication between sequential TACE-cryosurgery and cryo-alone groups n (%)**

	Patients (n = 420)	TACE- cryosurgery (n = 290)	Cryo-alone (n = 130)	P
Hepatic bleeding	16 (3.8)	5 (1.7)	11 (8.5)	0.001
Liver crack	2 (0.5)	0	2 (1.5)	
Thrombocytopenia and/or clotting dysfunction	30 (7.1)	21 (7.2)	9 (6.9)	0.907
Liver failure	16 (3.8)	12 (4.1)	4 (3.1)	0.599
Acute renal failure with myoglobinuria	3 (0.7)	2 (0.7)	1 (0.7)	
Agranulocytosis	4 (0.9)	4 (1.4)	0	
Pneumonia	25 (0.6)	17 (5.9)	8 (6.2)	0.907
Total	101 (24.0)	61 (21.0)	40 (30.7)	0.184

time of cryosurgery to the time of death or last follow-up with Kaplan-Meier method (*J Am Stat Assoc* 1958; 53: 457-481). Values were expressed as mean  $\pm$  SD. Variables were compared using Fisher's exact test (two-sided) or rank-sum test when appropriate.  $P < 0.05$  was considered statistically significant. SPSS 9.0 (SPSS, Chicago, Illinois) was used for data analysis.

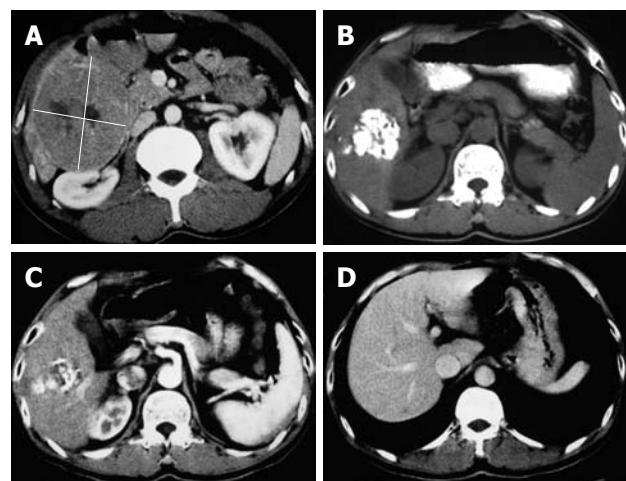
## RESULTS

### Survival

The patients were followed up for  $42 \pm 17$  mo (range, 24-70 mo) (Table 2). The local recurrence rate at the ablated area was 17% for all patients, and 11% and 23% for sequential and cryo-alone groups, respectively ( $P = 0.001$ ). The overall 1-, 2-, 3-, 4- and 5-year survival rate was 72%, 57%, 47%, 39% and 31%, respectively. The 1- and 2-year survival rates (71% and 61%) for sequential group were similar to those (73% and 54%) for cryo-alone group ( $P = 0.69$  and 0.147, respectively), while the 4- and 5-year survival rates were 49% and 39% for sequential group, higher than those (29% and 23%) for cryo-alone group ( $P = 0.001$ , Figure 1).

Eighteen patients with large HCC (> 5 cm in diameter) survived more than 5 years after sequential TACE-cryosurgery while no patient with large HCC survived more than 5 years after cryosurgery alone.

The CT of liver for selected patients is illustrated in Figures 2-4.

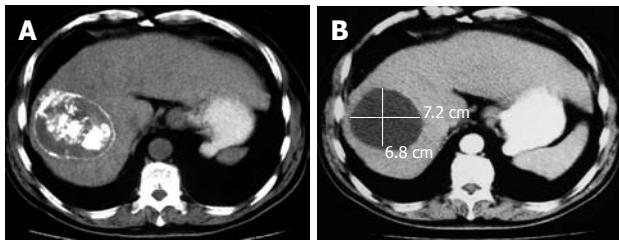
**Figure 2 Biopsy showing a HCC mass (11 cm x 9 cm) in the right liver lobe. The sequential changes (A-D) of the mass were disappeared after sequential therapy.**

### Complications

The main complications were hepatic bleeding, liver crack and failure, thrombocytopenia and/or clotting dysfunction, acute renal failure with myoglobinuria and pneumonia (Table 3). The overall complication rate was 24%, and the complication rate was 21% and 26%, respectively, for the sequential and cryo-alone groups ( $P = 0.184$ ). The incidence of hepatic bleeding was higher in cryo-alone group than in sequential group ( $P = 0.001$ ). Liver crack only occurred in the cryo-alone group. The incidence of thrombocytopenia and/or clotting dysfunction, liver failure and pneumonia was similar in the two groups ( $P = 0.599$ ). Acute renal failure with myoglobinuria was observed in two patients of the sequential group and in one patient of the cryo-alone group. Agranulocytosis occurred in only four patients of the sequential group. Two patients in the cryo-alone group died of liver crack and massive hepatic bleeding during pericyrosurgery.

## DISCUSSION

Surgical resection (hepatectomy) can achieve a long-term



**Figure 3 Histology showing the HCC mass at 3 mo (A) and 28 mo (B) after sequential therapy.** The cryoablated mass had cavitation formation. No cancer cells were identified by needle aspiration.

survival time in up to 30% of patients with HCC. Unfortunately, because of the limitations of surgical techniques and patient condition, only 25% or less of patients are estimated to be candidates for resection of HCC<sup>[12]</sup>. Since cryosurgery is a focal treatment, sparing more normal liver tissues than resection, it can be used for unresectable multiple lesions affecting both liver lobes and large tumors abutting major vessels<sup>[13,14]</sup>. It has been shown that mini-invasively percutaneous hepatic cryosurgery under guidance of real time ultrasound can achieve a long-term survival of patients with both primary and metastatic hepatic malignancies<sup>[15,16]</sup>.

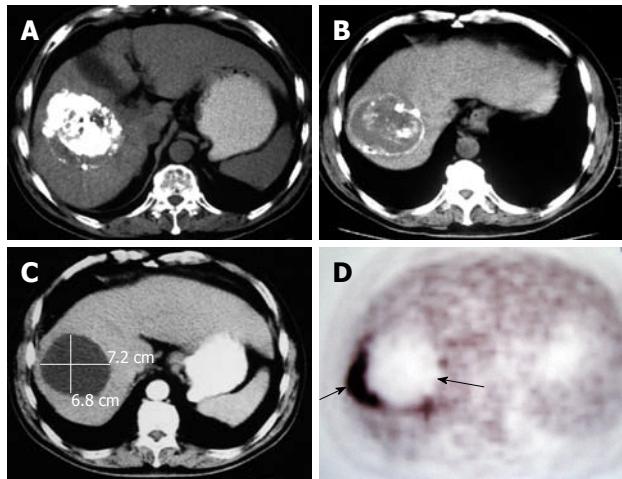
Cryoablation is effective for tumor mass, but post-cryosurgical recurrence is often observed due to insufficient freezing during cryosurgery<sup>[17]</sup>. Overcoming this problem is the key to the improvement of overall survival rate of patients.

TACE is used either as an adjuvant therapy or as a palliative procedure for patients with unresectable HCC<sup>[18,19]</sup>. It has been shown that TACE prior to surgical resection can not only prevent recurrence of HCC by controlling intrahepatic spread via the portal system, but also facilitate surgery by reducing tumor bulk<sup>[20]</sup>. Cryosurgery, in which tumors are frozen and then left *in situ* to be reabsorbed, is equivalent to the surgical resection of HCC in terms of total destruction of the tumor tissue, indicating that it is reasonable to perform TACE for tumors prior to cryosurgery.

In our study, the 1-, 2-, 3, 4- and 5-year survival rate was 71%, 61%, 52%, 49% and 39%, respectively, for the 290 patients after sequential TACE-cryosurgery, and 73%, 54%, 42%, 29% and 23%, respectively, for the 130 patients after cryo-alone. The 4- and 5-year survival rates were higher for the patients of sequential group than for those of cryo-alone group, suggesting that cryosurgery in combination with TACE can achieve a better long-term outcome, which is consistent with the reported findings<sup>[21]</sup>.

The relatively good outcome obtained in our study was due to the complementary effect of sequential TACE and cryosurgery.

Several factors can influence cryo-induced tumor cell destruction. Among these factors, temperature is a critical factor since a temperature lower than -40°C is assumed necessary to ensure tumor ablation. An ice-ball larger than the target lesion is thus necessary for complete tumor ablation, because the temperature at the



**Figure 4 A biopsy-proved HCC a female.** A: A space-occupying lesion in right lobe of the liver after TACE and prior to the cryosurgery; B: The cryoablated lesion at 3 mo and 3 years, respectively, after cryosurgery; C: Needle aspiration showing liquefaction of the cryoablated lesion and no cancer cells; D: PET-CT showing no metabolic activity in the cryoablated mass 5 years after therapy.

edge (a few millimeters in thickness) of an ice-ball is non-lethal<sup>[22]</sup>. An ice-ball encompassing the entire tumor mass 1 cm beyond the tumor border should be considered adequate for ablation. According to the MRI-estimated three-dimensional temperature distribution in liver cryolesions<sup>[23]</sup>, the mean value of distances calculated between the -40°C isotherm and cryolesion edge is a median of 4.1 mm and the largest distance defined for each cryo-lesion is a median of 8.1 mm. However, the distance is related to the volume of the cryo-treated tumor. The largest distance defined between the the -40°C isotherm and the edge of cryo-lesion may be close to 1 cm for a cryo-lesion volume less than 25 cm<sup>3</sup>, while a cryo-lesion larger than 25 cm<sup>3</sup>, the 1-cm ice-ball extension cannot ensure the temperature adequate for ablation in the entire volume enclosed by the rim zone. It has been shown that tumor size influences the tumor ablation rate, and lesions larger than 3 cm are more likely associated with a higher recurrence rate than smaller tumors<sup>[24]</sup>.

Therefore, the smaller the tumor is, the more effective the cryoablation is and the lower the recurrence rate is. Since TACE can shrink HCC mass, it can be expected to increase the efficacy of cryoablation for HCC, and to decrease its local recurrence if performed prior to cryoablation. In our study, the recurrence rate of HCC was 11% for the sequential group and 23% for the cryo-alone group, respectively, that is of special significance for large tumors close to large vessels, because the temperature cannot reach the edge zone of cryo-lesions due to its blood warming effect<sup>[25,26]</sup>. This opinion is supported by our study in which 18 patients with large HCC (> 5 cm in diameter) survived more than 5 years after sequential TACE while no patient with large HCC survived more than 5 years after cryosurgery alone.

The major complications of cryosurgery are hemorrhage and liver crack<sup>[27,28]</sup>. In our study, post-cryosurgery hepatic bleeding occurred in 16 patients.

Of them, 11 (8.5%) received cryo-alone and 5 (1.7%) underwent sequential TACE. The most dangerous liver crack was observed in only two patients of the cryo-alone group. Clavien *et al*<sup>[21]</sup> treated 15 HCC patients with cryosurgery and found that only one patient who did not undergo pre-cryosurgery TACE had hepatic bleeding, suggesting that TACE can reduce post-cryosurgery complications, such as bleeding and liver crack.

Pre-surgical TACE may be contraindicated in patients with resectable HCC and cirrhosis, because it causes progressive deterioration of liver function<sup>[29]</sup>. However, Kaibori *et al*<sup>[20]</sup> reported that TACE prior to HCC resection improves the prognosis of HCC patients with severe liver dysfunction, because decreasing resection of non-tumorous liver tissue avoids postoperative hepatic failure in patients with cirrhosis. In our study, liver failure occurred in 16 patients (3.8%), including 12 patients in the sequential group and four patients in the cryo-alone group, with no increased risk of liver failure caused by TACE. Other adverse effects of cryosurgery, such as thrombocytopenia and/or clotting dysfunction, acute renal failure with myoglobinuria, were observed in both groups of patients. In our study, four patients in the sequential group developed agranulocytosis due to a higher dose of chemotherapeutic agents used in TACE. The complication became less when the dose of chemotherapeutic agents was reduced.

In conclusion, TACE prior to cryosurgery can increase cryoablation efficacy and decrease its adverse effects, especially bleeding. Sequential use of TACE and cryosurgery may be a better procedure for improving the outcome of patients with unresectable HCC, especially large HCC. Further prospective study is required to fully evaluate its value.

## COMMENTS

### Background

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. Its treatment modalities, including surgical resection or liver transplantation, benefit only a small proportion of such patients. Cryosurgery, one of the proposed therapies for HCC, can improve the survival time of such patients.

### Research frontiers

Although cryosurgery is considered an effective treatment modality for unresectable HCC, it has been performed only for smaller tumors. Selected patients with stage III/IV HCC can be down staged with transarterial chemoembolization (TACE). Importantly, patients who are successfully down staged have a higher overall survival rate after resection of HCC. TACE prior to cryosurgery should be a good choice for HCC.

### Innovations and breakthroughs

Few reports are available on combined TACE and percutaneous cryosurgery. This study showed that pre-cryosurgical TACE could increase the cryoablation efficacy and decrease its adverse effects, especially bleeding, and improve the outcome of patients with unresectable HCC, especially large HCC.

### Applications

To the best of our knowledge, this is the first study comparing sequential TACE and cryosurgery for HCC patients. The results of this study show that sequential TACE and cryosurgery are a rather good treatment modality for HCC.

### Terminology

Cryosurgery, also known as cryotherapy or cryoablation, has gone through a long process of development. From the end of the 20th century, the development of imaging techniques and new freezing equipments has led to the emergence of modern cryosurgery. Liquid nitrogen operation system and

argon-helium surgical system are representative of two important stages of modern cryosurgery. Apart from liver cancer, cryosurgery has been successfully performed for a variety of tumors.

### Peer review

The study seems to be very interesting. The results, based on clinical observation and follow-up, suggest that sequential TACE and percutaneous cryosurgery are a better choice for unresectable HCC, especially large HCC.

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BRIEF ARTICLES

## CEUS: A new imaging approach for postoperative vascular complications after right-lobe LDLT

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### Abstract

**AIM:** To investigate contrast-enhanced ultrasound (CEUS) for early diagnosis of postoperative vascular complications after right-lobe living donor liver transplantation (RLDLT).

**METHODS:** The ultrasonography results of 172 patients who underwent RLDLT in West China Hospital, Sichuan University from January 2005 to June 2008 were analyzed retrospectively. Among these 172 patients, 16 patients' hepatic artery flow and two patients' portal vein flow was not observed by Doppler ultrasound, and 10 patients' bridging vein flow was not shown by Doppler ultrasound and there was a regional inhomogeneous echo in the liver parenchyma upon 2D ultrasound. Thus, CEUS examination was performed in these 28 patients.

**RESULTS:** Among the 16 patients without hepatic artery flow at Doppler ultrasound, CEUS showed nine cases of slender hepatic artery, six of hepatic arterial thrombosis that was confirmed by digital subtraction angiography and/or surgery, and one of hepatic arterial occlusion with formation of lateral branches. Among the two patients without portal vein flow at Doppler ultrasound, CEUS showed one case of hematoma compression and one of portal vein thrombosis,

and both were confirmed by surgery. Among the 10 patients without bridging vein flow and with liver parenchyma inhomogeneous echo, CEUS showed regionally poor perfusion in the inhomogeneous area, two of which were confirmed by enhanced computed tomography (CT), but no more additional information about bridging vein flow was provided by enhanced CT.

**CONCLUSION:** CEUS may be a new approach for early diagnosis of postoperative vascular complications after RLDLT, and it can be performed at the bedside.

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**Key words:** Contrast-enhanced ultrasound; Living donor liver transplantation; Postoperative complication; Vascular disease

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Luo Y, Fan YT, Lu Q, Li B, Wen TF, Zhang ZW. CEUS: A new imaging approach for postoperative vascular complications after right-lobe LDLT. *World J Gastroenterol* 2009; 15(29): 3670-3675 Available from: URL: <http://www.wjgnet.com/1007-9327/15/3670.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.3670>

### INTRODUCTION

A right-lobe living donor liver transplantation (RLDLT), with or without the middle hepatic vein (MHV), is now used generally for adult-to adult liver transplantation<sup>[1]</sup>. A right liver graft only involves part of the liver lobes from healthy donors, whose vessels are very slender and the reconstruction of one or more tributaries of the MHV (bridging vein) is sometimes involved. RLDLT can be complicated by a high incidence of postoperative vascular complications, which may cause graft dysfunction and liver failure. Therefore, early diagnosis of postoperative vascular complications is very important after RLDLT. As noninvasive examinations, 2D and Doppler ultrasound play important roles in

screening for postoperative complications, but vascular visualization is not always satisfactory<sup>[2-4]</sup>. Contrast-enhanced ultrasound (CEUS) has been applied gradually in recent years and has provided a great improvement for this defect, because of its fine vascular tracing and perfusion visualization<sup>[5-8]</sup>.

The present study analyzed retrospectively CEUS in patients who underwent RLDLT in West China Hospital, Sichuan University from January 2005 to June 2008, to investigate the diagnostic value of CEUS in postoperative vascular complications.

## MATERIALS AND METHODS

### Clinical data

The study was conducted under the approval of the Ethics Committee of West China Hospital, and written informed consent was obtained from all patients who received CEUS examination. We analyzed retrospectively the ultrasound results of 172 consecutive patients (137 men and 35 women, aged 18-63 years, mean 39.1 years), who underwent RLDLT from January 2005 to June 2008 in West China Hospital. Bypass of MHV tributaries was performed in 83 cases. The primary diseases included 98 with cirrhosis, 58 with malignant liver tumors, 12 with acute liver failure, one with hepatic echinococcosis, one with Budd-Chiari syndrome, and two cases liver re-transplantation.

### Instrument and methods

Phillips HDI5000 and HD 11 (Phillips Medical Systems, Bothell, WA, USA) with 2-5-MHz probes were used in our study as conventional ultrasound equipment. CEUS examinations were performed with Acuson Sequoia 512 with contrast pulse sequencing (Siemens Medical Solutions, Mountain View, CA, USA) or Phillips IU 22 (Phillips Medical Systems) with the pulse inversion technique. Gray-scale ultrasound was used to observe liver graft parenchyma, and portal and hepatic veins, and Doppler ultrasound was used to measure blood flow and its velocity in the hepatic artery, portal and hepatic veins, and bridging vein. CEUS was performed in patients without hepatic artery and/or portal vein flow and/or patients without bridging vein flow accompanied by liver regional inhomogenous echo. The aim of CEUS was to observe blood flow in the above vessels, as well as perfusion of the liver. Conventional Doppler ultrasound and CEUS were performed by two experienced ultrasound physicians with more than 5 years experience in liver transplantation. Ultrasonography was performed everyday within the first week after operation in the intensive care unit (ICU), and afterwards, the time and interval of ultrasound examination depended on laboratory results and clinical conditions. The contrast agent was SonoVue (Bracco Imaging, Milan, Italy), which consists of an inert gas, sulfur hexafluoride and phospholipid peplos with high flexibility. CEUS was performed using 1.2-2.4 mL SonoVue suspension by rapid bolus injection through the left ulnar vein,

followed by 10 mL saline flush and an ultrasonic inner time counter started simultaneously. Dynamic and static images were recorded to observe patency of the hepatic artery, portal and hepatic veins, and bridging vein, as well as perfusion of the liver parenchyma.

## RESULTS

Among these 172 patients, 16 patients' hepatic artery flow and two patients' portal vein flow was not observed by Doppler ultrasound, and 10 patients' bridging vein flow was not shown by Doppler ultrasound and there was a regional inhomogeneous echo in the liver parenchyma upon 2D ultrasound. Thus, CEUS examination was performed in these 28 patients.

Among the 16 patients without hepatic artery flow at Doppler ultrasound, CEUS showed a slender right hepatic artery in nine (Figure 1), hepatic artery thrombosis (HAT) in six (Figure 2), all confirmed by digital subtraction angiography and/or surgery, hepatic artery occlusion accompanied by lateral branches in one (Figure 3).

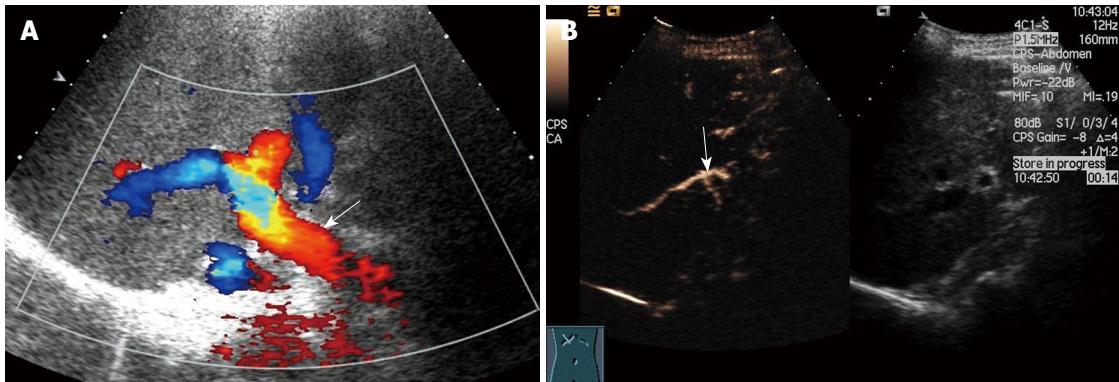
Among the two patients without portal vein flow at Doppler ultrasound, CEUS showed one with hematoma compression and one with portal vein thrombosis, both of which were confirmed by surgery.

The 10 patients without bridging vein flow and with regional liver inhomogenous echo at Doppler ultrasound were demonstrated by CEUS to have corresponding poor liver graft perfusion. The diagnosis was confirmed by enhanced CT in two cases. However, CT scan did not yield any additional information on bridging vein visualization (Figure 4).

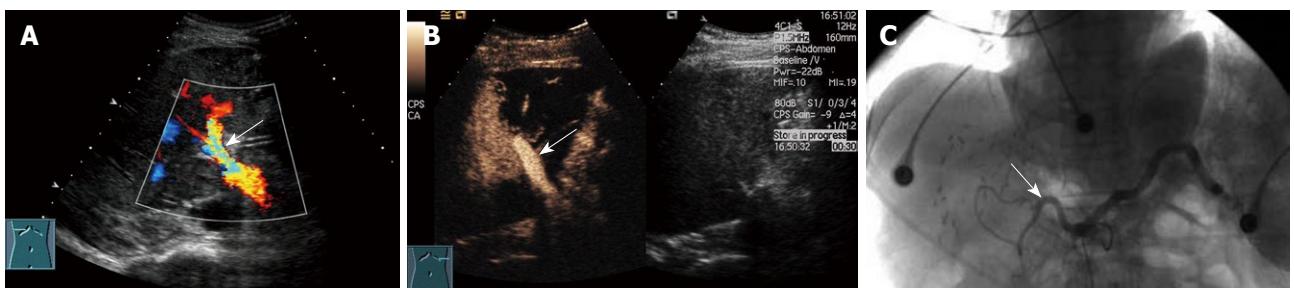
## DISCUSSION

### Hepatic artery complications after RLDLT

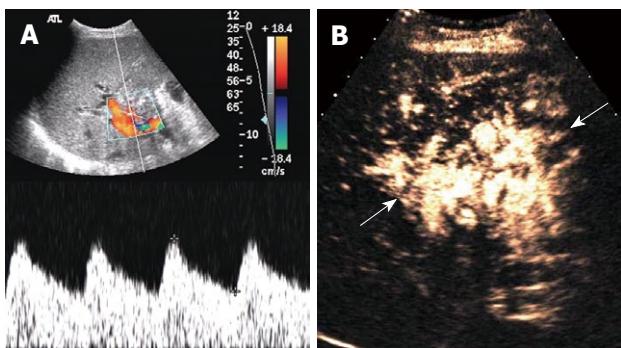
Hepatic artery complications, including HAT, hepatic artery stenosis (HAS) and pseudoaneurysm, are some of the most severe complications after liver transplantation, with an incidence of 5%-15%. HAT often occurs early after surgery, especially within 6 wk. Mortality from HAT is as high as 20%-60% and re-transplantation is usually required<sup>[2]</sup>. Therefore, early examination and diagnosis after surgery are crucial for graft survival and good prognosis. Ultrasound manifestations of HAT include the disappearance of blood flow at the porta hepatis, disappearance of or abnormal intrahepatic blood flow, such as the tardus-parvus spectrum wave form, possibly followed by secondary changes including biliary complications, hepatic infarction and abscess. HAT is highly suspected if no blood flow signal is present in the hepatic artery at Doppler ultrasound. However, false-positive results must be excluded, and the main reasons include instrumental insensitivity to blood flow, improper adjustment of parameters, incorrect detection approach, early transient artery spasm, and slender graft arteries. HAT sensitivity of Doppler ultrasound has been reported



**Figure 1 Slender hepatic artery in 54-year-old woman who received right-lobe living donor liver transplantation (RLDLT).** A: Longitudinal oblique Doppler Ultrasound (US) scan 2 mo after RLDLT did not reveal the right hepatic artery beyond the level of the right portal vein (arrow); B: Longitudinal oblique contrast-enhanced ultrasound (CEUS) scan obtained followed the Doppler scan. The right hepatic artery (arrow) was seen clearly 14 s after contrast material injection, but it was small.



**Figure 2 HAT in a 35-year-old man who underwent RLDLT.** A: Longitudinal oblique Doppler US scan 1 d after RLDLT did not reveal the right hepatic artery beyond the level of the right portal vein (arrow); B: Longitudinal oblique CEUS scan obtained following the Doppler scan. The right hepatic artery was still not seen after contrast material injection, but the right portal vein was demonstrated clearly (arrow). A complete unenhanced area indicates a lack of perfusion of the liver parenchyma in the anterior right lobe; C: Selective angiogram obtained after celiac artery injection confirmed complete thrombosis of the right artery at the level of the anastomosis (arrow).



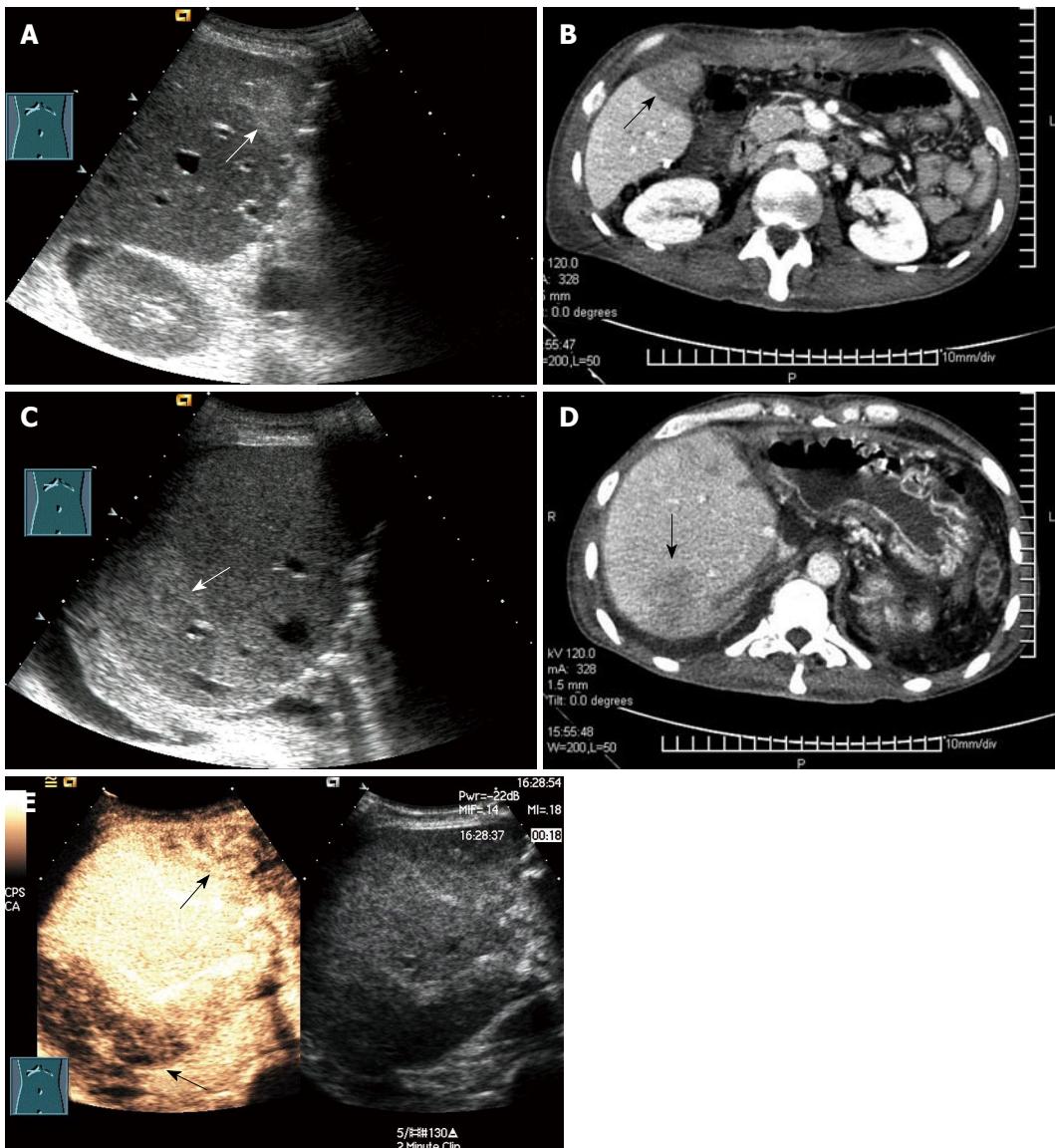
**Figure 3 Hepatic artery obstruction with collateral circulation in a 36-year-old man who underwent RLDLT.** A: Longitudinal oblique Doppler US scan 6 mo after RLDLT revealed a tardus-parvus spectrum at the porta hepatis; B: Longitudinal oblique CEUS scan obtained following the Doppler scan. A reticulate vessel instead of the right hepatic artery trunk was seen (arrows) at the porta hepatis after ultrasound agent injection.

to be 60%-90%<sup>[2-4,9-17]</sup>. Color Doppler technology has improved considerably during the past 10 years, and the possibility of non-flow visualization in the hepatic artery has decreased. In the present study, hepatic flow was not demonstrated in 1.3% (16/1231) of scans, similar to the study of Hom *et al*<sup>[5]</sup>. Visualization of the hepatic artery is improved significantly by CEUS. The accuracy of CEUS in diagnosing HAT was 100% in our study, similar to previous studies<sup>[5-8,18]</sup>.

The incidence of HAS is 5%-10%, which occurs mostly at the anastomotic stoma. Severe stenosis may result in similar outcomes to HAT, such as biliary ischemia, graft dysfunction and liver failure. Tardus-parvus changes ( $RI < 0.5$  and accelerating time  $> 0.08$  s) detected at Doppler ultrasound suggest HAS, and it is also suspected when regional blood flow velocity is  $> 200$  m/s at the anastomotic stoma. However, it is difficult to observe directly the stenosis site by ultrasound for intestinal gas or liver artery curvature. The value of CEUS in diagnosing HAS remains controversial. Some studies have revealed that CEUS may be more capable than conventional ultrasound of visualizing the trunk of the hepatic artery, possible sites of stenosis, and peripheral branch circulation<sup>[2-4,7,10-12,18]</sup>.

#### Portal vein complications

Portal vein complications include portal vein thrombus (PVT), stenosis and phlebolithoma. The incidence of PVT and stenosis is about 1%-2%. Monitoring of the portal vein after surgery is also important, because the blood supply from portal vein is essential for graft survival after liver transplantation. Once PVT or severe stenosis in the portal vein of liver grafts occurs after surgery, patients may suffer from primary graft dysfunction, visceral bleeding, bile leakage, ascites, splenectomy, and re-transplantation, re-operation or



**Figure 4** Regional congestion in a 35-year-old man who underwent RLDLT. A: Longitudinal oblique 2D US scan 1 d after RLDLT revealed regional echogenicity in segment V; B: Contrast-enhanced CT showed poor perfusion (arrow) in segment V 2 d after ultrasound examination; C: Longitudinal oblique 2D US scan revealed regional echogenicity in segment VII; D: Contrast-enhanced CT showed poor perfusion (arrow) in segment VII; E: Longitudinal oblique CEUS scan obtained following the Doppler scan. At CEUS, the poor perfusion area (arrows) was seen as an inhomogenous echo that corresponded to segment V and segment VII. This patient remained asymptomatic and formed collaterals between the tributary of the middle hepatic vein and the right hepatic vein at Doppler ultrasound follow-up.

interventional therapy are required. PVT is not difficult to diagnose using 2D and Doppler ultrasound. CEUS is helpful when the trunk or branches of the portal vein are not well visualized as a result of surgery or other causes<sup>[2-4,5,7,8,19-21]</sup>.

Caliber mismatch between the portal vein of the donor and recipient is very common, and is manifested as a hyperechoic ring at the anastomotic stoma and regional blood flow disorder by Doppler ultrasound. Currently, the diagnostic criteria for portal vein stenosis include: regional stenosis with a diameter of 2.5 mm, blood flow aliasing and acceleration at the stenosis site, blood flow velocity  $> 150$  cm/s at the stenosis site, or a prestenotic:stenotic blood flow velocity ratio of  $> 4:1$ , accompanied by symptoms of portal hypertension, including splenomegaly, ascites and branched circulation formation. CEUS is useful for diagnosis of portal vein

complications, because it can determine clearly the diameter of the portal vein and clarify the severity and range of portal vein stenosis<sup>[2-4,5,7,8,19-21]</sup>.

#### Outflow tract complications

Outflow tract in RLDLT includes the hepatic vein and bridging vein. Hepatic vein complications after surgery include thrombosis (HVT) and stenosis (HVS), with an incidence of about 1%. HVT is not difficult to diagnose by conventional ultrasound, but the diagnosis of HVS by Doppler ultrasound remains controversial. It has been reported that obvious stenosis can be observed by 2D ultrasound, when the ratio of prestenotic/stenotic blood flow velocity is higher than 3-4:1, the distal blood flow spectrum is straight and blood flow is decelerated or reversed, and the negative predictive value of phase loss in HVS is reported to be 98.4%. However, other

studies have concluded that ultrasound is inaccurate for the diagnosis of HAS, which should be combined with a pressure gradient for the hepatic vein and inferior vena cava, or depend on clinical manifestations<sup>[2-4,22-25]</sup>.

As RLDLT without the MHV has been performed increasingly in recent years, reconstruction and bypass of MHV tributaries have received more attention from clinicians, because "small-for-size syndrome" may be caused by early occlusion of bridging veins. However, these vessels are so tiny and deep that postoperative monitoring is difficult. CEUS improve the visualization of these bridging vessels and the perfusion of their drainage area<sup>[25-27]</sup>. However, there were only 10 patients who underwent bridging vessel monitoring by CEUS in our study, and thorough investigations with a larger sample size are required to clarify its diagnostic value.

Conventional 2D and Doppler ultrasound play important roles in screening and early diagnosis of vascular complications after RLDLT, but their inability to depict flow in some patients remains a substantial problem. Our initial experience shows that CEUS improves visualization of hepatic artery, portal and hepatic vein and bridging vein blood flow, as well as evaluation of perfusion of the liver graft parenchyma. Furthermore, CEUS can be performed at the bedside, and it has no iodine allergy or X-ray exposure. Therefore, CEUS provides a new approach in monitoring postoperative vascular complications after RLDLT, and more studies are needed for further evaluation.

## COMMENTS

### Background

Right-lobe living donor liver transplantation (RLDLT) is considered to be complicated liver surgery, with a high incidence of postoperative vascular complications that may cause graft dysfunction and liver failure. Noninvasive 2D and Doppler ultrasound play important roles in screening for postoperative complications, but vascular visualization is not always satisfactory. Contrast-enhanced ultrasound (CEUS) provides a great improvement for this defect because of its fine vascular tracing and perfusion visualization. Therefore, the authors investigated if CEUS improved the diagnosis of postoperative vascular complications after RLDLT.

### Research frontiers

CEUS has been applied gradually in recent years and focuses mainly on characterization of focal liver lesion and ablation monitoring. CEUS has the ability to show macro- as well as microvasculature, but there have been more studies based microvasculature.

### Innovations and breakthroughs

CEUS can improve flow visualization of the hepatic artery, portal and hepatic veins and bridging vein, as well as evaluation of perfusion of liver graft parenchyma after RLDLT.

### Applications

RLDLT is now generally used for adult-to adult liver transplantation worldwide. CEUS provides a new approach in monitoring postoperative vascular complications after RLDLT.

### Peer review

This is a good descriptive study in which the authors analyzed the value of CEUS in vascular complications after ALDLT. The results suggest that CEUS can be performed at the bedside; it has no iodine allergy, no X-ray exposure, and can be repeated with little interference to the vessels. Thus, CEUS will be a new advanced and noninvasive technique for vascular complications in RLDLT.

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S- Editor Li LF L- Editor Kerr C E- Editor Yin DH



BRIEF ARTICLES

# ***Astragalus mongholicus* polysaccharide inhibits lipopolysaccharide-induced production of TNF- $\alpha$ and interleukin-8**

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## **Abstract**

**AIM:** To explore the effect of *Astragalus mongholicus* polysaccharide (APS) on gene expression and mitogen-activated protein kinase (MAPK) transcriptional activity in intestinal epithelial cells (IEC).

**METHODS:** IEC were divided into control group, lipopolysaccharide (LPS) group, LPS+ 50  $\mu$ g/mL APS group, LPS+ 100  $\mu$ g/mL APS group, LPS+ 200  $\mu$ g/mL APS group, and LPS+ 500  $\mu$ g/mL APS group. Levels of mRNAs in LPS-induced inflammatory factors, tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-8, were measured by reverse transcription-polymerase chain reaction. MAPK protein level was measured by Western blotting.

**RESULTS:** The levels of TNF- $\alpha$  and IL-8 mRNAs were significantly higher in IEC with LPS-induced damage than in control cells. APS significantly abrogated the LPS-induced expression of the *TNF- $\alpha$*  and *IL-8* genes. APS did not block the activation of extracellular signal-regulated kinase or c Jun amino-terminal kinase, but inhibited the activation of p38, suggesting that APS inhibits LPS-induced production of TNF- $\alpha$  and IL-8 mRNAs, possibly by suppressing the p38 signaling pathway.

**CONCLUSION:** APS-modulated bacterial product-mediated p38 signaling represents an attractive strategy for prevention and treatment of intestinal inflammation.

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**Key words:** *Astragalus mongholicus* polysaccharide; Intestinal epithelial cells; Tumor necrosis factor- $\alpha$ ; Interleukin-8; Extracellular signal-regulated kinase; C Jun amino-terminal kinase; p38 kinase

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Yuan Y, Sun M, Li KS. *Astragalus mongholicus* polysaccharide inhibits lipopolysaccharide-induced production of TNF- $\alpha$  and interleukin-8. *World J Gastroenterol* 2009; 15(29): 3676-3680 Available from: URL: <http://www.wjgnet.com/1007-9327/15/3676.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.3676>

## **INTRODUCTION**

Intestinal epithelia cells (IEC) are the first line of defense against noxious intraluminal agents, including microorganisms and toxic antigens<sup>[1]</sup>. Although IEC are less responsive to polysaccharide than monocytes/macrophages, it has been shown that endotoxin triggers a proinflammatory gene transcriptional program in some IEC<sup>[2]</sup>, including the rat small intestinal cell line IEC-6<sup>[1,3,4]</sup>. Luminal endotoxin may participate in various intestinal inflammatory disorders. Modulation of bacteria- and bacterial product-induced gene expression in the intestine may have a significant impact on intestinal inflammatory disorders<sup>[5]</sup>.

*Astragali Radix*, root of *Astragalus membranaceus* Bunge, is a popular herb that has been used for thousands of years in treatment of a variety of diseases in oriental medicine. *Astragalus mongholicus* polysaccharide (APS) is the main ingredient of *Astragali Radix*. Studies have

revealed the anti-inflammatory, antioxidant, and immune regulatory roles of APS<sup>[6]</sup>. However, knowledge of how APS exerts its anti-inflammatory effects is still limited. Lee *et al*<sup>[7]</sup> reported that *Astragali Radix* appears to exert immune modulating effects by regulating the expression of cytokines, such as interleukin (IL)-1, IL-6 and inducible nitric oxide synthase (iNOS), as well as the production of nitric oxide (NO). In this study, the effect of APS on LPS-induced mitogen-activated protein kinase (MAPK) signaling and pro-inflammatory gene expression in IEC-6 cells was investigated, showing that APS prevents the activation of p38MAPK signaling in IEC-6 cells *in vitro*.

## MATERIALS AND METHODS

### Materials

APS was isolated from a 6-year-old *Astragalus membranaceus* sample purchased from the Chinese Medicinal Herbs Company (Beijing, China), with a purity of 98.5%. IEC-6 cells were purchased from the Chinese Academy of Medical Sciences, Center for Biological Detection (Beijing, China). Lipopolysaccharide (LPS, *Escherichia coli* O55:B5) and insulin (I5500) were purchased from Sigma (USA). Phospho-specific rabbit polyclonal antibodies against Thr180 and Tyr182 dual-phosphorylated p38, Thr183 and Tyr185 dual-phosphorylated c Jun amino-terminal kinase (JNK), Thr202 and Tyr204 dual-phosphorylated extracellular signal-regulated kinase (ERK)/2 and total p38, ERK1/2, JNK were purchased from Cell Signaling Technology (USA). A rabbit polyclonal antibody against actin and a peroxidase (HRP)-labeled anti-rabbit IgG antibody were purchased from Sigma (USA).

### Culture and treatment of IEC

The rat small intestinal cell line IEC-6 was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 0.01 mg/mL insulin. IEC-6 cells were grown in 6-well plates at a density of  $5 \times 10^5$  cells per well and cultured in DMEM at 37°C in a humidified atmosphere containing 50 mL CO<sub>2</sub> for 24 h. After incubation, non adherent cells were removed and adherent cells were pretreated for 1 h with APS at different concentrations (50, 100, 200 and 500 µg/mL). The cells were then stimulated with LPS (10 µg/mL) and harvested at the indicated time points.

### RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) analysis

IEC-6 cells were cultured in DMEM containing LPS with or without various concentrations of APS, for 1 h to allow detection of tumor necrosis factor (TNF)-α mRNA, and for 2 h to allow detection of IL-8 mRNA. Cells were washed in PBS and used for RNA isolation. Total RNA was isolated using Trizol reagent according to its manufacturer's instructions. RT-PCR was carried out using 1 µg of total RNA from IEC-6 cells and an oligo(dT)<sub>12-18</sub> primer.

The sequences of primers for amplification of cDNAs of rat TNF-α-U, TNF-α-L, IL-8-L, GAPDH-U and GAPDH-L are 5'-TTCGGGGTGATCGGTCCAA-3', 5'-AGCATCTCGTGTGTTCTGA-3', 5'-CCTGAAGA CCCTACCAAG-3', AGGCTCCATAAATGAAAGA-3', 5'-ATCACTGCCACTCAGAAGAC-3', 5'-TGAGGG AGATGCTCAGTGTT-3', respectively. GAPDH was used as an invariant housekeeping internal control gene. Twenty-five cycles of amplification were performed for all reactions. The length of PCR products of TNF-α, IL-8 and GAPDH was 750, 494 and 580 bp, respectively.

### Western blotting analysis

IEC-6 cells were stimulated with LPS (10 µg/mL) for various periods of time (0-1 h). The cells were cultured in a medium containing LPS with or without various concentrations of APS for 1 h to detect phosphorylated-p38, ERK1/2, JNK, and total p38, ERK, and JNK, and lysed with a SDS sample buffer. The supernatants were analyzed by 10% SDS-PAGE. Proteins were transferred to nitrocellulose membranes, which were blocked with 10% nonfat dry milk in TBST containing 20 mmol/L Tris (pH 8.0), 137 mmol/L NaCl and 10% Tween-20, and blotted with the relevant primary antibody, then with a horseradish peroxidase-conjugated secondary antibody. Bound proteins were detected by enhanced chemiluminescence according to its manufacturer's instructions.

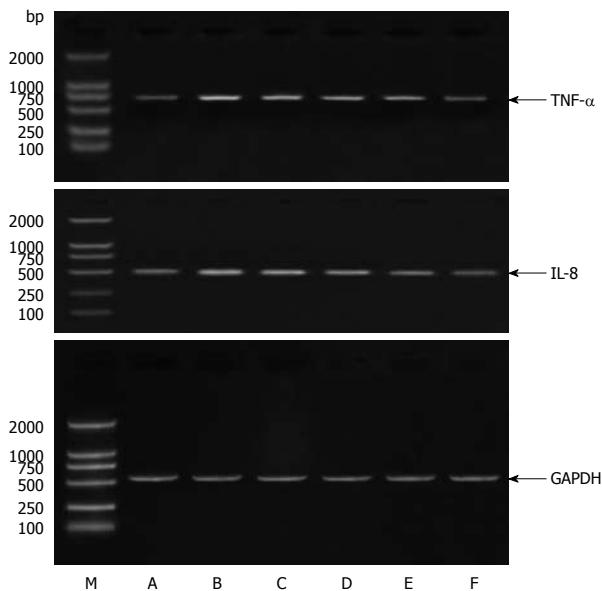
### Statistical analysis

Statistical analysis was performed using SPSS 11.5. All data were expressed as mean ± SE. Statistical significance of differences among values was determined by ANOVA and LSD was used for inter-group comparison. *P* < 0.05 was considered statistically significant.

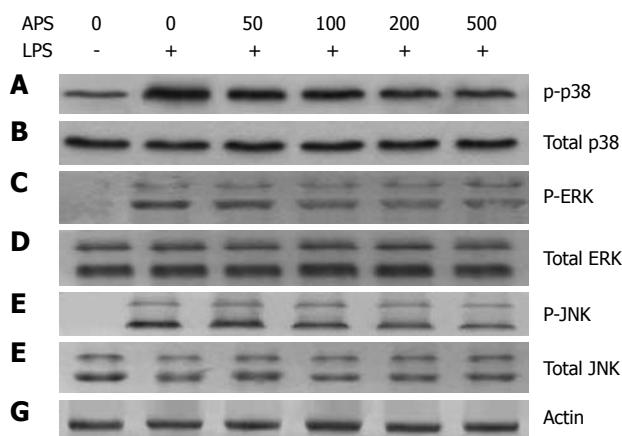
## RESULTS

### APS abrogated LPS-induced TNF-α and IL-8 gene expression in IEC-6 cells

The effects of APS on LPS-induced TNF-α and IL-8 gene expression in the intestinal cell line IEC-6 were evaluated. Stimulation of IEC-6 cells by LPS markedly increased the production of TNF-α and IL-8. The effect of APS on the levels of TNF-α and IL-8 mRNAs in IEC-6 cells was detected after LPS stimulation. IEC-6 cells were pretreated with APS at different concentrations (50, 100, 200 and 500 µg/mL) for 24 h, stimulated with LPS (10 µg/mL) for 1 h. TNF-α and IL-8 gene expressions were detected by RT-PCR. As shown in Figure 1, RT-PCR analysis revealed that TNF-α and IL-8 mRNAs were induced readily in IEC-6 cells by LPS. However, this induction was inhibited by APS in a concentration-dependent manner, namely 50 and 500 µg/mL APS partially and significantly suppressed the production of TNF-α and IL-8 mRNAs in the presence of LPS-activated IEC-6 cells compared with LPS stimulation in the absence of APS (*P* < 0.01).



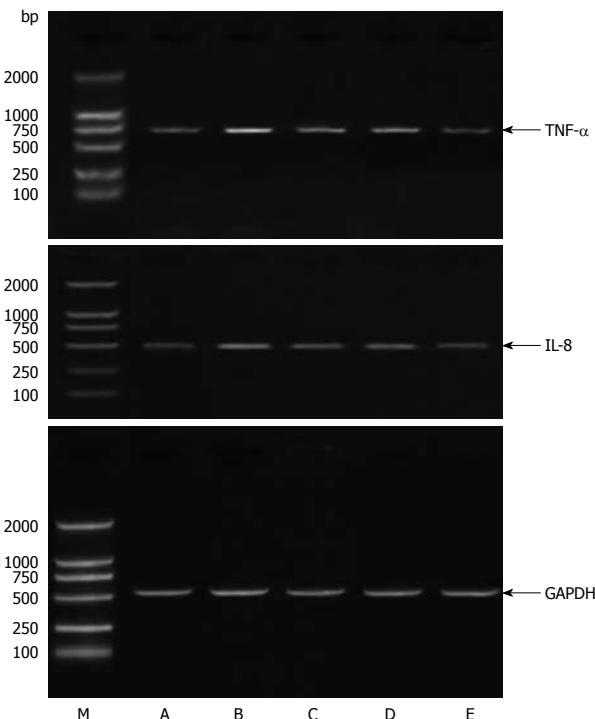
**Figure 1** *Astragalus mongholicus* polysaccharide inhibits TNF- $\alpha$  and IL-8 production in LPS-stimulated rat small intestinal cells. Intestinal epithelial cells (IEC) were treated with APS for 1 h, and cultured in a medium containing 10  $\mu$ g/mL LPS with APS at different concentrations for 1 h to detect TNF- $\alpha$  and IL-8 mRNAs in the cells by RT-PCR. M: Marker. A: Control group; B: LPS+ 0  $\mu$ g/mL APS group; C: LPS+ 50  $\mu$ g/mL APS group; D: LPS+ 100  $\mu$ g/mL APS group; E: LPS+ 200  $\mu$ g/mL APS group; F: LPS+ 500  $\mu$ g/mL APS group.



**Figure 3** *Astragalus mongholicus* polysaccharide inhibits p38 phosphorylation but not ERK1/2 or JNK activation in LPS-stimulated rat small intestinal cells. IEC were treated as described in Figure 1. After incubation in a medium containing 10  $\mu$ g/mL LPS with APS at different concentrations for 1 h, Western blotting analysis was performed to detect phosphorylated p38 (A), total p38 (B), phosphorylated ERK (C), total ERK (D), phosphorylated JNK (E), total JNK (F) and actin (G).

#### APS inhibited both TNF- $\alpha$ and IL-8 production by LPS-activated IEC-6 cells in a time-dependent manner

IEC-6 cells were pretreated with APS (500  $\mu$ g/mL) for 24 h, stimulated with LPS (10  $\mu$ g/mL) for 1-4 h. The expression of the TNF- $\alpha$  and IL-8 genes was detected by RT-PCR. As shown in Figure 2, LPS-induced TNF- $\alpha$  mRNA expression was inhibited 10.3% and 25.5% by APS treatment at 1 and 4 h post-stimulation, respectively. LPS-induced IL-8 mRNA expression was also inhibited 15.3% and 18.8% by APS treatment at 1 and 4 h post-stimulation, respectively.



**Figure 2** *Astragalus mongholicus* polysaccharide inhibits TNF- $\alpha$  and IL-8 production in LPS-stimulated rat small intestinal cells. IEC were treated with APS (500  $\mu$ g/mL) for 1 h, and cultured in a medium containing 10  $\mu$ g/mL LPS for up to 4 h to detect TNF- $\alpha$  and IL-8 mRNAs in the cells by RT-PCR. M: Marker. A: Control group; B: LPS+ 0  $\mu$ g/mL APS group; C: LPS+ 50  $\mu$ g/mL APS group; D: LPS+ 100  $\mu$ g/mL APS group; E: LPS+ 200  $\mu$ g/mL APS group; F: LPS+ 500  $\mu$ g/mL APS group.

#### APS inhibited p38 phosphorylation but not ERK1/2 or JNK activation in LPS-activated IEC-6 cells

Activation of MAPK p38 can mediate the production of pro-inflammatory cytokines when IEC-6 cells are activated. To further understand the mechanisms underlying the APS-mediated anti-inflammation in IEC-6 cells, we examined whether APS inhibits LPS-triggered activation of MAPK signaling, including phosphorylation of p38, ERK1/2 and JNK. The levels of phosphorylated (activated) p38, ERK1/2 and JNK were analyzed in LPS-stimulated IEC-6 cells following treatment with or without APS. p38, ERK1/2 and JNK were strongly activated in IEC-6 cells stimulated with LPS. However, APS treatment decreased LPS-induced p38 phosphorylation. Moreover, p38 phosphorylation was inhibited by LPS in a concentration-dependent manner. Fifty micrograms per millilitre APS partially blocked LPS-induced p38 phosphorylation and 500  $\mu$ g/mL APS significantly inhibited p38 phosphorylation in LPS-stimulated IEC-6 cells (Figure 3) while the total protein level of p38, ERK1/2 or JNK remained unchanged, indicating that APS inhibits the activation of p38 but not ERK1/2 and JNK in IEC-6 cells after LPS stimulation.

## DISCUSSION

The search for active compounds in natural products used in traditional medicine has attracted great interest,

because traditional herbal drugs have many benefits, few side effects and low cytotoxicity<sup>[8-11]</sup>. Isolation, identification and characterization of these compounds, and evaluation of their potential benefits to humans, have become an important field in pharmaceutical research<sup>[12]</sup>.

It has been reported that APS has a variety of pharmacological properties. Traditionally, APS is used to treat weakness, wound, anemia, fever, multiple allergies, chronic fatigue, and loss of appetite<sup>[13]</sup>. APS is used as a diuretic and tonic herbal medicine in Asian countries, to enhance physical strength and endurance, strengthen the immune system, decrease blood pressure, and promote excretion and circulation<sup>[6,14,15]</sup>. Clinically, APS is used to treat chronic phlegmatic disorders and general gastrointestinal disturbances including stomach ulcer and diarrhea<sup>[16,17]</sup>. The mechanism by which APS mediates the above-mentioned effects is unclear. Studies on the use of APS in treatment of various human diseases showed that this herb may act as an immune regulator that can enhance strength, immunity and circulation<sup>[18-21]</sup>. It has been reported that APS also appears to exert an immune modulating effect by regulating the expression of cytokines such as IL-1, IL-6 and iNOS, as well as the production of NO<sup>[6]</sup>. In this study, APS inhibited the production of both TNF- $\alpha$  and IL-8 in LPS-stimulated IEC-6 cells in a concentration-dependent manner (Figure 1). Since excessive production of TNF- $\alpha$  and IL-8 induces tissue injury, septic shock and inflammatory intestinal disease, APS can be developed into a drug for intestinal injury.

MAPKs (ERK, p38, JNK)<sup>[11,22-24]</sup> and NF- $\kappa$ B<sup>[25-27]</sup> positively control TNF- $\alpha$  and IL-8 expression in LPS-activated IEC-6 cells, via a unique signaling pathway. Inhibition of any of the three MAPK pathways is sufficient to block the TNF- $\alpha$  and IL-8 induced by LPS in IEC-6 cells<sup>[28-30]</sup>. In this study, whether APS exerts its effects on TNF- $\alpha$  and IL-8 by interfering with the activation of ERK, p38 and JNK was tested, showing that APS cannot block the activation of ERK or JNK. Therefore, these two pathways do not mediate any inhibitory effect of APS on TNF- $\alpha$  and IL-8 production by LPS-stimulated IEC-6 cells. In our study, APS inhibited the activation of p38 and the expression of the TNF- $\alpha$  and IL-8 genes, suggesting that inhibition of the activation of p38 but not ERK and JNK may inhibit the production of TNF- $\alpha$  and IL-8.

In summary, APS inhibits the production of both TNF- $\alpha$  and IL-8 in LPS-stimulated IEC-6 cells by suppressing p38 signaling.

## COMMENTS

### Background

*Astragalus mongholicus* polysaccharide (APS) with a variety of pharmacological properties is a component isolated from *Astragali Radix*, a traditional Chinese herbal medicine. Studies have revealed its anti-inflammatory, antioxidant, and immune regulatory effects. However, knowledge about how APS exerts its anti-inflammatory effects is still limited.

### Research frontiers

*Astragali Radix* appears to exert its immune modulating effects by regulating the

expression of cytokines, such as interleukin (IL)-1, IL-6 and inducible nitric oxide synthase, as well as the production of nitric oxide. Thus, whether APS affects the production of tumor necrosis factor (TNF)- $\alpha$  and IL-8 in lipopolysaccharide (LPS)-activated intestinal epithelial cells (IEC)-6 cells by interfering with mitogen-activated protein kinase (MAPK) signaling was investigated in this study.

### Innovations and breakthroughs

This is the first study to investigate the effect of APS on LPS-induced MAPK signaling and pro-inflammatory gene expression in IEC-6 cells. APS was found to inhibit the production of both TNF- $\alpha$  and IL-8 in LPS-induced IEC-6 cells in a concentration-dependent manner, and excessive production of TNF- $\alpha$  and IL-8 was observed to induce tissue injury, septic shock and inflammatory intestinal disease.

### Applications

APS can be developed into a drug for intestinal injury.

### Terminology

MAPK phosphorylates serine and threonine residues of proteins in cells. MAPK is also an important signal regulator linking cell surface receptors to changes in gene expression. In mammalian cells, at least three members of the MAPK family including extracellular signal-regulated kinase (ERK), c Jun amino-terminal kinase (JNK), and p38 have been cloned.

### Peer review

In this study, the authors detected the protective effects of a purified herbal product on LPS-induced intestinal inflammatory mucosal injury and conducted their study in IEC-6 cells and measured the levels of TNF- $\alpha$  and IL-8 mRNAs as well as proteins in the p38 signaling pathway. The results presented in this paper show that purified herbal polysaccharide can inhibit LPS-induced production of TNF- $\alpha$  and IL-8 by suppressing p38, P-ERK and P-JNK. In general, the experiments in this study were carefully designed and carried out. Data description is clear and the results are adequately discussed.

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# Bilhemia after trans-jugular intra-hepatic porto-systemic shunt and its management with biliary decompression

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## INTRODUCTION

Transjugular intrahepatic portosystemic shunt (TIPS) is a frequently performed procedure to achieve decompression of the portal venous system in the treatment of (1) uncontrolled acute variceal bleeding, (2) prevention of recurrent variceal bleeding, (3) refractory ascites, and (4) hepatic hydrothorax<sup>[1]</sup>. Early complications (within 24-48 h) include intraperitoneal bleeding, shunt occlusion, vascular fistulae, and hepatic infarction. We report a case of bilhemia (bile mixing with blood) after TIPS which was successfully managed with endoscopic decompression of the biliary system.

## CASE REPORT

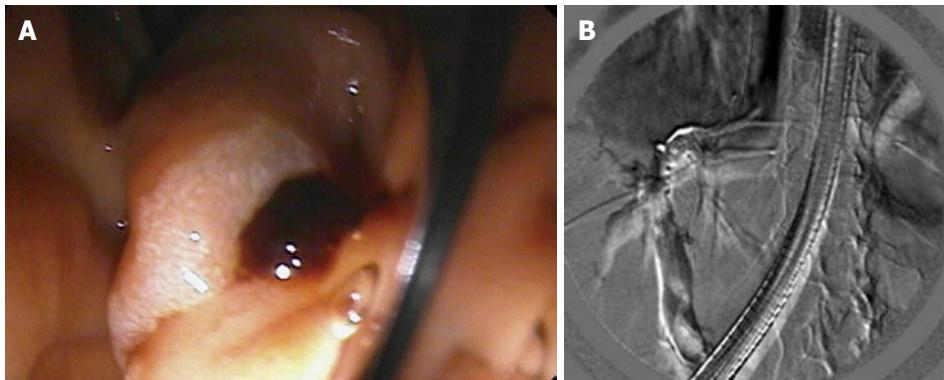
A 73 year-old Caucasian female with sarcoidosis-associated cirrhosis underwent TIPS for the treatment of refractory hepatic hydrothorax. The diagnosis of cirrhosis was made in 2002 based on liver biopsy findings. The patient had a history of cholecystectomy in the remote past. Evaluation of liver function tests in 2002-2003 showed total bilirubin (TB) 1.8 mg/dL, alkaline phosphatase (ALP) 560 IU/L, aspartate aminotransferase (AST) 87 IU/L, alanine aminotransferase (ALT) 63 IU/L, total proteins (TP) 7.1 g/dL and albumin 3.4 g/dL. Ascites was managed with diuretics and salt restriction until December 2008 when she developed shortness of breath. Due to closure of our institution during Hurricane Ike, she was referred to another hospital. On work-up she was found to have pleural effusion on the right side. The pleural fluid was transudate with a serum-fluid albumin gradient > 1.1, consistent with hepatic hydrothorax. As she required repeated thoracocentesis, pleurodesis was tried using talc, however, this was unsuccessful. In mid January, an

## Abstract

Bilhemia or bile mixing with blood is a rare clinical problem. The clinical presentation is usually transient self-resolving hyperbilirubinemia, progressive and rapidly rising conjugated hyperbilirubinemia, or recurrent cholangitis. Endoscopic retrograde cholangio-pancreatography (ERCP) plays an important role in diagnosis and management. Biliary decompression with endoscopic sphincterotomy is useful in treating these patients. If not recognized and treated in time, the condition can be fatal in a significant proportion of patients. This usually occurs after blunt or penetrating hepatic trauma due to a fistulous connection between the biliary radicle and portal or hepatic venous radical. Cases have been described due to iatrogenic trauma such as liver biopsy and percutaneous biliary drainage. However, the occurrence after trans-jugular intra-hepatic porto-systemic shunt (TIPS) is very rare. We report a case of bilhemia presenting as rapidly rising bilirubin after TIPS. The patient was managed successfully with ERCP and removal of a blood clot from the common bile duct.

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**Key words:** Bilhemia; Biliary-venous fistula; Portal vein-biliary fistula; Trans-jugular intrahepatic shunt



**Figure 1** Endoscopic retrograde cholangiogram with the endoscopic view showing a blood clot at the ampulla (A), and a fluoroscopic image showing the blood clot within the common bile duct (B).

indwelling pleural catheter was placed in her right pleural cavity. In early February 2009, she came back to us for follow up after our institution reopened. The catheter was draining about 500-1000 cc every day. The patient did not show any clinical evidence of infection. Placement of an indwelling catheter or a chest tube is not recommended for the management of hepatic hydrothorax<sup>[2]</sup>. Therefore, it was decided to control her hydrothorax with TIPS to be followed by the removal of the chest catheter at a later date.

Laboratory parameters prior to TIPS were: TB 0.4 mg/dL, albumin: 2.8 g/dL, ALP 259 IU/L, ALT-33 IU/L, AST52 IU/L, international normalized ratio (INR) 1.1, hemoglobin 11.7 g/dL and serum creatinine 0.7 mg/dL. During the procedure while attempting to cannulate the hepatic vein, the interventional radiologist noticed the presence of bile in the catheter. However, the shunt was placed between the right hepatic vein and the right main branch of the portal vein using a 1 cm × 7 cm polytetrafluoroethylene covered stent (Gore Viatorr, USA). The procedure was successful in achieving decompression of the portal venous system with a reduction of the portosystemic gradient from 16 before TIPS to 0 after the procedure. Post-procedure the patient remained in hospital to determine the amount of drainage from the pleural catheter in order to decide on its removal. The patient was doing well until the second day after the procedure, when she was noted to be diffusely yellow. Laboratory evaluation at this time showed hemoglobin to be 11.7 g/dL, white blood cell count 11.9/cubic mL, INR 1.2, creatinine 0.7 mg/dL, TB 15.9 mg/dL, conjugated bilirubin 15.2 g/dL, AST 183 IU/L, ALT 71 IU/L, and ALP 409 IU/L. She denied any abdominal pain or fever and there was no change in her mental status. The next day her bilirubin increased to 22.4 mg/dL. A CT scan of the abdomen showed dilatation of the common bile duct (CBD) and intra-hepatic biliary radicles along with a filling defect in the CBD suggestive of sludge or a blood clot. An emergency endoscopic retrograde cholangio-pancreaticogram (ERCP) was performed at this stage. The endoscopic examination showed clotted blood at the ampulla. Cannulation and contrast injection of the CBD showed a dilated CBD (to 12 mm) with a luminal filling defect due to a blood clot (Figure 1). The blood clot was removed using a balloon. An adequate sphincterotomy was successfully performed for further decompression of the biliary system. On follow up, the TB rapidly decreased to 18.1 mg/dL the next day and to 3 mg/dL within 48 h. She was discharged from hospital in good condition.

The pleural catheter drained about 500 cc on day 1 and had to be flushed with TPA for adequate drainage. As the catheter was still draining approximately 500 cc/d, she was discharged home with this catheter in place.

On follow up, one week after discharge, the patient was doing well. As the diuretics were not reinstated after the TIPS, the patient developed significant leg edema. She denied any respiratory or cardiac symptoms. As the chest catheter did not drain any fluid for 2-3 d it was removed by the interventional radiologist. On the clinic visit her bilirubin had normalized to 0.1 mg/dL. After restarting her diuretics (furosemide 40 mg/d and spironolactone 100 mg/d), the leg edema improved and she lost 16 lbs of bodyweight (137 to 121 lbs) over a 2-wk period.

## DISCUSSION

Conjugated hyperbilirubinemia can result due to hepatocellular or biliary disease. Diversion of portal venous blood (about 70% of total hepatic blood supply) to the systemic circulation after TIPS can potentially result in liver dysfunction. This is the basis of hepatic encephalopathy, a common complication after TIPS<sup>[1]</sup>. However, this is unlikely in our patient for the following reasons: (1) occurrence of liver dysfunction after TIPS is related to pre-TIPS liver function<sup>[2]</sup>. Our patient with a pre-TIPS model of end-stage liver disease score of 7 was not at risk for post-TIPS liver dysfunction despite reducing the porto-systemic gradient from 16 to 0 mmHg, (2) patients with ischemic hepatocellular liver damage usually have transaminases > 1000 IU/L and our patient had very minimal elevations in liver enzymes, (3) there was no change in mental status which would have been a significant feature in the patient's presentation if this level of bilirubin was due to hepatocellular dysfunction, and (4) ability to handle and conjugate the increased load of bilirubin by a well compensated liver.

The presence of a blood clot in the CBD on ERCP examination makes biliary disease the likely cause of hyperbilirubinemia. However, bleeding into the bile duct or hemobilia does not explain the clinical picture in the absence of biliary colic and/or gastrointestinal bleeding. Moreover, the hemoglobin remained stable. In addition, a rapid rise in bilirubin (1-18 mg/dL within 48 h and to 22.4 mg/dL within 72 h of the procedure) does not occur with obstruction of the bile ducts

including hemobilia. However, this has been described in cases of bilhemia secondary to the development of biliary-venous fistula<sup>[3]</sup>. It is likely that hyperbilirubinemia in our patient was the result of biliary-venous fistula resulting in bilhemia. Aspiration of bile by the interventional radiologist while trying to puncture the right hepatic vein raises this suspicion further. After the development of a fistula, the direction of flow depends upon the pressures on either side. Normal pressure in the hepatic vein is 0-5 mmHg and in the CBD is 10-12 mmHg, and in the portal system in this case the pressure was 16 mmHg<sup>[3]</sup>. Therefore, if the pressure in the venous side is higher than in the CBD, this will result in hemobilia and bilhemia will be a major manifestation if the pressure is higher in the CBD. Lack of visualization of the fistula despite an occlusive cholangiogram could either be due to its presence in the small peripheral biliary radicle or to spontaneous closure by the time cholangiogram was performed.

How do we explain the presence of a blood clot in the CBD? We propose that the following sequence of events occurred: Initially the bile duct radicle was inadvertently punctured creating a fistula with one of the small portal venous radicles. This initiated bleeding into the bile duct due to a higher portal pressure initiating hemobilia. However, successful placement of the TIPS decompressed the portal venous system. This favored leakage of bile into the blood causing bilhemia. In fact, the blood clot resulting from the clinically insignificant bleeding exaggerated the biliary pressure, further favoring leakage of bile into the blood.

Although, the term bilhemia was introduced in 1975, the first case was described in 1952<sup>[4]</sup>. Most cases of bilhemia are reported after hepatic trauma and its occurrence after TIPS is a rare event<sup>[5-7]</sup>. The patient can be asymptomatic or present with transient hyperbilirubinemia with spontaneous improvement due to closure of the fistula. If the shunt is infected, recurrent cholangitis and bacteremia could dominate the clinical picture<sup>[5-7]</sup>. To our knowledge, this is the first report of portal vein-biliary fistula causing bilhemia and presenting with rapidly rising bilirubin within 48 h of the TIPS procedure. This condition can be lethal in about 50% of cases, mostly due to fat embolism from a sudden gush of un-dissolved bile into the bloodstream<sup>[4,8]</sup>. ERCP is the most useful tool to diagnose this condition as it follows the direction of flow across the fistula<sup>[8]</sup>. Scintigraphy may be used if endoscopic expertise is unavailable<sup>[8]</sup>.

Treatment of patients with bilhemia should aim to achieve decompression of the biliary system and to

reduce the pressure within the CBD. This will allow drainage of bile towards the gut and prevent leakage into the blood. This is best achieved by endoscopic biliary sphincterotomy. In a series of 20 patients with biliary leaks (four with bilhemia), endoscopic sphincterotomy successfully managed all patients but one who died of bile thromboembolism<sup>[9]</sup>. In the event of a higher location or lack of available technical expertise for ERCP, percutaneous drainage of the biliary system is an alternative<sup>[8]</sup>. Unfortunately, if the shunt is infected, liver transplantation is the only option to salvage these patients as when the TIPS is placed it can not be removed<sup>[5,7]</sup>. In our patient, the presence of a blood clot added to the severity of the problem and its removal and the performance of endoscopic sphincterotomy achieved successful biliary decompression resulting in a positive patient outcome.

In summary, the occurrence of jaundice after TIPS raises the suspicion of a biliary-venous fistula. A high index of suspicion is required for successful management and the prevention of mortality. ERCP with endoscopic sphincterotomy is an effective modality for managing this potentially lethal complication.

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CASE REPORT

## What is a reliable CT scan for diagnosing splenosis under emergency conditions?

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## INTRODUCTION

Splenosis is a condition in which splenic tissue is present in a non-anatomical position due to its auto-transplantation after splenic trauma or surgery causing injury to the splenic capsule. It can be found as a single mass or multiple intraperitoneal or pelvic masses that may or may not be associated with pain. In some cases, it can manifest as an acute abdominal pain secondary to the torsion of the splenic pedicle. These splenic implants can mimic neoplasms and only specific examinations can confirm the diagnosis. Although ultrasonography, computed tomography (CT) and magnetic resonance imaging (MRI) can help in localizing and establishing the size and structure of these implants and in determining their relationship with adjacent organs, they are non-specific examinations. Only by using specific agents that are selectively sequestered by the reticulo-endothelial system [such as <sup>99m</sup>Technetium-labelled heat-denatured autologous red blood cells or <sup>111</sup>Indium-labeled platelets, single photon emission computed tomography scans and recently ferumoxide-enhanced MRI (small iron oxide particles SPIO)] allow a specific diagnosis of intra-abdominal splenosis. However, these examinations cannot be performed in an emergency situation.

Several authors report the role of these advanced imaging techniques in the diagnosis of splenosis, however, there exist no studies in the literature reporting the use of imaging techniques in emergency situations.

Here we describe the role of contrast-enhanced CT in the diagnosis of splenosis in an emergency situation.

## CASE REPORT

A 23-year-old male patient was admitted to the emergency

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**Key words:** Splenosis; Ectopic splenic tissue; Computed tomography scan; Splenectomy; Emergency



**Figure 1** Post-contrast CT scans (after intravenous injection of 130 mL of contrast media, Ultravist, 3 mL/s flow rate). Lesions are homogeneously hyperdense (92 HU) in the arterial phase (no typical striped enhancement pattern).



**Figure 2** Isodensity in the early portal venous phase.



**Figure 3** Intraoperative picture revealing a large red mass adherent to the bladder and prostate gland and a small red mass implanted on the rectum.

department of our hospital with acute abdominal pain. He presented with fever, periumbilical colic-like pain, nausea and vomiting with absolute constipation. Clinical examination revealed a distended abdomen that was tender but not peritonitic and feverous. At auscultation, peristalsis was very slow. Digital rectal examination revealed a large lobulated mass imprinting the anterior wall of the rectum, while testicular palpation did not reveal abnormal findings. Laboratory results revealed the presence of leukocytosis. The patient had a history of childhood splenectomy for a blunt abdominal trauma caused by a horse's kick. No other significant medical data were reported.

An abdomino-pelvic CT scan revealed a small bowel obstruction and two well-demarcated rounded solid masses (10 and 3.5 cm) located in the rectovesical pouch posterior to the prostate gland and the seminal vesicles. These lesions did not show any macroscopic contiguity with the surrounding organs (i.e. the prostate gland, the bladder, the seminal vesicles and the rectal ampulla). No lymphadenopathy (iliac or obturator group) or signs of invasion of the adipose tissue of the rectovesical pouch were found. These features excluded malignant behaviour of the lesions.

Pre-contrast CT images showed these masses were isodense to liver. In particular, a quantitative analysis of the density value was made by the positioning of multiple regions of interest (ROIs) into the lesions, demonstrating Hounsfield Unit (HU) values ranging between 55 and 60. In the post-contrast CT scans (after intravenous in-

jection of 130 mL of contrast media, Ultravist, 3 mL/s flow rate), these lesions became homogeneously hyperdense (92 HU) in the arterial phase (no typical striped enhancement pattern) (Figure 1) and isodense in the early portal venous phase (Figure 2). Quantitative analyses of the different density values in the arterial and early portal venous phases demonstrated that these lesions were highly vascularised (92 and 97 HU, respectively). The enhancement pattern was similar to that of parenchymatous organs, such as the liver. The origin of the masses as being from the prostate or seminal vesicles was thereby ruled out because of the early and homogeneous enhancement. On the basis of both the CT findings and clinical data, the splenic origin of these lesions was hypothesized.

The patient underwent surgery to resolve the intestinal occlusion. An adhesiolysis for small bowel obstruction secondary to adhesive intestinal syndrome was conducted. A mass was found in the rectovesical pouch attached to the bladder and to the prostate gland and was macroscopically compatible with splenic tissue. Another similar smaller mass was found adherent to the rectum (Figure 3). This mass was successfully removed. Histopathologic examination demonstrated the benign nature of the lesion, which consisted of splenic tissue. The larger mass was not removed as it was not associated with any clinical symptom.

## DISCUSSION

Although splenic implants are generally asymptomatic, they can lead to recurrent episodes of abdomino-pelvic pain or small bowel obstruction secondary to adhesive bands to the splenic implants. Splenosis can be confused with other intra-abdominal masses. Differential diagnoses of splenosis include endometriosis, peritoneal mesothelioma, renal neoplasms, abdominal lymphomas and peritoneal metastatic implants<sup>[1]</sup>. Splenosis should be suspected in patients with a history of splenic trauma. Non-invasive diagnosis of splenosis needs integration of radiological and scintigraphic methods<sup>[2]</sup>.

In patients with bowel obstruction, a history of splenic trauma and an occasional finding of an abdominal mass, contrast-enhanced CT scan is an efficient tool for the diagnosis of splenosis. In pre-contrast enhanced CT scans, normal splenic tissue appears homogenous

with a density ranging between 56 and 65 HU, which is similar or slightly lower than that of normal liver tissue. After high flow-rate (3-4 mL/s) administration of contrast media, early post-contrast scans show an inhomogeneous impregnation with a cord-like pattern. This is probably related to the existence of a double blood-circulation inside the red pulp where areas of early enhancement represent fast circulation with direct connections between arterioles and venules, and areas of late enhancement represent a slower circulation with connections between the arterioles and the venules through the Billroth-channels<sup>[3,4]</sup>. The splenic parenchyma reaches a homogeneous enhancement approximately 1 min after contrast agent administration with density values ranging between 75 and 97 HU<sup>[5]</sup>.

Although differential diagnosis includes a variety of other conditions presenting with a pelvic mass, the relation with the surrounding tissues and the contrast-enhancement characteristics can help in the diagnosis of splenosis. In particular, in the case we present, the absence of infiltration of the prostate gland, seminal vesicles and rectum ruled out the malignant nature of the masses. Furthermore, the enhancement pattern made a malignancy less probable, such as a sarcoma that would have presented as an inhomogeneous enhancement on

both pre- and post-contrast images. The hypothesis of an ectopic splenic mass was made after an accurate evaluation of the CT images and correlation with clinical history.

In the case, we present demonstrates the importance of contrast-enhanced CT imaging with ROI analysis in the differential diagnosis of splenosis from other pelvic masses in emergency conditions.

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## Obstructive ileus due to a giant fibroepithelial polyp of the anus

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### Abstract

Fibroepithelial polyps or hypertrophied anal papillae are essentially skin tags that project up from the dentate line and the junction between the skin and the epithelial lining of the anus. They are usually small in size, but sometimes they become enlarged, causing unexpected medical conditions. An extremely rare case of a giant hypertrophied anal papilla complicated by obstructive ileus is reported. Fibroepithelial anal polyp, despite its size, should be included in the differential diagnosis of a smooth mass located near the anal verge, especially in a patient with a history of chronic anal irritation or infection.

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**Key words:** Anus; Fibroepithelial polyp; Hypertrophied anal papillae; Ileus; Intestinal obstruction

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### INTRODUCTION

Fibroepithelial polyps of the anus, also referred to as hypertrophied anal papillae, are structures formed by hyperplasia of connective tissue in the vicinity of the anal columns. They are usually relatively small in size and asymptomatic. Enlargement of anal papillae is thought to be due to a hyperplastic response of the modified ectoderm to chronic irritation, injury or infection. Following repeated inflammatory episodes they can hypertrophy into the rectum and be confused with adenomatous polyps. Fibroepithelial polyps of the anus should be included in the differential diagnosis of a smooth mass located near the anal verge, especially in a patient with a history of chronic anal irritation or infection. To our knowledge, this is the first case of a giant fibroepithelial polyp of the anus complicated by obstructive ileus: there is only one similar case study in the medical literature regarding a giant hypertrophied anal papilla complicated by massive anal bleeding and prolapse<sup>[1,2]</sup>.

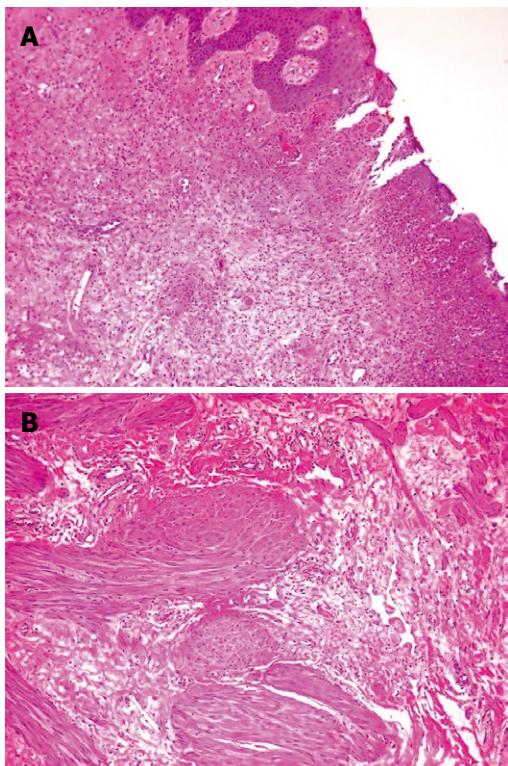
### CASE REPORT

A 67-year-old woman was admitted to the emergency room with a diffuse, cramping abdominal pain of progressive onset, tendency to vomit and constipation for 5 d. She had experienced similar episodes of intermittent abdominal pain, anal bleeding and persistent perianal pain due to long-standing hemorrhoidal disease over the last 7 years. Despite having been aware of the presence of chronic constipation, she had not sought timely medical treatment. Her family history was unremarkable, as was her medical history, except for clonazepam intake due to epileptic episodes during her childhood.

On physical examination, she had reduced bowel sounds, while the upper abdomen was distended with mild tenderness on palpation. Digital examination was negative for the presence of blood in the rectum, but revealed a giant elastic mass with convoluted grooves on a smooth surface in the distal rectum. This lesion was located 2 cm above the dentate line, obstructing the intestinal lumen. Preoperative examinations, consisting of a full blood count, serum kidney and liver functions, as well as cancer markers (CEA, CA 19-9), were within



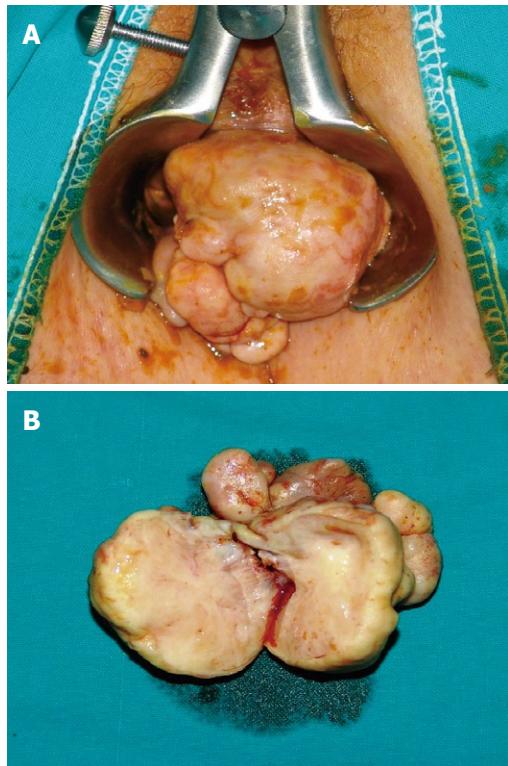
**Figure 1** Computed tomography of the pelvis showing the mass in the rectum.



**Figure 3** Histological features of the fibroepithelial polyp. A: Photomicrograph of resected polyp showing fibrous tissue covered by squamous epithelium, small vessels and the ulceration of fibrous stroma (HE,  $\times 100$ ); B: Deeper layer of the polyp showing scattered smooth muscle fibers within the fibrous stroma (HE,  $\times 100$ ).

normal limits. Plain abdominal X-rays revealed the presence of dilated small intestine with air-fluid levels. A computed tomography of the abdomen and pelvis demonstrated the presence of a mass in the rectum (Figure 1). Anal examination under anesthesia was carried out. A giant (measuring 15 cm  $\times$  12 cm) smooth mass attached to a wide pedicle extending over the entire posterior wall of the anal canal, above the dentate line, was easily observed (Figure 2). During the proctoscopy, biopsy specimens and frozen section analysis confirmed the benign nature of the lesion.

The huge mass was uneventfully removed by local excision with the aid of an electrothermal bipolar



**Figure 2** Clinical appearance of the giant fibroepithelial anal polyp. A: The clinical aspect of the mass during surgery; B: The gross macroscopic specimen.

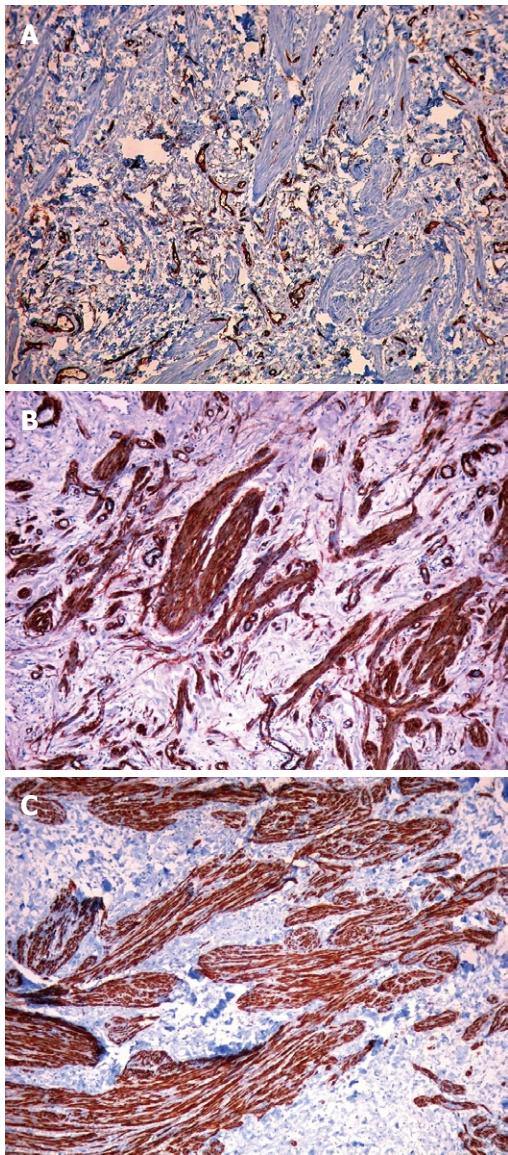
vessel sealer (LigaSure Precise<sup>®</sup>). The histological assay of the resected specimen showed fibrous tissue covered by multilayered squamous epithelium, many small dilated vessels filled with red blood cells, scattered smooth muscle fibers and a superficial ulceration of the fibrous stroma (Figure 3). In all immunohistochemical sections from the giant fibroepithelial polyp, spindle and stellate mononucleated stromal cells were positive for CD34, SMA (Smooth Muscle Actin) and desmin (Figure 4).

The histological and immunohistochemical findings therefore established the diagnosis of fibroepithelial polyp of the anus. The patient presented no postoperative complications and was discharged after 3 d of hospitalization.

## DISCUSSION

Fibroepithelial polyps of the anus, also known as hypertrophied anal papillae, are common lesions that have attracted little attention in the medical literature. They are enlarged, benign, polypoid projections of the anal squamous epithelium and the subepithelial connective tissue. These lesions are present in 45% of patients who undergo proctoscopic examination and are considered to be acquired triangular protrusions that arise from the base of the rectal columns of Morgagni at the dentate line<sup>[2]</sup>. As regards our patient, this is a report of a unique case of a very oversized fibroepithelial polyp of the anus which finally led to intestinal obstruction.

Clinically, these polyps may have the appearance of hemorrhoids, but they do not display thick-walled veins or any evidence of hemorrhage and organizing thrombi.



**Figure 4** Immunohistochemical analysis of the polyp. A: Stromal cells strongly express SMA; B: Stromal cells strongly express desmin; C: Stromal cells strongly express CD34.

In persons with poor anal hygiene, local disease or bowel dysfunction, these structures may become inflamed and edematous. They are usually small in size, asymptomatic and can be regarded as normal anatomic variations. Most anal papillae are 2-5 mm at their greatest dimension, although rarely they may exceed 2 cm. Enlargement of a fibroepithelial polyp to more than 3 cm in diameter is rare, and it is therefore necessary to differentiate such lesions from malignant tumors including leiomyosarcoma, anorectal carcinoma and malignant lymphoma.

Hypertrophied anal papillae are liable to trauma during the passage of stools and may become inflamed. Furthermore, they produce symptoms by projecting at the anal orifice during defecation and sometimes require digital repositioning, strongly simulating a rectal prolapse. Pruritus, foreign body sensation, mucus discharge, sense of incomplete evacuation and discomfort while sitting are the prevailing symptoms associated with this medical condition. The location of a smooth mass just

inside the anal verge should suggest the possibility of a fibroepithelial polyp, especially in a patient with a history of chronic anal irritation or locoregional infection<sup>[2,3]</sup>.

Endoscopically, a fibroepithelial polyp can be easily distinguished from an adenomatous polyp by several distinctive characteristics: (1) its mucosa is whitish compared with the reddish appearance of an adenomatous polyp, (2) the “stalk” of a hypertrophied anal papilla originates from the squamous side of the dentate line, (3) closure of the biopsy forceps on a fibroepithelial polyp results in pain, and (4) biopsy of these structures always demonstrates squamous epithelium. On the other hand, radiographic demonstration (on barium enema examination or computed tomography) of a fibroepithelial polyp is uncommon and occurs only when it becomes large enough to prolapse into the rectum. The differential diagnosis should always include internal hemorrhoids, rectal polyp, anal carcinoma and submucosal anorectal tumor<sup>[4,5]</sup>.

Groisman *et al*<sup>[6]</sup> studied the histological, immunohistochemical and ultrastructural features of a series of 40 fibroepithelial polyps of the anus. The authors concluded that fibroepithelial polyps of the anus are benign lesions characterized by the presence of mononucleated and multinucleated, sometimes atypical, CD34+ stromal cells showing fibroblastic and myofibroblastic differentiation. They also suggested that polyps harboring atypical cells are those of large size. The morphologic resemblance between these lesions and normal anal mucosa supports the hypothesis that fibroepithelial polyps may represent a reactive hyperplasia of the subepithelial connective tissue of the anal mucosa. Mast cell infiltration, by means of their fibrogenic, fibrolytic and angiogenic activities, may play an important role in the pathogenesis of these structures.

The presence of smooth muscle bundles and hyalinized vascular changes at the base of anal fibroepithelial polyps has also been reported. The rectal muscularis mucosa may be found in the upper part of the anal transitional zone. An increase in smooth muscle bundles could be the result of the hyperplastic response which caused anal fibroepithelial polyps to form a central core, and to become enlarged<sup>[7]</sup>. In our case study, the presence of abundant smooth muscle fibers in the stroma merely emphasizes the extreme enlargement of the polyp.

Another prominent histological feature of fibroepithelial polyps of the anus, but of uncertain significance, is the eosinophilic epithelial vacuolation. This is characterized by the accumulation of PAS-positive homogeneous eosinophilic material in superficial keratinocytes. This change may be a non-specific reaction to mechanical trauma, particularly given its propensity to afflict the apex of polyps. Pathologists should be very careful to avoid a misdiagnosis of extramammary Paget disease or oral white sponge nevus, which may sometimes affect anal epithelium<sup>[8]</sup>.

A fibroepithelial polyp that starts projecting into the anal canal requires attention and proper therapeutic strategy. Complete removal by crushing of the base and excision after using an electrothermal bipolar vessel

sealing system (EBVS-LigaSure), electrocauterization or ultrasonic energy (Harmonic Scalpel) has been suggested by many authors. Some other surgeons have found that the use of radio frequency devices is a quick, easy and significantly complication-free procedure for these pathological entities. This type of device can ablate the papillae instantly, while the fibrous polyps can be excised after coagulation of the bases and thereafter the pedicles<sup>[9]</sup>.

Although in our case we had no other option but to remove this giant polyp due to the intestinal obstruction it caused, in general, complete removal of these structures, regardless of the method used, offers definite therapeutic benefits and results in improved patient satisfaction.

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# Xanthogranulomatous cholecystitis mimicking gallbladder carcinoma with a false-positive result on fluorodeoxyglucose PET

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## INTRODUCTION

Patients with abnormal thickening of the gallbladder wall caused by benign lesions such as chronic cholecystitis and adenomyomatosis are often encountered in daily clinical practice. Sometimes, it is difficult to differentiate these from gallbladder carcinoma by the conventional imaging techniques of ultrasonography, computed tomography (CT) and magnetic resonance imaging (MRI). Recently, several reports have demonstrated that fluorine-18 fluorodeoxyglucose positron emission tomography (FDG-PET) is useful in differentiating between benign and malignant lesions in the gallbladder<sup>[1-5]</sup>. However, 18F-FDG is not specific for malignant lesions, and can accumulate in inflammatory lesions with increased glucose metabolism. We present a patient with irregular thickening of the gallbladder wall which was diagnosed as a gallbladder carcinoma by ultrasonography and CT. FDG-PET showed increased activity. However, postoperative pathological examination revealed a xanthogranulomatous cholecystitis (XGC) with no evidence of malignancy. In this case, FDG-PET, ultrasonography and CT resulted in a misdiagnosis of gallbladder carcinoma.

## Abstract

Recently, several reports have demonstrated that fluorine-18 fluorodeoxyglucose positron emission tomography (FDG-PET) is useful in differentiating between benign and malignant lesions in the gallbladder. However, there is a limitation in the ability of FDG-PET to differentiate between inflammatory and malignant lesions. We herein present a case of xanthogranulomatous cholecystitis misdiagnosed as gallbladder carcinoma by ultrasonography and computed tomography. FDG-PET also showed increased activity. In this case, FDG-PET findings resulted in a false-positive for the diagnosis of gallbladder carcinoma.

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**Key words:** Fluorodeoxyglucose F18; Positron-emission tomography; Xanthogranulomatous cholecystitis; Gallbladder cancer

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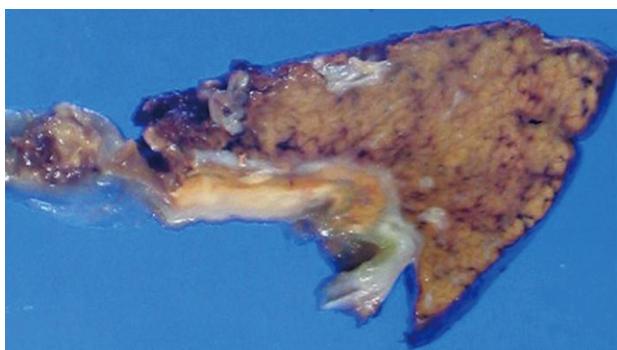
Makino I, Yamaguchi T, Sato N, Yasui T, Kita I. Xanthogranulomatous cholecystitis mimicking gallbladder carcinoma with a false-positive result on fluorodeoxyglucose PET. *World J*

## CASE REPORT

A 76-year-old man receiving treatment for rheumatoid arthritis with prednisolone 7.5 mg/d underwent blood examination in a routine checkup. An increased serum level of  $\gamma$ -glutamyltranspeptidase was found. He received abdominal ultrasonography for further examination. It showed an irregular thickening of the gallbladder wall on the hepatic side (Figure 1). No gallstones were detected. A CT scan also revealed an irregular thickening of the wall of the gallbladder body on the hepatic side. The border between the thickened gallbladder wall and liver parenchyma was indistinct (Figure 2A and B). There was no dilatation of the intrahepatic and extrahepatic bile duct. No lymphadenopathy was detected. FDG-PET/CT scan showed increased activity



**Figure 1** Abdominal ultrasonography showed an irregular thickening of the gallbladder wall on the hepatic side. No gallstones were detected.

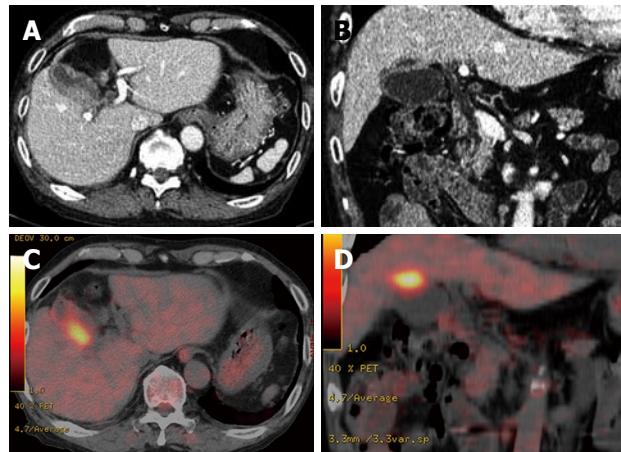


**Figure 3** Cut surface of the specimen. A yellowish tumor about 2 cm in diameter was observed between the mucosa of the body of the gallbladder and the liver parenchyma in the cut specimen.

in the thickened wall of the gallbladder. The increased uptake area appeared to extend into the liver parenchyma (Figure 2C and D). Endoscopic retrograde cholangiopancreatography showed no abnormality of the biliary tract including the cystic duct. An anomalous pancreaticobiliary ductal junction was not found. The result of laboratory examination showed no significant abnormality except for an increased serum level of  $\gamma$ -glutamyltranspeptidase (207 IU/L). C-reactive protein (CRP) was within the normal range (0.09 mg/dL). Tumor markers [carcinoembryonic antigen, carbohydrate antigen (CA19-9), and DUPAN2] were not elevated. We diagnosed the lesion preoperatively as a gallbladder carcinoma with direct invasion to the liver bed. We performed subsegmentectomy of the liver S4a + S5 and lymph node dissection of the hepatoduodenal ligament without extrahepatic bile duct resection. Gross examination of the specimen demonstrated no obvious tumor on the mucosa of the gallbladder. There were no gallstones and only a small amount of biliary sludge in the gallbladder. A yellowish tumor about 2 cm in diameter was observed between the mucosa of the body of the gallbladder and the liver parenchyma in the cut specimen (Figure 3). Pathological examination revealed a XGC with no evidence of malignancy. The postoperative course was uneventful. He is now in good health 8 mo after surgery.

## DISCUSSION

XGC is an unusual inflammatory disease of the



**Figure 2** Abdominal computed tomography (CT) and fluorine-18 fluorodeoxyglucose positron emission tomography (FDG-PET). A, B: Abdominal CT scan revealed an irregular thickening of the wall of the gallbladder body on the hepatic side. The border between the thickened gallbladder wall and liver parenchyma was indistinct; C, D: FDG-PET/CT scan showed increased activity in the thickened wall of the gallbladder. The increased uptake area appeared to extend into the liver parenchyma.

gallbladder manifested by abnormal thickening of the wall. It was first reported and named by McCoy<sup>[6]</sup> in 1976 and described as a distinct pathological condition by Goodman and Ishak<sup>[7]</sup> in 1981. It is a variant of cholecystitis characterized by intense acute or chronic inflammation, severe proliferative fibrosis with formation of multiple yellow-brown intramural nodules and foamy histiocytes. It is speculated that XGC may result from extravasation of bile into the gallbladder wall with involvement of Rokitansky-Aschoff sinuses or through a small ulceration in the mucosa. The inflammatory process often extends into neighboring organs, such as the liver, omentum, duodenum, and colon<sup>[6-12]</sup>. The clinical importance of XGC lies in the fact that it can be confused radiologically with a gallbladder carcinoma. Several reports demonstrated the radiological features of XGC. However, as some are nonspecific, it is often difficult to distinguish XGC from gallbladder carcinoma by the conventional imaging techniques of ultrasonography, CT and MRI<sup>[13-18]</sup>. Moreover, the fact that XGC can infrequently be associated with gallbladder carcinoma<sup>[19-20]</sup> makes the differentiation more difficult. Consequently, patients with XGC have sometimes undergone excessive surgical resection with preoperative diagnosis of advanced gallbladder carcinoma<sup>[21-22]</sup>.

Recently, several reports have demonstrated that FDG-PET is useful in differentiating between benign and malignant lesions in the gallbladder. They reported the sensitivity and specificity of FDG-PET for the diagnosis of gallbladder carcinoma as 75%-100% and 75%-89%, respectively<sup>[1-5]</sup>. However, 18F-FDG is not specific for malignant lesions and can accumulate in inflammatory lesions with increased glucose metabolism. Indeed, false-positive results in benign lesions, including chronic cholecystitis, tuberculosis, and adenomyomatosis of the gallbladder have been reported<sup>[4,23,24]</sup>. Nishiyama *et al*<sup>[25]</sup> evaluated the correlation between CRP and

18F-FDG uptake in gallbladder lesions. They reported that the specificity of FDG-PET for the diagnosis of gallbladder carcinoma was 80% in the group with normal CRP levels; on the other hand, it decreased to 0% in the group with elevated CRP levels. They recommended FDG-PET for patients with normal CRP levels to differentiate between malignant and benign lesions. In our case, although the patient had no obvious symptoms, such as fever and abdominal pain, and the laboratory data showed no significant inflammatory reaction, such as increased white blood cell counts and elevated CRP level throughout his clinical course, FDG-PET examination resulted in a false-positive diagnosis. It was speculated that the inflammatory reaction in the gallbladder might have been concealed by the effect of the steroid he had daily taken for the treatment of rheumatoid arthritis. From now on, FDG-PET may be applied for the diagnosis of gallbladder carcinoma more frequently. We should consider the possibilities of false-positive results as a result of inflammatory lesions, because of the frequent occurrence of inflammatory diseases in the gallbladder.

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CASE REPORT

## Bell's palsy and choreiform movements during peginterferon α and ribavirin therapy

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### INTRODUCTION

Neuropsychiatric side effects of long-term recombinant interferon- $\alpha$  (IFN- $\alpha$ ) therapy consist of a large spectrum of symptoms. Organic personality syndrome, organic affective syndrome, psychotic manifestations, and seizures are more common side effects of IFN- $\alpha$ , whereas cranial neuropathy and movement disorders are less common<sup>[1]</sup>.

We present a patient with chronic hepatitis C who showed an increase in choreiform movements associated with Huntington's disease (HD), and also developed Bell's palsy during treatment with pegylated interferon (peginterferon) and ribavirin.

### CASE REPORT

A 54-year-old woman presented with anti-hepatitis C virus (HCV) positivity and elevated alanine aminotransferase (ALT) levels in February 2007. After documentation of PCR-based HCV viremia, she underwent liver biopsy. As pathological examination revealed stage III fibrosis, it was planned to give her antiviral therapy that consisted of peginterferon- $\alpha$  and ribavirin. However, she had a history of minor choreiform limb movements for the past few years, but no cognitive impairment was observed. Her mother and sister had similar movements, and her mother had dementia consistent with HD. She also had been taken L-thyroxine for hypothyroidism for a few years. Although genetic analysis for exclusion of HD was proposed before starting antiviral therapy, the patient preferred to start therapy without genetic analysis for HD. She commenced peginterferon- $\alpha$ 2b 100  $\mu$ g once weekly and ribavirin 1000 mg/d in May 2007. In the third month of therapy, she had a 2 log decrease in HCV viral load.

After the fourteenth dose of peginterferon, she suffered pain behind her right ear, followed by dropping of the right side of the face. She attended the emergency

### Abstract

Neuropsychiatric side effects of long-term recombinant interferon- $\alpha$  therapy consist of a large spectrum of symptoms. In the literature, cranial neuropathy, especially Bell's palsy, and movement disorders, have been reported much less often than other neurotoxic effects. We report a case of Bell's palsy in a patient with chronic hepatitis C during peginterferon- $\alpha$  and ribavirin therapy. The patient subsequently developed clinically inapparent facial nerve involvement on the contralateral side and showed an increase in choreic movements related to Huntington's disease during treatment.

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**Key words:** Interferon  $\alpha$ ; Bell's palsy; Neurotoxicity; Huntington's disease

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service of a local hospital, and she was started on anti-inflammatory therapy, but not on steroid therapy, because of the possibility of increasing HCV viral load. She then attended an outpatient clinic of the neurology department in our hospital. Neurological examination revealed right-sided Bell's palsy of House-Brackmann grade V<sup>[2]</sup>. The right facial nerve had 90% degeneration on electroneurography (ENG). No motor unit potentials were identified by needle electromyography (EMG). Contrast-enhanced magnetic resonance imaging (MRI) of the inner ear showed abnormal enhancement of the intracanalicular portion of the right facial nerve. Serological examination of some viral diseases revealed that herpes simplex virus (HSV) type I IgG and anti-cytomegalovirus IgG were positive, whereas IgM tests for these viruses were negative. IgG and IgM tests for HSV type II were also negative. She stopped antiviral therapy and was given anti-inflammatory therapy. An operation for facial nerve palsy was advised by the consultant in otorhinolaryngology, but hypothyroidism, most likely caused by inappropriate usage of oral thyroid hormone therapy, was found on preoperative screening tests. Two months after the facial symptoms began, she was euthyroid, and she underwent surgery for facial nerve decompression. She promptly experienced a virological and biochemical relapse after cessation of antiviral therapy. Three and a half months after facial paralysis, she chose to continue antiviral therapy, but this time peginterferon- $\alpha$ 2a 180  $\mu$ g was reinstated along with ribavirin. Two and a half months after the operation, facial movements were shown to be improved and graded as House-Brackman 3 on the right side. The right facial nerve had 48% degeneration upon ENG. We also observed reinnervation potentials on needle EMG. MRI of facial nerves performed after 5 mo of the second round of antiviral therapy revealed contrast enhancement of bilateral facial nerves, even though she had no clinical findings on the left side of her face. At the same time, the patient reported a noticeable increase in her choreiform movements, which were not found to be related to L-thyroxin overdose, after performing thyroid function tests. Upon neurological examination, clear choreiform movements of her upper and lower limbs were observed. There was no cognitive deterioration. Genetic analysis revealed increased CAG repeat length (23/43) consistent with HD. Her movement disorder did not deteriorate further and she did not experience facial palsy on the other side despite continuing therapy, but she was treated with systemic steroids during the last 2 wk of therapy to prevent overt facial palsy. She completed the 48-wk course of antiviral therapy. Although her choreic movements decreased upon completion of antiviral therapy, a prominent improvement in her movement disorder was observed with tetrabenazine (50 mg/d), which was started 3 mo after completion of antiviral therapy.

## DISCUSSION

Bell's palsy is often idiopathic, but has been linked to

some viral infections, particularly with herpes viruses<sup>[3,4]</sup>. Other infections, such as human immunodeficiency virus infection and Lyme disease, may also lead to idiopathic facial paralysis. Neither acute nor chronic HCV infection has been implicated previously in Bell's palsy, but IFN- $\alpha$  may have a role<sup>[5]</sup>.

IFN- $\alpha$ , or peginterferon, together with ribavirin are the current treatment regimen for chronic hepatitis C. Large studies have shown that whereas flu-like symptoms and reversible hematological cytopenia are common side effects of this treatment regimen, more serious side effects are rare. In a large retrospective study from Italy, with a total of 11 241 patients who received IFN- $\alpha$ , new neurological problems including seizures and neuropathy occurred in less than 10 patients<sup>[6]</sup>. In a study of 677 Japanese patients treated with high-dose IFN, only one suffered a neurological complication, and developed sudden hearing loss<sup>[7]</sup>. However, psychiatric symptoms such as depression or anxiety have been seen commonly during IFN treatment. The reported frequency of neurotoxicity, including psychiatric side effects during IFN treatment ranges from 25% to 33%<sup>[8]</sup>. In another study from Italy, 108 out of 441 patients treated with IFN plus ribavirin for hepatitis C failed to finish combination therapy because of adverse effects. Ten patients suffered from neuropsychiatric problems: six presented with depression or anxiety, one with erectile dysfunction, one with seizures, one with vertigo and one with peripheral neuropathy<sup>[9]</sup>. There are also reports of IFN therapy leading to ischemic optic neuropathy, retinopathy, peripheral neuropathy, oculomotor neuropathy and trigeminal sensory neuropathy<sup>[10]</sup>.

We found only six cases of Bell's palsy associated with IFN, in our literature search<sup>[5,10-12]</sup>. However, one case was associated with peginterferon- $\alpha$ <sup>[10]</sup>. Ogundipe *et al*<sup>[11]</sup> firstly documented two cases of Bell's palsy that developed during IFN- $\alpha$  and ribavirin therapy for chronic HCV. In both cases, facial palsy developed a number of months after commencing therapy and resolved, one with and one without cessation of therapy. The authors have suggested that a neuropathic effect of IFN- $\alpha$ 2b on the facial nerve may result in facial paralysis. In another report including two patients with chronic HCV infection, Bell's palsy occurred during treatment with IFN- $\alpha$ 2b and ribavirin at week 7 in one patient and week 12 in another<sup>[12]</sup>. Facial paralysis resolved in both cases after withdrawal of antiviral therapy. However, one of these patients experienced Bell's palsy shortly after an outbreak of HSV oral stomatitis. Thus, it is difficult to implicate exclusively combination therapy as the etiology for this case of Bell's palsy.

Hoare *et al*<sup>[5]</sup> have reported that three patients with chronic HCV infection also developed Bell's palsy during combination therapy, with spontaneous resolution after withdrawal of treatment. Two of the patients had immune-mediated baseline disease, ulcerative colitis and focal segmental glomerulonephropathy, which may have increased their risk of complications such as Bell's palsy.

The present case is, to the best of our knowledge, the second case of Bell's palsy associated with peginterferon

therapy reported in the literature. Bilateral facial nerve involvement is another interesting feature of our case. Hypothyroidism may be another cause for the primary right-sided palsy. However, subclinical involvement of the other facial nerve, which was found to be contrast-enhanced by MRI, during the second round of antiviral therapy, supported the suggestion of IFN-induced neurotoxicity.

Some adverse effects of IFN are thought to be caused by immune dysregulation, and a good example is autoimmune thyroiditis<sup>[13]</sup>. Previous studies on Bell's palsy have shown that it may be the result of an attack on cranial nerve VII by lymphocytes that sensitized to the Schwann cell membrane of the nerve itself<sup>[14]</sup>. Thus cell-mediated immunity, augmented by peginterferon and ribavirin, may target Schwann cells of cranial nerve VII and consequently lead to facial paralysis. Another possible mechanism might involve microvascular lesions that result from vasculitis or vasospasm, because there is evidence of IFN- $\alpha$ -induced retinal abnormalities, including ischemic lesions, cotton wool exudate, capillary occlusion or retinal hemorrhage<sup>[15,16]</sup>.

To the best of our knowledge, four cases of chorea, associated with IFN- $\alpha$  were have been reported in the literature so far<sup>[17-20]</sup>. These were not associated with HD. Choreaform movements may increase during IFN- $\alpha$  therapy, since it affects dopaminergic pathways. Long-term IFN- $\alpha$  therapy acts as a dopamine antagonist by altering dopaminergic pathways through an opioid-receptor-associated action, and may cause choreic movements by dysfunction of basal ganglia-thalamocortical loops<sup>[17]</sup>.

Bell's palsy and movement disorders are rare complications of IFN therapy. We suggest that the underlying mechanisms involve autoimmunity or neurotoxic effects of IFN on the neuroendocrine system and neurotransmitters, thus physicians should be aware of these rare neurotoxic side effects of IFN- $\alpha$ .

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## Recurrent giant fibrovascular polyp of the esophagus

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### INTRODUCTION

Giant fibrovascular polyps of the esophagus are rare. Even though they are benign, they may be lethal due to either bleeding or asphyxiation if regurgitated. Recurrence of these giant polyps are rare and treatment is often difficult, specialized and may require combination of a few treatment approaches and modalities including endoscopy and open surgical techniques. We illustrate this problem with a case report and review the literature.

### Abstract

Giant fibrovascular polyps of the esophagus and hypopharynx are rare benign esophageal tumors. They arise most commonly in the upper esophagus and may, rarely, originate in the hypopharynx. They can vary significantly in size. Even though they are benign, they may be lethal due to either bleeding or, rarely, asphyxiation if a large polyp is regurgitated. Patients commonly present with dysphagia or hematemesis. The polyps may not be well visualized on endoscopy and imaging plays a vital role in aiding diagnosis as well as providing important information for pre-operative planning, such as the location of the pedicle, the vascularity of the polyp and the tissue elements of the mass. They can also be recurrent in rare cases, especially if the resection margins of the base are involved. We review the recent literature and report a case of a 61-year-old man with a recurrent giant esophageal fibrovascular polyp with illustrative contrast barium swallow, CT and intra-operative images, who required several surgeries *via* a combination of endoscopic, trans-oral, trans-cervical, trans-thoracic and trans-abdominal approaches.

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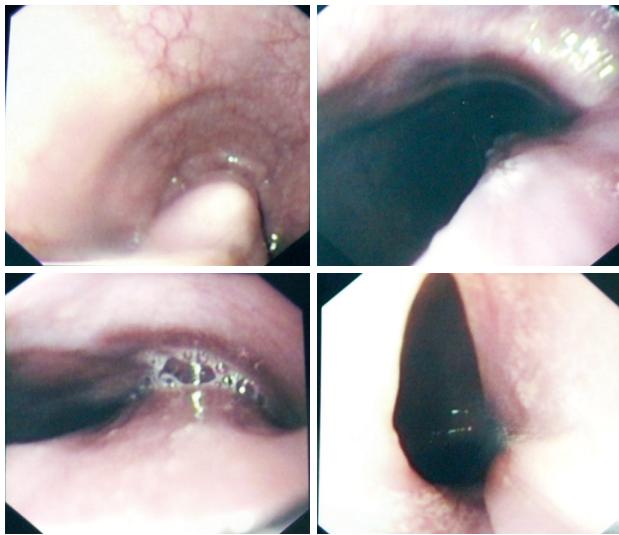
**Key words:** Giant fibrovascular polyp; Esophagus; Esophageal polyp; Fibroepithelial polyps

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### CASE REPORT

A 61-year-old man with a medical history of hypertension and hyperlipidemia presented with melena and symptomatic anemia. His initial hemoglobin was 6.2 g/dL. He was transfused appropriately and underwent an emergent esophago-gastro-duodenoscopy (OGD). The endoscopy revealed a large polyp, arising from the upper esophagus settling in the upper stomach (Figure 1). The endoscopic biopsy revealed benign squamous mucosa and granulation tissue. He was offered surgical excision of the polyp in view of the bleeding episodes from the ulcerated polyp but he declined surgery. He was initially able to regurgitate polyps out into the mouth, but he gradually progressed to mild intermittent dysphagia and agreed to surgical intervention 6 mo after initial presentation.

Under general anesthesia, laryngoscopic and esophagoscopic evaluation revealed two polyps with broad long stalks arising from the upper third of the oesophagus. The decision was made to proceed with a left cervical esophagotomy. There was difficulty in retracting the polyps cranially *via* the left cervical approach. A decision was then made to perform a gastrotomy *via* a laparotomy in an attempt to retract and pull the polyps into the stomach. The large polyps were noted at the cardio-esophageal junction but it was not possible to pull the polyps with their long stalks into the stomach for adequate resection. In view of these difficulties, we proceeded with esophagotomy *via* a left thoracotomy approach to deliver and excise the polyps.



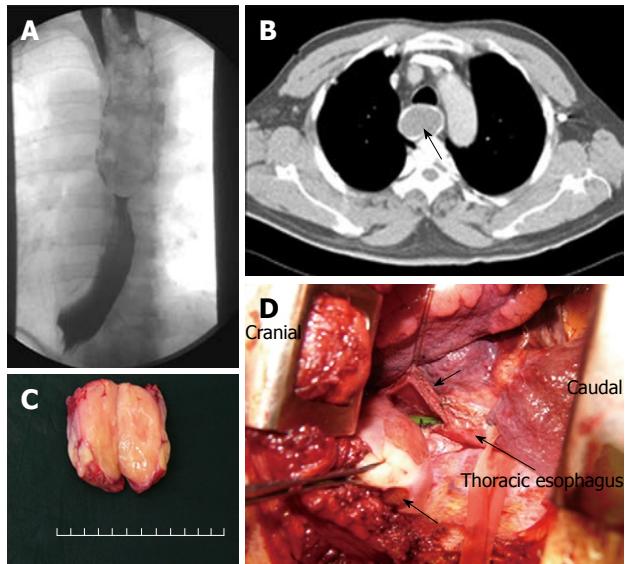
**Figure 1** Endoscopy showing the long broad pedicle and broad base.

Final histology revealed two fibrovascular polyps with ulceration measuring  $4\text{ cm} \times 4\text{ cm}$  and  $6\text{ cm} \times 6\text{ cm}$ , respectively. There was no evidence of malignancy. He was discharged in good health and followed up in the outpatient clinic at 3-6 mo intervals. He developed gradual intermittent dysphagia again 2 years later. A flexible OGD was performed and it revealed a recurrent fibrovascular polyp. In this admission, peroral excision of the recurrent polyp was performed with the aid of an ENDO GIA™ 30 stapler (Ethicon, Cincinnati, OH). The esophageal lumen was then inspected endoscopically and found to be free from bleeding, mucosal tears, or perforation prior the end of the procedure.

Histology was consistent with a previous report of a giant fibrovascular polyp, measuring  $5\text{ cm} \times 6\text{ cm}$ . There was no evidence of malignancy, however, the resection margin was noted to be involved. He was discharged in good health and followed up in the outpatient clinic at 3-6 mo intervals.

He re-presented approximately 2 years later, again for similar symptoms of intermittent dysphagia. An examination under anesthesia with a direct laryngoscopy and a rigid esophagoscopy revealed another polyp with a long stalk arising from the upper esophagus. In view of the recurrent nature and the previous surgeries, a barium swallow and CT of the neck and thorax were performed to aid surgical planning (Figure 2).

In this second recurrent episode, intra-operative findings revealed a  $6\text{ cm} \times 7\text{ cm}$  polyp with a long stalk measuring about 5 cm and a broad base of 1 cm width originating from the upper third of the esophagus. A trans-cervical esophagotomy was performed initially but due to the size of the polyp, it was not possible to deliver the polyp and an upper esophagotomy *via* a right thoracostomy approach was performed to deliver and resect the polyp. Excision of the polyp *via* the thoracic esophagotomy and excision of fibro-vascular stalk *via* the cervical esophagotomy was performed (Figure 2). Histology was consistent with that of a giant



**Figure 2** Recurrent Giant Esophageal polyp in 61-year-old man. A: Barium swallow study; B: CT Thorax, showing the homogenous nature of the polyp (arrow); C: Bi-valved bulk of the polyp without its pedicle, showing the fatty nature of the polyp; D: Intra-operative trans-thoracic view showing the polyp's head and an ulcer (bottom arrow), esophagotomy (top arrow) with the nasogastric tube *in-situ* (middle arrow).

fibrovascular polyp with no evidence of malignancy. He was discharged and is well to date.

## DISCUSSION

Fibrovascular polyps of the esophagus are rare benign tumors, comprising about 1% of all benign esophageal tumors, however, they are the most common intraluminal benign tumors of the esophagus<sup>[1]</sup>. Giant fibrovascular polyps are defined as polyps larger than 5 cm in maximum diameter. To date, there are just over 100 reported cases in the literature and the largest single series consists of 16 patients<sup>[2,3]</sup>. They are slow growing, pedunculated tumor masses that often arise from the upper esophagus, near the level of the cricopharyngeus at the pharyngo-esophageal junction, and this area has also been termed as the Laimer-Haeckermann triangle (also known as the Laimer's triangle)<sup>[4]</sup>. The pathogenesis of these polyps is thought to originate from the loose and redundant submucosal tissue near the Laimer's triangle. This relatively mobile tissue due to lack of muscular support, through years of esophageal peristalsis traction and swallowing, is dragged along, elongated and enlarged intraluminally.

The histology of the fibrovascular polyp consists of a mixture of lipomatous tissue among dense or loose fibrous elements, accompanied by an abundant network of vessels and covered by a normal squamous epithelium. The squamous epithelium may ulcerate and bleed especially in the larger tumors. Particularly in giant polyps, different histological components may vary and one may predominate leading to different terminology of these lesions as fibroepithelial polyps, fibrolipomas, fibromyxomas, lipomas, fibromas. These are all now collectively classified by World Health Organisation as

fibrovascular polyps. The more common differential diagnosis includes leiomyomas, leiomyosarcomas, squamous papillomas, lymphomas, spindle cell carcinomas and hemangiomas amongst others.

Malignant transformation is rare but has been reported in esophageal polyps. The lipomatous components can undergo sarcomatous changes, the squamous mucosa can develop into squamous carcinomas and small polyps have developed into adenocarcinoma<sup>[1]</sup>.

Due to the indolent nature of these polyps and the potential space the esophagus provides, these fibrovascular polyps can grow up to considerable sizes without causing many symptoms till late, measuring as big as 26 cm in largest diameter<sup>[5]</sup>. The majority of fibrovascular polyps occur in elderly men aged between 60 and 70 years old, but they have been reported in a 5-month-old infant<sup>[5,6]</sup>.

Though biologically benign, these giant fibrovascular polyps can have dramatic and even life-threatening presentations. Due to their size and mobility, they can be regurgitated and can cause asphyxiation or require emergent airway management<sup>[1,7]</sup>. In the literature, dysphagia was the most common complaint (present in 87%), followed by respiratory symptoms (25%) and regurgitation of the polyp into the pharynx or mouth (12%). The other reported non-specific symptoms included epigastric pain, odynophagia, non-exertional substernal chest pain, loss of weight, persistent cough and in our case, gastrointestinal bleeding<sup>[1,8]</sup>.

In the absence of an obvious regurgitation of the polyp during examination, diagnosis can be a challenge<sup>[8]</sup>. During endoscopy, the lesion may be missed especially if the origin of the stalk is not visualized, as the polyp may occupy the esophageal lumen and the surface of the polyp can resemble normal esophageal mucosa<sup>[5]</sup>. Diagnosis can be made by a combination of clinical history and various investigations such as endoscopy and imaging studies like barium swallow studies, endoscopic ultrasonography (EUS), CT and magnetic resonance imaging (MRI). Barium studies are commonly used and can show up the characteristic appearance of a smooth intra-luminal sausage-shaped mass with bulbous tips, with varying degrees of lobulation (Figure 1)<sup>[9]</sup>. It must be noted that, on occasion, these polyps may be opposed against the esophageal wall and give a false impression of a normal barium swallow. EUS can be a useful adjunct as it provides information on the size, origin of the stalk and vascularity of the polyp. In addition, EUS-fine needle aspiration may provide a more diagnostic histological sample than a superficial biopsy from an endoscopic approach. CT has been recommended by some; in addition to providing information on the components of the tumor, at an early arterial phase, especially in large polyps, feeding vessels can be visualized which can aid in surgical planning. In such cases, where a large feeding vessel is demonstrated, open surgery is advocated instead of endoscopic techniques e.g. snare polypectomy<sup>[10]</sup>. MRI is also a useful adjunct in diagnosis and surgical planning as it provides multiple planes of section and high soft tissue differentiation resolution<sup>[11]</sup>.

The mainstay of treatment once diagnosis is achieved is surgical excision in view of the potential risk of respiratory compromise, bleeding (as in this patient) and the debilitating symptoms. Surgery also serves to exclude cancer and avoid the small risk of malignant degeneration. Depending on the size, the location of the stalk's base and its mobility, different approaches have been practiced. The different methods include simple endoscopic excision techniques using electrocautery or even Nd:YAG laser ablation in a case, cervical esophagotomy, trans-thoracic esophagotomy and esophagectomy<sup>[4]</sup>. Smaller polyps, less than 2 cm in diameter with a thin pedicle, can be removed endoscopically without many complications but this is not recommended in larger tumors (length > 8 cm). Due to the thick vascularized pedicle, hemostasis is most safely achieved by open surgical techniques<sup>[5]</sup>. As the origin of the pedicle of the polyp in the majority of the cases lies in the upper third of the esophagus, cervical esophagotomy seems like the approach of choice. However, it is important to take note, at this point, the value and accuracy of pre-operative assessment of the base of the pedicle and the bulk of the tumor mass. Occasionally, a thoracotomy should be considered as it may be required for difficult, large lesions or lesions with a low origin. On occasion, a trans-abdominal approach via laparotomy may be a useful option as well, to aid in the delivery of the bulky polyp head through a gastrotomy<sup>[5]</sup>.

We advocate an individualized surgical strategy according to the characteristics of each polyp and recommend that in difficult cases [recurrent cases, patients with more than one polyp or patients with a large polyp (> 5 cm) with a long stalk (> 5 cm)] esophagotomy via thoracotomy should be considered for good control of hemostasis as well as for providing adequate exposure for resection of the pedicle's origin and any redundant mucosa around the pedicle. Recurrence of giant fibrovascular polyps is rare but has been described<sup>[2,12,13]</sup>. We believe that residual tissue around the pedicle's base may cause recurrent polyp formation which we hypothesize is the reason for the recurrent nature of polyp formation in our patient.

We therefore suggest appropriate pre-operative counseling and investigations such as obtaining additional informed consent for thoracotomy and laparotomy procedures. We also recommend patients who smoke or have significant respiratory morbidity should have a pre-operative lung function assessment. Peri-operatively it is also essential to inform the anesthetist and operating theatre staff and prepare the patient in anticipation for a thoracotomy or a laparotomy should the situation arise.

In summary, giant fibrovascular polyps are rare esophageal tumors and recurrences are even more uncommon. Although most are benign, surgical excision is recommended in view of potential deadly complications e.g. asphyxiation, bleeding, malignancy. Adequate pre-operative investigations should aim to identify the pedicle's origin and the bulk of the polyp to aid the planning of the surgical approach

to ensure adequate hemostasis and clear resection of the base to prevent recurrences. The surgical options include endoscopic resection, open surgery *via* trans-cervical, trans-thoracic or trans-abdominal approaches. This should be tailored on a case-to-case basis and a combination of the approaches should be considered in difficult cases.

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## Heterotopic pancreas in the gastrointestinal tract

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### INTRODUCTION

Heterotopic pancreas is defined as pancreatic tissue found outside the usual anatomical location of the pancreas. It is usually found in the upper gastrointestinal tract. The incidence of heterotopic pancreas is low. Preoperative diagnosis is difficult. Although echogastriccopy is helpful for diagnosis<sup>[1]</sup>, it is difficult to distinguish from gastrointestinal stromal tumor (GIST). Frozen sections may help in deciding the extent of resection intraoperatively. We report two cases of heterotopic pancreatic lesions in the duodenum and gastric antrum.

### CASE REPORT

#### Case 1

A 60-year-old man presented to the gastroenterology outpatient clinic complaining of joint pain all over the body of 2 years duration, and abdominal distension for 1.5 years. He had a history of dermatomyositis. Physical examination revealed some symptoms of carcinoid syndrome including face rubeosis, abdominal bulge, and abdominal distension after walking for several minutes. Laboratory findings were that routine blood examination was normal, blood clotting and erythrocyte sedimentation rate were normal, postprandial blood sugar was 11.10 mmol/L, anti-dsDNA antibody, anti-O antibody and antinuclear antibody were normal, serum rheumatoid factor was 34.00 IU/mL, total cholesterol was 6.1 mmol/L, triglyceride was 2.85 mmol/L, 24-h urinary protein was normal, tumor markers were normal except that CA72-4 was a little high. Epigastric CT revealed a duodenal bulb submucosal lump that showed exophytic growth, which was possibly a benign GIST. Gastroscopy revealed chronic atrophic gastritis. Echogastriccopy revealed a mass in the duodenal submucosa, which might have been GIST. The patient underwent exploratory laparotomy after general anesthesia. During the operation, surgeons discovered that there was a tenacious mass in the duodenal bulb subserosa, with a diameter of 1.5 cm, and it did not encroach on mucosal

### Abstract

Heterotopic pancreas is defined as pancreatic tissue found outside the usual anatomical location of the pancreas. It is often an incidental finding and can be found at different sites in the gastrointestinal tract. It may become clinically evident when complicated by pathological changes such as inflammation, bleeding, obstruction, and malignant transformation. In this report, a 60-year-old man with carcinoid syndrome caused by heterotopic pancreatic tissue in the duodenum is described, along with a 62-year-old man with abdominal pain caused by heterotopic pancreatic tissue in the gastric antrum. The difficulty of making an accurate diagnosis is highlighted. The patients remain healthy and symptom-free after follow-up of 1 year. Frozen sections may help in deciding the extent of resection intraoperatively. Although heterotopic pancreas is rare, it should be considered in the differential diagnosis of gastrointestinal stromal tumor.

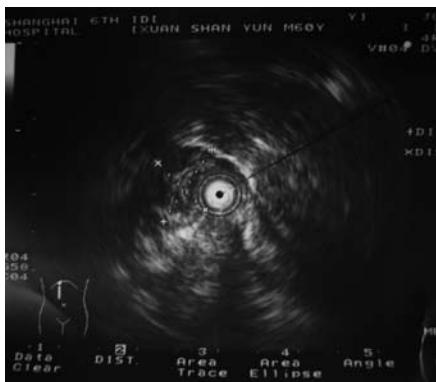
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**Key words:** Heterotopic pancreas; Gastrointestinal stromal tumor; Carcinoid syndrome; Ultrasonography; Gastroscopy

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**Figure 1** Endoscopy showing a solid tumor mass under the mucosal membrane in the gastric antrum.



**Figure 3** Echogastroscope revealing low-echogenicity mass under the gastric wall submucosal muscularis propria, with a clear boundary and uneven internal echogenicity.

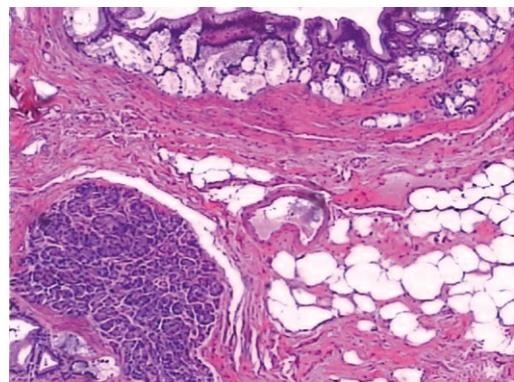
membrane. Local excision was carried out, and frozen sections showed heterotopic pancreas in the duodenal bulb. After surgery, paraffin sections showed that heterotopic pancreas in the duodenal bulb with papillary hyperplasia of the pancreatic duct epithelium. He was discharged 10 d after surgery without face rubeosis or abdominal distention after walking. He remained in good health during follow-up.

## Case 2

A 62-year-old man complained of repeated, vague right upper quadrant pain for 2 years. Physical examination and laboratory findings were unremarkable. Endoscopy showed a solid tumor mass in the gastric antrum (Figure 1). Epigastric zone CT also showed a mass in the gastric antrum (Figure 2). Echogastroscope showed that there was a solid tumor mass under the mucosal membrane in the gastric antrum (Figure 3), which was suggestive of GIST (a low-echogenicity mass under the gastric wall submucosal muscularis propria, with a clear boundary and uneven internal echo, and measuring 24.6 mm × 16.5 mm). Tumor markers were normal. During the operation, surgeons discovered that there was a tenacious mass with a diameter of 2 cm in the gastric antrum, which was considered to be GIST. Partial gastrectomy was performed because endoscopy could not rule out the possibility of malignancy. Frozen and paraffin sections showed heterotopic pancreas in the gastric antrum



**Figure 2** CT reconstruction showing a mass with a diameter of 2 cm in the gastric antrum.



**Figure 4** Lobules of pancreatic tissue with ducts located within the smooth muscle of the pylorus (HE,  $\times 100$ ).

(Figure 4). He was discharged 14 d after surgery without abdominal pain or complications. He remained in good health during follow-up.

## DISCUSSION

Jean-Schultz was the first to report that heterotopic pancreas is pancreatic tissue found outside the usual anatomical location of the pancreas<sup>[2]</sup>. It is a congenital abnormality. Its precise incidence rate has not been reported either in China or abroad, but it is very low. The incidence rate is 0.11%-0.21% at autopsy, with a male to female ratio of 3:1<sup>[3]</sup>. Heterotopic pancreas can exist at any position in the abdominal cavity. It is usually found in the upper gastrointestinal tract, with > 90% of the cases involving the stomach, duodenum or jejunum. Unusual locations are the colon, spleen or liver<sup>[4,5]</sup>. Heterotopic pancreas is usually buried in the submucosa, which makes it difficult to distinguish from GIST<sup>[4,6-8]</sup>. The diameter of heterotopic pancreatic tissue is generally about 1-2 cm, and in our cases, the diameter was 1.5-2 cm.

Patients with heterotopic pancreas can be normal, or present with abdominal pain and distension. In addition, it can manifest clinically in some rare diseases of the pancreas including pancreatitis, islet cell tumor, pancreatic carcinoma, and pancreatic cyst<sup>[9]</sup>. In our study, one of the patients revealed some symptoms of carcinoid syndrome, which is rare, including face rubeosis, abdominal

bulging, and abdominal distention after walking for several minutes. There is no specific examination and diagnostic method at present, and it is difficult to diagnose this disease definitely before laparotomy<sup>[2,10,11]</sup>. Echogastroscope, CT and gastroscopy can be helpful in diagnosis. The literature shows that the rate of diagnosis of this disease is high with echogastroscope<sup>[1]</sup>. In our two patients, echogastroscope and epigastric CT before laparotomy did not reveal heterotopic pancreas, and diagnosis was dependent on frozen or paraffin sections postoperatively. Heterotopic pancreas should be considered in the differential diagnosis of GIST. Medical treatment is not effective for heterotopic pancreas, and surgical excision is the first and best choice<sup>[12-14]</sup>. It is often impossible to distinguish gastric heterotopic pancreas from primary or metastatic cancer because endoscopic biopsies are often unremarkable. Therefore, frozen sections should be taken rapidly and routinely so as to confirm the diagnosis and avoid unwanted radical surgery such as Whipple's procedure or subtotal gastrectomy. Heterotopic pancreas may manifest some symptoms of carcinoid syndrome, and surgical treatment may eliminate such symptoms. Asymptomatic heterotopic pancreas is hard to diagnose. The treatment of asymptomatic histologically verified gastric heterotopic pancreas is debatable<sup>[15]</sup>.

In summary, the incidence of heterotopic pancreas is low and preoperative diagnosis is difficult. Although echogastroscope is helpful for diagnosis<sup>[1]</sup>, it is difficult to distinguish from GIST. Frozen sections should be taken so as to distinguish heterotopic pancreas from malignant tumor.

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CASE REPORT

## Primary gastrointestinal stromal tumor of the liver: A case report

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## INTRODUCTION

Gastrointestinal stromal tumor (GIST) is an uncommon tumor and can be seen mostly in gastrointestinal tract origin<sup>[1]</sup>. Sporadic primary GISTs unrelated to the tubular gastrointestinal tract, such as the omentum, mesentery or retroperitoneum, gallbladder, uterus and liver<sup>[1-4]</sup>, have been reported suggesting that these tumors are more widespread than commonly appreciated. Herein, we report a case of primary GIST of the liver evaluated by contrast-enhanced ultrasound (CEUS) and enhanced spiral computed tomography (CT) with histological and immunohistochemical diagnosis.

## CASE REPORT

A 17-year-old man, who was a HBV carrier without symptoms, was found to have a mass in the anterior segment of the right liver lobe by B-mode ultrasound examination at a local hospital and was then transferred to our hospital. Abdominal physical examination found no palpable mass. Laboratory studies showed positive HBsAg, HBe-Ab as well as HBc-Ab, and negative HBs-Ab, HBeAg, HCV, CEA, AFP, CA 19-9, CA 12-5, and CA 15-3. Blood chemistry was normal except for an alanine aminotransferase level of 60 U/L which was slightly increased. The fecal occult blood test was negative. Chest X-ray was also negative.

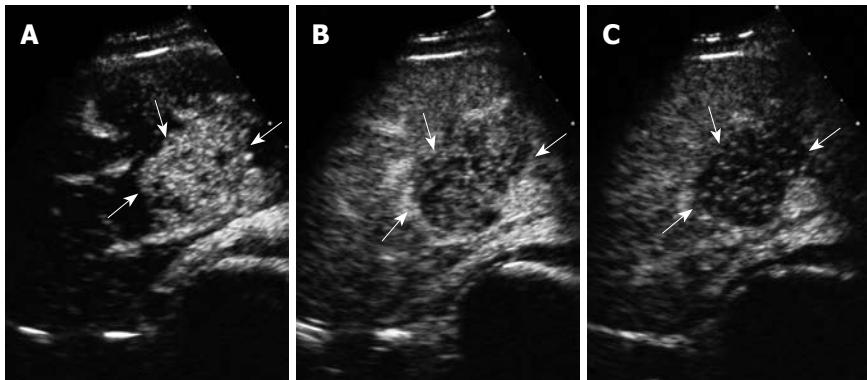
On CEUS examination using a 3.5 MHz convex transducer and a harmonic tissue program (Sequoia 512, Acuson Corporation, USA), a solid, oval-shaped, hyperechogenic 5.1 cm × 3.8 cm × 4.6 cm mass in the anterior segment of the right lobe next to the liver hilum was detected, showing vascularization on color Doppler imaging. Contrast-enhanced sonography with pulse inversion sequence imaging was performed which

## Abstract

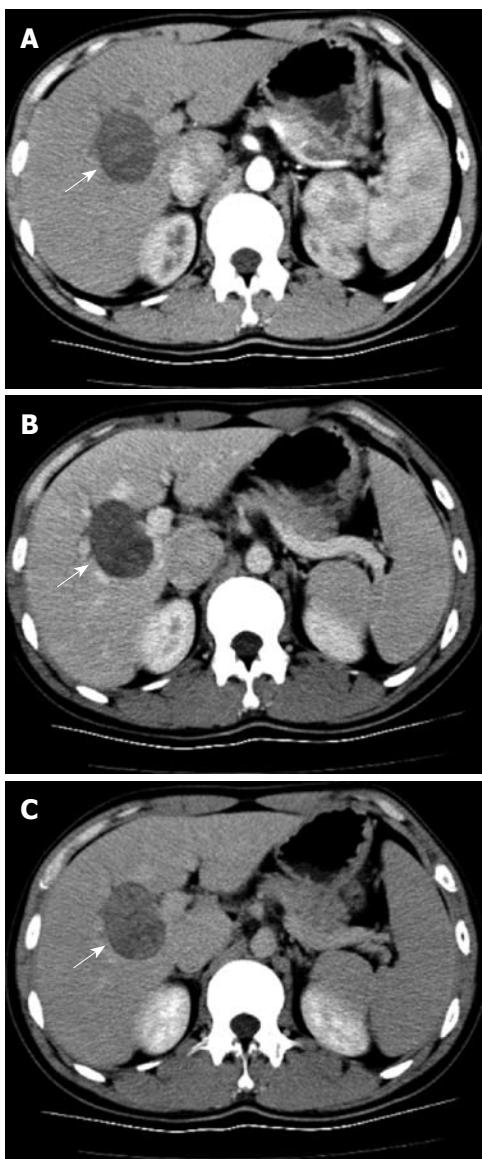
We report a case of primary gastrointestinal stromal tumor (GIST) of the liver. A 17-year-old man with a solid mass in the anterior segment of the right liver was asymptomatic with negative laboratory examinations with the exception of positive HBV. Contrast-enhanced ultrasound (CEUS) revealed a hypervascular lesion in the arterial phase and hypoechoic features during the portal and late phases. However, enhanced spiral computed tomography (CT) showed hypoattenuation in all three phases. Following biopsy, immunohistochemical evaluation demonstrated positive CD117. Different imaging features of primary GISTs of the liver are due to pathological properties and different working systems between CEUS and enhanced spiral CT.

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**Key words:** Contrast-enhanced ultrasound; Enhanced spiral computed tomography; Gastrointestinal stromal tumor; Liver

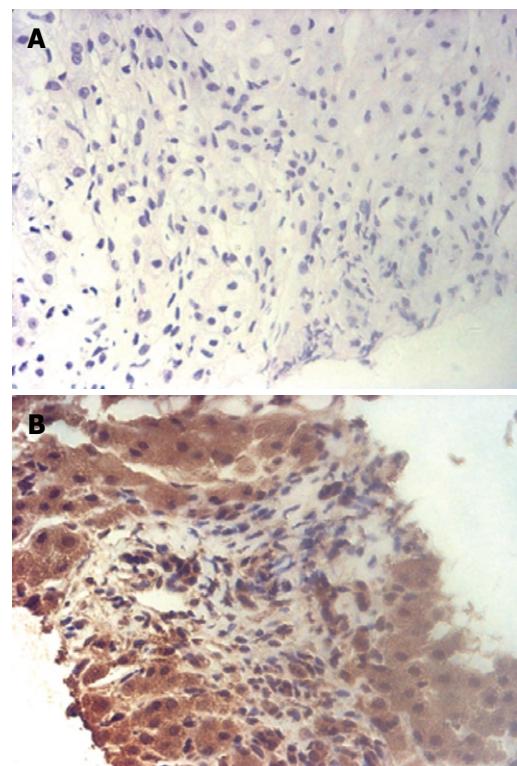


**Figure 1** Real-time CEUS showing a solid, oval-shaped, hyperechogenic  $5.1 \text{ cm} \times 3.8 \text{ cm} \times 4.6 \text{ cm}$  mass in the anterior segment of the right liver lobe. A: Hypervascularity in the arterial phase; B: Partial wash-out in the portal venous phase; C: Hypoechoic feature in the late phase.



**Figure 2** Transverse sequential triple-phase enhanced spiral CT showing a hypovascular mass in the anterior segment of the right liver lobe. A: The arterial-dominant phase; B: The portal-dominant phase; C: The late phase.

Showed contrast enhancement in the lesion appeared 14 s after a bolus injection of 2.4 mL SonoVue® (Bracco Diagnosis, Italy) and peaked at 27 s. Contrast enhancement subsequently decayed 30 s after injection to the end of the delay phase and the lesion was hypoechoic compared with that of the adjacent liver parenchyma (Figure 1).



**Figure 3** Histologic and immunohistochemical examinations of the tumor biopsy specimen. A: Neoplastic spindle cells in an irregular pattern, with normal hepatic parenchyma in peripheral areas (HE, original magnification,  $\times 200$ ); B: Diffuse and strong positivity for CD117 (original magnification,  $\times 200$ ).

Enhanced spiral CT (Aquilion, 16-slice, Toshiba, Japan) was performed 2 wk after CEUS and sequential triple-phase scanning began 20 s after 80 mL Iopromide (Ultravist, Schering, Berlin, Germany), an iodinated contrast, was injected at a flow rate of 3 mL/s. A hypodensity mass was demonstrated in the right liver lobe with slight enhancement in the arterial phase, which gradually increased in the portal phase and reached a maximum in the late phase (Figure 2).

Thereafter, biopsy was performed under the guidance of ultrasound. Histologic examination of the biopsy specimen showed that the tumor consisted of irregular neoplastic spindle cells and no mitoses were found (Figure 3A). Immunohistological staining showed positive reactivity for CD117 (Figure 3B), vimentin and CD34, and staining for keratin, HHF35, a-SMA, desmin, NF and S-100 was negative. With no other

findings in the abdominal and pelvic cavity on CEUS and enhanced spiral CT studies, the final diagnosis of a GIST originating from the liver was made. The patient was subsequently treated with radiofrequency ablation therapy. CEUS examination 3 mo later demonstrated no vascularity in the tumor.

## DISCUSSION

GIST is one of the soft tissue sarcomas arising from a pacemaker cell known as the interstitial cell of Cajal<sup>[5]</sup>, and can be seen mostly in gastrointestinal tract origin but predominantly occurs in the stomach (60% to 70%) and small intestine (25% to 35%), with rare occurrences in the colon and rectum (5%), esophagus (< 2%) and appendix<sup>[1]</sup>. GISTs originating from outside the gastrointestinal tract are extremely unusual. Regardless of where they occur, almost all GISTs have the same biologic behavior with the expression of the c-kit (CD117) protein, which is a growth factor transmembrane receptor with tyrosine kinase activity and differentiates these tumors from other mesenchymal tumors<sup>[1-4]</sup>.

The diagnosis of GIST mainly depends on histologic and immunohistochemical appearance, usually after surgery. GIST is mainly composed of spindle cells, which are randomly arranged in a variety of patterns. Immunohistochemical profiles show positivity for CD117, CD34, and vimentin; however, they are usually negative for keratin, S-100 protein, and smooth muscle actin. Tumor size and mitotic counts are used as prognostic factors, and mitotic counts exceeding 5 per 50 high power fields or size larger than 5 cm can predict future recurrence or metastasis to other areas with a predilection for the liver or the peritoneal cavity<sup>[1,6]</sup>. In our case, the histologic and immunohistochemical findings strongly supported the diagnosis of GIST. Owing to the fact that almost all GISTs in the liver are malignant, and metastatic lesions usually arise from the gastrointestinal tract, we carefully scanned the abdominal and pelvic cavities and showed no other positive findings. In addition, no mitoses were noted on pathological evaluation. The final diagnosis was primary GIST of the liver which was hypothesized to originate from progenitors of mesenchymal cells capable of differentiation toward a pacemaker cell phenotype<sup>[3,4]</sup>. The tumor was graded as low-grade malignant GIST due to its size.

Imaging modalities play an important role in detecting tumor localization, staging tumor status, assessing response to treatment and in monitoring tumor recurrence following treatment. Imaging features have revealed that the majority of GISTs manifest themselves as having an exophytic and cavitary nature with heterogeneous enhancement of contrast agents<sup>[6,7]</sup>. In Vanel's<sup>[6]</sup> report, 13 cases of hepatic metastases from GISTs were heterogeneous hypodense lesions with progressive, concentric enhancement. In our case, hypoattenuation with a gradual contrast enhancement pattern on enhanced spiral CT was coincident with those of metastatic GISTs of the liver. However, hypervascularity with quick wash-in

and wash-out on CEUS resembled malignant tumors of the liver, and because the patient was a HBV carrier, the initial diagnosis was highly suspicious for hepatocellular carcinoma. An analogous case appeared in Nicolau's report<sup>[8]</sup>, where the CEUS pattern in one benign solitary fibrous tumor (SFT) presented as arterial enhancement with quick wash-out in the portal and late phases which led to the misdiagnosis of a malignant tumor. It is worth noting that SFT is also one of the soft tissue sarcomas which are thought to have many clinical and pathologic features in common and the tumors are generally rich in vasculature even in benign stages<sup>[9,10]</sup>.

Although imaging findings did not seem to be a determinant factor in the diagnosis of this case, when the medical records were reviewed some clues were revealed. In this patient, a solitary parenchymatous tumor was found in the liver, with negative tumor markers on laboratory testing and no other findings on medical examination except positivity for HBV. This implied that it would be necessary to make a differential diagnosis from a relatively large spectrum of tumors. It should be borne in mind that some soft tissue sarcomas, such as GISTs, have prominent vascular features and the pharmacokinetics and pharmacodynamics of the contrast agent entering these types of tumors may be similar to those in malignant tumors.

Discrepancies between CEUS and enhanced spiral CT may be due to the distinct vascular architecture of GISTs and different working systems existing in the two imaging modalities. Contrast enhancement in CT or MR imaging can not be performed in real-time. Single frames are obtained during each of the phases of the liver. The time window of contrast-enhanced CT to image the liver lesion during the hepatic arterial-dominant phase is so short (approximately 20-30 s) that it results in the possibility of missing the early arterial enhancement. Since CEUS performed in real-time has the ability to continuously evaluate the entire arterial, portal, and late phases, if a lesion shows rapid changing enhancement, it is possible that this change in enhancement may be better visualized in CEUS than in contrast enhanced CT or MR imaging in the early phase. In Murphy-Lavallee's<sup>[11]</sup> studies, most hepatic metastases, including those thought to be hypovascular, showed transient arterial hypervascularity followed by rapid and complete wash-out within the conventional arterial phase on CEUS. The time to peak arterial enhancement of these tumors ranged from 8 to 27 s, and the beginning of wash-out ranged from 13 to 50 s.

Conclusively, primary GIST of the liver is extremely rare. Despite some discordance between CEUS and enhanced spiral CT in our case, their imaging features still seem to mimic those of the malignant tumors of the liver, irrespective of whether they are primary or metastatic lesions. To date, there are only two cases of primary GIST of the liver reported in the English literature, and to our knowledge, the case presented here is the first report of both CEUS and enhanced spiral CT evaluations before the final diagnosis. The present case may be taken as a warning for future clinical practice.

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## Meetings

### Events Calendar 2009

January 12-15, 2009  
Hyatt Regency San Francisco, San Francisco, CA  
Mouse Models of Cancer

January 21-24, 2009  
Westin San Diego Hotel, San Diego, CA  
Advances in Prostate Cancer Research

February 3-6, 2009  
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)  
Second AACR Conference  
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009  
Hyatt Regency Boston, Boston, MA  
Translation of the Cancer Genome

February 8-11, 2009  
Westin New Orleans Canal Place, New Orleans, LA  
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009  
Hong Kong Convention and Exhibition Centre, Hong Kong, China  
19th Conference of the APASL  
<http://www.apasl2009hongkong.org/en/home.aspx>

February 27-28, 2009  
Orlando, Florida  
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009  
Vienna, Austria  
EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease  
[www.easl.ch/vienna2009](http://www.easl.ch/vienna2009)

March 13-14, 2009  
Phoenix, Arizona  
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009  
Marriott Wardman Park Hotel  
Washington, DC  
13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009  
Glasgow, Scotland  
British Society of Gastroenterology (BSC) Annual Meeting  
Email: bsg@mailbox.ulcc.ac.uk

April 8-9, 2009  
Silver Spring, Maryland  
2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009  
Colorado Convention Center, Denver, CO  
AACR 100th Annual Meeting 2009

April 22-26, 2009  
Copenhagen, Denmark  
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)  
<http://www.easl.ch/>

May 17-20, 2009  
Denver, Colorado, USA  
Digestive Disease Week 2009

May 29-June 2, 2009  
Orange County Convention Center Orlando, Florida  
45th ASCO Annual Meeting  
[www.asco.org/annualmeeting](http://www.asco.org/annualmeeting)

May 30, 2009  
Chicago, Illinois  
Endpoints Workshop: NASH

May 30-June 4, 2009  
McCormick Place, Chicago, IL  
DDW 2009  
<http://www.ddw.org>

June 17-19, 2009  
North Bethesda, MD  
Accelerating Anticancer Agent Development

June 20-26, 2009  
Flims, Switzerland  
Methods in Clinical Cancer Research (Europe)

June 24-27 2009  
Barcelona, Spain  
ESMO Conference: 11th World Congress on Gastrointestinal Cancer  
[www.worldgicancer.com](http://www.worldgicancer.com)

June 25-28, 2009  
Beijing International Convention Center (BICC), Beijing, China  
World Conference on Interventional Oncology  
<http://www.chinamed.com.cn/wcio2009/>

July 5-12, 2009  
Snowmass, CO, United States  
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009  
Aspen, CO, United States  
Molecular Biology in Clinical Oncology

August 1-7, 2009  
Vail Marriott Mountain Resort, Vail, CO, United States  
Methods in Clinical Cancer Research

August 14-16, 2009  
Bell Harbor Conference Center, Seattle, Washington, United States  
Practical Solutions for Successful Management  
<http://www.asge.org/index.aspx?id=5040>

September 23-26, 2009  
Beijing International Convention Center (BICC), Beijing, China  
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists (IASGO)  
<http://iasgo2009.org/en/index.shtml>

September 27-30, 2009  
Taipei, China  
Asian Pacific Digestive Week  
<http://www.apdwcongress.org/2009/index.shtml>

October 7-11, 2009  
Boston Park Plaza Hotel and Towers, Boston, MA, United States  
Frontiers in Basic Cancer Research

October 13-16, 2009  
Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States  
Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009  
Versailles, France  
Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009  
Boston, MA, United States  
The Liver Meeting

November 15-19, 2009  
John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States  
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009  
London, UK  
Gastro 2009 UEGW/World Congress of Gastroenterology  
[www.gastro2009.org](http://www.gastro2009.org)



### Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.



## Instructions to authors

### GENERAL INFORMATION

*World Journal of Gastroenterology* (*World J Gastroenterol*) ISSN 1007-9327, DOI: 10.3748 is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1126 experts in gastroenterology and hepatology from 60 countries.

The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

Maximization of the value of the readers can be comprehended in two ways. First, the journal publishes articles that can be directly read or downloaded free of charge at any time, which attracts more readers. Second, the readers can apply the knowledge in clinical practice without delay after reading and understanding the information in their fields. In addition, the readers are encouraged to propose new ideas based on those of the authors, or to provide viewpoints that are different from those of the authors. Such discussions or debates among different schools of thought will definitely boost advancements and developments in the fields. Maximization of the value of the authors refers to the fact that these journals provide a platform that promotes the speed of propagation and communication to a maximum extent. This is also what the authors really need. Maximization of the value of the society refers to the maximal extent of the social influences and impacts produced by the high quality original articles published in the journal. This is also the main purpose of many journals around the world.

The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastrolesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.

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- 3 Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of

balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 4 **Diabetes Prevention Program Research Group.** Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

**Books**

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

**Patent** (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc, assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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## Management of hepatitis C virus infection in HIV/HCV co-infected patients: Clinical review

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understanding of liver disease in co-infected patients is needed to derive new strategies for improving outcome and survival.

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**Key words:** Hepatitis C virus; Human immunodeficiency virus; Coinfection; Pegylated interferon; Ribavirin

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### Abstract

Nearly one fourth of individuals with human immunodeficiency virus (HIV) infection have hepatitis C virus (HCV) infection in the US and Western Europe. With the availability of highly active antiretroviral therapy and the consequent reduction in opportunistic infections, resulting in the prolongation of the life span of HIV-infected patients, HCV co-infection has emerged as a significant factor influencing the survival of HIV patients. Patients with HIV/HCV co-infection have a faster rate of fibrosis progression resulting in more frequent occurrences of cirrhosis, end-stage liver disease, and hepatocellular carcinoma. However, the mechanism of interaction between the two viruses is not completely understood. The treatment for HCV in co-infected patients is similar to that of HCV mono-infection; i.e., a combination of pegylated interferon and ribavirin. The presence of any barriers to anti-HCV therapy should be identified and eliminated in order to recruit all eligible patients. The response to treatment in co-infected patients is inferior compared to the response in patients with HCV mono-infection. The sustained virologic response rate is only 38% for genotype-1 and 75% for genotype-2 and -3 infections. Liver transplantation is no longer considered a contraindication for end-stage liver disease in co-infected patients. However, the 5 year survival rate is lower in co-infected patients compared to patients with HCV mono-infection (33% vs 72%, P = 0.07). A better

### INTRODUCTION

Co-infection with hepatitis C virus (HCV) and human immunodeficiency virus (HIV) is common as both viruses share similar modes of transmission. The management of HCV infection in the HIV infected population poses a serious challenge for physicians. Approximately two thirds of co-infected patients do not receive anti-HCV treatment for reasons such as poor compliance with highly active anti-retroviral therapy (HAART), decompensated liver disease, co-morbidities, active substance use, ongoing alcohol use, and advanced HIV disease<sup>[1,2]</sup>. Thus, only a minority of such patients receive anti-HCV treatment<sup>[2-4]</sup>. In order to improve the outcome in co-infected patients, it is important to have a good understanding and knowledge of HAART and its interaction with drugs used for HCV treatment.

### EPIDEMIOLOGY AND PREVALENCE OF HCV IN HIV INFECTED INDIVIDUALS

In the US, about 25% to 35% of patients with HIV are infected with HCV, with a higher prevalence (40%) in US military veterans<sup>[5,6]</sup>. This translates to nearly 300 000 people in the US who are co-infected with HIV and HCV. The co-infection rate is higher when transmission occurs through the parenteral route

compared to when the infection is acquired by sexual contact. The prevalence of HCV in HIV patients who acquire infection through intravenous drug abuse (IVDA) or multiple blood transfusions is 75%-90%<sup>[3,7]</sup>. Although sexual contact is a common route of HCV transmission<sup>[8,9]</sup>, it is difficult to determine its true significance because other variables are frequently also involved. Bollepalli *et al*<sup>[10]</sup> reported that IVDA, snorting drugs, sharing toothbrushes/razors, being in prison, and tattooing are significant non-sexual risk factors, while sex for money or drugs, sex with IVDA users, and men who have sex with men (MSM) are significant sexual risk factors for co-infection. However, on multivariate analysis, only IVDA emerged as a risk factor for co-infection ( $P = 0.001$ )<sup>[10]</sup>.

## SIGNIFICANCE OF HCV AND HIV CO-INFECTION

With the availability of HAART, the outcome of patients infected with HIV has improved remarkably in the last decade<sup>[11,12]</sup>. Since HIV patients are now surviving longer, liver disease due to HCV co-infection has emerged as a significant problem. The presence of HIV alters the natural history of HCV infection. After acquiring HCV, the infection has a tendency to chronicity in over 90% amongst HIV patients due to the lack of critical CD4 T cell responses against HCV<sup>[13,14]</sup>. Once chronic HCV infection is established, fibrosis progression is much faster, resulting in a higher frequency of cirrhosis and its complications compared to HCV mono-infection<sup>[15,16]</sup>. A meta-analysis of 8 studies involving 1871 HCV positive patients (601 co-infected with HIV) showed a relative risk of 2.92 (95% CI 1.70-5.01) for more severe disease, 2.07 (95% CI 1.40-3.07) for histological cirrhosis and 6.14 (95% CI 2.86-13.20) for decompensated liver disease<sup>[15]</sup>. Similarly, there is a higher incidence of hepatocellular carcinoma (HCC) in co-infected patients<sup>[17,18]</sup>. Co-infected patients with HCC are younger ( $42 \pm 10$  year *vs*  $69 \pm 9$  year,  $P < 0.001$ ) and have a shorter duration of HCV infection ( $18 \pm 3$  year *vs*  $28 \pm 11$  year,  $P < 0.05$ ) compared to patients with HCC in HCV mono-infection. Tumors at presentation in co-infected patients have an infiltrative pattern, are at an advanced stage, and have more frequent extra-nodal metastasis<sup>[18]</sup>.

The mechanism of interaction between the two viruses and their impact on liver injury is not completely understood. HCV is not directly cytopathic and the pathogenesis of liver injury is believed to be immune mediated<sup>[19]</sup>. It can be argued that HIV patients should develop less severe liver injury because of immune-suppression, while the use of HAART with resultant immune restoration should lead to increased liver damage. However, such a scenario is not observed in clinical practice. Puoti *et al*<sup>[20]</sup> showed that a CD4 count of  $< 500$  was an independent predictor ( $P = 0.04$ ) for stage 3-4 disease in a study on 204 patients with chronic HCV infection (84 with HIV co-infection).

A reduction in fibrosis progression, as well as liver disease, has been observed with successful control of the HIV infection<sup>[21,22]</sup>. In a retrospective analysis of 381 patients, Verma *et al*<sup>[21]</sup> showed that the fibrosis progression rate and cirrhosis in co-infected patients was significantly reduced in patients who received HAART as the initial therapy compared to those who received NRTIs followed by HAART (50% *vs* 68%,  $P < 0.006$ ). It has been shown that co-infected patients are more susceptible to liver toxicity and steatosis from HAART, and more so with the use of NRTIs<sup>[23]</sup>. Despite immune suppression, HCV-specific CD8 and CD4 cell responses have been observed in the livers of patients with HIV and HCV co-infection<sup>[24]</sup>. Moreover, the HCV viral load is higher in co-infected patients compared to HCV mono-infection<sup>[25,26]</sup>. Whether high HCV load is associated with more severe liver injury is not known. The lack of an animal model prevents development of a better understanding of the pathogenesis of liver disease in co-infected patients.

## MANAGEMENT OF HIV/HCV CO-INFECTION

### Timing of treatment

With improved survival of HIV patients in the HAART era, liver disease has emerged as an important cause of morbidity and mortality in co-infected patients. In an analysis of the adverse events of anti-HIV drugs, 1246 patients out of a total of 23441 died over a 5-year follow-up period; liver failure constituted 15% of the mortality and over 50% of deaths occurred despite adequate HIV suppression<sup>[27]</sup>. Therefore, effective treatment for both viruses is essential for a favorable outcome in co-infected patients.

The treatment of HCV in HIV infected patients is a challenging undertaking. Management should ideally be provided in a center with experience in treating such patients, preferably under the care of a multidisciplinary team comprised of hepatologists, infectious disease physicians, psychiatrists, pharmacists, social workers, and a substance abuse program.

All HIV patients should be tested for HCV as soon as HIV is diagnosed. An HIV patient negative for anti-HCV, but with significant risk factors for HCV, should be tested for HCV-RNA as a minority of HIV patients may have viremia despite a negative anti-HCV test<sup>[28]</sup>. Assessment of viral load of HIV and HCV along with a CD4 count should be obtained. The current recommendation is to suppress HIV before starting anti-HCV therapy<sup>[29]</sup>.

### Selection of patients

Patients positive for HCV RNA with ALT elevation and/or stage 2 disease (or more) on liver biopsy should be considered for treatment. Patients with mild disease (stage 1 fibrosis) may also be considered if they are good treatment candidates (low viral count, genotype-2 and -3). About 25% patients with mild disease on the initial

**Table 1** Baseline assessment before starting treatment in HCV-HIV co-infected patients

Assessment of liver diseases status
CTP staging
HCV-RNA
HCV genotype
AFP and USG/CT scan for HCC
HBV markers (HBsAg and anti-HBc)
HBV-DNA for patients with isolated anti-HBc to detect low level viremia
Anti-HAV IgG
Assessment of HIV disease status
Current and past opportunistic infections
HIV-associated malignancy
CD4 count
HIV viral load
Details of HAART
Assessment for problems precluding therapy or requiring control before therapy
TSH
Screening for depression or other psychiatric diseases
Complete blood count
Blood sugar
History of significant cardiac, renal, or pulmonary disease
Fundus examination for retinopathy
Beta HCG to exclude pregnancy in females
Urine toxicology screening to exclude concurrent active substance abuse
Social support and treatment compliance

HIV: Human immunodeficiency virus; HCV: Hepatitis C virus; CTP: Child Turcotte Pugh; AFP:  $\alpha$ -feto protein; USG: Ultrasonogram; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; HCC: Hepatocellular cancer; HAART: Highly active antiretroviral therapy; TSH: Thyroid stimulating hormone; HCG: Human chorionic gonadotropin.

liver biopsy develop significant fibrosis over an average period of 3 years. The occurrence of ALT elevation during the follow up period is an independent predictor of disease progression<sup>[30]</sup>. Therefore, if the treatment was deferred at the initial evaluation, regular clinical and laboratory follow-up should be pursued. A repeat liver biopsy should be performed after 3 years, especially in patients with ALT elevation on follow up.

#### Assessment of liver fibrosis before treatment

Assessment of fibrosis allows treatment to be deferred in patients with mild disease due to genotype-1 or -4 infections. As mentioned earlier, treatment may be considered for genotype-2 or -3 disease as the response to therapy in this population is very good. Liver biopsy is the gold standard for the assessment of fibrosis and cirrhosis. However, liver biopsy has certain disadvantages. Being an invasive procedure, serious complications such as bleeding can occur<sup>[31]</sup>. Sampling error due to tissue fragmentation and small sample size may provide inaccurate results. Moreover, the procedure adds to the overall cost of the treatment<sup>[32]</sup>.

Non invasive markers of fibrosis are available and may replace liver biopsy in the future. Various surrogate serum markers such as AST to platelet ratio index (APRI), Fibrotest score (comprised of serum bilirubin,  $\alpha$ -2 macroglobulin, Apolipoprotein A1, haptoglobin, and gamma glutamyl transpeptidase activity), SHASTA index (hyaluronic acid, AST, and albumin levels), Forn's

index (platelet, GGT, age, and cholesterol), and FIB-4 (age, AST, ALT, and platelets) have been tested in HIV-HCV co-infected patients with generally favorable results<sup>[33]</sup>. The measurement of liver stiffness by elastography (Fibroscan or Elastoscan) using an ultrasonic transducer is a rapid, simple, non-invasive, and reproducible technique. It is also more accurate than serum markers in the diagnosis of cirrhosis in co-infected patients<sup>[34]</sup>.

#### Pre-treatment assessment

A detailed clinical evaluation with history and physical examination is essential before starting anti-HCV therapy (Table 1). Patients with decompensated liver disease; i.e., with presence of ascites, variceal bleeding, jaundice, and encephalopathy (Child Turcotte Pugh or CTP stage B and C), do not tolerate interferon (IFN) based therapy well<sup>[35]</sup>. Patients should have a hemoglobin level > 11 g/dL in females and > 12 g/dL in males, a platelet count > 75 000/cm and an absolute neutrophil count (ANC) > 1500 before starting treatment. A baseline fundus examination is recommended as pegylated interferon (PEG-IFN) can cause retinopathy with visual disturbances<sup>[36]</sup>. Combination of Didanosine (DDI) and Zidovudine (AZT) should be avoided to prevent hepatic and mitochondrial toxicity<sup>[37,38]</sup>.

#### Therapeutic regimen for treating HCV

The introduction of interferon (INF) in 1991 has revolutionized the treatment of HCV infection. In HIV/HCV co-infected patients, results obtained with PEG-IFN + ribavirin (RBV)<sup>[39-54]</sup> were superior to those with PEG-IFN alone, standard IFN alone<sup>[48]</sup>, or standard IFN + RBV (Table 2). A meta-analyses of 6 randomized controlled studies showed superior sustained virologic response (SVR) with PEG-IFN + RBV compared with IFN + RBV and PEG-IFN monotherapy. The response was more favorable in males, patients with CD4 > 500, HCV load < 100 000, and in non drinkers<sup>[55]</sup>. Currently, PEG-IFN and RBV combination is the standard of care for the treatment of HCV in HIV patients.

#### Response rate

In an intention to treat analysis, the end of treatment response (ETR); i.e., negative HCV-RNA at the end of treatment, and SVR; i.e., negative HCV-RNA 24 wk after discontinuation of treatment with PEG-IFN + RBV, in IFN naïve HIV/HCV co-infected patients were 29%-62% and 17%-53%, respectively (Table 2). The large difference in the response rates is due to variable frequency of baseline characteristics, such as the presence of cirrhosis, frequency of genotype-1 (GT-1), baseline HIV parameters, dose of RBV, and treatment discontinuation rate. The ETR and SVR rates were superior for the GT-2/3 (44%-81% and 24%-62%, respectively) compared to GT-1/4 (19%-47% and 9%-38%, respectively)<sup>[44,46,47]</sup>. In a recent study, the treatment response rates were similar with the use of PEG-IFN 2a and 2b<sup>[56]</sup>.

The highest SVR rate of 53% was reported by Hopkins *et al*<sup>[49]</sup>, which may be related to the lower

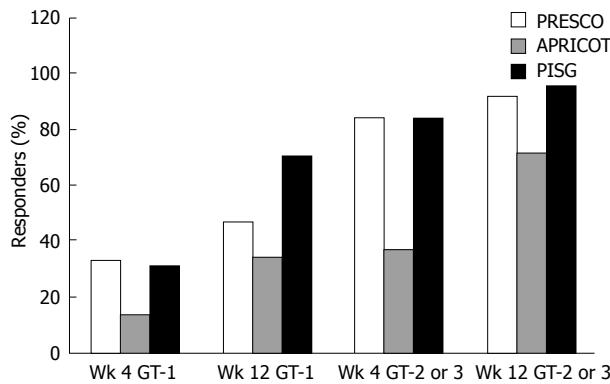
**Table 2 Trials of anti-HCV treatment in HIV patients**

Author (yr)	Country	n	Schedule	Duration	Overall response (%)		GT-2/3		GT-1/4	
					ETR	SVR	ETR	SVR	ETR	SVR
Pérez-Olmeda et al <sup>[39]</sup> (2003)	Spain	68	PEG-IFN 150 µg/wk × 12 wk, then 100 µg/wk + RBV 400 mg bid	6 mo for GT-1/4 and 12 mo for GT-3	30.3	24.2	81 <sup>1</sup>	52.3	30.3	24.2
Cargnel et al <sup>[40]</sup> (2005)	Italy Multi Center	135	PEG-IFN 1.5 µg/kg per week + RBV 400 bid	48 wk	28.9	21.7	43.7	34.4	18.7	9.4
Bräu et al <sup>[41]</sup> (2004)	United States	107	IFN α-2b 3 mu tiw + RBV 800 mg /d vs IFN α 2b 3 mu tiw + placebo × 16 wk, then RBV 800 mg/d	48 wk	16.7 18.9	9.1 11.3	16.2 50	10.8 41.7	14.7 10	8.8 2.5
Laguno et al <sup>[42]</sup> (2004)	Spain	95	IFN α 2b 3 mu tiw + RBV 800-1200 mg/d vs PEG-IFN 100-150 µg/wk + RBV 800-1200 mg/d	48 wk for GT-1/4 and 24 wk for GT-2/3	30 <sup>1</sup> 52	21 <sup>1</sup> 44	67 68	47 53	11 41	7 38
Myers et al <sup>[43]</sup> (2004)	Canada	32	PEG-IFN α 2b (1.5 µg/kg per week) + weight based RBV (1000 mg for 75 kg or less or 1200 mg for > 75 kg) in IFN non responders	48 wk	19	16		29		9
Chung et al <sup>[44]</sup> (2004) ACTG	United States	133	PEG-IFN α 2a 180 µg/wk + RBV (400 mg/d × 4 wk- 600 mg/d × 4 wk - 1000 mg/d vs IFN α 2a 6 mu tiw × 12 wk, then 3 mu tiw + RBV as above	48 wk	41 <sup>1</sup> 12	27 <sup>1</sup> 12	80 <sup>1</sup> 33	73 <sup>1</sup> 33	33 <sup>1</sup> 6	29 6
Moreno et al <sup>[45]</sup> (2004)	Spain	35	PEG-IFN 50 µg/wk + RBV 800 mg/d	48 wk		31		70		25
Torriani et al <sup>[46]</sup> (2004)	APRICOT	868	PEG-IFN α 2a 180 µg/wk + RBV 800 mg/d vs PEG-IFN α 2a + Placebo vs IFN α 2a 3 mu tiw + RBV	48 wk	47	40 <sup>1</sup> 31	64 20	62 <sup>1</sup> 57	38 36	29 21
Carrat et al <sup>[47]</sup> (2004) RIBAVIC	France Multi Center	412	PEG-IFN α 2b 1.5 µg/kg per week + RBV 800 mg/d vs IFN α 2b 3 mu tiw + RBV 800 mg/d	48 wk	35 <sup>1</sup> 21	27 <sup>1</sup> 20	43.7 47.4	25.6 43.4	16.8 <sup>1</sup> 6.2	16.8 <sup>1</sup> 6.2
Khalili et al <sup>[48]</sup> (2005)	United States	154	PEG-IFN α 2a 180 µg/wk vs PEG-IFN + Placebo	48 wk	55 3	35 0	NA			
Hopkins et al <sup>[49]</sup> (2006)	United Kingdom	45	PEG-IFN α 2b 1.5 µg/kg per week + RBV (1000-1200 mg/d)	24 wk for GT-2/3 and 48 wk for GT-1	62	53	82 75 <sup>1</sup>	25	19	
Moreno et al <sup>[50]</sup> (2006)	Spain	70 (HCV) 36 (HCV + HIV)	PEG-IFN α 2b 1.5 µg/kg per week + RBV 10.6 mg/kg per day	48 wk	46	37 <sup>1</sup> 25	All patients had GT-1 or 4 infection and the overall ETR and SVR were 39 and 30% respectively			
Santin et al <sup>[51]</sup> (2006)	Spain Multi Center	60	PEG-IFN α 2b 80-150 µg/wk + RBV 800-1200 mg/d	24 wk for GT-2/3 and 48 wk for GT-1/4	33	27	53 42	24	20 <sup>1</sup>	
Voigt et al <sup>[52]</sup> (2006)	Germany Multi Center	122	PEG-IFN α 2b 1.5 µg/kg per week + RBV 800 mg/d	24 wk for GT-2/3 and 48 wk for GT-1/4	52	25	72 44	41	18 <sup>1</sup>	
Fuster et al <sup>[53]</sup> (2006)	Spain Multi Center	110	PEG-IFN 180 µg/wk + RBV 800 mg/d	24 wk for GT-2/3 and 48 wk for GT-1/4	53	42	68 55	47	33	
Righi et al <sup>[54]</sup> (2008)	Italy	43	PEG-IFN 2a 180 µg/wk or 2b 1.5 µg/kg per week + RBV (10.6 mg/kg per day)	24 wk for GT-2/3 and 48 wk for GT-1a	51	30	38	24		Despite 51% of GT-3 infection, lower response due to high dropout rate of 63% (voluntary in 52%)

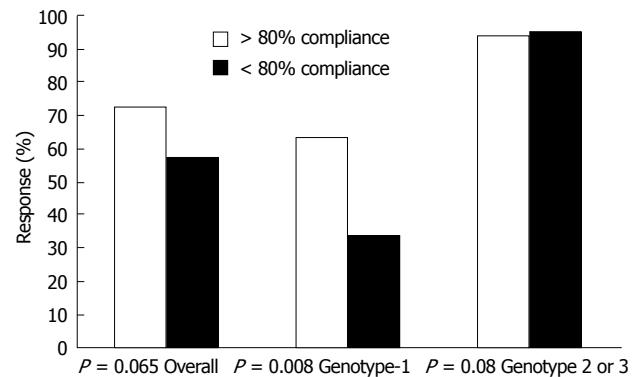
<sup>1</sup>Responses are on intent to treat analysis. ETR: End of treatment response (HCV RNA < 50 copies/mL); SVR: Sustained virologic response (HCV RNA < 50 copies/mL 6 mo after discontinuing treatment); PEG-IFN: Pegylated interferon; RBV-ribavirin; GT: Genotype; tiw: Three times a week; ACTG: AIDS clinical trial group; APRICOT: AIDS pegasys ribavirin international coinfection trial.

proportion of GT-1 patients (33%) compared with other studies (48%-77%) (Table 2). The pooled SVR rate with the use of PEG-IFN + RBV obtained from 7 randomized controlled trials or prospective cohort studies involving 784 HIV + HCV co-infected patients was 33.3% (27.3%-44.2%)<sup>[57]</sup>. The SVR rate in HCV mono-infection with PEG-IFN + RBV was much better (52% for GT-1 and 80% for GT-3)<sup>[58]</sup>. A head-to-head

comparison of the two groups also showed superior ETR and SVR rates for GT-1 infection (46% vs 25%,  $P < 0.05$  and 37% vs 17%,  $P < 0.05$ , respectively)<sup>[50]</sup>. These inferior response rates in co-infected patients can be explained to some extent by the greater number of patients with cirrhosis (43% vs 11%,  $P = 0.0001$ ), and lower doses of RBV used ( $12.75 \pm 1.46$  vs  $14.10 \pm 1.88$ ,  $P = 0.0001$ ) in these patients.



**Figure 1** Patients who consumed > 80% of their anti-HCV medications showed a trend towards better sustained virologic response (SVR) compared to those with < 80% drug compliance (72% vs 57%,  $P = 0.06$ ). The SVR was much better in GT-1 infections (63% vs 34%,  $P = 0.008$ ) but not in GT-2 or -3 infections (94% vs 95%,  $P = 0.88$ ). Wk: Week.



**Figure 2** Results of virologic response in different studies. PRES CO study 33% and 84%, respectively, at weeks 4 and 12 for GT-1 infections; APRICOT study 13% and 34%; PISG study 31% and 71% respectively. Similar figures for GT-2 and -3 infections were 47% and 92%, 37% and 72%, 84% and 96%, respectively.

**Table 3** Barriers against anti-HCV therapy in co-infected patients

Patient-related factors	
Active substance abuse	
Concern regarding adverse effects	
Cost of treatment	
Lack of social support	
Lack of transport	
Number of pills and dosing frequency (including HAART)	
Physician-related factors	
Lack of experience	
Lack of awareness	
Lack of motivation for referral	
Fear of adverse effects	

HAART: Highly active anti-retroviral therapy.

Another important reason for the lower SVR in the co-infected population is the high treatment dropout rate (12%-51%) (Table 3). The importance of medication compliance was shown in a recent retrospective analysis on HCV mono-infected patients<sup>[59]</sup>. Patients who consumed > 80% of the medications (PEG-IFN + RBV), had a superior SVR compared to those with < 80% compliance (Figure 1). The difference in SVR was noted for GT-1 but not for GT-2/3 infection. The treatment discontinuation rates were 39% in both the APRICOT and RIBAVIC trials, and 12% in the ACTG study<sup>[44,46,47]</sup>. Discontinuation of treatment due to adverse effects was observed in 13%, 17%, and 12% of patients, respectively. These figures are similar to discontinuation rates in the HIV negative population<sup>[60]</sup>. Other reasons for treatment discontinuation were patient refusal (4%), lack of follow up (4%), and violation of the study protocol (1%) in the APRICOT study. Another reason was relapse of substance abuse in 1%-4% patients<sup>[41,48]</sup>.

The barriers to anti-HCV treatment may be patient as well as physician related (Table 3). Education and motivation of patients with intensive training and provision of better infrastructure for the treating physicians may improve the outcome. Most studies to date have excluded patients with active substance abuse (Table 3). A recent NIH consensus conference has

recommended that active substance abuse should not be considered a contraindication to anti-HCV treatment<sup>[61]</sup>. Instead, greater integration of HCV treatment in HIV clinics and detoxification programs and provision of additional social support should be encouraged<sup>[62]</sup>. In a retrospective analysis of 73 patients with HCV infection (32% with HIV) treated at a methadone maintenance treatment program, encouraging ETR and SVR rates of 55% and 45%, respectively, were obtained<sup>[63]</sup>.

### Drug doses

The recommended dose of PEG-IFN 2a is 180 µg/wk and for PEG-IFN 2b is 1.5 µg/kg per week (Table 2). Because of the wider systemic distribution of PEG-IFN 2b, it is essential to adjust the dose for body weight, as compared to PEG-IFN 2a, which is restricted to blood and interstitial fluids only. In most studies, RBV has been used at a dose of 800 mg/d or 10.6 mg/kg per day in co-infected patients (Table 2). Virologic response at weeks 4 and 12 were superior with the use of 1000-1200 mg of RBV compared to 800 mg/d of RBV (Figure 2). Improved SVR rates, similar to that seen in HCV mono-infection, were observed when RBV was used at a dose of 1000-1200 mg<sup>[49,64,65]</sup>. Based on the current data, it is recommended that weight-based RBV (1000 mg/d for < 75/kg and 1200 mg/d for 75 kg and above) should be used in co-infected patients.

### Duration of therapy

The use of PEG-IFN + RBV for 48 wk in GT-1/4 and 24 wk in GT-2/3 infections yielded ETR in 53%-82% and SVR in 42%-75% patients (Table 2). On the other hand, treatment for 48 wk for all GTs achieved an ETR in 50%-80% and SVR in 44%-73% co-infected patients<sup>[44,46,47]</sup>. In the recently completed PRES CO trial, PEG-IFN and weight-based RBV were used for 48 wk or 72 wk for GT-1/4 and 24 wk or 48 wk for GT-2/3<sup>[65]</sup>. The overall SVR was 49.6% (72.4% in GT-2/3 and 35% in GT-1/4,  $P < 0.001$ ). In patients with GT-2/3, a higher relapse rate was seen in patients treated for 24 wk compared to those receiving 48 wk of treatment (40% vs

**Table 4** Morbidity and mortality with PEG-IFN + RBV: results of three large trials

Parameter	Sub-parameter	ACTG (n = 66)	APRICOT (n = 286)	RIBAVIC (n = 194)
Treatment related adverse events	Influenza like symptoms	31 (47)		172 (89)
	Fatigue		128 (44)	
	Pyrexia		128 (44)	
	Headache		111 (39)	
	Myalgia		103 (36)	
	Nausea		85 (30)	
	Diarrhea		81 (28)	
	Depression	7 (11)	76 (26)	46 (24)
	Weight loss		82 (28)	46 (24)
	Injection site reaction			44 (21)
	Anorexia			38 (20)
	Irritability			32 (16)
	Bronchitis/cough			26 (13)
	Insomnia		76 (26)	19 (10)
	Elevated lipase/amylase	9 (14)		
	Glucose: high or low	19 (28)		
HIV-related adverse events	Lipodystrophy			37 (19)
	Oral candidiasis		2 (< 1)	30 (15)
	AIDS defining event		2 (< 1)	
Specific serious adverse events	Psychiatric disorders			8 (4)
	Liver failure		1 (< 1)	4 (2)
	Liver decompensation		5 (2)	
	Pneumonia/sepsis		2 (1)	6 (3)
	Symptomatic increased lactate		4 (1)	9 (5)
	Pancreatitis		2 (1)	
	Lactic acidosis	0 (0)	2 (1)	
	ELAT > 10 ULN	20 (30)		16 (8)
	Neutropenia < 500/ $\mu$ L	5 (8)		10 (5)
	Anemia	2 (3)	6 (2)	
	Thrombocytopenia	1 (2)	1 (< 1)	
	Drug abuse		4 (1)	
	Deep vein thrombosis		3 (1)	
	Bacterial infection		3 (1)	
	Gastroenteritis		2 (1)	
Any serious event	Total		50 (17)	68 (35)
	Treatment-related		24 (8)	30 (15)
Deaths	Total	1 (2)	4 (1)	5 (3)

**Table 5** Hepatotoxicity of highly active anti-retroviral therapy drugs and guidelines for their use in patients with liver disease

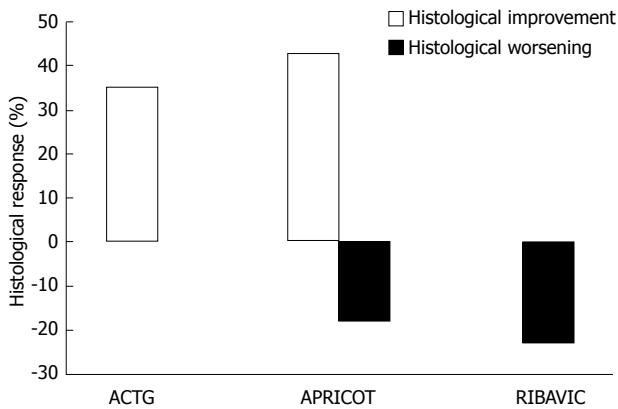
Group	Drug	Dose adjustment
NRTI	Didanosine	Use cautiously
NNRTI	Delavirdine, Efavirenz, Nevirapine	Caution with hepatic impairment
PIs	Lopinavir, Nelfinavir, Ritonavir, Saquinavir	Caution with hepatic impairment
	Atazanavir	Reduce 25% of dose in patients with CTP stage B and C
	Indinavir	Reduce dose by 25% in CTP stage B and C
	Tipranavir	Avoid in patients with CTP stage B or C
	Fosamprenavir	Avoid in patients with CTP stage C
	Darunavir	Avoid in patients with CTP stage C

10%,  $P = 0.02$ ). Patients treated for 48 wk were 5.4 times more likely to achieve SVR. If these data are confirmed in other studies, the standard of care in co-infected patients will be 48 wk of treatment with PEG-IFN and weight based RBV, irrespective of the GT.

#### Safety and tolerability of medications

Adverse effects are seen in the majority of patients (Tables 4 and 5). The most common adverse effects are influenza-like symptoms (Table 4). About one third of patients experience a drop in hematologic parameters. In most cases, the adverse effects are mild, but, treatment

discontinuation was needed in 12%-17% of patients and dose modification of PEG-IFN and RBV, due to either adverse events or a change in laboratory values (neutropenia or thrombocytopenia with PEG-IFN and anemia with RBV), was required in 25%-33% patients<sup>[35,47]</sup>. RBV-induced anemia is exacerbated with the concomitant use of AZT<sup>[38]</sup>. This was confirmed in the PRESCO study where RBV-induced anemia was observed in only 2.6% patients, despite using weight-based higher doses of RBV<sup>[65]</sup>. Every effort should be made to maintain patients on the medications, while simultaneously managing the hematologic abnormalities



**Figure 3** A beneficial histological response was seen in 35% and 43%, respectively, in patients with no sustained virologic response (SVR) in the ACTG and APRICOT trials. Worsening histology was seen in 18% patients with no SVR in the APRICOT study; similar data were not available in the ACTG study. In the RIBAVIC study, histological worsening was seen in 23% without SVR while histology remained stable in the remaining patients.

using blood products and growth factors. In a recent study, the use of Eicosapentaenoic acid prevented RBV dose reduction during first 12 wk of treatment<sup>[66]</sup>.

#### Patient monitoring during therapy

To ensure the safety of treatment, patients should have regular assessment with complete blood count and liver tests. Efficacy of treatment is assessed by quantitative HCV-RNA at weeks 4, 12, 24, 48, and 72. The goal of therapy is to achieve a SVR as this is associated with < 1% recurrence of infection at 5 years<sup>[67]</sup>. Assessment of a 2 log reduction in the HCV viral load at week 12 is crucial; lack of such a reduction has a 98%-100% probability of not achieving a SVR. Recently, data have emerged that patients who become HCV-RNA negative at wk 4 (rapid virologic response or RVR) have a SVR rate of 82%, while 99% of those who fail to achieve a 1-log reduction at week 4 are unlikely to have a SVR<sup>[68]</sup>. However, the current recommendation is to stop further treatment in patients who fail to achieve a 2-log reduction at week 12 of treatment.

#### Management of non-responders and relapsers

The role of induction therapy using a higher IFN dose was tested in the CORAL-1 study<sup>[69]</sup>. The use of PEG-IFN  $\alpha$ -2a at a dose of 270  $\mu$ g for 4 wk followed by 180  $\mu$ g for 8 wk was compared with the standard dose of 180  $\mu$ g for 12 wk. Both groups received 1000-1200 mg/d of weight-based RBV. Although the induction dose was safe and well tolerated, it failed to provide any efficacy benefit. Treatment for an extended duration was assessed in another study in which patients who failed to achieve a 2-log reduction at 12 wk were randomized to receive either 48 wk or 72 wk of PEG-IFN + RBV. This approach also lacked efficacy as only one (2.5%) patient achieved an SVR.

The lack of an animal model has hampered the development of a vaccine against HCV. Several new drugs for treating HCV are under various stages of development<sup>[70]</sup>. These include improved IFN

molecules (albumin fused with IFN- $\alpha$ ,  $\Omega$  IFN, sequential treatment with IFN- $\alpha$  and IFN- $\gamma$ ), improved RBV molecules (Viramidine with a lower potential for hemolysis, and Levovirin), immunomodulators (histamine dihydrochloride, thymosin  $\alpha$ , and isatoribine), and directly acting anti-HCV agents (HCV protease inhibitors, HCV entry inhibitors, ribozymes, and antisense nucleotides)<sup>[70]</sup>.

The anti-fibrotic effect of IFN, with the potential of reducing fibrosis and HCC, is the basis for its use as a maintenance therapy. A beneficial histological response (HR) was observed in all 3 trials with co-infected patients who did not achieve a SVR (Figure 3). In the ACTG and APRICOT studies, HR was defined as an improvement of  $\geq 2$  points in the HAI<sup>[35,44]</sup>, and an improvement of  $\geq 1$  point in the Metavir score in the RIBAVIC study<sup>[47]</sup>. Currently, other studies (SLAM-C and ENDURE) are in progress to confirm the role of maintenance therapy in HIV patients who fail anti-HCV treatment<sup>[71]</sup>.

#### General advice

Patients should be counseled on treatment compliance, and should be encouraged to consume a healthy diet. Patients should abstain from alcohol as HCV and alcohol have a synergistic effect on liver injury<sup>[72]</sup>. Patients with active substance abuse use should be enrolled in drug detoxification programs. Safe sex practices should be encouraged amongst high risk populations, such as multiple sexual partners, active sexually transmitted disease, and MSM. Patients should be instructed to report to their physician if they experience any unusual symptoms, and should avoid herbal or over the counter medications. Patients who are negative for anti-HBs and antibodies to hepatitis A virus (HAV) should be vaccinated against HAV and HBV<sup>[73]</sup>.

## MANAGEMENT OF SPECIAL GROUPS

#### *Patients with normal aminotransferase (PNAL)*

PNAL is defined as the presence of at least 3 normal ALT values, each at least 2 mo apart, over a period of 12 mo. The prevalence of PNAL in co-infected patients is 8%-30%<sup>[74-76]</sup>. Although, the prevalence of stage 3-4 disease in patients with HCV mono-infection and PNAL was lower compared to patients with elevated ALT (5% vs 20%,  $P = 0.01$ ), the prevalence was similar in co-infected patients (37% vs 32%,  $P = 0.6$ )<sup>[77]</sup>. Five year follow-up of 27 co-infected patients with PNAL showed intermittent or persistent elevation of ALT in more than one third of the patients, with the development of decompensated cirrhosis in 2 patients and death in one patient<sup>[75]</sup>.

Treatment of every co-infected patient with PNAL is a daunting task, with considerable expenditure of time and cost. Therefore, it is important to identify factors that may induce rapid FPR in these patients. Currently, it is recommended that treatment should be considered in highly motivated patients and/or those with GT-2/3 infection<sup>[29]</sup>.

### **Management of acute HCV infection**

In the US, about 30 000 cases of acute HCV infection occur annually with increasing incidence in MSM<sup>[78-80]</sup>. The diagnosis is important since unrecognized infection tends to become chronic in 90%-95% co-infected patients<sup>[79]</sup>. The criteria for diagnosis are: elevation of ALT > 10 times the upper limit of normal (ULN), a positive HCV-RNA test and exposure to HCV during the preceding 2-12 mo<sup>[78]</sup>. A high index of suspicion is required, and acute HCV should be excluded before attributing the elevated of ALT to HAART.

The current data on the treatment of acute hepatitis C in HIV patients is limited to a few case studies with SVR of 59% to 91%<sup>[81-83]</sup>. The large difference in the response rates may be due to factors such as clinical presentation (anicteric vs symptomatic) and compliance with therapy. The timing of treatment in co-infected patients is not clear. In HCV mono-infection, a 12 wk waiting period is recommended to allow spontaneous clearance of the virus. Consideration should be given to early treatment of acute HCV in HIV patients because of a higher frequency of chronic disease. However, issues such as the timing of treatment, treatment with PEG-IFN alone, and the role of extended treatment can only be determined by properly conducted controlled trials. Until then, it is recommended that acute HCV infection in HIV positive subjects should be treated with PEG-IFN and RBV for a total of 24 wk, irrespective of the GT<sup>[14]</sup>.

### **Management of HIV- HCV co-infected patients with end-stage liver disease**

With the reduction in occurrence of opportunistic infections with the use of HAART, HCV-induced end-stage liver disease (ESLD) is now one of the leading causes of death (denominator effect) in these patients<sup>[84]</sup>. Indeed, HCV is considered as an OI in HIV patients<sup>[85]</sup>. The development of HCC is an important reason for high mortality in co-infected patients, and its incidence has increased from 4.7% in 1995 to 25% in 2000<sup>[86]</sup>. Whether co-infected patients with cirrhosis and end-stage liver disease (ESLD) should be screened for HCC every 6 mo or earlier is not clear. In one study, 5 of 8 patients diagnosed with advanced HCC were tumor negative 6 mo earlier<sup>[18]</sup>. Until more data on the screening interval and the factors that influence progression to HCC becomes available, we recommend that co-infected patients should be screened for HCC every 6 mo<sup>[87]</sup>.

Previously, HIV infection was considered a contraindication for liver transplantation (LT). However, in the HAART era, the outcome after LT has improved considerably, with survival at 1 year and 3 years of 87%-91% and 64%-73% ,respectively<sup>[88]</sup>. A recent retrospective analysis of the United Network for Organ Sharing (UNOS) database showed that although the 2 year survival of HIV patients ( $n = 138$ ) was inferior to HIV -ve patients (70% vs 81%), the survival was similar in HIV -ve patients and HIV +ve patients without HCV or HBV co-infection<sup>[89]</sup>. Similar poor outcome in HIV patients with concomitant HCV infection has been

observed in other studies<sup>[88,90-95]</sup>. Survival of co-infected patients was inferior to HCV mono-infected patients at 1 year, 3 years, and 5 years (68%-80% vs 76%-87%, 56%-57% vs 72%-81%, and 33%-51% vs 72%-81%, respectively)<sup>[88,93,94]</sup>. Poor survival with co-infection has been attributed to the recurrence of HCV, which runs an aggressive course, with frequent development of fibrosing cholestatic hepatitis<sup>[93]</sup>. Moreover, the treatment of recurrent HCV is associated with inferior results (SVR of 27%-28%) compared with treatment of post-LT HCV recurrence in mono-infected patients<sup>[93,96]</sup>. Other factors affecting post-LT survival were intolerance to HAART, CD4 < 200, HIV viral load > 400 cpm, African American race, and model of end-stage liver disease (MELD) score of > 20<sup>[88,93]</sup>. Whether such patients should be treated with pre-emptive anti-HCV therapy or maintenance treatment is unclear. Moreover, the ethical consideration of performing LT in HIV/HCV patients is questionable given the short supply of organ donors in the US<sup>[97]</sup>. An NIH sponsored prospective study on OLT in co-infected patients reported 1 year and 3 year patient survival of 91% and 64%, with graft survival of 82% and 64%, respectively<sup>[98]</sup>. Based on these data, it can be concluded that OLT should be performed only on selected HIV patients in centers with adequate expertise.

The criteria for LT are the same as for HIV -ve patients, including abstinence from drugs for  $\geq 2$  years and abstinence from alcohol for  $\geq 6$  mo. HIV should be adequately controlled with VL < 400 cpm and CD4 > 100. OIs precluding LT are multifocal leucoencephalopathy and multi-drug resistant fungal infections<sup>[99]</sup>. The major issues after LT in co-infected patients are the interaction of HAART with immunosuppressive drugs and recurrence of HCV disease. Most of the immunosuppressive medications are metabolized by the cytochrome P450 system. Protease inhibitors, such as ritonavir, can reduce metabolism of calcineurin inhibitors (CIs), such as cyclosporine and tacrolimus. Therefore, the doses of CIs need to be reduced by about 50%-75% with concomitant administration of PIs. On the other hand, drugs like mycophenolate mofetil interact with nucleoside analogues with decreases in efficacy of zidovudine and stavudine and increases in activity of abacavir and didanosine<sup>[100]</sup>.

### **FUTURE DIRECTIONS**

Based on the literature, recommendations on management of HCV in HIV patients can be made (Table 6). However, there are still many gaps in knowledge and new areas for future research. To better understand the pathogenesis of co-infection, there is an urgent need for *in vitro* and *in vivo* models of dual HIV/HCV infection. The issue of non-invasive means of assessing hepatic fibrosis requires further study. Strategies for the treatment of acute hepatitis C in HIV patients are unclear and involve issues such as the timing of treatment and treatment regimens. The response rate with PEG-IFN and RBV combination for chronic

**Table 6 Recommendations for management of HCV in HIV patients**

All HIV patients should be screened for concomitant HCV infection  
Effective management of HCV is crucial to improve the survival of HIV patients  
Patients with stage 2 or more disease are candidates for therapy provided their HIV disease is controlled  
Pegylated interferon and weight based ribavirin combination is recommended  
Liver transplantation is no longer a contra-indication in the presence of HIV and should be considered in appropriate patients  
Patients with cirrhosis should be screened for esophageal varices and for hepatocellular carcinoma

HCV in HIV patients is low. The safety and efficacy of newer anti-HCV drugs needs to be determined. It should be noted that patients with PNAL have significant disease and therefore should be considered for treatment. However, whether prolonged PEG-IFN therapy or maintenance IFN therapy has any role in non-responders remains to be determined. Currently, co-infected patients with ESLD are screened for HCC every 6 mo, however, advanced HCC has been known to occur within the 6 mo interval. The cost-efficacy of more frequent screening is unknown. In order to improve the post-OLT survival of co-infected patients, strategies to prevent and treat HCV recurrence in this setting are crucial.

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TOPIC HIGHLIGHT

Peter V Draganov, MD, Assistant Professor, Series Editor

## Endoscopic management of biliary strictures after liver transplantation

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### Abstract

Bile duct strictures remain a major source of morbidity after orthotopic liver transplantation (OLT). Biliary strictures are classified as anastomotic or non-anastomotic strictures according to location and are defined by distinct clinical behaviors. Anastomotic strictures are localized and short. The outcome of endoscopic treatment for anastomotic strictures is excellent. Non-anastomotic strictures often result from ischemic and immunological events, occur earlier and are usually multiple and longer. They are characterized by a far less favorable response to endoscopic management, higher recurrence rates, graft loss and need for re-transplantation. Living donor OLT patients present a unique set of challenges arising from technical factors, and stricture risk for both recipients and donors. Endoscopic treatment of living donor OLT patients is less promising. Current endoscopic strategies for biliary strictures after OLT include repeated balloon dilations and placement of multiple side-by-side plastic stents. Lifelong surveillance is required in all types of strictures. Despite improvements in incidence and long term outcomes with endoscopic management, and a reduced need for surgical treatment, the impact of strictures on patients after OLT is significant. Future considerations include new endoscopic technologies and improved stents, which could potentially allow for a decreased number of interventions, increased intervals before retreatment, and decreased reliance on percutaneous and surgical modalities. This

review focuses on the role of endoscopy in biliary strictures, one of the most common biliary complications after OLT.

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**Key words:** Anastomotic strictures; Bile duct diseases; Endoscopic retrograde cholangiopancreatography; Orthotopic liver transplantation; Surgical anastomosis

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### INTRODUCTION

Complications of the biliary tract have been considered the technical ‘Achilles heel’ of orthotopic liver transplantation (OLT) because of their high frequency, need for long term treatment and potential detrimental effects on graft and patient survival. While in early reports, morbidity rates of up to 50% and mortality of 25%-30% were reported, with improvements in organ selection, retrieval, preservation and standardization of the methods of biliary reconstruction, the incidence of these complications have been reduced dramatically<sup>[1-9]</sup>. The biliary tract, however, still remains the most common site for postoperative complications in OLT<sup>[4,10]</sup>. Biliary complications, which most frequently include bile duct strictures and leaks, also include casts, sludge, stones, sphincter of Oddi dysfunction, mucoceles, and hemobilia in 10%-25% of cases and result in death in up to 10% of cases<sup>[1,2,6,8,9,11-17]</sup>. These high rates of post-transplant biliary complications, may point to an inherently sensitive nature of the biliary epithelium to ischemic damage in comparison to hepatocytes and vascular endothelium<sup>[18]</sup>. Beyond graft survival, biliary complications also have a major impact on the quality of life for an OLT recipient, as they entail frequent readmission, repeated imaging, invasive procedures, and occasional reoperation, all adding to the

significant monetary cost of OLT and to the emotional toll these patients suffer<sup>[5]</sup>. While surgical treatment used to be the standard of care at one time, the non-operative management of biliary complications following OLT has become standard practice with primarily endoscopic techniques as the preferred diagnostic and therapeutic modalities, obviating the need for surgery in a majority of patients<sup>[9,19,20]</sup>.

This article will review the role of endoscopy in the diagnosis, treatment options, outcome, and future therapy considerations of biliary strictures after OLT, from the perspective of the practicing gastroenterologist.

## INCIDENCE OF BILIARY STRICTURES

While there has been a decreasing trend in recent years, bile duct strictures are still frequent and account for approximately 40% of all biliary complications after LT, occurring with an incidence of 5%-15% after deceased donor OLT and 28%-32% after right-lobe live donor OLT, with higher incidences seen in reports with a longer follow-up<sup>[7,14,21-33]</sup>. Strictures can occur with either type of biliary anastomosis, although according to some series, strictures were more common with Roux-en-Y hepaticojjunostomy or choledochojejunostomy reconstructions than duct-to-duct anastomoses<sup>[1,14,21]</sup>. Although strictures can present at any time, the overwhelming majority occur within the first year after OLT<sup>[1,23]</sup>. The mean interval at the time of presentation is 5-8 mo after OLT<sup>[3,7,23,34,35]</sup>. Recent studies suggest that their prevalence continues to increase with time<sup>[36]</sup>. Strictures which occur early mostly result from technical problems, whereas later strictures mainly arise from vascular insufficiency and problems with healing and fibrosis<sup>[37,38]</sup>.

## CLASSIFICATION OF BILIARY STRICTURES

Biliary duct strictures can be classified according to their location; strictures localized at the site of the biliary anastomosis or anastomotic strictures are usually single while non-anastomotic strictures in other locations of the biliary tree are most often multiple<sup>[36,39-42]</sup>. These 2 types of biliary strictures differ in incidence, etiology, presentation, natural history and response to therapy rendering their distinction clinically relevant<sup>[25]</sup>. Biliary strictures tend to be a common problem after living donor OLT in both the donor and the recipient and are discussed separately.

### Anastomotic strictures

**Pathogenesis and risk factors:** Anastomotic strictures occur in 5%-10% of cases, are most often isolated, short in length, and are a result of fibrotic healing within the first year after transplantation<sup>[1,7,36]</sup>. Among the etiological factors for anastomotic strictures which appear early in the postoperative period, technical issues appear to be the most important: improper surgical techniques, small

caliber of the bile ducts, a mismatch in size between the donor and recipient bile ducts, inappropriate suture material, tension at the anastomosis, excessive use of electrocauterization for control of bile duct bleeding, and infection<sup>[25]</sup>. Bile leak is an independent risk factor for the development of anastomotic strictures<sup>[17]</sup>. Later onset anastomotic strictures, most likely indicate fibrotic healing arising from ischemia at the end of the donor or recipient bile duct<sup>[34,36,38,40,43-46]</sup>. Anastomotic strictures are reported to be more common after hepaticojjunostomy than after direct duct-to-duct anastomosis<sup>[1,14]</sup>. Duct-to-duct biliary anastomosis has the additional advantage of easy endoscopic access to the biliary system and preservation of the sphincter of Oddi which in theory avoids reflux of contents into the bile duct<sup>[44]</sup>. While biliary complications including bile leaks and cholangitis appear to be higher in patients with T-tubes, biliary strictures appear to be increased following duct-to-duct anastomosis in non-T-tube recipients as compared to anastomosis over a T-tube<sup>[15,47-50]</sup>. Anastomotic strictures are more frequent than non-anastomotic strictures in living donor OLT as compared to recipients of deceased donor OLT<sup>[51-53]</sup>.

**Presentation:** The majority of anastomotic strictures occur within the first 12 mo after OLT. Patients may be asymptomatic at presentation, with elevations of serum aminotransferases, bilirubin, alkaline phosphatase and/or gamma-glutamyl transferase levels. Occasionally, patients have non-specific symptoms such as fever and anorexia, right upper quadrant pain, pruritus, and/or jaundice. A high index of suspicion must be maintained, as pain may be absent in the transplant setting because of immunosuppression and hepatic denervation<sup>[41,44,54]</sup>.

**Diagnosis:** Once abnormalities in liver function chemistries raise the possibility of biliary strictures, further imaging, either noninvasive or invasive, should be performed. Biopsy may only rarely suggest the presence of pathology and thus has a limited role<sup>[55]</sup>.

Initial evaluation should include liver ultrasound (US) with Doppler evaluation of the hepatic vessels. If hepatic artery stenosis or occlusion is suspected on Doppler US, hepatic angiography is usually indicated. Unfortunately, in liver transplant patients, abdominal US may not be sufficiently sensitive (sensitivity of 38%-66%) to detect biliary obstruction<sup>[4]</sup>. The absence of biliary dilation has been found to be an unreliable indicator of adequate biliary drainage<sup>[49]</sup>. The size of the duct is also unreliable in following up these patients or in assessing the response to treatment. Indeed, US has been shown to have a high false negative rate in liver transplant recipients. Furthermore, there appears to be a significant lack of correlation between ductal dilation on US and the cholangiographic and clinical picture<sup>[23,56]</sup>. It is not clear why the donor bile ducts do not respond to distal obstruction by displaying the same degree of proportional dilation as non transplanted livers, however the presence of fibrosis leading to less pliable ducts has been suggested as a possible etiology<sup>[57]</sup>. Therefore, the absence of biliary

dilation identified on US should not preclude further evaluation with more sensitive techniques if the suspicion of biliary stricture remains strong.

Scintigraphy of the hepatobiliary tract with 99-technetium labeled iminodiacetic acid identifies strictures with 75% sensitivity and 100% specificity but a lack of therapeutic benefit limits its clinical use<sup>[58,59]</sup>. Biliary scintigraphy is therefore rarely performed when biliary stricture is suspected but still remains an excellent test to detect biliary leaks<sup>[58]</sup>.

If there is strong clinical suspicion or an US indicates a possible bile duct obstruction, a cholangiogram should be obtained and is considered the reference standard for the diagnosis of biliary tract complications<sup>[2,3,7,8,36,44,54]</sup>. While endoscopic retrograde cholangiopancreatography (ERCP) or percutaneous transhepatic cholangiography (PTC) remain the gold standard, particularly when there is a very high pretest probability of a biliary stricture and need for intervention, magnetic resonance cholangiopancreatography (MRCP) has gained increasing acceptance as a reliable technique in detecting biliary complications. In a study of 64 consecutive patients with suspected biliary complications, MRCP had a sensitivity of 95%, a positive predictive value of 98% and an overall accuracy of 95% compared to ERCP as the reference standard<sup>[60]</sup>. MRCP is currently considered an optimal noninvasive diagnostic tool for the assessment of biliary complications after OLT<sup>[25]</sup>. Once MRCP expertise becomes more widely available, it should have an even more prominent role in limiting the role of invasive cholangiography for therapeutic purposes. The chief disadvantage of MRCP, beyond lack of availability, is the lack of its therapeutic ability. However, it can still be used as a second step after ultrasound in patients for whom the use of ERCP or PTC carries a higher procedural risk.

Use of ERCP *vs* PTC depends on the type of biliary reconstruction, the likelihood of therapeutic intervention and the available expertise. ERCP has the advantage over PTC as it is not only more physiological but also less invasive. In most large tertiary care centers, ERCP is seen as the best diagnostic and therapeutic intervention in patients with duct-to-duct anastomosis. PTC is most often reserved for patients in whom ERCP is unsuccessful and in patients with Roux-en-Y hepaticojjunostomy or choledochojejunostomy. While an inherent limitation of ERCP is the problem of access in patients with Roux-en-Y reconstructions, in high volume centers with experienced endoscopists, newer approaches of ERCP can be successfully performed using the variable stiffness colonoscope, double balloon enteroscope, single balloon enteroscope, and spiral overtube<sup>[61-64]</sup>.

The characteristic cholangiographic appearance of an anastomotic stricture is that of a thin, short, localized, isolated narrowing in the area of the biliary anastomosis. In some patients, a transient narrowing of the anastomosis may become evident within the first 1-2 mo after OLT as a result of postoperative edema and inflammation<sup>[56]</sup>.

**Management:** Over the past 2 decades, there has been

a transition in the primary management of anastomotic strictures from predominantly surgical management to primarily endoscopic management. Percutaneous therapy, although it has a success rate of 40%-85% is still considered a second-line option because of its invasive nature and the associated complications of hemorrhage, bile leaks and significant morbidity<sup>[5]</sup>. Surgical revision is now reserved for patients who have failed the endoscopic and transhepatic measures, and retransplantation is the final option when all else fails<sup>[1,21,23,65]</sup>.

Balloon dilation alone without stent placement is only successful in approximately 40% of cases<sup>[66]</sup>. Balloon dilation with additional stent placement however, appears to be more successful with a durable outcome in 75% of patients with anastomotic strictures<sup>[32,66]</sup>. Placement of not one, but multiple side-by-side plastic stents further increases successful outcomes in 80%-90% of patients<sup>[67-69]</sup>. There is some experience in temporary placement of covered self-expanding metal stents to reduce the need for repeated stent exchanges, but the data are limited<sup>[70]</sup>. A smaller subset of patients, with transient narrowing of the anastomosis within the first 1-2 mo after OLT, presumably due to postoperative edema and inflammation, may respond with a single intervention of endoscopic balloon dilatation and plastic stent placement without need for further treatment<sup>[36]</sup>.

Most patients with anastomotic strictures require ongoing ERCP sessions every 3 mo with balloon dilation of 6-10 mm and multiple stents of 7 Fr to 10 Fr repeated for 12-24 mo<sup>[54,68,69]</sup>. Stents are exchanged at 3-monthly intervals to avoid stent occlusion and bacterial cholangitis. An increasing number of stents can be used at each session to achieve a maximum diameter. The majority of patients require several endoscopic interventions, with a mean of 3 to 5 with long term success rates in the range of 70%-100%<sup>[3,32,36,37,68,69,71-74]</sup>. A protocol of accelerated dilation every 2 wk, and a shortened stenting period of an average of 3.6 mo, showed some encouraging results with a high 87% success rate<sup>[68]</sup>. In patients with duct-to-duct anastomosis, endoscopic management is hence first line, and it appears that while repeat endoscopic treatment is needed, shorter intervals in between treatments may ultimately reduce the time needed for successful long term outcomes (Figure 1).

The major drawbacks of endoscopic dilation with placement of one or more stents as a standard of care in the management of anastomotic strictures, are the need for multiple procedures repeated over extended periods of time, and the risk of cholangitis resulting from stent occlusion.

When endoscopic access to the anastomotic stricture is unobtainable, as in Roux-en-Y reconstructions, another option to be considered is a combined approach where access to the biliary tree is obtained *via* a percutaneous transhepatic route followed by "rendezvous" endoscopy<sup>[62,75]</sup>. Management including using percutaneous transhepatic drainage achieves success rates of 50%-70%<sup>[74]</sup>. The role of surgical revision is confined to endoscopic failures, and the long term results are good with no effect on patient or graft survival<sup>[7,11,76]</sup>.



**Figure 1** Cholangiograms showing anastomotic stricture (A) dilated and stented with 3 plastic stents (B) leading to resolution of the stricture (C).

**Natural history:** Patients with anastomotic strictures require long term surveillance since strictures often recur. Anastomotic strictures identified within 6 mo after OLT usually have a good response to short term stenting (3-6 mo), with the lowest recurrence rates<sup>[36]</sup>. Anastomotic stricture recurrence, however, is particularly high among patients with an initial delayed presentation beyond 6 mo after OLT, and very tight strictures<sup>[5]</sup>. Surveillance is lifelong in all anastomotic stricture patients with a need for periodic evaluation of liver enzymes and imaging. In an illustrative study, patients who developed biliary strictures after OLT and were initially treated endoscopically with balloon dilation and plastic stents, had a recurrence rate of 18% with a mean time to recurrence of 110 d<sup>[71]</sup>. However, patients appear to respond well to repeated endoscopic treatment after recurrence<sup>[68]</sup>. Overall, when anastomotic strictures are treated appropriately, the long term results in terms of patient and graft survival are equivalent to those for matched controls without anastomotic strictures<sup>[2,3,77]</sup>.

#### Non-anastomotic strictures

**Pathogenesis and risk factors:** Non-anastomotic strictures account for 10%-25% of all stricture complications after OLT, with an incidence of 1%-19%; these are often multiple, longer and occur earlier than anastomotic strictures. Multiple factors contribute to the occurrence of non-anastomotic strictures with the main categories of risk factors including ischemia-related injury with or without hepatic artery thrombosis, immunologically-induced injury including chronic ductopenic rejection, and cytotoxic injury induced by bile salts<sup>[1,23,25,29,35,39,77]</sup>. Ischemic and immunological injuries to the biliary epithelium are the most important contributors. Ischemic injury may result from arterial insufficiency and hepatic artery thrombosis, or other forms of ischemia because of donation after cardiac death, prolonged use of vasopressors in donors, older donor age or longer cold and warm ischemia times<sup>[1,3,26,27,78-85]</sup>. Immunological injury is assumed to be a risk factor based on the relationship of non-anastomotic strictures with ABO incompatibility, polymorphism in genes encoding chemokines, and pre-existing immunologically-mediated disease seen in recipients with underlying disease such as primary sclerosing cholangitis (PSC) or autoimmune hepatitis<sup>[78]</sup>. Less important and

inconsistent are the reported associations with hepatitis C and cytomegalovirus<sup>[21,39,86]</sup>.

**Presentation:** Non-anastomotic strictures tend to occur earlier than anastomotic strictures, with a mean time to stricture development of 3-6 mo<sup>[23,39]</sup>. Buis *et al*<sup>[78]</sup> further reported that non-anastomotic strictures secondary to ischemic causes presented within 1 year of transplant, whereas the occurrence after 1 year was more often related to immunological causes as the risk factors. Patients appear to present with similar non-specific symptoms as patients with anastomotic strictures<sup>[42]</sup>.

**Diagnosis:** Diagnosis of non-anastomotic strictures is made with the same modalities as those described for anastomotic strictures. Non-anastomotic strictures can occur proximal to the anastomosis in the extra- or intra-hepatic bile ducts. These strictures tend to be multiple, and longer in length<sup>[23,29,39]</sup>. There may be multiple strictures involving the hilum and intrahepatic ducts causing a cholangiographic appearance that resembles PSC. Biliary sludge can accumulate proximal to the strictures leading to the formation of casts<sup>[42]</sup>. Biliary sludge and casts can rapidly accumulate even after the biliary tree has been cleared and adequate drainage has been achieved. This most likely arises from ongoing sloughing of the biliary epithelium as a result of the underlying ischemic or immunologic injury<sup>[5]</sup>.

**Management:** Non-anastomotic strictures are more difficult to treat than anastomotic strictures with more complications of cholangitis, and overall less favorable outcomes including increased graft loss and death. Only 50%-75% of patients have a long term response to endoscopic therapy with dilatation and stent placement compared to 70%-100% in patients with anastomotic strictures as described above<sup>[2,3,23,54,77]</sup>. Accumulation of biliary sludge and casts renders therapy particularly difficult because of rapid stent occlusion. Non-anastomotic strictures require an increased number of interventions compared to patients with anastomotic strictures<sup>[23]</sup>. Time to response with non-anastomotic strictures is more prolonged than with anastomotic strictures<sup>[23]</sup>. In an illustrative study, the median time of response was 185 d for non-anastomotic strictures *vs* 67 d for anastomotic strictures<sup>[77]</sup>. Also, treatment of non-anastomotic

strictures did not result in significant long term improvement of liver chemistry<sup>[23]</sup>. It does not appear that the poor response of non-anastomotic treatment to treatment varies with etiology<sup>[39]</sup>.

Endoscopic therapy of non-anastomotic strictures typically consists of extraction of the biliary sludge and casts which are routinely present, and balloon dilation of all accessible strictures followed by placement of plastic stents with replacement every 3 mo<sup>[39]</sup>. Balloon dilation of all strictures is frequently not feasible because of the multifocal distribution of the strictures and their predilection for the smaller intrahepatic ducts. Furthermore, rapid stent occlusion with recurrent cholangitis is an ongoing challenge when managing non-anastomotic strictures. Finally, ischemic events which are associated with diffuse intrahepatic bile duct strictures are associated with poor graft survival, and in most instances may require early retransplantation in suitable cases. Hence, endoscopic therapy is also first line in non-anastomotic strictures and may occasionally be a definite solution, but appears to play a more prominent role as a bridge to liver retransplantation<sup>[46,85]</sup>.

**Natural history:** Patients with non-anastomotic strictures require lifelong surveillance since strictures are likely to recur. Complications with cholangitis after treatment are not uncommon, with subsequent repeated need for hospitalization. Most importantly, non-anastomotic strictures may result in significantly increased graft loss; up to 30%-50% of patients undergo retransplantation or die as a consequence of this complication despite endoscopic therapy<sup>[23,39,44,54,87]</sup>.

Surgical revision may ultimately be required in patients with strictures that are refractory to endoscopic or percutaneous treatment. A Roux-en-Y hepaticojejunostomy is usually performed in patients with duct-to-duct anastomosis. In those who already have a Roux-en-Y hepaticojejunostomy anastomosis, a revision may be required by repositioning the bile duct of the graft to a better vascularized area<sup>[42]</sup>.

### **Living donor liver transplantation associated biliary strictures**

Biliary complications are more common in living donor OLT patients compared to deceased donor OLT patients, occurring in up to 32% of patients compared to 10%-15% of patients who undergo deceased donor OLT<sup>[51,88]</sup>. Studies have shown that despite the difference in incidence, the types of complications are similar with biliary strictures as the most common<sup>[54]</sup>. Living donor OLT patients present a unique problem, as not only are recipients at increased risk of biliary duct strictures, but donors too are at risk of these complications. Living donor OLT patients present with the usual factors putting them at risk of biliary strictures, but also with factors unique to surgical techniques required for living donor OLT. The presence of bile leaks has emerged as one of the most important factors in the causation of these strictures, in addition to elderly donors and small duct size (< 4 mm) in duct-to-duct anastomoses<sup>[22,42,66,87,88]</sup>. It appears

that rates of complications with bile duct strictures after Roux-en-Y reconstructions and duct-to-duct anastomoses are similar<sup>[22]</sup>. Presentation is not unlike that in deceased donor OLT patients, however a recent report highlighted the use of a serum bilirubin over 1.5 mg/dL as a better indirect marker of biliary stasis in these patients than alkaline phosphatase which may be overly sensitive<sup>[89]</sup>.

**Recipient-associated biliary strictures:** Bile duct strictures occur in an estimated 1%-9% of these patients<sup>[88,90]</sup>. Ischemic biliary injury in recipients of living donor OLT is a well recognized risk factor which has been associated with extensive dissection of the right hepatic artery and bile duct in the donor in early cases of living donor OLT. In living donor transplantation the incidence of anastomotic strictures appears to be consistently higher than non-anastomotic strictures as compared with recipients of whole liver grafts<sup>[51-53]</sup>. This is considered to be related to the blood supply of the anastomosis and often the presence of multiple and small caliber donor ducts. In terms of management, the therapeutic value of ERCP and PTC in living donor OLT is still under evaluation<sup>[5,54]</sup>. Methods of treatment and success rates of long term endoscopic intervention therapy are difficult to interpret because of the presence of multiple ductal anastomoses, smaller size, peripheral location and increase risk for devascularization<sup>[51-53]</sup>. To date, only 6 published trials have evaluated the efficacy of endoscopic therapy in anastomotic strictures after living donor OLT<sup>[24,30,35,52,84,87,91]</sup>. Endoscopic treatment success rates appear significantly less than for anastomotic strictures in deceased donor OLT at 60%-75%<sup>[52,84,87,91]</sup>. Just as in deceased donor OLT patients, the combination of balloon dilatation and stenting is more effective than either modality alone<sup>[91]</sup>. Technically, living donor OLT presents a challenge with the most common reason for failure being the inability to traverse the stricture and complex peripheral anastomosis, rendering plastic stent placement difficult<sup>[52]</sup>. A subset of patients with a sharp angulation or "crane neck" deformity of the bile duct may make strictures resistant to endoscopic treatment<sup>[53]</sup>. Percutaneous transhepatic therapy appears safe and effective when ERCP fails<sup>[53,92,93]</sup>. Non-anastomotic strictures in living donor OLT have even lower success rates ranging from 25% to 33%, significantly below the 50%-75% seen in patients with deceased donor OLT<sup>[31,52,53,92,93]</sup>. Overall, surgical revision is carried out more frequently in recipients after living donor OLT than after deceased donor OLT<sup>[87]</sup>.

**Donor associated biliary strictures:** In living donor OLT, not only recipients but also donors are at risk of biliary complications including biliary duct strictures. While the overall incidence of bile duct injury in liver donors is low, biliary strictures are increasingly recognized complications in this subset of patients and have even been reported much beyond the early postoperative period<sup>[94-97]</sup>. In a survey of 1508 donors in Asia, complications were seen more commonly with right-lobe as compared to left-lobe or left lateral segment donation<sup>[90]</sup>.

The general principles of management discussed earlier for all biliary strictures apply to living donors with biliary strictures. Similar outcomes were noted for both donors and recipients with biliary strictures with surgical revisions needed more often than for deceased donor OLT<sup>[87]</sup>.

## FUTURE DIRECTIONS

Innovations in ERCP techniques are likely to change the management of biliary duct strictures in the future. Technical reasons for failure of therapeutic ERCP indicate that the presence of a stricture that is too tight to allow access to the central duct system is the most common cause of ERCP failure in anastomotic strictures, non-anastomotic strictures and biliary strictures after living donor OLT<sup>[36,42]</sup>. Use of new intraductal endoscopy technologies such as the SpyGlass direct visualization system (Boston Scientific, Natick, Mass.), which allows visualization of the inner wall of the biliary tree and can act as the guidance system for passage of the guidewire through a tight stricture, has shown some early promise in this area<sup>[5,98-100]</sup>. A recent case report detailed the use of methylene blue-aided chromoendoscopy *via* peroral cholangioscopy to successfully optically diagnose extensive ischemic-type non-anastomotic biliary lesions after transplant<sup>[101]</sup>. Also, new types of balloons and stents will also have a significant role in improvement of management of biliary strictures. Preliminary evidence shows that peripheral cutting balloons may be more effective in biliary strictures not responsive to standard measures<sup>[102]</sup>. Plastic stents and catheters presently used carry a high risk for occlusions. Use of larger metallic open mesh or partially covered stents increases the probability of patency but carries several disadvantages including ingrowths of tissue from reactive hyperplasia, their permanency and the potential difficulty of removing them at surgery. Therefore uncovered and partially covered self-expandable metal stents cannot be recommended for therapy of biliary strictures. Newer removable fully covered metal stents could offer potential therapeutic options for patients with biliary strictures with increased duration of patency than plastic stents<sup>[25]</sup>. More distant in the future are bioabsorbable stents which could be deployed and would remain in place for several months with subsequent biodegradation. These innovations will hopefully allow treatments with single interventions rather than the current practice of repeated interventions and thus have a significant positive impact on the quality of life of the patients treated.

## CONCLUSION

Despite advances in organ procurement and surgical techniques, biliary complications remain a significant source of morbidity in patients after OLT, with biliary strictures still among the most common of these complications. In recent years, endoscopy has played a progressively greater role in the diagnosis and

management of biliary stricture complications after transplantation. Repeated endoscopic dilation with placement of one or more plastic stents is currently the standard first line management of biliary strictures avoiding the need, particularly in anastomotic strictures, for percutaneous transhepatic approaches and surgical management in most cases. Percutaneous and surgical modalities are now reserved for patients in whom endoscopic treatment fails and for those with multiple inaccessible intrahepatic strictures or Roux-en-Y anastomoses. Even in these latter cases, with the recent advances of deep small bowel enteroscopy techniques, the role of endoscopic therapy is expanding. Indeed, as technology progresses with newer endoscopic techniques including intraductal endoscopy, this will allow for enhanced access and visualization of complex strictures as well as improved stenting modalities, and offer hope that the burden to OLT patients related to biliary stricture complications will be reduced even further in the future.

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REVIEW

## Disparities in colorectal cancer in African-Americans vs Whites: Before and after diagnosis

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### Abstract

There are differences between African-American and white patients with colorectal cancer, concerning their characteristics before and after diagnosis. Whites are more likely to adhere to screening guidelines. This is also the case among people with positive family history. Colorectal cancer is more frequent in Blacks. Studies have shown that since 1985, colon cancer rates have dipped 20% to 25% for Whites, while rates have gone up for African-American men and stayed the same for African-American women. Overall, African-Americans are 38% to 43% more likely to die from colon cancer than are Whites. Furthermore, it seems that there is an African-American predominance in right-sited tumors. African Americans tend to be diagnosed at a later stage, to suffer from better differentiated tumors, and to have worse prognosis when compared with Whites. Moreover, less black patients receive adjuvant chemotherapy for resectable colorectal cancer or radiation therapy for rectal cancer. Caucasians seem to respond better to standard chemotherapy regimens than African-Americans. Concerning toxicity, it appears that patients of African-American descent are more likely to develop 5-FU toxicity than Whites, possibly because of their different dihydropyridine dehydrogenase status. Last but not least, screening surveillance seems to be higher among white than among black long-term colorectal cancer survivors. Socioeconomic and educational status account for most of these differences whereas little evidence exists for a genetic contribution in racial disparity. Understanding the nature of racial differences in colorectal cancer allows tailoring of screening and treatment interventions.

### INTRODUCTION

Colorectal cancer is the third most common malignancy and third most frequent cause of cancer-related death in the United States, with 148 810 new cases and 49 960 deaths anticipated in 2008<sup>[1]</sup>. Screening for this type of malignancy reduces mortality through detection of cancer at an earlier, more treatable stage as well as by identification and removal of the precursor lesion, the adenomatous polyp. Today there is a range of options for colorectal cancer screening in the average-risk population, with current technology falling into 2 general categories: stool tests, which include tests for occult blood or exfoliated DNA, and structural exams, which include flexible sigmoidoscopy, colonoscopy, double-contrast barium enema, and computed tomographic colonography. Several treatment options are available after diagnosis depending on the stage including radiation for rectal cancer, surgery and systemic chemotherapy for colon and rectal cancer.

This is a review of the bibliography concerning the differences between African Americans and Caucasians before and after the diagnosis of colorectal cancer. Issues such as screening trends, biologic background, racial variation of colorectal cancer risk and sub site specific risk, prognosis, treatment and surveillance are examined. In addition, socioeconomic and educational disparity has been taken into consideration.

## RACIAL DIFFERENCES BEFORE DIAGNOSIS

### **Colorectal screening disparities between African American and White populations**

Being African American is associated with a lower screening rate for colorectal cancer<sup>[2-12]</sup>. Perceived positive beliefs or barriers about colorectal cancer screening, physician recommendation and knowledge of screening tests and colorectal cancer risk are responsible for a great deal of the difference. Socioeconomic status which is composed of education and income also account partly for the observed difference. African Americans and Caucasians seem to prefer different screening options. In addition, screening disparity exists among Blacks and Whites with positive family history. However, not all studies confirm these data.

**Knowledge about colorectal cancer and screening:** African-Americans' perceptions of sigmoidoscopy and colonoscopy differ from fecal occult blood testing (FOBT) with respect to perceived benefits *vs* barriers. Specifically, barriers are significantly and negatively associated with FOBT and sigmoidoscopy, whereas there is a significant positive association between perceived benefits and sigmoidoscopy or colonoscopy but not with FOBT<sup>[13]</sup>. Higher educational status and greater knowledge of flexible sigmoidoscopy predicted greater adherence to screening flexible sigmoidoscopy guidelines, whereas greater knowledge of FOBT and doctor recommendation predicted greater adherence to FOBT screening guidelines in an African American population in East Harlem<sup>[14]</sup>. Knowledge status about colorectal cancer is lower in African Americans than in Whites<sup>[15]</sup>. In addition, African Americans who adhered to the screening guidelines tended to be more knowledgeable about colorectal cancer and to hold more positive beliefs about the benefits of screening than those who were not up-to-date with screening. Moreover, they tended to receive more physician recommendation and to have better insurance status<sup>[16]</sup>. Low-income African Americans are optimistic and hopeful about early colorectal cancer detection and believe that thorough and accurate screening is valuable. Lack of colorectal cancer knowledge and fear are major barriers to screening for this population<sup>[17]</sup>. African American descent, communication with the health care provider<sup>[18]</sup>, knowledge about colorectal cancer<sup>[18]</sup> and physician recommendation<sup>[19]</sup> predicts adherence to the screening guidelines. Perceived absolute risk, comparative risk or colorectal cancer concerns, predict planning to get an FOBT in the next 2 years among low income African Americans<sup>[20]</sup>. It appears that doctor's recommendation, awareness of screening, older age, greater education and perceived susceptibility account for the differences in colorectal cancer screening among Blacks and Whites in a study<sup>[11]</sup>. African American women who perceive fewer barriers, more benefits, and have increased confidence

in the accuracy of screening are more likely to undergo screening<sup>[21]</sup>.

**Socioeconomic contribution to the racial screening disparity:** Having either a screening sigmoidoscopy or colonoscopy is positively associated with educational status, being married, higher household income, recent medical visit, higher age and public or private insurance among African Americans<sup>[5]</sup>. White race and higher socioeconomic status are associated with higher rate of physician recommendation of screening<sup>[6]</sup>. A study showed that screening rate varies less by race than by region<sup>[22]</sup>. This study concentrated on Southern US regions where there are high concentrations of African Americans as well as high levels of unemployment and poverty. Screening disparities between Blacks and Whites were eliminated after adjusting for socioeconomic status as this was defined by income and education<sup>[23]</sup>. Medicare coverage of colonoscopy since 2001 did reduce racial screening disparities between elderly Whites and Blacks<sup>[24]</sup>.

**Blacks and Whites undergo different screening tests:** It appears that preferred screening techniques vary according to race. African Americans tend to receive significantly less frequent screening colonoscopy than Whites. On the other hand, African Americans are more likely to receive a screening sigmoidoscopy at regular intervals<sup>[5]</sup>. Whites are more likely, in a statistically significant manner, to undergo an endoscopy as a colorectal cancer screening test than African Americans<sup>[9]</sup>. Racial/ethnic minorities are significantly less likely than Whites to prefer computed tomography colonography (CTC) over optical colonoscopy (OC) (Whites, 65.7%; Blacks, 45.1%; Hispanics, 35.8%; and other, 35.7%;  $P < 0.001$ ). Racial/ethnic minorities are less satisfied with CTC (Whites,  $8.4 \pm 1.7$ ; Blacks,  $7.8 \pm 1.7$ ; Hispanics,  $7.4 \pm 1.8$ ; and other,  $7.5 \pm 2.1$ ;  $P = 0.001$ ) and are significantly less willing to undergo CTC again in the future (Whites, 95.5%; Blacks, 80.3%; Hispanics, 84.9%; and other, 85.7%;  $P = 0.006$ )<sup>[25]</sup>. Among tests that examine the entire colon, barium enema is more commonly used among Blacks, whereas colonoscopy is more commonly among Whites<sup>[26]</sup>.

**Screening among people with positive family history:** Screening for colorectal cancer differs between Blacks and Whites with a positive family history for colorectal cancer. Among people with multiple affected first degree relatives (FDRs), or relatives diagnosed before age 50 years, African Americans were less likely than Whites to follow the screening guidelines after adjusting for age, sex, educational status, annual income, insurance status, total number of affected and unaffected FDRs, and time since last medical visit. Specifically, 27.3% of the African-Americans reported having had a colonoscopy during the last five years *vs* 43.1% of Whites [ $P < 0.001$ , odds ratio (OR) = 0.51, 95% CI 0.38-0.68]<sup>[27]</sup>. Another study confirmed this

outcome (27.9% of Whites *vs* 9.3% of Blacks with a positive family history had undergone an endoscopic screening procedure in the last 10 years,  $P = 0.03$ ). After adjusting for age, family history, gender, educational level, insurance status, and usual source of care, Whites were more likely to be current with early initiation endoscopic screening recommendations than African Americans ( $OR = 1.38$ , 95% CI 1.01-1.87)<sup>[28]</sup>. Family history did not predict screening in African Americans when the analysis was controlled for age, education, and insurance. African Americans who have a family history are less likely to screen compared with their white counterparts and compared with African Americans who are at average risk for colorectal cancer ( $P < 0.05$ )<sup>[29]</sup>. Tailored intervention increased screening *via* FOBT in a statistically significant manner in the Caucasian but not in the non-Caucasian population of first degree relatives of people affected with colorectal cancer<sup>[30]</sup>.

**Studies that do not confirm higher screening rates in the White population *vs* Blacks:** Few studies have shown either a higher screening rate in the African-American than in the white population, or no racial difference. In a Veteran medical centre, patients' files were retrospectively analyzed and it was shown that Blacks are more likely to receive either an FOBT within the last year or a flexible sigmoidoscopy/colonoscopy within the last five years than their white counterparts although physician recommendation did not vary among the racial groups<sup>[31]</sup>. No screening differences were noticed between African Americans, European Americans and Native Americans<sup>[32]</sup>. Adherence to the Medicare-covered intervals for colorectal cancer screening tests is low (56.8% for Whites, 39.1% for African Americans), and did not significantly differ by race after adjustment. African Americans were, however, significantly less likely to have ever been tested ( $OR = 0.48$ , 95% CI 0.33-0.70) and more likely to have had an endoscopic test than an FOBT in this study ( $OR = 3.06$ , 95% CI 1.70-5.51)<sup>[33]</sup>. After adjusting for age, having a regular doctor and participation in general medical exams, Blacks and Whites did not vary significantly in their current colorectal cancer screening status, with an OR of 1.1 (95% CI 0.7-1.6)<sup>[34]</sup>. No racial difference was observed in the percentage of people with a positive FOBT who underwent a colonoscopy in the following 12 mo<sup>[35]</sup>. A study among low income women showed that African-American descent predicts a lower likelihood of reporting having had screening colonoscopy within the past 10 years ( $OR = 0.46$ ,  $P < 0.001$ ) although following the screening guidelines did not vary by race<sup>[36]</sup>.

**Findings from colonoscopy screenings:** A study<sup>[37]</sup> carried out and led by David Lieberman, MD, of Portland VA Medical Center collected information from colonoscopy screenings of 5464 African-Americans and 80 061 Whites from 67 screening centers around the United States as published in the September 24, 2008 issue of *The Journal of the American Medical Association*.

The researchers found that "asymptomatic black men and women undergoing colonoscopy screening are more likely to have one or more polyps sized more than 9 mm compared with white individuals. The differences were especially striking among women. These findings emphasize the importance of encouraging all black men and women to be screened."

**The findings:** Nearly 8% of African-American patients had one or more polyps larger than 9 mm; 6% of Whites had one or more polyps larger than 9 mm; African-American women had a 62% greater risk of having such a polyp in the colon when compared with white women; African-American men had a 16% greater chance of having large polyps when compared with white men.

#### **Sub site location of colorectal cancer according to race**

It appears that colon cancer sub site varies according to race. Differences in location among African-Americans and non-Hispanic Whites implicate different screening guidelines in the two racial groups.

A study showed that the proportion of sigmoid colon cancer is 15.6%-21.3% lower in African Americans than non Hispanic Whites over three successive time periods between 1973 and 2002, whereas the diagnosis of descending colon cancer is 40.5%-45.3% higher in the African-American than the white subgroup<sup>[38]</sup>. Regression analysis in this study confirmed that tumors sited proximal to the sigmoid colon or to the splenic flexure are more common in Blacks than in Whites. African Americans are less likely to have colonic polyps ( $OR = 0.77$ , 95% CI 0.70-0.84) and more likely to have colonic tumors than Whites ( $OR = 1.78$ , 95% CI 1.14-2.77), but they are more likely to have polyps in the proximal colon ( $OR = 1.30$ , 95% CI 1.11-1.52) and colonic tumors in the proximal colon ( $OR = 4.37$ , 95% CI 1.16-16.42)<sup>[39]</sup>. Advanced proximal colon cancer is more frequent in African-Americans<sup>[40-42]</sup>. Blacks are more likely to receive a diagnosis of proximal colon cancer than distal colorectal cancer but within the same sub site, they are less likely than Whites to receive a diagnosis of localized disease<sup>[41]</sup>. African Americans tend to have more proximal tumors than Whites<sup>[43]</sup>. Another study confirmed African American predominance in proximal colorectal cancer and white predominance in distal tumors<sup>[44]</sup>. Carcinoma *in situ* has the same pattern of distribution in Blacks and Whites as invasive colorectal carcinoma (white predominance in distal disease and black predominance in proximal disease)<sup>[45]</sup>.

However, an older study, showed no racial variation in cecum or ascending colon cancer incidence, black predominance in transverse or descending colon cancer and white predominance in sigmoid and rectal cancer<sup>[46]</sup>.

#### **Racial variation of colorectal cancer risk**

African-Americans are at increased risk of developing colon cancer<sup>[47]</sup>, especially distant disease<sup>[48]</sup>, compared with Whites, but rectal cancer rate was shown to be higher among Whites when compared with Blacks<sup>[47]</sup>. Black seniors are less likely to be diagnosed with early

stage disease than their white counterparts and this association is greater in areas with high racial segregation and low income<sup>[49]</sup>. Between the years 1992-2001 a decline is observed in colorectal cancer incidence among Whites (1.2% per year in white men and 0.7% per year in white women) but not among Blacks<sup>[50]</sup>. Colorectal cancer was found to be more common in the black population than in the other racial groups and colorectal cancer screening beginning at the age of 50 is more cost-effective in the black population<sup>[51]</sup>. Colorectal cancer incidence was higher in the white than the black race until the mid 1980s whereas the opposite is true in the following years. Furthermore, the same study showed that proximal and transverse colon cancers are more common in Blacks than in Whites and distal colon and rectal cancer are more common in Whites than in Blacks. Colorectal cancer is of lower grade and of greater stage in Blacks than in Whites. In both racial groups, there is a decline in the incidence of distant disease, more in Whites than in Blacks. In the former, the incidence of localized and regional disease was found to be increasing, whereas in the latter this incidence is decreasing<sup>[42]</sup>. Colorectal cancer risk in people younger than 50 years old is higher in African Americans than in Whites<sup>[52]</sup>. In addition, the high incidence and younger age at presentation of colorectal cancer in African Americans warrants initiation of colorectal cancer screening at the age 45 year rather than 50 year<sup>[53]</sup>.

**Nutritional habits and colorectal cancer risk:** African-Americans were found to consume less micronutrients than Whites. High intake of beta carotene, vitamin C and calcium are associated with a lower risk of colorectal cancer in the white population whereas high intake of vitamin C and E are inversely associated with colorectal cancer risk in the African-American population<sup>[54]</sup>. Usage of non-steroidal anti-inflammatory drugs is associated with a reduced risk of colorectal cancer and this association did not vary among African Americans and Whites<sup>[55]</sup>. Fiber consumption is significantly associated with 50%-60% reduced risk of colorectal cancer in African Americans and non-significantly with 30% reduced risk of colorectal cancer in Whites<sup>[56]</sup>. Hydrogen response to 10 g of oral lactulose is significantly higher in the African-American than in the Caucasian American population implicating a difference in colonic bacterial metabolism between the two groups<sup>[57]</sup>. African Americans in all age groups seem to consume fewer mean daily servings of total dairy, milk, cheese, and yogurt than non-African Americans, and have lower mean intakes of calcium, magnesium, and phosphorus<sup>[58]</sup>.

**Racial polymorphism variation:** Cigarette smoking was found to be positively associated with colorectal cancer risk in the white but not in the black population<sup>[59]</sup>. This study also examined the possible association between GSTM1 and GSTT1 polymorphisms with colon cancer. There is a trend towards increased risk of colon cancer for individuals with GSTM1 null (African Americans, OR = 1.43, 95% CI 0.98-2.09; Whites, OR = 1.19, 95%

CI 0.90-1.58) and a decreased risk of colon cancer for individuals with GSTT1 null (African Americans, OR = 0.59, 95% CI 0.40-0.86; Whites, OR = 0.72, 95% CI 0.53-1.00). There are weak interactions between GSTT1 null and cigarette smoking in Whites, and GSTM1 null genotype and cigarette smoking in African Americans. African Americans are more likely to have the BLFA haplotype of the vitamin D receptor (6.5% in the white population vs 41.2% in the African American population) which was found to be associated with increased risk of colorectal cancer (OR = 2.4, 95% CI 1.38-4.38)<sup>[60]</sup>. Adjusted ORs for the combined effects of codon 677 CC and codon 1298 AA genotypes (these codons being part of the 5-10 methylenetetrahydrofolate reductase gene) and folate intake < 400 µg/d are 1.9 (95% CI 1.1-3.4) in African Americans and 2.5 (95% CI 1.2-5.2) in Whites<sup>[61]</sup>. Arachidonate lipoxygenase (ALOX) and cyclooxygenase (COX) are considered important in the development of colon cancer. ALOX5 Glu254Lys, COX2 C-645T and Val511Ala allele frequencies vary among Caucasians and African-American controls ( $P < 0.001$ ). The ALOX5-1752 and -1699 polymorphisms are in linkage disequilibrium ( $P < 0.001$ ) and lower colon cancer risk in Caucasians in ALOX5 haplotype analyses ( $P = 0.03$ ). Furthermore, an inverse association is observed between A alleles at positions -1752 and -1699 of ALOX5 and colon cancer risk in Caucasians, but not in African-Americans. Caucasians with A alleles at ALOX5-1752 have a reduced odds of colon cancer vs those with G alleles [OR (GA vs GG) = 0.63, 95% CI 0.39-1.01; OR (AA vs GG) = 0.33, 95% CI 0.07-1.65,  $P$  (trend) = 0.02]. The same is observed for ALOX5 G-1699A [OR (GA vs GG) = 0.59, 95% CI 0.37-0.94; OR (AA vs GG) = 0.27, 95% CI 0.06-1.32,  $P$  (trend) = 0.01]<sup>[62]</sup>. Serum 25-(OH)D levels are higher in non-Hispanic Caucasians than in subjects of other ethnicities ( $P < 0.001$ ) in both people with and without colorectal adenomas. Serum 25-(OH)D is inversely associated with colorectal adenomas showing a 26% decrease in the rate of colorectal adenoma with each 10 ng/mL increase in serum 25-(OH)D<sup>[63]</sup>. N acetyltransferase 2 rapid/intermediate genotype is associated with increased colon cancer risk in Whites (OR = 1.4, 95% CI 1.0-1.8), when compared with the slow genotype, which is not true for Blacks<sup>[64]</sup>. N-myc downstream-regulated gene 1 (NDRG1) expression is correlated to histopathological type, Dukes' stage and HIF-1 alpha expression in US-Caucasian patients but not in US-African American patients. Interestingly, Kaplan-Meier survival analysis demonstrated that NDRG1 expression correlated significantly with poorer survival in US-African American patients but not in other patient groups<sup>[65]</sup>.

## POSTDIAGNOSIS DISPARITIES BETWEEN WHITES AND AFRICAN AMERICANS

### **Racial prognosis difference due to socioeconomic status, stage and other tumour characteristics**

Colorectal cancer mortality declined in Whites but

increased in Blacks from 1950 to 1992<sup>[66]</sup>. However, mortality has declined in both Blacks and Whites from 1990 to 1998, although the rate for Blacks has remained high<sup>[67]</sup>. Disparity in survival between Blacks and Whites is confirmed by another study<sup>[68]</sup>. There is no difference in survival between Blacks and Whites with colorectal cancer among patients with the same stage receiving the same treatment<sup>[69]</sup>. Recurrence-free survival is modestly lower in African-Americans than in Whites with operable rectal cancer [hazard ratio (HR) 1.25, 95% CI 0.94-1.66] whereas racial disparity in mortality rate is larger (HR 1.45, 95% CI 1.09-1.93)<sup>[70]</sup>. Furthermore, after adjusting for socioeconomic status, mortality in stage II and III colon cancer patients was only marginally higher in African Americans than in Whites<sup>[71]</sup>. An older study showed that among patients with colon cancer, black to white mortality was 1.5 (95% CI 1.2-1.9) and 1.2 after adjustment for stage (95% CI 1.0-1.5)<sup>[72]</sup>. In the same study, differences in mortality was observed in stages II and III (HR 1.8, 95% CI 1.0-3.1 and HR 1.5, 95% CI 1.2-2.3, respectively) but not in stage IV. Furthermore, another study confirmed that Blacks are more likely to die from colorectal cancer than Whites (relative risk = 1.34, 95% CI 1.26-1.42)<sup>[73]</sup>. In this study, socioeconomic status and stage accounted for half of the disparity for all stages and socioeconomic status accounted for all the disparity in stage III of colon and stage II and III of rectal cancer. In addition, comorbidity did not contribute to the disparity, but treatment (surgery, radiation, chemotherapy) explained a very small proportion of the black-white difference. The fully-adjusted relative mortality rate comparing Blacks to Whites was 1.14 (1.09-1.20) for all-cause mortality and 1.21 (1.14-1.29) for colorectal cancer specific mortality. A large meta-analysis (including articles in English from 1966 to August 2007) showed that racial disparities in survival for colon cancer between African-Americans and Caucasians are only marginally significant after adjusting for socioeconomic factors and treatment (HR 1.13, 95% CI 1.01-1.28)<sup>[74]</sup>. On the other hand, another study showed that there is higher stage-specific mortality in Blacks compared with Whites and socioeconomic status accounts for some but not all of this disparity<sup>[75]</sup>. Increased mortality rate in Blacks compared with Whites is the outcome of another study<sup>[76]</sup>. After adjustment for age, sex, histology, site within the colon, and stage, African Americans are more likely to die compared with Caucasian patients with colorectal cancer (colon: HR 1.19, 95% CI 1.14-1.25; rectum: HR 1.27, 95% CI 1.17-1.38). However, after further adjustment for socioeconomic status and treatment, the risk of death for African-Americans compared with Caucasians is substantially diminished (colon: HR 1.08, 95% CI 1.03-1.13; rectum: HR 1.11, 95% CI 1.02-1.20)<sup>[77]</sup>. Survival in patients suffering from colorectal cancer with more than 16 years of education increased from 1993 to 2001 [2.4% ( $P < 0.001$ ), 4.8% ( $P = 0.011$ ), 3.0% ( $P < 0.001$ ), and 2.6% ( $P = 0.030$ ) annually among white men, black men, white women, and black women, respectively]. Among patients with less than 16 years of

education, an increase in mortality was observed only in black men (2.7% per year;  $P < 0.001$ ) whereas the death rate remained stable in the other groups<sup>[78]</sup>. Blacks with colonic adenocarcinoma were found to have a reduced 5- (OR = 1.67, 95% CI 1.21-2.33) and 10- (OR = 1.52, 95% CI 1.12-2.07) year survival than Whites after surgery<sup>[79]</sup>. The strongest and statistically significant association was observed only among patients with stage II. No racial differences in overall survival were observed among patients with rectal cancer. In this study no neo-adjuvant or adjuvant treatment was given to the patients. Among patients who underwent an operation for primary colorectal carcinoma and did not receive pre-surgical or post-surgical chemotherapy, 54% of African Americans and 21% of Caucasians with high grade tumors died within the first year after surgery ( $P = 0.007$ ). African Americans with high-grade tumors were 3 times (HR 3.05, 95% CI 1.32-7.05) more likely to die of colon carcinoma within 5 years post-surgery, compared with Caucasians with high-grade tumors. There were no survival differences by race among patients with low-grade tumors<sup>[80]</sup>.

Another study showed no association between socioeconomic status and survival in patients with colorectal cancer treated in city and university hospitals<sup>[81]</sup>. However, Blacks are more likely to be treated in city than in university hospitals (53% vs 20.6%,  $P < 0.001$ ). Patients treated in city hospitals had a worse prognosis than those treated in university hospitals and Blacks had a worse prognosis in both city and university hospitals in this study.

### Racial variation in treatment

In adjusted comparisons with white patients with colorectal carcinoma, African American patients reported more problems with coordination of care ( $P < 0.001$ ), psychosocial care ( $P = 0.03$ ), access to care ( $P = 0.03$ ), and health information ( $P < 0.001$ )<sup>[82]</sup>.

**Surgical treatment of colorectal cancer in African-Americans and Whites:** Among patients who underwent surgery for rectal cancer, the rate of sphincter-ablating procedure was 37% for Whites and 43% for Blacks [adjusted odds ratio (AOR) 1.42, 95% CI 1.23-1.65]<sup>[83]</sup>. Blacks had a higher risk of dying from colorectal cancer (HR 1.17, 95% CI 1.06-1.30) in a study and adjustment for tumour stage reduced the hazard ratio to 1.11 and surgical treatment further reduced hazard ratio to 1.06<sup>[84]</sup>. Black patients were more likely than Whites not to receive surgical treatment in stage I (OR = 2.08, 95% CI 1.41-3.03 among males; OR = 2.38, 95% CI 1.69-3.45 among females) and IV (OR = 1.25, 95% CI 1.01-1.56 among males; OR = 1.41, 95% CI 1.14-1.72 among females) colon cancer and most stages of rectal cancer, and they were more likely to refuse recommended treatment<sup>[85]</sup>. Black patients were less likely to undergo surgery than Whites (86% vs 91%,  $P = 0.02$ ) but the same study showed no racial differences in overall survival<sup>[86]</sup>. However, long term survival after rectal cancer surgery was shorter for Blacks

than for Whites [five-year survival rates were 41% and 50%, respectively ( $P < 0.0001$ )]. In this study, African Americans were more likely to be treated by low volume surgeons and not to receive adjuvant chemotherapy<sup>[87]</sup>.

**Chemotherapy and radiation therapy in white and black patients with colorectal cancer:** African Americans were treated less frequently with chemotherapy and radiation therapy compared with their Caucasian counterparts, in a retrospective analysis of data coming from a single institution<sup>[88]</sup>. African Americans with stage III colon cancer are less likely to receive adjuvant chemotherapy after surgery and they gain less benefit from adjuvant chemotherapy than Whites<sup>[89]</sup>. There is no statistically significant difference between black and white patients with stage II and III rectal carcinoma in the frequency of consultation with a medical oncologist (73.1% for Blacks *vs* 74.9% for Whites, difference = 1.8%, 95% CI 5.9%-9.5%,  $P = 0.64$ ) or radiation oncologist (56.7% *vs* 64.8%, difference = 8.1%, 95% CI 0.5%-16.7%,  $P = 0.06$ ), but Blacks are less likely than Whites to consult with both a medical oncologist and a radiation oncologist (49.2% *vs* 58.8%, difference = 9.6%, 95% CI 0.9%-18.2%,  $P = 0.03$ ). Among patients who visited an oncologist, black patients are less likely than white patients to receive chemotherapy (54.1% *vs* 70.2%, difference = 16.1%, 95% CI 6.0%-26.2%,  $P = 0.006$ ), radiation therapy (73.7% *vs* 83.4%, difference = 9.7%, 95% CI 0.4%-19.8%,  $P = 0.06$ ), or both (60.6% *vs* 76.9%, difference = 16.3%, 95% CI 4.3%-28.3%,  $P = 0.008$ ). Patient and provider characteristics have minimal influence on the racial disparity in the use of adjuvant therapy<sup>[90]</sup>. The same was shown for stage III colon cancer in elderly patients: consultation with a medical oncologist was equal among black and white patients, but the former were less likely to receive chemotherapy (59.3% *vs* 70.4%, difference = 10.9%, 95% CI 5.1%-16.4%,  $P < 0.001$ ). Disparity was higher among patients aged 66-70 (black patients 65.7%, white patients 86.3%, difference = 20.6%, 95% CI 10.7%-30.4%,  $P < 0.001$ ) which was confirmed by regression analysis and decreased in older patients. Disparity in this age group was partially due to patient, physician, hospital and environmental factors (accounted for 50%), surgical length of stay, neighbourhood socioeconomic factors (27%) and health system factors (12%)<sup>[91]</sup>. Furthermore, 53% of Whites and 56% of Blacks received no radiation therapy for stage II to III rectal cancer (AOR, 1.30; 95% CI, 1.15-1.47) in a study<sup>[83]</sup>. White patients received standard adjuvant therapy more frequently than African-Americans (OR = 1.75; 95% CI 1.09-2.83)<sup>[92]</sup>.

On the other hand, race was not associated with receipt of adjuvant chemotherapy in patients with stage III colon cancer<sup>[93]</sup>. This is confirmed by another study in which after adjustment for socioeconomic status, race was not associated with receiving adjuvant treatment or radiation in stage III colon and stage II, III rectal cancer<sup>[94]</sup>.

#### Racial variation in response to chemotherapy:

**Table 1 Differences in toxicity of the standard treatments for colorectal cancer between whites and AAs**

Regimens	Toxicity
Irinotecan and oxaliplatin	FOLFIRI, FOLFOX and IROX are less toxic to AAs than Whites
Fluoropyrimidines	5-FU is less toxic to AAs than Whites
Cetuximab	Whites are more prone to hypersensitivity reactions

AAs: African Americans.

In at least one study<sup>[95]</sup>, it was shown that Caucasian patients with metastatic colorectal cancer respond better to standard chemotherapy combinations (IFL, FOLFOX or IROX) than African-Americans (response rate for African Americans 30%, for Caucasians 41%,  $P = 0.015$ ). This was shown in multivariate analysis as well (29% response rate for African Americans *vs* 41% for Caucasians,  $P = 0.012$ ). The difference was noted in each treatment arm. However, no association was noted between race and time to progression or overall survival. The same study showed racial variation in pharmacogenomic parameters. Specifically, UGT1A1 6/7 and 7/7 polymorphisms were more common in African Americans, whereas UGT1A1 6/6 was more common in Caucasians ( $P = 0.0081$ ). In addition, ercc2-d A/A and A/B was more common in Caucasians and ercc2-d B/B more common in African Americans ( $P = 0.0002$ ). GSTM1-0 was more frequently absent in Caucasians and more frequently present in African Americans ( $P = 0.001$ ). Finally xrcc1-r399q C/C was more frequent in African Americans than in Caucasians, whereas in the other genotypes the opposite was true ( $P = 0.0006$ ). Moreover, a study showed that there is a higher risk of neutropenia in UGT1A1 7/7 patients especially in patients treated with IROX<sup>[96]</sup>. No association was found between this genotype and overall survival, time to progression, response rate or diarrhoea. Among previously treated patients with metastatic colorectal cancer who received bevacizumab or FOLFOX4 or the combination of these regimens, African Americans differed significantly from Whites in response rate and overall survival, whereas there was no significant difference in progression-free survival (RR: 10.2% *vs* 11.8%,  $P = 0.03$ ; OS: 10.2 mo *vs* 11.2 mo,  $P = 0.03$ ; PFS 4.2 mo *vs* 5.0 mo)<sup>[97]</sup>.

**Disparities in treatment related toxicity between the two ethnic groups:** The data concerning toxicity difference between Caucasians and African Americans are summarized in Tables 1 and 2.

#### Stage and grade at diagnosis

Two studies showed that black patients with colorectal carcinoma are diagnosed at a more advanced stage than their white counterparts<sup>[76,77]</sup>. Blacks are more likely to be diagnosed at a younger age and at a more advanced stage than white patients with rectal cancer<sup>[83]</sup>.

Apart from socioeconomic status, black-white

**Table 2 Differences in toxicity of the standard treatments for colorectal cancer between Whites and African Americans**

Regimens	Toxicity	Difference	Reference
Irinotecan and oxaliplatin	FOLFIRI, FOLFOX and IROX are less toxic to AAs than Whites	34% vs 48%, $P = 0.004$ , for severe toxicity	[95]
Fluoropyrimidines	5-FU regimens in the adjuvant setting differed between AA and Whites, with AA experiencing statistically significantly lower rates	5% vs 17%, $P = 0.004$ for diarrhea Diarrhea ( $P < 0.001$ ) Nausea ( $P < 0.001$ ) Vomiting ( $P = 0.01$ ) Stomatitis ( $P < 0.001$ ) Overall toxicity ( $P = 0.005$ )	[98]
DPD deficiency	AA, particularly AA women, have significantly reduced DPD enzyme activity compared with Whites, which may predispose this population to less 5-FU toxicity	-	[99]
Cetuximab	In a retrospective analysis, it was shown race was strongly associated with HSR to cetuximab among patients with CRC and head and neck cancer, with Whites experiencing HSR more frequently than AA	(Fisher exact) $P = 0.017$	[100]

HSR: Hypersensitivity reactions; AA: African American.

differences in tumour grade among patients with colon cancer were found. Specifically, Blacks appeared less likely to suffer from poorly differentiated tumors-grade 3 (OR = 0.44; 95% CI 0.22-0.88) with lymphoid reaction (OR = 0.49; 95% CI 0.26-0.90) when compared with Whites. This outcome remained statistically significant after adjusting for age, sex, metropolitan area, socioeconomic status, body mass index, and health care access and utilization. A trend without statistical significance was shown towards less high-grade (grade 3) nuclear atypia, mitotic activity, and tubule formation in Blacks compared with Whites. These findings were confirmed in patients with advanced but not with early disease. In addition, no differences were found in blood and lymphatic vessel invasion, mucinous histology, necrosis or fibrosis.

Blacks were more likely to suffer from better differentiated tumors in the proximal, but not in the distal colon. These outcomes suggest that factors other than tumour differentiation contribute to the different survival between Blacks and Whites along with socioeconomic status disparity<sup>[101]</sup>. Another study confirmed that African Americans are more likely to be diagnosed with more advanced and better differentiated tumors than Whites<sup>[43]</sup>.

African-Americans were more likely to present with life-threatening symptoms at the time they were diagnosed with colorectal cancer than Whites. This association was found to be independent of socioeconomic status. In addition, African Americans were more likely to die during their hospitalization when compared to Whites in both overall and high socioeconomic status<sup>[102]</sup>.

#### Quality of life in long term colon cancer survivors

African Americans are more likely to report better quality of life and psychological well being (marginally statistically significant,  $P = 0.07$ ) as long-term colon cancer survivors<sup>[103]</sup>. Among colon cancer survivors, African Americans presented with higher fruit/vegetable consumption than Whites<sup>[104]</sup>. White colorectal cancer survivors are more likely to undergo colon examination surveillance than African Americans 1, 2

and 5 years after diagnosis<sup>[105]</sup>. After adjusting for socio-demographic, hospital and clinical characteristics, Blacks are 25% less likely than Whites to receive surveillance<sup>[106]</sup>. This finding was not statistically significant in another study (relative risk 0.70,  $P = 0.14$ )<sup>[107]</sup>.

## CONCLUSION

African Americans are at increased risk of developing advanced colorectal cancer and are less likely to be up to date with colorectal cancer screening guidelines when compared with Whites. They also tend to be diagnosed with more proximal, more advanced and better differentiated tumors than Whites. In addition, they seem to have a worse prognosis and to receive appropriate treatment to a lesser extent. Socioeconomic factors, educational status, different beliefs and physician recommendation account for most of the disparity. However, little evidence exists concerning genetic differences which would explain the variety in colorectal cancer predisposition, sensitivity to carcinogens, response and toxicity of treatment between Blacks and Whites. In our opinion further research on these issues should be performed. Therefore, continued research efforts are necessary to disentangle the clinical, social, biological, and environmental factors that constitute this racial disparity. In addition, results across data sources should be considered when evaluating racial differences in cancer outcomes.

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REVIEW

## Transition of children with inflammatory bowel disease: Big task, little evidence

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### INTRODUCTION

The relapsing and remitting nature of inflammatory bowel disease (IBD) with its high morbidity makes transition of children with IBD into adult care a mandatory step in their care<sup>[1]</sup>. As young people move to maturity, their medical and psychological needs change and it is crucial for them to receive age-appropriate medical care<sup>[2]</sup>. This highlights the importance of what is called health-care transition. Preparing older children and young adults for this process is very important as they need to develop a sense of independence and maturity<sup>[3]</sup>.

There have been several definitions of the process of transition of medical care but the most quoted one is that by Blum *et al*<sup>[3]</sup> “the purposeful planned movement of adolescents and young adults with chronic physical and medical conditions from child-centered to adult-oriented health care systems”.

The aim of the transition program is to achieve for each chronically ill patient a continuum of care that includes normalization of social and emotional development and acquisition of independent living skills<sup>[5]</sup>.

Several hurdles may interfere with this process. As the patients and their families are used to dealing with certain faces, they may be reluctant to move to an adult health care system. A strong sense of bonding usually develops over years of care under pediatric service. On the other hand, pediatric caregivers may also find it difficult to give up their patients after years of care and strong ties. They may feel that the adult caregivers are unable to provide the same quality of, not only medical, but also psychological care during this critical period<sup>[6]</sup>.

In addition, the adult caregivers may feel that patients and their families with pediatric-onset disease may be too demanding. The adult caregivers may assume that patients should take a large part of responsibility for the details of their care. Adult care normally lacks the multidisciplinary team approach that pediatric service usually offers<sup>[7,8]</sup>.

### WHAT IS THE BEST STRATEGY OF TRANSITIONING CHILDREN WITH IBD?

Most of the available pediatric IBD transition literature is

### Abstract

Children with chronic long-term disorders need to move to the adult practice at some point in their life. Establishing a smooth and efficient transition process is a complicated task. Transition of medical care to adult practice is defined as the purposeful planned movement of adolescents and young adults with chronic physical and medical conditions from child-centered to adult-oriented health care systems. This step is of the utmost importance for several reasons. There is an obvious deficiency of research in this area especially when it comes to pediatric inflammatory bowel disease (IBD). There is a considerable difference in individual practice among different centers. Also, age of transition varies among different countries and sometimes, even within the same country, transition age may vary among different provinces and districts! Interestingly, local politics and many factors other than children's welfare often play a role in deciding the age that older children move to adult practice at. This review discusses transition of children with IBD in view of the available evidence.

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**Table 1** Checklist of tasks for the patients and the medical team based on chronological age as suggested by Hait *et al*<sup>[8]</sup>

Age	Patient	Medical team
11-13	Able to articulate his or her GI condition Able to name medications, doses, side effects Knows strategies to take his or her medications Able to use and read a thermometer Articulates impact of IBD on school and daily life	Introduces idea of future independent visits Asks parents to remain in waiting room for a portion of the visit Anticipatory guidance about fitness, sexuality, and substance abuse
14-16	Able to identify medical team Knows names and purposes of procedures and tests done on him or her Knows his or her medical history Knows names of IBD social support groups and community organizations Understands the medical risk of no adherence Understands the impact of drugs and alcohol on the illness	Directs all questions and explanations to patient Patient ask for input first Explores family's apprehensions about patient taking in primary role Clarifies to patient what must legally be disclosed to parents
17-19	Knows how to gather information about IBD Demonstrates consistent ability to book own appointments, fill prescriptions, contact medical team Able to name his or her insurance coverage and plans for next 2 years of coverage Carries insurance information in wallet/purse/backpack	Initiates conversation about potential barriers to transition Identifies potential adult GI providers Encourages patient to meet and interview providers Reminds patient and family that at age 18 the patient has the right to make his or her own health choices
20-23	Has had a telephone conversation with potential adult GI providers Initial visit with adult GI is scheduled while IBD stable	Provides patient with medical summary and checklist (see Table 2) Transfers medical records to adult provider

IBD: Inflammatory bowel disease; GI: Gastrointestinal.

in the form of some guidelines based on personal experience. The North American Society of Pediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN) indicates its recommendations for this process as<sup>[6]</sup>: (1) The pediatric gastroenterologist should start seeing adolescent patients without their parents in order to build some sort of relationship that promotes independence, so the patient will have this well-established before going to adult practice. Nevertheless, it is not clear at what age pediatric gastroenterologists should start implementing this recommendation. (2) Introduction of the patients subject to transition, and their families, to a gastroenterologist who is trained in internal medicine. Emphasis placed on benefits of transition to the patients and their families, including exposure to a physician experienced in aspects that pediatricians are not exposed to such as pregnancy, fertility and cancer surveillance. (3) Selection of an adult gastroenterologist who cares for young adults and identifies what this sector of patients may need that differs from newly diagnosed young adults. (4) The transition process should include a detailed letter from the pediatric to the adult gastroenterologist with a copy to the family. (5) The timing of the transition should be flexible according to patients' individual needs, e.g. the pediatric gastroenterologist should continue to follow those patients with delayed puberty who still have some potential to grow.

In a recent report, Hait *et al*<sup>[8]</sup> recommended some tasks and knowledge that pediatric patients at different age groups should be able to accomplish and be familiar with through the help of the medical team (Table 1).

This work has not been validated.

In a similar report, Pinzon *et al*<sup>[7]</sup> also recommended a certain amount of information pediatric patients should know in certain age groups (Table 2).

Dabadie *et al*<sup>[9]</sup> have recently conducted a survey on a small sample of young adults with IBD. Their mean age of transition was  $17.9 \pm 0.9$  years. Eighty five percent of patients and 74% of their parents felt they were ready to move to adult practice. The transition model they were using involved having one joint pediatric-adult care visit before moving completely to adult care. The majority of patients felt comfortable with this model<sup>[9]</sup>.

Many other centers are using the same model. In fact some hospitals may have more than one visit per patient to the joint clinics before transitioning children to adult practice. Most of these clinics take place in hospitals where both adult and pediatric gastroenterology departments exist. The problem with this system is the diversity of patients' location especially in large countries. As the number of adult gastroenterology centers exceeds that of pediatric centers, some patients may choose to move to an adult gastroenterologist near where they live, in which case they would not see the same gastroenterologist from the transition clinic. Available health resources may interfere with arranging transition clinics where patients reside.

Another survey by Hait *et al*<sup>[10]</sup> was done among 1132 adult gastroenterologists in the Netherlands. The response rate was only 34% but the majority of these gastroenterologists reported a lack of medical knowledge among young adults with IBD. They also reported receiving inadequate

**Table 2 Clinical transition framework<sup>[9]</sup>**

	<b>Early (10 to 12 years of age )</b>	<b>Middle (13 to 15 years of age)</b>	<b>Late (16 to 18 years of age )</b>
Self-advocacy	Describe GI condition	Name and describe the role of those involved in care	Review successful changes achieved in life
Independent behaviors	Learn about transition process Name medications and doses Choose method to remember to take medication	Learn about rights and responsibilities Describe tests and reasons for them Plan and prepare for appointments Discuss differences in pediatric and adult care Encourage self-reporting	Review GI conditions and ways to stay informed Demonstrate knowledge in dealing with own care needs (e.g. booking appointments, refilling prescriptions)
Sexual health	Discuss puberty and changes related to GI condition and medication side effects	Discuss safer relationships and dating Name places for reliable sexual health information	Aware of sexual capability and ability to have children Understands sexual behaviors and its risks
Social supports	Describe role of family in transition process	Discuss family medical history Discuss ways to benefit from peer support	Enrolls in GI support associations Identifies contacts in case of need of emotional support
Educational/vocational planning	Discuss school attendance, goals and strengths	Review restrictions for education or work Discuss volunteering opportunities at school or community	Aware of work opportunities Discuss further plans for insurance coverage
Health and lifestyles	Review healthy active living Tobacco use or other substances	Review ways of coping with stress Learn of risks associated with driving and GI condition	Describes exercise/activity routines Discuss importance of planning ahead for trips and being away from home

Adapted from the ON-TRAC abo model at the British Columbia Children's and women' Health Center.

medical history from their pediatric colleagues.

We recently started conducting a survey looking at the level of disease-related knowledge among older children and young adults with IBD. We asked patients and their parents to complete the same questionnaire forms independently. The preliminary results showed a significant lack of knowledge among those children and a significant knowledge deficit between children and their parents. This was the case despite the presence of a meticulous program for educating newly diagnosed children with IBD. The problem is that patients in this age group are often highly dependant on their parents on selecting and filtering all the information they need. Consequently we started seeing older patients with IBD on their own without their parents early before transitioning them to our adult colleagues. We are also evaluating a new strategy for patients' education to see if the outcome can be improved.

## LESSONS FROM OTHER SUBSPECIALTIES

Taking in consideration the huge advances in patient care and management, children with chronic disease are expected to live longer. Consequently, the area of transition has been explored in most other pediatric subspecialties.

In a recent multicentre audit from the UK, Robertson *et al*<sup>[11]</sup> reported some improvements in their transition program after highlighting the aspects of care provisions in transition through a national UK service framework of children. Improvements took place in the documentation of transition issues and in some educational needs of patients and their parents that were related to their disease. The concept of independent clinic visits was introduced at an earlier age (mean of 15.8 years vs 16.8 years before provision).

A service model for adults with congenital heart disease was recently published stressing the importance of close communication between local district general hospitals, tertiary specialist centers and primary care clinics. The authors suggested that transition clinics should start at the age of 12 years<sup>[12]</sup>.

McDonagh and Kelly summarized determinants of the timing of transition as follows<sup>[13]</sup>: (1) Chronologic age; (2) Maturity; (3) Current medical status; (4) Adherence to therapy; (5) Independence in health care; (6) Self-advocacy skills; (7) Preparation; (8) Readiness of the young person and (9) Availability of an appropriate adult specialist.

Clearly it is not an easy task for pediatric gastroenterologists to determine the exact maturity status for each child with IBD. Moreover, even when we feel that some children are not mature enough to move to adult service at the age of 16-17 years; it is not easy to convince health authorities to keep seeing these children in pediatric services. In fact, the differences between transition services in different health care systems were proven to inhibit a smooth and successful transition process in a recent study by Reiss *et al*<sup>[14]</sup>.

There have been some attempts to create a self-efficacy/independence scale to facilitate assessing older children with chronic disease for maturity. Schlosser and Havermans created and validated a self-efficacy scale for children with bronchial asthma between 10-18 years of age<sup>[15]</sup>. The scale consisted of 38 items partly taken from a self-efficacy scale by Grossman *et al*<sup>[16]</sup> for diabetics. Older children scored higher on the total scale. One major flaw in this study was the lack of adjustment for obvious confounders like social class or IQ. These points were partially taken into consideration in another independence-scale that was designed for children with cystic fibrosis (SCIS). The scale consisted of 44 items. An initial interview was done to screen for children with an average or high cog-

nitive function<sup>[17]</sup>. The study was conducted among CF patients aged from 14-17 years. Based on age, two different intellectual screening tools were used. There was a positive correlation between SCIS scores, patient's age and the number of years since diagnosis<sup>[17]</sup>. A similar study with a larger sample size was conducted among patients with diabetes not only for children but also for parents assessing independence of their children<sup>[18]</sup>. Approximately 25% of parents of children 6 years-old, 50% of parents of children 8 years-old and 75% of parents of 14 year-old children reported that their children had mastered their disease-related skills<sup>[18]</sup>.

Another tool was developed by the Boston Children's Hospital, USA to allow older children to evaluate their readiness for transition. This tool consisted of 15 questions<sup>[19]</sup>. Validation of this tool remains questionable.

Two case studies were recently published demonstrating the concept of "a medical home"<sup>[20]</sup>. This concept was highlighted in a statement by the American Academy of Pediatrics. It was stated that all children and youth with special health care needs should have a medical home where health care services are accessible, family centered, continuous, comprehensive, coordinated and compassionate<sup>[21]</sup>. The authors tried to set a model for transitioning older children with chronic, disabling medical problems using the above concept.

Currently there is a residency program in the United States (combined Medicine-Pediatrics Residency Program) that helps in qualifying physicians to look after children with chronic disorders during this critical period<sup>[22]</sup>. Training includes pediatric and adult subspecialty experience. Spreading this program to other countries may help in smoothing the transition process.

## CONCLUSION

Transitioning adolescents with IBD to adult gastroenterologists requires an understanding of the specific issues and challenges involved in the management of pediatric IBD<sup>[23]</sup>. Currently there is no consistency in transition practice among different centers.

The best strategy for health education or patients' transition is yet to be determined. Assessing children with IBD for maturity could be a challenge in view of the lack of validated scales. Current guidelines and suggestions are based on personal opinions. More research addressing best strategy for education, transition and building bridges with adult gastroenterologists are needed.

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ORIGINAL ARTICLES

# Characterization of focal liver lesions with SonoVue®-enhanced sonography: International multicenter-study in comparison to CT and MRI

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one focal liver lesion detected in baseline ultrasound (US) were examined with conventional US, contrast-enhanced US ( $n = 134$ ), contrast-enhanced CT ( $n = 115$ ) and/or dynamic contrast-enhanced MRI ( $n = 70$ ). The lesions were classified as malignant, benign or indeterminate and the type of lesion was determined. The final diagnosis based on the combined information of all imaging examinations, clinical information and histology ( $n = 32$ ) was used. Comparisons were made to see whether the addition of contrast-enhanced US led to the improvement of the characterization of doubtful focal liver lesions.

**RESULTS:** In comparison with unenhanced US, SonoVue® markedly improves sensitivity and specificity for the characterization (malignant/benign) of focal liver lesions. In comparison with CT and/or dynamic MRI, SonoVue®-enhanced sonography applied for characterization of focal liver lesions was 30.2% more sensitive in the recognition of malignancy and 16.1% more specific in the exclusion of malignancy and overall 22.9% more accurate. In the subgroup with confirmative histology available ( $n = 30$ ), sensitivity was 95.5% (CEUS), 72.2% (CT) and 81.8% (MRI), and specificity was 75.0% (CEUS), 37.5% (CT) and 42.9% (MRI). The sensitivity and specificity of CEUS for the identification of focal nodular hyperplasia (FNH) and hemangiomas was 100% and 87%, resulting in an accuracy of 94.5%.

**CONCLUSION:** SonoVue®-enhanced sonography emerges as the most sensitive, most specific and thus most accurate imaging modality for the characterization of focal liver lesions.

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**Key words:** Contrast-enhanced sonography; Contrast media; Dynamic sonography; Liver lesion; Liver disease; Liver; Oncologic imaging; Sonography

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Trillaud H, Bruel JM, Valette PJ, Vilgrain V, Schmutz G, Oyen R, Jakubowski W, Danes J, Valek V, Greis C. Characterization

## Abstract

**AIM:** To evaluate in a multicenter study whether the sonographic characterization of focal liver lesions can be improved using SonoVue®-enhancement; and to compare this method with computed tomography (CT) and magnetic resonance imaging (MRI).

**METHODS:** One hundred and thirty four patients with

of focal liver lesions with SonoVue®-enhanced sonography: International multicenter-study in comparison to CT and MRI. *World J Gastroenterol* 2009; 15(30): 3748-3756 Available from: URL: <http://www.wjgnet.com/1007-9327/15/3748.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.3748>

## INTRODUCTION

Whereas un-enhanced ultrasound and color Doppler ultrasonographic examination are widely used to screen for liver lesions, these techniques have limited performance in the characterization of solid focal tumors<sup>[1-3]</sup>. Thus contrast enhanced computed tomography (CT) or magnetic resonance imaging (MRI) is required to assess the malignancy of the tumors as basis for therapy decisions. The characterization of lesions with contrast enhanced CT or MRI is based on the vascularity and enhancement pattern within the lesion<sup>[4-7]</sup>. SonoVue® is a microbubble contrast agent of the 2nd generation, allowing the assessment of vascularity and enhancement pattern of focal lesions with ultrasound in real-time, using low mechanical index scanning technology<sup>[8-24]</sup>. Low-mechanical index (MI) real-time ultrasound in combination with SonoVue® allows the continuous assessment of tumor vascularity and enhancement during the different vascular phases (arterial, portal and late phase) with better temporal resolution than with CT or MRI<sup>[18-19]</sup>. Safety and effectiveness of this agent have been proved in numerous clinical studies. In this clinical study, we compared the diagnostic performance of this technique in a multicenter study using different systems with conventional unenhanced ultrasound as well as with contrast-enhanced CT and MRI, being the current state-of-the-art methods for characterization of focal liver lesions. Contrast specific US scanning modes, equipments, manufacturers, transducers and range of low mechanical index are extremely varied in clinical practice and validation of SonoVue®-enhanced sonography in a multicenter and multi-equipments study is an essential requirement before introducing this method in routine clinical practice.

Our purpose was to evaluate in a controlled multicenter study whether the characterization of focal liver lesions can be improved by using SonoVue®-enhanced sonography, as compared with unenhanced sonography, contrast-enhanced CT and contrast-enhanced MRI.

## MATERIALS AND METHODS

### Study population

The study population consisted of 179 patients [85 men and 94 women, mean age 55.3 years (range 19-93)] with one single focal liver lesion, detected with unenhanced sonography (screening). The lesion had to be clearly identifiable in the different modalities (US, CT, MRI) for matching of results. Exclusion criteria were age < 18 years, pregnant or lactating women, acoustic window insufficient for adequate sonographic examination of the liver, a contra-indication to SonoVue® or any of the

diagnostic examinations, and/or inability to give informed consent.

This study was a phase IIIb, multicenter, multinational, open label within patient comparison in 9 centers (5 in France, 2 in Czech Republic, 1 in Belgium and 1 in Poland), performed between December 2003 and February 2005. The study was performed according to Good Clinical Practice (GCP) and the ethical principles of the declaration of Helsinki, in particular approval by the responsible Ethical Committees was obtained and all patients gave written informed consent. Due to the fact that a new imaging modality (dynamic low-MI real time sonography) was used, a technical run-in phase was performed to allow establishment of adequate machine settings with 45 patients (5 in each center). The following 134 patients were part of the main phase and constituted the intent-to-treat population. Of these 134 patients 7 patients were excluded due to the following reasons: missing reference examination (CT and MRI) in 3 patients, reference examination outside of acceptable time window ( $\pm$  3 mo) in 2 patients, ultrasound examination not possible due to technical reasons in 1 patient and final reference diagnosis not supported by valid reference examinations in 1 patient. Therefore, the protocol-correct population used for efficacy analysis consisted of 127 patients [54 men and 73 women, mean age  $54.8 \pm 19.9$  years (range 19-93), mean height  $168.7 \pm 8.5$  cm (range 154-190), mean weight  $69.7 \pm 13.7$  kg (range 43-106)].

### Imaging procedures

All imaging examinations were performed by experienced radiologists. For the contrast-enhanced ultrasound examinations a technical training was performed, including an educational lecture, local setup of contrast-specific software and standard settings in cooperation with contrast ultrasound application specialists and examination of 5 run-in patients under technical supervision. Different US systems were used (Table 1). The sequence of the different imaging procedures (i.e. US, CT and MRI) was not fixed by the study protocol and could be arranged to the organizational situations in the different study center. All examinations should have been performed within 14 d, however in individual cases a time range of up to 3 mo was accepted if there was no treatment or indication of significant changes in between, to avoid repeated radiation exposure.

### Baseline ultrasound

First, an unenhanced sonography was performed to verify the presence of one single focal liver lesion with grey-scale imaging (B-mode). Each focal lesion was measured, localized (Couinaud segments) and the characteristic features (border, shape, echogenicity) were described. The vascularisation was assessed by color Doppler ultrasound. Representative sequences of the examination were stored digitally.

### Contrast-enhanced ultrasound (CEUS)

Each patient received at least 2 bolus injections of

**Table 1** US equipment and contrast-specific modes

Equipment manufacturer	Scanning mode	No. of patients scanned	Transducer	Mechanical index
Elegra, Siemens	Ensemble contrast imaging (ECI)	25	Convex array 3.5C40H	0.10-0.30
Aplio, Toshiba	Contrast tissue discrimination (CTD)	24	Convex array PVT375AT	0.1
HDI 5000, Philips	Pulse inversion harmonic contrast imaging (PI)	18	Convex array C5-2	0.04-0.12
Technos MPX, Esaote	Contrast tuned imaging (CnTI)	18	Convex array CA430	0.09-0.10
Logic 9, GE	Coded phase inversion (PI)	16	Convex array 3.5C	0.09-0.17
SSD-5500, Aloka	Extended pure harmonic detection (E-PHD)	16	Convex array UST 9126	0.09-0.20
Sequoia, Siemens	Cadence contrast pulse sequencing (CPS)	7	Convex array 4C1-S	0.15-0.24
IU 22, Philips	Pulse inversion (PI)	3	Convex array C5-2	0.06-0.07

SonoVue®, a first one for characterization of the detected focal lesion and another one for detection of additional lesions. Contrast-enhanced sequences were obtained using dedicated low-MI contrast-imaging software (MI < 0.2). Standard pre-settings were used, with the possibility to adjust settings to the individual patient. SonoVue® was injected intravenously as a bolus of 2.4 mL, using a 20 gauge cannula placed in the antecubital vein, followed by a flush of 5 mL normal saline solution. Digital cine clips representing the dynamic contrast enhancement within the lesion and the surrounding liver tissue were recorded continuously, starting 5 s before the SonoVue® injection and covering the arterial (i.e. 10-45 s inject.), portal (i.e. 60-90 s inject.) and late (i.e. 120-150 s inject.) phase. In case of a suspected hemangioma, additional 15 s scans were performed after 240 s and 300 s. The injection could be repeated in case of technically insufficient quality, using the same dose (2.4 mL) or double dose (4.8 mL), up to a total dose of 9.6 mL SonoVue®. All sequences were recorded and stored digitally. Intratumoral vascular geometry and enhancement pattern of the lesions were described.

#### Analysis of malignancy and lesion type

The lesion was classified separately for unenhanced and SonoVue®-enhanced sonography as benign, indeterminate or malignant. In addition, the particular type of lesion was determined according to pre-defined criteria (Table 2).

With unenhanced sonography the classification was based on echogenicity, morphological criteria, and color Doppler signals reflecting abnormal vascularity, using the following criteria: lesion size, lesion depth, border definition (clear or blurred), shape (round, oval or irregular) and echogenicity (hypoechoic, isoechoic or hyperechoic).

With contrast-enhanced sonography, the classification was based on the dynamic enhancement pattern reflecting vascularity and perfusion pattern of the lesion, using the following criteria: vascularity (no intratumoral vessel, straight feeding vessels, irregular feeding vessels, radial intratumoral vessels/spoke and wheel sign or basket-like

vessels around the lesion), enhancement pattern in arterial and portal-venous phase (hypoenhancing, isoenhancing or hyperenhancing) and homogeneity of enhancement (homogeneous or non-homogeneous). The characteristic enhancement pattern used for classification were in accordance with pattern described earlier for CT, MRI and contrast-enhanced US<sup>[17]</sup>.

#### CT and MRI examination

All patients received at least one triple phase contrast-enhanced reference examination with CT or MRI. Single-slice CT was available in 37/127 patients, multi-slice CT in 78/127 patients and dynamic MRI in 70/127 patients. In all (except one) patients having just a single-slice CT, an additional dynamic MRI examination was performed to be sure to have a proper representation of the different enhancement phases in the reference examination. In the one patient with just a single slice CT without additional MRI, all imaging examinations resulted in the same diagnosis (HCC), which was confirmed by histology, so that the reference examination was considered to be acceptable. In patients having a CT and MRI examination available, a combined tomographic diagnosis (consensus) was made.

#### Histology

In 32 patients, a histological examination of the lesion was available. Samples for histology could be obtained by biopsy or surgical resection, according to the clinical situation and requirements. Due to ethical reasons, no tissue samples were taken for study reasons only without clinical indication<sup>[25]</sup>. From 32 histology specimen 2 were classified as indeterminate, so that in 30 patients a histological diagnosis could be obtained.

#### Final reference-standard diagnosis

After all examinations were completed, a final diagnostic assessment was performed, taking into consideration all-available imaging examinations and clinical information (i.e. US, CT, MRI, clinical data, biochemical markers and histology, if available) of the respective patient. This was considered to reflect the true-disease-state of the

**Table 2 Classification and diagnostic criteria for the assessment of focal liver lesions**

<b>Lesion type</b>	<b>Arterial phase</b>	<b>Portal venous phase</b>	<b>Sinusoidal phase</b>
Malignant lesions			
Hepatocellular carcinoma (HCC)	Hyperenhanced, often with prominent delineation of feeding vessels around and inside of the lesion, however in well differentiated tumors sometimes only weak arterial enhancement	Iso- or hypoenhanced, usually rapid contrast wash-out	Mostly hypoenhanced, however in well differentiated tumors some portal venous enhancement may be present
Cholangiocarcinoma (CCC)	Moderately hyperenhanced	Iso- or hypoenhanced	Mostly hypoenhanced
Hypervascular metastases (MET)	Hyperenhanced, often restricted to the margin of the lesion	Iso- or hypoenhanced	Always hypoenhanced (black spots)
Hypo-vascular metastases (MET)	Not enhanced or only few isolated spots inside of the lesion	Hypoenhanced	Always hypoenhanced (black spots)
Other malignant			
Benign lesions			
Hemangioma (typical)	Peripheral nodular enhancement, sharp margin in high-flow hemangiomas: complete filling of the lesion during arterial phase	Slow centripetal progression of the enhancement ('iris diaphragm sign'), leading to an iso- or hyperenhanced appearance; fill-in can be very slowly (lasting minutes) or rather fast (lasting less than a minute)	More or less complete enhancement, prolonged compared to surrounding liver tissue and therefore hyperenhanced at later time points; enhancement may be incomplete in case of (partial) thrombosis
Hemangioma (atypical)	Peripheral nodular enhancement, sharp margin or no enhancement (complete thrombosis) or complete enhancement (high flow hemangioma)	Slow centripetal progression of the enhancement ('iris diaphragm sign'), leading to an iso- or hyperenhanced appearance; fill-in can be very slowly (lasting minutes) or rather fast (lasting less than a minute)	More or less complete enhancement, prolonged compared to surrounding liver tissue and therefore hyperenhanced at later time points; enhancement may be incomplete in case of (partial) thrombosis
Focal nodular hyperplasia (FNH)	Hyperenhanced, with fast centrifugal filling of the lesion; usually a central vessel and radial vascular branches can be delineated, especially in larger lesions ('spoke and wheel sign')	Iso- or hyperenhanced	Iso- or hyperenhanced, central scar may become visible
Hepatic adenoma	Hyperenhanced, frequently with fast centrifugal filling and rapid contrast wash-out; no radial vascular structures visible	Iso- or hyperenhanced; hypoenhanced areas in case of central bleeding or scar	Iso- or hyperenhanced; hypoenhanced areas in case of central bleeding or scar; no central scar or radial intralesional structures
Large regenerating or dysplastic nodules	Isoenhanced	Isoenhanced	Isoenhanced
Focal fatty accumulation	Isoenhanced	Isoenhanced	Isoenhanced
Focal fatty sparing	Isoenhanced	Isoenhanced	Isoenhanced
Cyst	No enhancement	No enhancement	No enhancement
Other benign			

patient and used as gold standard. For the final reference diagnosis, malignancy and lesion type was determined using the same classification as described above.

### Statistical analysis

All continuous variables were presented with its summary statistics (*n*, mean, standard deviation and range). Categorical data were given by frequency distribution tables.

Sensitivity, specificity and accuracy for the assessment of malignancy were calculated for unenhanced ultrasound, SonoVue®-enhanced ultrasound and combined CT/MRI, using the final reference diagnosis as gold standard. Indeterminate classifications were rated as incorrect classifications. Patients for whom the final reference diagnosis was indeterminate (*n* = 4) were excluded from these analyses. Differences between unenhanced ultrasound, SonoVue®-enhanced ultrasound and combined CT/MRI were analysed by using McNemar test two-sided test. *P* < 0.05 was considered as statistically significant.

## RESULTS

Complete data for unenhanced and SonoVue®-enhanced ultrasound were obtained in 127 patients. Four patients were excluded because no decisive final reference diagnosis could be obtained (indeterminate character of the lesion), so that in 123 patients the accuracy of the ultrasound examinations *versus* the final reference diagnosis could be calculated.

According to the final reference diagnosis, 68 focal liver lesions were benign and 55 were malignant. The detailed lesion characters of target lesions are presented in Table 3. The number of correctly classified lesions was significantly higher with SonoVue®-enhanced ultrasound compared to unenhanced ultrasound (benign lesions: 60/68 vs 25/68, malignant lesions: 54/55 vs 22/55). This results in a sensitivity, specificity and accuracy of 98.2%, 88.2% and 92.7% for CEUS compared to 40.0%, 36.8% and 38.2% for unenhanced ultrasound, respectively. In comparison unenhanced US

**Table 3** Lesion character of target lesion: SonoVue®-enhanced sonography vs other imaging modalities and final reference diagnosis n (%)

	Unenhanced sonography	SonoVue®-enhanced sonography	CT and/or MRI	Histology	Final reference diagnosis
n	127	127	115	31	127
Benign	25 (19.7)	61 (48.0)	41 (35.7)	8 (25.8)	68 (53.5)
Hemangioma (typical)	10 (40.0)	11 (18.0)	12 (29.3)	1 (12.5)	10 (14.7)
Hemangioma (atypical)	5 (20.0)	16 (26.2)	12 (29.3)	1 (12.5)	19 (27.9)
Focal nodular hyperplasia	6 (24.0)	20 (32.8)	9 (22.0)	3 (37.5)	24 (35.3)
Hepatic adenoma	0	2 (3.3)	2 (4.9)	2 (25.0)	3 (4.4)
Regenerating or dysplastic nodules	1 (4.0)	2 (3.3)	1 (2.4)	0	2 (2.9)
Focal fatty accumulation	0	2 (3.3)	1 (2.4)	0	2 (2.9)
Focal fatty sparing	2 (8.0)	3 (5.0)	1 (2.4)	0	3 (4.4)
Cyst	0	0	0	0	0
Other benign	1 (4.0)	5 (8.2)	3 (7.3)	1 (12.5)	5 (7.4)
Malignant	25 (19.7)	57 (44.9)	38 (33.0)	21 (67.7)	55 (43.3)
Hepatocellular carcinoma	14 (56.0)	34 (59.7)	21 (55.3)	13 (61.9)	33 (60.0)
Cholangiocarcinoma	0	0	0	1 (4.8)	1 (1.8)
Hypervascular metastasis	2 (8.0)	9 (15.8)	4 (10.5)	2 (9.5)	7 (12.7)
Hypovascular metastasis	9 (36.0)	10 (17.5)	10 (26.3)	3 (14.3)	10 (18.2)
Other malignant lesion	0	4 (7.0)	3 (7.9)	2 (9.5)	4 (7.3)
Indeterminate	77 (60.6)	9 (7.1)	34 (29.6)	2 (6.5)	4 (3.2)

CT: Computed tomography; MRI: Magnetic resonance imaging.

was significantly ( $P < 0.0001$ ) less sensitive, specific and accurate than SonoVue®-enhanced ultrasound.

In 2 patients, no CT/MRI evaluation of the target lesion could be obtained. Therefore, a comparison of the accuracy of SonoVue®-enhanced ultrasound and combined CT/MRI examination could be performed in 121 patients.

Correct classifications of benign lesions could be obtained with CEUS in 59/67 lesions and with CT/MRI in 50/67 lesions. Correct classifications of malignant lesions could be obtained with CEUS in 53/54 lesions and with CT/MRI 37/54 lesions. This results in a sensitivity, specificity and accuracy of 98.2%, 88.1% and 92.6% for CEUS compared to 68.5%, 74.6% and 71.9% for CT/MRI. In comparison CT/MRI was significantly less sensitive ( $P < 0.0001$ ), less specific ( $P < 0.029$ ) and less accurate ( $P < 0.0001$ ) than SonoVue®-enhanced ultrasound. In the subgroup of patients with histological diagnosis as part of the final reference standard ( $n = 30$ ) the excellent performance of SonoVue®-enhanced ultrasound could be confirmed. Sensitivity, specificity and accuracy of the 3 modalities were 95.5%, 75.0% and 90.0% for CEUS, 72.7%, 37.5% and 63.3% for CT and 81.8%, 42.9% and 66.6% for MRI, respectively.

Looking on the false positive classifications (malignant or indeterminate instead of benign) of the different methods CEUS was indeterminate in 5 patients and wrongly classified two FNH (as HCC and hypervascular metastasis) and one adenoma (as hypovascular metastasis). CT was indeterminate in 17 patients and missed the lesion in 2 patients. MRI was indeterminate in 11 patients. With regard to false negative findings (benign or indeterminate instead of malignant), CEUS was indeterminate in 1 patient with an HCC. CT was indeterminate in 17 patients and misdiagnosed 1 hypovascular metastasis as hemangioma. MRI was indeterminate in 9 patients, missed one hypervascular metastasis and misdiagnosed one

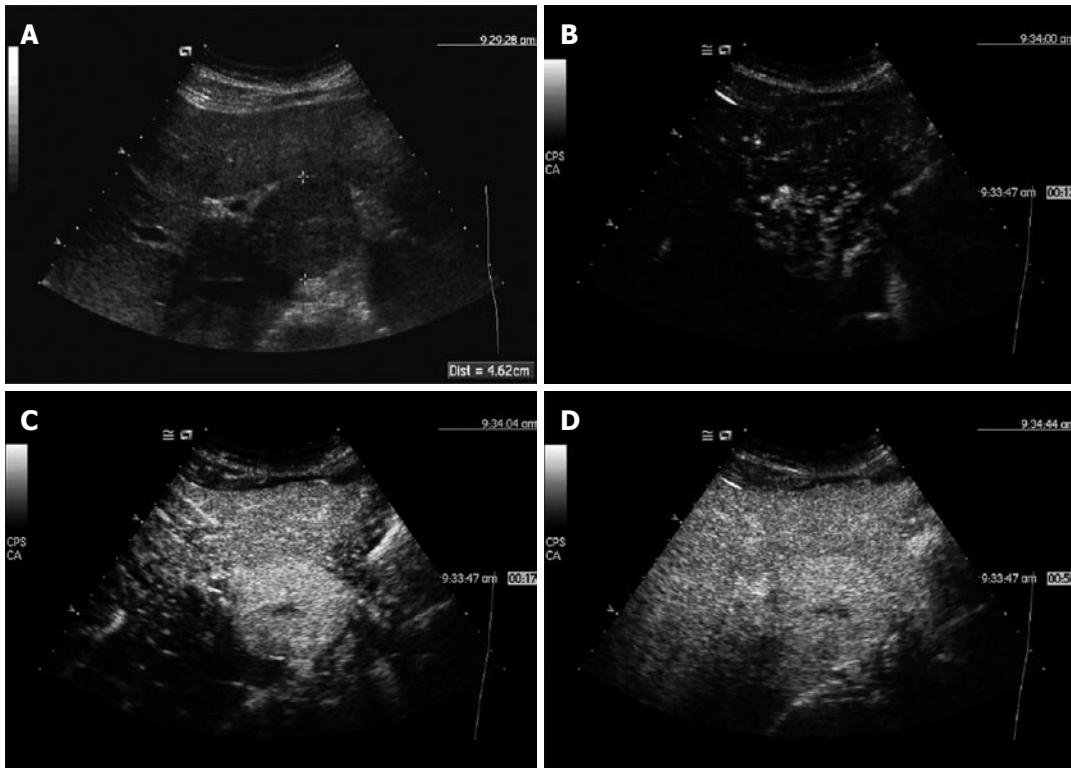
hypovascular metastasis as hemangioma.

Safety assessment was performed in all patients receiving SonoVue® ( $n = 179$ ). Four adverse events were reported, 3 with mild and 1 with moderate intensity. Three of these adverse events were local reactions at the injection site (2 pain and 1 pruritic rash), from which 2 (1 pain and 1 rash; 1.1% of the patients population) were considered to be possibly related to the contrast agent. One adverse event was systemic (nausea), but this was considered related to a recent cholecystectomy and not to the study agent. The related adverse events were of mild intensity and both patients recovered spontaneously without sequelae.

## DISCUSSION

Characterization of a focal liver lesion requires the assessment of morphological characteristics as well as vascularity and enhancement patterns within the lesion. Therefore, the administration of a contrast agent, demonstrating the intratumoral vascularity and blood flow, gives essential information for the characterization of focal liver lesions. In CT and MRI, the acquisition of contrast-enhanced images in different phases is a well-established standard procedure<sup>[4-6]</sup>. In ultrasound, the standard approach for focal lesion characterization includes only the use of color-coded Doppler imaging, which is not able to demonstrate microvascular flow and dynamic enhancement patterns<sup>[1-3]</sup>. With the introduction of second generation ultrasound contrast agents like SonoVue®, in combination with appropriate scanner technology (low-MI real-time contrast imaging), the assessment of intratumoral vascularity and dynamic enhancement pattern became possible, comparable to the information obtained by CT and dynamic MRI<sup>[17,26-28]</sup>.

Contrast-enhanced imaging with ultrasound has two major advantages: (1) the microbubble contrast agents



**Figure 1 45-year-old female patient.** A: 4.6 cm isoechoic lesion in segment 1 found in B-Mode sonography; B: Contrast-enhanced sonography with SonoVue® showed an early arterial enhancement with radial intratumoral vessels seen 13 s after injection; C: Strong enhancement of the whole lesion after 17 s, with a central scar becoming visible; D: In the portal phase, the lesion becomes isoenhancing with the surrounding normal liver tissue. The enhancement pattern is typical for a focal nodular hyperplasia (FNH).

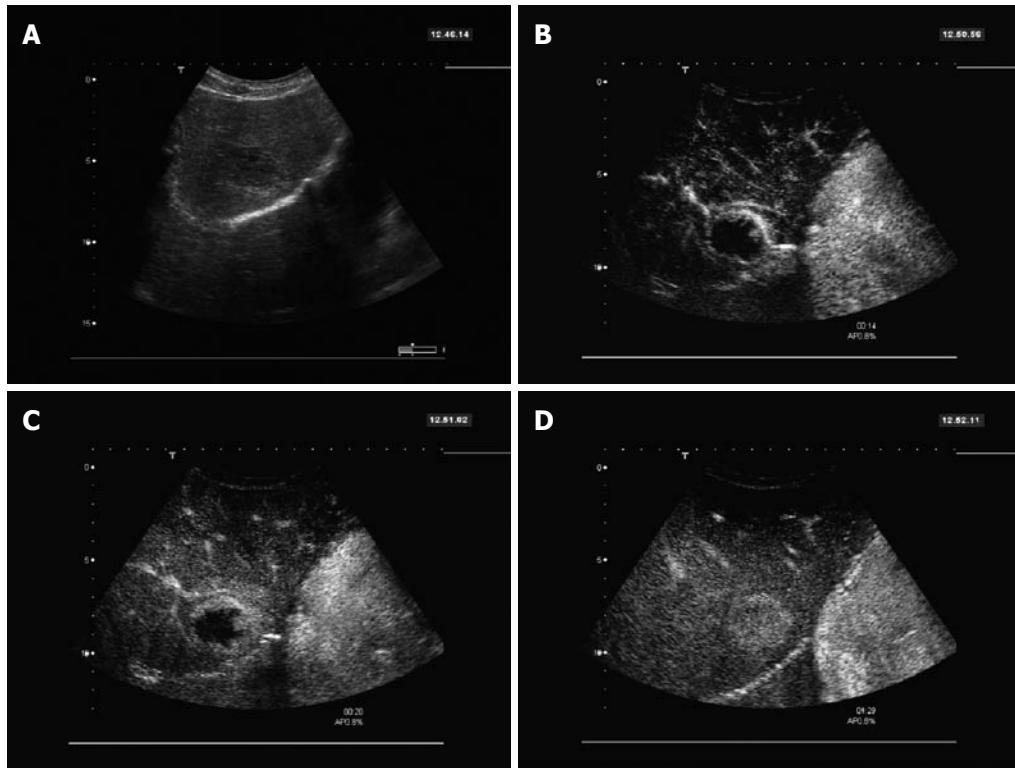
are real blood-pool agents not leaving the intravascular space and (2) a continuous imaging over the whole enhancement period with high temporal resolution is possible, not limited to distinct, pre-defined time points<sup>[18,19]</sup>. Furthermore contrast-enhanced ultrasound has many other advantages, such no exposure to radiation, and absence of nephrotoxic contrast agents and large availability of machines.

There are already several studies published, demonstrating the safety and efficacy of this method for the diagnosis of focal liver lesions, however most of these studies were single-center studies performed with just one type of machine<sup>[9,10,26]</sup>. This clinical study investigated the diagnostic performance of SonoVue®-enhanced ultrasound in the characterization of focal liver lesion in an international multicenter and multi-equipment setting in comparison to established CT and MRI examinations, using a final reference diagnosis based on all available imaging, histology and clinical information as gold standard.

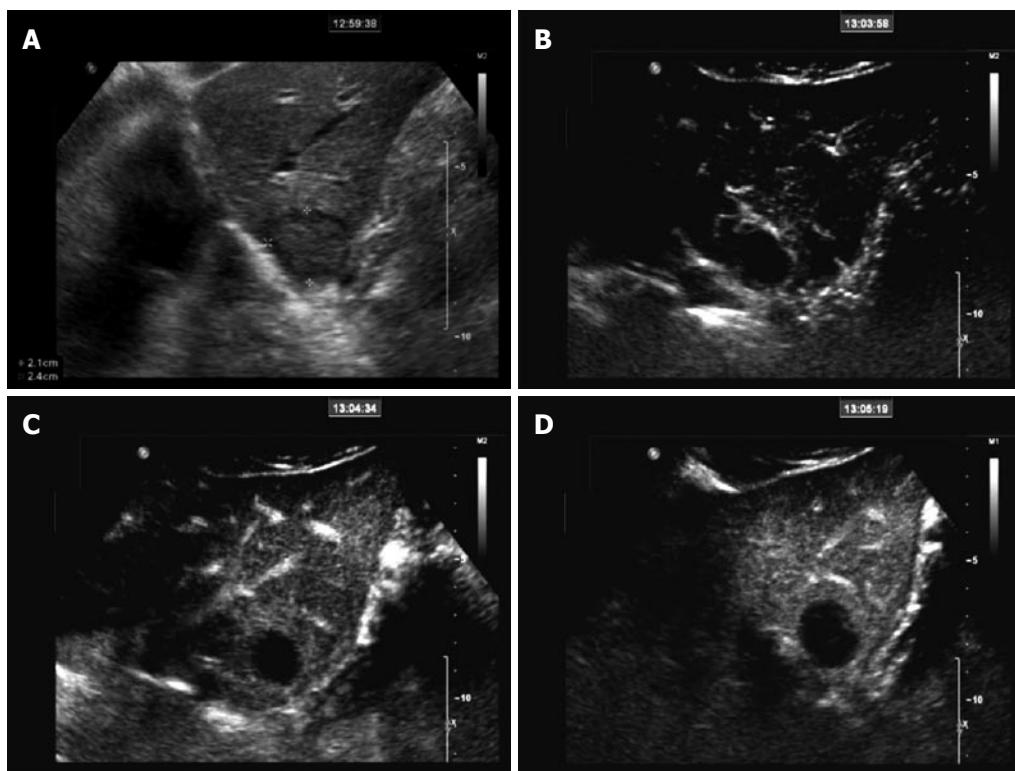
The results of this study confirm the excellent performance of real-time contrast-enhanced ultrasound for focal liver lesion characterization which is clearly superior to that of unenhanced ultrasound, as shown already in other studies with SonoVue® and other microbubble contrast agents<sup>[10-17,26-29]</sup>. Furthermore, this study demonstrates, that the diagnostic accuracy of SonoVue®-enhanced ultrasound is even better than that of contrast-enhanced CT and MRI. The markedly improved accuracy of SonoVue®-enhanced compared

to unenhanced sonography (+ 54.5%) is statistically significant ( $P < 0.0001$ , McNemar's test) and of high clinical relevance. Usually, sonography is the first-line examination for the assessment of focal liver lesions and the high number of 'indeterminate' evaluations found with unenhanced ultrasound causes the high number of follow-up examinations found in today's clinical practice, along with all the costs and discomfort for the patient. The comparable low accuracy for unenhanced sonography in our study could be explained by the high number of patients with malignant lesions (43.3%) in our study population, which was intended to obtain a balanced study population for characterization but does not reflect the distribution in an unselected study population. In our study population, benign lesions with typical B-Mode appearance (cysts, typical hemangiomas, typical FNH), which are easy to characterize based on morphological criteria, were less frequent, making the characterization more difficult compared to an unselected patient population. However, even in an unselected patient population unenhanced sonography is known to be clearly less accurate than the 92.7% accuracy obtained in our study with SonoVue®-enhanced Sonography<sup>[1-3]</sup>.

For the diagnosis of benign lesions (especially FNH and hemangioma), correct diagnosis is the only issue since usually no further activities are required. Therefore, incorrect classification of a malignant lesion is the main concern. In our study, there was only one false-negative characterization with SonoVue®-enhanced sonography



**Figure 2 36-year-old female.** A: 4.0 cm hypoechoic lesion in segment 8 found in B-Mode sonography; B: Contrast-enhanced sonography with SonoVue® showed a peripheral enhancement with nodular contrast accumulations 14 s after injection; C: Slow progression of the enhancement from the periphery towards the center of the lesion, with a broader peripheral enhancement zone seen at 20 s; D: After 1.5 min, the lesion is completely filled with contrast and appears hyperenhanced compared to the surrounding normal liver tissue. The enhancement pattern is typical for a hemangioma.



**Figure 3 44-year-old male patient with a history of rectal carcinoma.** A: 2.4 cm isoechoic lesion in segment 2/3 found in B-Mode sonography; B: Contrast-enhanced sonography with SonoVue® showed a distinct rim enhancement in the peripheral zone of the lesion 13 s after injection, representing the abnormal arterial supply of the lesion; C: Early washout of the contrast enhancement in the portal phase and lack of any portal enhancement resulted in a hypo enhancement of the lesion compared to the surrounding normal liver tissue; D: In the late phase, complete lack of enhancement within the lesion (black spot) allows a clear discrimination of the lesion from the strongly enhanced normal liver tissue. The enhancement pattern is typical for a hypovascular metastasis.

(1 out of 55 patients with malignancies), a lesion which was diagnosed as indeterminate instead of malignant. The tumor was an HCC with hypoenhancement in arterial and portal venous phase. The final diagnosis could only be obtained by histology, since also with CT just an indeterminate diagnosis could be made due to the non-characteristic enhancement pattern. For exclusion of malignancy we observed 5 indeterminate and 3 false malignant classifications out of 68 patients with benign lesion. All of these lesions corresponded to atypical enhancement patterns (4 FNH, 1 adenoma, 2 hemangioma, and 1 hepatic fibrosis).

SonoVue®-enhanced ultrasound turned out to be even more sensitive, specific, and accurate for the characterization of doubtful focal liver lesions compared to CT/MRI. Percentage point differences were 29.7% for sensitivity (recognition of malignancy), 13.6% for specificity (exclusion of malignancy), and 20.8% for accuracy. Sensitivity was nearly perfect for the SonoVue®-enhanced sonography (98.2%) in comparison to the much lower sensitivity of CT/ MRI (68.5%). The higher accuracy of SonoVue®-enhanced sonography may be explained by the real-time capabilities of dynamic low-MI contrast sonography, allowing a continuous assessment of the whole enhancement period, whereas in CT and MRI spiral scans at distinct time points (usually early arterial, late arterial, portal-venous and - in MRI - late phase)<sup>[4-6]</sup>. Especially the very early contrast period (8-12 s post injection) is usually missed with CT/MRI, but may provide crucial diagnostic information especially in highly arterialized lesions. For example, this early arterial phase was particularly useful for the FNH demonstrating the centrifugal hyper-enhancement with a central vessel and radial vascular branches (Figure 1), for haemangioma showing the very early nodular enhancement (Figure 2) or for hypovascular metastasis to demonstrate few isolated spots in the lesions with marked wash-out in portal and late phase (Figure 3). Furthermore, the blood-pool characteristics of SonoVue® prevent the extravasation of the contrast agent (present in conventional CT and MRI contrast agents), which may obscure vascular flow information especially at later time points (equilibrium phase in CT/MRI)<sup>[26]</sup>. The data collected in this study suggest that contrast-enhanced sonography is the best imaging modality for the characterization (malignant/benign) of focal liver lesions.

A limitation of this study is the lack of an off-site assessment with blinded and separated unenhanced and combined unenhanced/contrast-enhanced sequences, to evaluate the accuracy of the pure image sequences without any clinical patient information and with validated blinding with regard to reference examinations. Information about patient's characteristics (e.g. laboratory values) may influence the pre-test probability for malignancy and thus facilitate the classification by imaging. However, this reflects the situation in routine clinical practice. The improvement of the sonographic characterization with SonoVue® can be explained by the additional information on vascularity and perfusion obtained with the analysis of the dynamic enhancement pattern of the

lesions, which is standard in CT and MRI examinations (generally using contrast enhanced sequences for characterization) but not in conventional sonography (just assessing morphological criteria and Doppler signals in large vessels). An unenhanced examination should be considered as inadequate for the characterization of focal liver lesions, which can't be clearly classified as benign due to unequivocal morphological criteria. Contrast-enhanced ultrasound should be the preferred first-line examination for the characterization of such lesions, followed by supplementary CT/MRI examinations or histological confirmation, if required.

## COMMENTS

### Background

The characterization of liver lesions with imaging is based on the examination of the vascularity and enhancement pattern within the lesion. SonoVue® is an Ultrasonographic contrast agent allowing the assessment of vascularity and enhancement pattern of focal lesions with ultrasound in real-time.

### Research frontiers

The authors demonstrate the higher accuracy of contrast-enhanced sonography related to the real-time capabilities of dynamic contrast sonography, allowing a continuous assessment of the whole enhancement period, whereas in computed tomography (CT) and in magnetic resonance imaging (MRI) spiral scans at distinct time points (usually early arterial, late arterial, portal-venous and - MRI - late phase).

### Innovations and breakthroughs

There are already several studies published, demonstrating the safety and efficacy of contrast-enhanced ultrasonography for the diagnosis of focal liver lesions. However most of these studies were single-center studies performed with just one type of machine. This clinical study investigated the diagnostic performance of contrast-enhanced ultrasound in the characterization of focal liver lesion in an international multicenter and multi-equipment setting in comparison to established CT and MRI examinations.

### Applications

Contrast-enhanced ultrasound should be the preferred first-line examination for the characterization of liver lesions, followed by supplementary CT/MRI examinations or histological confirmation, if required.

### Terminology

SonoVue® is an ultrasonographic contrast agent composed by micro bubbles. It is used with specific contrast-enhanced sequences obtained using low-mechanical index (MI) imaging software ( $MI < 0.2$ ). Low-mechanical index or low acoustic pressure is used to do not destroy the microbubbles too fast to allow multiphasic imaging. The real-time capability of sonography in comparison with CT or MRI is related to a better temporal resolution of this technique.

### Peer review

The authors evaluated in a controlled multicenter study whether the characterization of focal liver lesions can be improved by using SonoVue®-enhanced sonography, as compared with unenhanced sonography, contrast-enhanced CT and contrast-enhanced MRI. Contrast-enhanced sonography emerges as the most sensitive, most specific and thus most accurate imaging modality for the characterization of focal liver lesions.

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# Lentivirus-mediated shRNA interference targeting STAT3 inhibits human pancreatic cancer cell invasion

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**CONCLUSION:** The STAT3 signaling pathway may provide a novel therapeutic target for the treatment of pancreatic cancer since it inhibits the invasion ability of pancreatic cancer cells.

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**Key words:** Signal transducer and activator of transcription 3; RNA interference; Lentivirus vector; Pancreatic cancer cells; Invasion

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Yang G, Huang C, Cao J, Huang KJ, Jiang T, Qiu ZJ. Lentivirus-mediated shRNA interference targeting STAT3 inhibits human pancreatic cancer cell invasion. *World J Gastroenterol* 2009; 15(30): 3757-3766 Available from: URL: <http://www.wjgnet.com/1007-9327/15/3757.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.3757>

## Abstract

**AIM:** To investigate RNA interference targeting signal transducer and activator of transcription-3 (STAT3) on invasion of human pancreatic cancer cells.

**METHODS:** We constructed three plasmids of RNA interference targeting the STAT3 gene. After LV (lentivirus)-STAT3siRNA (STAT3 small interfering RNA) the vector was transfected into the human pancreatic cell line, SW1990 and cell proliferation was measured by the MTT assay. Flow cytometry was used to assess cell cycle. Vascular endothelial growth factor (VEGF) and matrix metalloproteinase-2 (MMP-2) mRNA and protein expression were examined by quantitative PCR and western blotting, respectively. The invasion ability of SW1990 cells was determined by cell invasion assay.

**RESULTS:** We successfully constructed the LV-STAT3siRNA lentivirus vector and proved that it can suppress expression of STAT3 gene in SW1990 cells. RNA interference of STAT3 by the LV-STAT3siRNA construct significantly inhibited the growth of SW1990 cells, in addition to significantly decreasing both VEGF and MMP-2 mRNA and protein expression. Moreover, suppression of STAT3 by LV-STAT3siRNA decreased the invasion ability of SW1990 cells.

## INTRODUCTION

Pancreatic cancer is a highly lethal disease that is usually diagnosed at an advanced stage for which there is little or no effective therapy. It remains the fourth most common cause of cancer-related death in the western world<sup>[1]</sup>. The annual incidence rate of pancreatic cancer is almost identical to the mortality rate; approximately 37 000 new cases are diagnosed each year in the United States, and approximately 33 000 patients die from this disease<sup>[2]</sup>. Due to the aggressive natural history of this disease, less than 10% of these cases constitute candidates for surgical resection at the time of diagnosis. Although an adjuvant treatment regimen after surgical resection seems to prolong survival, the precise treatment protocol including drug-of-choice is still debated and the focus of several ongoing clinical trials<sup>[3]</sup>. Effective systemic therapy capable of reversing the aggressive biology of this disease is currently not available. Thus, understanding the molecular mechanisms of pancreatic cancer is one of the most important issues for treatment.

Signal transducer and activator of transcription 3 (STAT3) is an oncogene that is activated by phosphorylation of a conserved tyrosine residue in response to extracellular signals and oncogenes. Once tyrosine is phosphorylated, STAT3 monomers form dimers through reciprocal phosphotyrosine-SH2 interactions. The dimers are phosphorylated STAT3 (P-STAT3), which translocate to the nucleus and bind to cognate DNA sequences, regulate the transcription of target genes and modulate fundamental cellular processes, such as proliferation and differentiation<sup>[4]</sup>. Inappropriate and constitutive activation of STAT3 may be responsible for pancreatic cancer progression through regulating the expressions of target genes, such as Bcl-xL, Mcl-1, Bcl-2, Fas, cyclin D1, survivin, c-Myc, VEGF, MMP-2 and MMP-9<sup>[5-7]</sup>. Many studies have indicated that the constitutive activation of STAT3 influences invasion and metastasis. Specifically, the level of activated STAT3 protein has been found to be associated with invasion in thymic epithelial tumors<sup>[8]</sup>, colorectal adenocarcinoma<sup>[9]</sup>, and cutaneous squamous cell carcinoma<sup>[10]</sup>. Conversely, inhibition of the STAT3 signaling pathway suppresses cancer cell growth, invasion and induces apoptosis in various cancers<sup>[11]</sup>. Thus, the STAT3 signaling pathway may be one of the common pathways involved in regulating cancer invasion.

Small hairpin RNA (shRNA) expression vector systems have been established to induce RNA interference (RNAi) in mammalian cells<sup>[12]</sup>. Although these vectors provide certain advantages over chemically synthesized siRNAs, some disadvantages remain, including transient shRNA expression and low transfection efficiency, especially in non-dividing primary cells. To overcome these limitations, shRNA delivery systems using retroviral vectors<sup>[13]</sup>, adenoviral vectors<sup>[14]</sup> and, more recently, lentiviral vectors<sup>[15]</sup> have been reported and proven to be safe for humans. Lentivirus vectors encoding antisense targeting sequence have been used in clinical trials with no obvious side effects<sup>[16,17]</sup>. Lentivirus-delivered shRNAs are capable of specific, highly stable and functional silencing of gene expression in a variety of human cells including primary non-dividing cells and also in transgenic mice<sup>[18,19]</sup>.

In our study, we constructed a lentivirus vector mediating RNAi targeting of STAT3 (LV-STAT3siRNA). The efficacy of LV-STAT3siRNA plasmids in interference with STAT3 was confirmed by real-time PCR and western blotting. We found that LV-STAT3siRNA suppressed growth and invasion by markedly decreasing the expression of VEGF and MMP-2 in SW1990 cells, but LV-Con (control) had no effect on SW1990 cells. Since the STAT3 signaling pathway is critical for growth and the invasive behavior of pancreatic cancer, silencing of the STAT3 gene with RNAi may provide a novel strategy for investigation of the role of STAT3 gene in the invasion of human pancreatic cancers.

## MATERIALS AND METHODS

### Construction and production of lentivirus vectors

We designed and cloned a shRNA template into a

lentivirus vector. A third generation self-inactivating lentivirus vector containing a CMV-driven GFP reporter and a U6 promoter upstream of the cloning restriction sites (*Hpa* I and *Xba* I) was used. The introduction of oligonucleotides encoding shRNAs (Figure 1A) between these restriction sites enables the production of the shRNA *in vivo*. Three coding regions corresponding to targeting human STAT3 starting at positions 466, 1638, and 1061 in the sequence (GenBank Accession: NM 39276) were selected as siRNA target sequences under the guide of siRNA designing software offered by Genscript. We constructed three shRNA-STAT3 lentivirus vectors, namely LV-STAT3siRNA-1, LV-STAT3siRNA-2 and LV-STAT3siRNA-3, respectively (Table 1). Briefly, oligonucleotides were annealed, digested and inserted between the *Hpa* I and *Xba* I restriction sites of the plasmid vector. Some mutations were introduced in the sense sequence of the hairpin structure to facilitate sequence and avoid destruction by bacteria during amplification in the bacterial host. Correct insertions of shRNA cassettes were confirmed by restriction mapping and direct DNA sequencing. We constructed a STAT3 over-expression vector, pEGFP-N1-STAT3 (Figure 1B), and co-transfected with recombinant lentivirus vectors into 293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). To detect the interference effects of different target, STAT3 protein expression was determined using western blotting. Recombinant lentivirus vectors and control lentivirus vectors were produced by co-transfecting 293T cells with the lentivirus expression plasmid and packaging plasmids (pHelper 1.0 including *gag/pol* and pHelper 2.0 including VSV-g). Infectious lentivirus vectors were harvested at 48 h post-transfection, centrifuged to remove cell debris, and filtered through 0.45 μm cellulose acetate filters. The infectious titer was determined by hole-by-dilution titer assay. The virus titers produced were approximately 10<sup>9</sup> transducing U/mL medium.

### Cell culture and infection

The human pancreatic cancer cell line, SW1990, and 293T cells were purchased from the American Type Culture Collection. Cells were grown in 5% CO<sub>2</sub> saturated humidity, at 37°C and cultured as monolayers in RPMI 1640 supplemented with penicillin/streptomycin, 2 mmol/L glutamine and 10% FBS. Cells were subcultured at 1 × 10<sup>5</sup> cells per well into six-well tissue culture plates. After 24 h culture, cells were infected with recombinant lentivirus vectors at a multiplicity of infection (MOI) of 40.

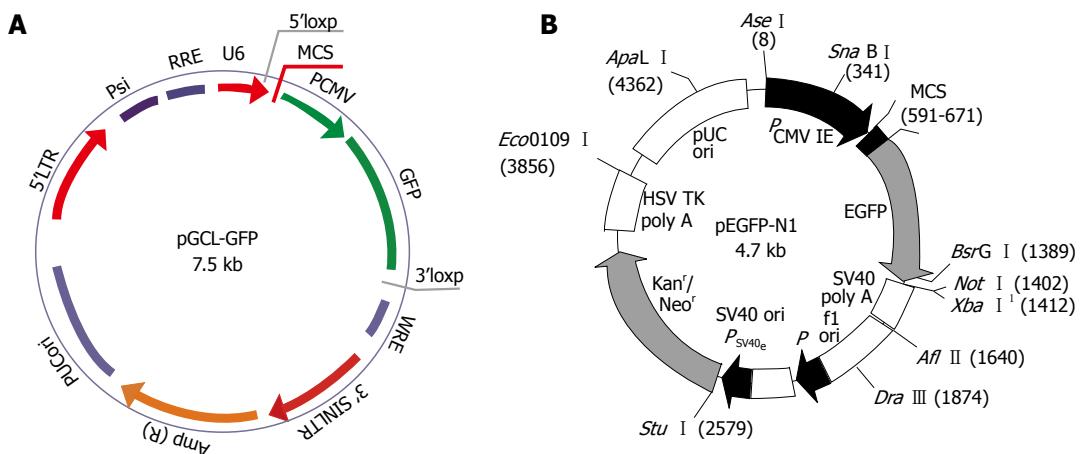
### Cell proliferation assay

Cell proliferation was determined by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Pancreatic cancer cells were seeded in 96-well culture plates in culture medium at an optimal density (5 × 10<sup>3</sup> cells per well) in triplicate wells for the LV-STAT3siRNA, LV-Con and parental cells groups. After 1, 2, 3 and 4 d, cells were stained with 20 μL MTT (5 mg/mL) (Sigma, St Louis, MO, USA) at 37°C for 4

**Table 1** Interfering sequence specified for STAT3 gene

Number	Sequence
LV-STAT3siRNA-1	Oligo1: 5'TaaTGTCTCTATCAGCACAAATTCAAGAGAATTGTGCTGATAGAGAACATTTTTC3' Oligo2: 5'TCGAGAAAAAAaaTGTCTCTATCAGCACAAATTCTCTGAAATTGTGCTGATAGAGAACATTA3'
LV-STAT3siRNA-2	Oligo1: 5'TaaCATCTGCCTAGATCGGCTATTCAAGAGATAGCCGATCTAGGCAGATGTTTTTC3' Oligo2: 5'TCGAGAAAAAAaaCATCTGCCTAGATCGGCTATCTTGAATAGCCGATCTAGGCAGATGTTA3'
LV-STAT3siRNA-3	Oligo1: 5'TaaCTTCAGACCCGTCAACAAATTCAAGAGATTGTTGACGGGCTGAAGTTTTTC3' Oligo2: 5'TCGAGAAAAAAaaCTTCAGACCCGTCAACAAATTCTTGAATTGTTGACGGGCTGAAGTTA 3'

Three coding regions corresponding to targeting human STAT3 starting at 466, 1638, and 1061 in the GenBank (NM 139276). Interfering sequence specified for STAT3 gene, namely LV-STAT3siRNA-1, LV-STAT3siRNA-2 and LV-STAT3siRNA-3, respectively.



**Figure 1** Structure of vectors. A: Lentivirus vector pGCL-GFP containing a CMV driven GFP reporter and a U6 promoter upstream of cloning restriction sites (*Hpa*I and *Xho*I) to allow the introduction of oligonucleotides encoding shRNAs. The multiple cloning site (MCS) is located between the U6 promoter and CMV. B: The pEGFP-N1 vector expressed green fluorescent protein following transfection into mammalian cells. MCS is located between the immediate early promoter of CMV and the EGFP. \*Methylated in the DNA provided by CLONTECH.

h and subsequently made soluble in 150 µL of DMSO. Absorbance was measured at 490 nm using a microtiter plate reader. Cell growth curves were calculated as mean values of triplicates per group.

### Flow cytometry

The cell cycle effects of LV-STAT3siRNA on human pancreatic cancer cells were analyzed by flow cytometry. Briefly,  $1 \times 10^5$  cells were trypsinized, washed with PBS, centrifuged at 800 r/min and fixed with 70% cold ethanol overnight. Ethanol was aspirated after centrifugation at 1000 r/min, and the cells were incubated in a solution of propidium iodide (PI) (100 µg), 0.5% Triton-X100 and RNase (2000 µg/mL) for 15 min and analyzed by flow cytometry (FCM).

### Quantification by real-time PCR

Total RNA was isolated using TRIzol LS (Invitrogen, Carlsbad, CA, USA). The concentration and purity of RNA was determined using a spectrophotometer. M-MLV reverse transcriptase (Promega, Madison, WI, USA) was used to create cDNA for further analyses. Quantitative real-time polymerase chain reaction (RT-PCR) assays were carried out using SYBR Green Real-Time PCR Master Mix (Toyobo, Osaka, Japan) and real-time PCR amplification equipment.

The PCR primers used to detect STAT3 and β-actin were as follows: STAT3, sense strand 5'-CCAAGGA

GGAGGCATTG-3', antisense strand 5'-ACATCG GCAGGGTCAATGG-3', with a product length of 147 bp; β-actin, sense strand 5'-TCGTGCGTG ACATTAAGGAG-3', antisense strand 5'-AAGGTA GTTTCGTGGATGCC-3', with a product length of 214 bp. The thermal profile consisted of one cycle at 95°C for 15 min, followed by 45 cycles at 95°C for 15 s and 60°C for 30 s. The expression of STAT3 was determined by normalization of the threshold cycle (C<sub>t</sub>) of these genes to that of the control housekeeping gene (β-actin). The delta Ct was determined using the following equation:  $(\Delta C_t) = (C_t \text{ of STAT3}) - (C_t \text{ of } \beta\text{-actin} \text{ in each group})$ . The  $\Delta C_t$  value obtained was used to find the relative expression of the STAT3 gene according to the following formula: Relative expression =  $2^{-\Delta C_t}$ ,  $\Delta C_t$  = (mean  $C_t$  of STAT3 genes in LV-STAT3siRNA groups) - ( $C_t$  of STAT3 genes in LV-Con group or parental SW1990 group).

The PCR primers used to detect MMP-2, VEGF and β-actin were as follows: MMP-2 sense strand 5'-TAGCATGTCCCTACCGAGTCT-3', antisense strand 5'-ATTGGATGGCAGTAGCTGC-3', with a product length of 151 bp; VEGF sense strand 5'-CTGTCTGGGTGCATTGGA-3', antisense strand 5'-ATTGGATGGCAGTAGCTGC-3', with a product length of 152 bp; β-actin sense strand 5'-CACCAACTGGGACGACAT-3', antisense strand 5'-ATCTGGGTCACTTCTCGC-3', with a product

length of 138 bp. PCR parameters were as follows: 95°C for 20 min, then 95°C for 30 s, 56°C for 30 s, 72°C for 40 s for 40 cycles, 72°C for 10 min. A standard calibration curve for the expression of each mRNA was generated using 8-fold dilutions of a control RNA sample. MMP-2 and VEGF mRNA expression was calculated as a ratio compared with that of β-actin.

### **Western blotting**

Whole-cell protein extracts and nuclear protein extracts from pancreatic cancer cells were prepared with RIPA Lysis Buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA), respectively, according to the manufacturers' instructions. Protein concentrations were determined using an assay kit (Bio-Rad, Hercules, CA, USA).

Lysates containing 100 μg of protein were mixed with loading buffer with 5% β-mercaptoethanol and heated for 5 min at 100°C. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes by semi-dry blotting.

Membranes were incubated in blocking buffer [tris-buffered saline (TBS), 0.1% Tween 20, and 5% non-fat dry milk] for 1 h at room temperature, followed by hybridization with anti-P-STAT3 (tyr-705) antibody (Cell Signaling Technology, 1:1000 dilution), anti-STAT3 antibody (Cell Signaling Technology, 1:1000 dilution), anti-MMP-2 antibody (Santa Cruz Biotechnology, 1:500 dilution), anti-VEGF antibody (Santa Cruz Biotechnology, 1:500 dilution), GADPH antibody (Lab Vision, Fremont, CA, USA, 1:100 dilution) or anti β-actin antibody (Lab Vision, Fremont, CA, USA, 1:100 dilution) at 4°C overnight.

After 3 washes in TBS/0.1% Tween 20, the membranes underwent hybridization with a horseradish peroxidase-conjugated secondary antibody rabbit IgG (Santa Cruz Biotechnology, 1:5000 dilution) for 1 h at room temperature. After 3 washes in TBS/0.1% Tween 20, signals were detected by chemiluminescence using luminol reagent (Santa Cruz Biotechnology).

### **Invasion assay**

The cell invasion assay was performed using a specialized invasion chamber that included a 24-well tissue culture plate with 12 cell culture inserts (Chemicon, Temecula, CA, USA). The inserts contained an 8 μm pore size polycarbonate membrane with a precoated thin layer of basement membrane matrix (ECMatrix). Briefly, media supplemented with 10% fetal bovine serum was added to the lower chamber as a chemo-attractant. After reaching 60%-70% subconfluence, pancreatic cancer cells were trypsinized, re-suspended in DMEM at 1 × 10<sup>6</sup> cells/mL, and 300 μL of the cell suspension was added to each upper compartment.

After 24 h incubation at 37°C, non-invasive cells were removed from the upper surface of the membrane using a moist cotton-tipped swab. Invasive cells on the lower surface of the membrane, which had invaded the ECMatrix and had migrated through the polycarbonate

membrane, were stained with the staining solution for 20 min and rinsed with distilled water several times. Invasiveness was quantitated by selecting ten different views (400 times) and calculating the number of invading cells.

### **Statistical analysis**

All assays were conducted three times and found to be reproducible. Data were expressed as mean ± SD and the statistical correlation of data between groups was analyzed by Student's *t* test, where *P* < 0.05 were considered significant. These analyses were performed using SPSS 11.0 software.

## **RESULTS**

### **Selection of the most effective STAT3-specific siRNA expression vector**

Three plasmids containing shSTAT3 (pGCL-GFP-STAT3siRNA) and pEGFP-N1-STAT3 were co-transfected into 293T cells, respectively. GFP expression in 293T cells was observed under a fluorescent microscope 36-48 h after transfection with pEGFP-N1-STAT3 and pGCL-GFP-STAT3siRNA. Results of the western blotting assay showed that LV-STAT3siRNA-1 and LV-STAT3siRNA-2 could significantly suppress the expression of STAT3 at the protein level in 293T cells. According to the results of western blotting assay, LV-STAT3siRNA-2 was the most effective lentivirus vector and, thus, was used in the following research (Figure 2).

### **Expression of STAT3 suppressed by LV- siSTAT3-2 in SW1990 cells**

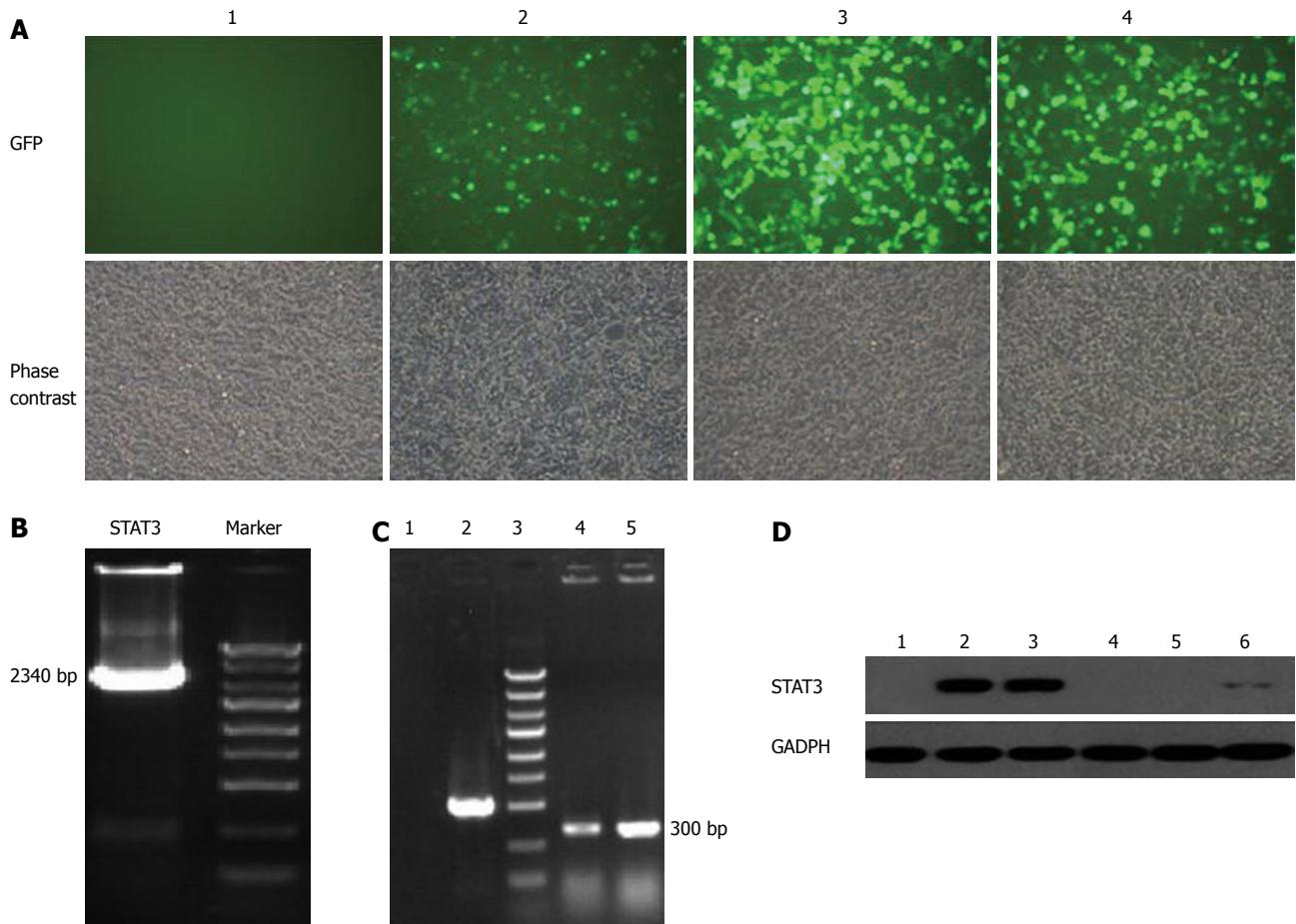
To determine the effect of LV-STAT3siRNA on the expression of STAT3, GFP expression was observed under a fluorescent microscope in SW1990 cells 72 h after infection with LV- siSTAT3-2 at an MOI of 40. Next, real-time PCR and western blotting were performed to determine the mRNA and protein levels of STAT3 in LV-STAT3siRNA-2, LV-Con and parental cell groups. These analyses demonstrated that LV-STAT3siRNA-2 significantly inhibited expression of STAT3 mRNA (*P* = 0.006, *P* = 0.007) and protein compared with SW1990 cells and the LV-Con group (Figure 3).

### **Effects of LV-shSTAT3 on cell growth of SW1990 cells**

Cell proliferation was monitored for four days after SW1990 cells were infected with LV-STAT3siRNA-2 and LV-Con. The growth of cells infected with LV-STAT3siRNA-2 was markedly inhibited compared with LV-Con and parental SW1990 (Figure 4).

### **Effects of LV-shSTAT3 on cell cycle in SW1990 cells**

To investigate the effects of LV-STAT3siRNA-2 on cell cycle, G1, G2 and S phase cells were detected by flow cytometric analysis. The total S phase plus G2 phase fraction was used to measure cell proliferation. SW1990 cells were infected with LV-STAT3siRNA-2 and cell proliferation after 72 h was detected by flow



**Figure 2 Selection of the most effective STAT3 specific siRNA expression vector in 293T cells.** A: Phase contrast and GFP expression under a fluorescent microscope was taken after 36–48 h in 293T cells, 1: 293T cells; 2: Transfection of pEGFP-N1 vector in 293T cells; 3: Transfection of pEGFP-N1-STAT3; 4: Co-transfected of pEGFP-N1-STAT3 and pGCL-GFP-siRNA vectors in 293T cells (original magnification  $\times 200$ ). B: STAT3 gene was cloned from the cDNA library with production of 2340 bp by PCR. C: pEGFP-N1-STAT3 was constructed after restricted enzyme cutting and connection with production of 300 bp by PCR. 1: pEGFP-N1 vector; 2: A special sequence was inserted in pEGFP-N1 vector; 3: Marker; 4-5: STAT3 gene over-expression vector pEGFP-N1-STAT3. D: Protein level of STAT3 in 293T cells was detected by western blotting. Lanes 1-6: 293T cells, STAT3 gene over-expression vector, non-silence control, LV-STAT3siRNA-1, -2, and -3 respectively. LV-STAT3siRNA-1 and LV-STAT3siRNA-2 can significantly knock down expression of STAT3 at the protein level.

**Table 2 Cell cycle detected by flow cytometry (%)**

Group	G1 fraction	G2 fraction	S fraction	G2 + S fraction
SW1990	$35.83 \pm 5.85$	$49.29 \pm 2.80$	$14.45 \pm 3.24$	$63.74 \pm 5.66$
LV-Con	$43.34 \pm 2.14$	$51.88 \pm 3.35$	$4.79 \pm 1.27$	$56.66 \pm 2.14$
LV-STAT3siRNA-2	$52.36 \pm 1.02$	$43.34 \pm 1.14$	$4.30 \pm 2.08$	$47.63 \pm 1.20^{a,c}$

Cell cycle detected by flow cytometry. The G2 + S phase fraction of the LV-STAT3siRNA-2 group was the lowest compared with LV-Con group and parental SW1990 group. This result was consistent with that of the MTT assay. <sup>a</sup> $P < 0.05$  vs SW1990 group, <sup>c</sup> $P < 0.05$  vs LV-Con group.

cytometry. Consistent with the MTT assay (Table 2), LV-STAT3siRNA-2 caused a significant reduction in cell proliferation compared with the control, LV-Con ( $P = 0.003$ ) and parental SW1990 groups ( $P = 0.008$ ).

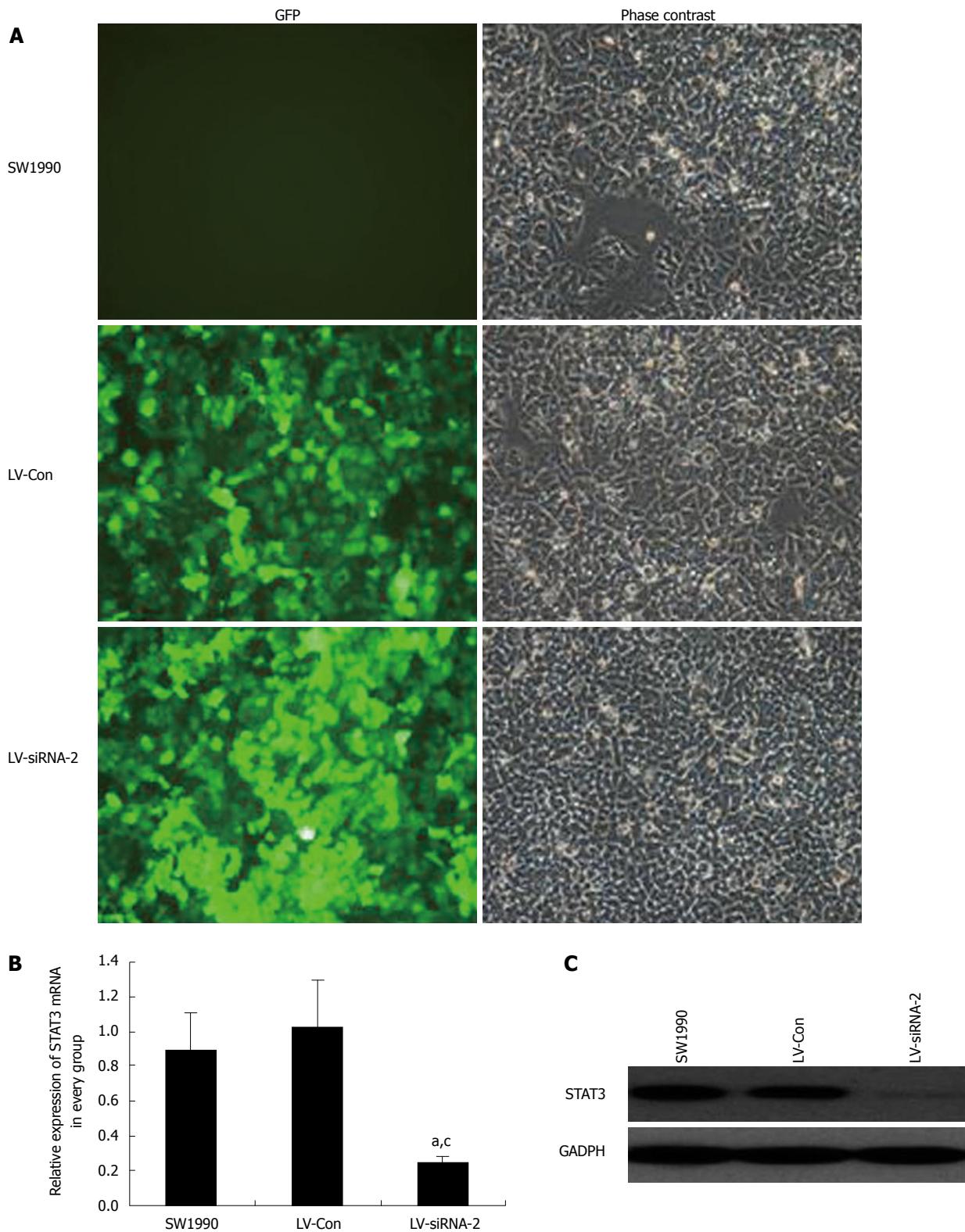
#### Effects of LV-shSTAT3 on VEGF and MMP-2 expression in SW1990 cells

The expression of P-STAT3, MMP-2 and VEGF was analyzed at the mRNA level by real-time PCR (Figure 5A and B). Protein expression was evaluated by western blotting (Figure 5C and D) in the human pancreatic cancer cell lines. Silencing of STAT3 expression by stable

transfection of LV-STAT3siRNA-2 significantly decreased the expression of MMP-2 ( $P = 0.004$ ,  $P = 0.008$ ) and VEGF ( $P = 0.006$ ,  $P = 0.0015$ ) mRNA compared with the control, SW1990 and LV-Con groups. LV-STAT3siRNA-2 could markedly downregulate protein levels of P-STAT3 ( $P = 0.001$ ,  $P = 0.000$ ), VEGF ( $P = 0.031$ ,  $P = 0.025$ ), and MMP-2 ( $P = 0.007$ ,  $P = 0.026$ ) in SW1990 cells compared with the SW1990 and LV-Con groups.

#### Effects of LV-shSTAT3 on invasion ability in SW1990 cells

An *in vitro* cell invasion assay was performed and the

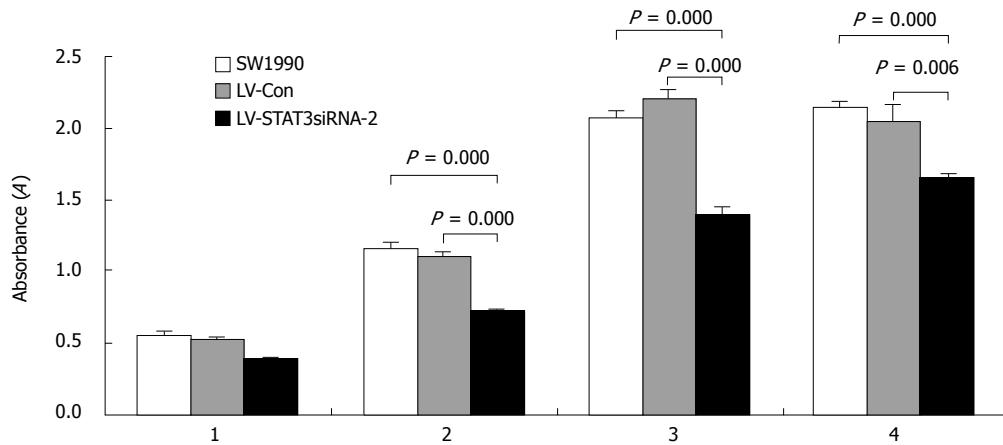


**Figure 3 Expression of STAT3 suppressed by LV-siSTAT3-2 in SW1990 cells.** A: SW1990 cells were infected with LV-Con or LV-STAT3siRNA-2; The cells were infected (MOI = 40), GFP expression and the phase contrast images were taken after 72 h (original magnification  $\times 200$ ). B: mRNA level of STAT3 after SW1990 cells were treated with LV-STAT3siRNA-2 and LV-Con detected by real-time PCR. LV-STAT3siRNA-2 significantly inhibited expression of STAT3 mRNA in SW1990 cells,  $^aP < 0.05$ , compared with SW1990 cells,  $^cP < 0.05$ , compared with LV-Con group. C: Western blotting analysis showed that STAT3 protein was markedly inhibited by LV-STAT3siRNA-2 in SW1990 cells.

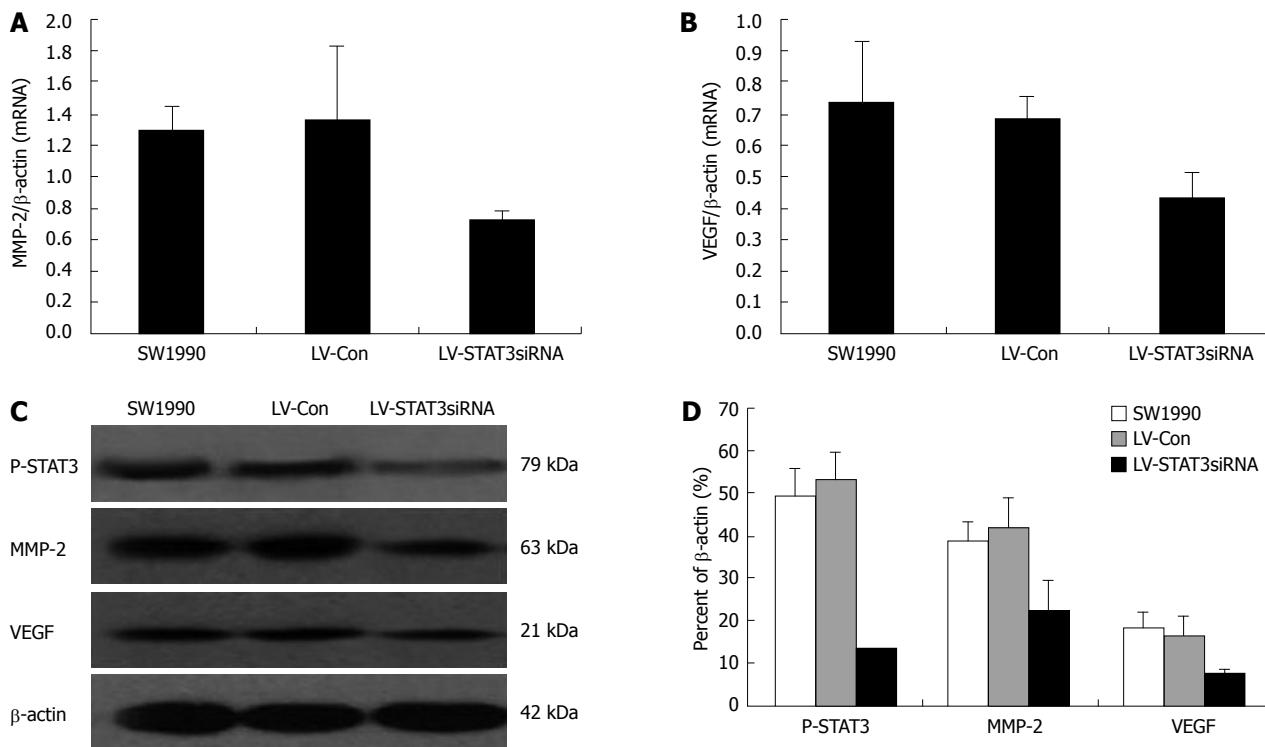
number of invading cells counted. The control group, LV-Con, had no effect on invasion of SW1990 cells, however, LV-STAT3siRNA-2 markedly reduced the invasion ability compared with LV-Con ( $P = 0.004$ ) and parental SW1990 ( $P = 0.001$ ) (Figure 6).

## DISCUSSION

STAT3 is a key signal transduction protein that mediates signaling by numerous cytokines, peptide growth factors, and oncproteins. The Janus kinase (JAK)/STAT3



**Figure 4 Pancreatic cancer cells growth was detected by MTT assay.** SW1990 cells growing in 96-well plates were infected with LV-STAT3siRNA-2 and LV-Con 1, 2, 3, 4 d respectively, and the MTT assay revealed that the cell growth was significantly suppressed by LV-STAT3siRNA-2 in SW1990 cells compared with LV-Con group and parental SW1990 group. Data are mean  $\pm$  SD.

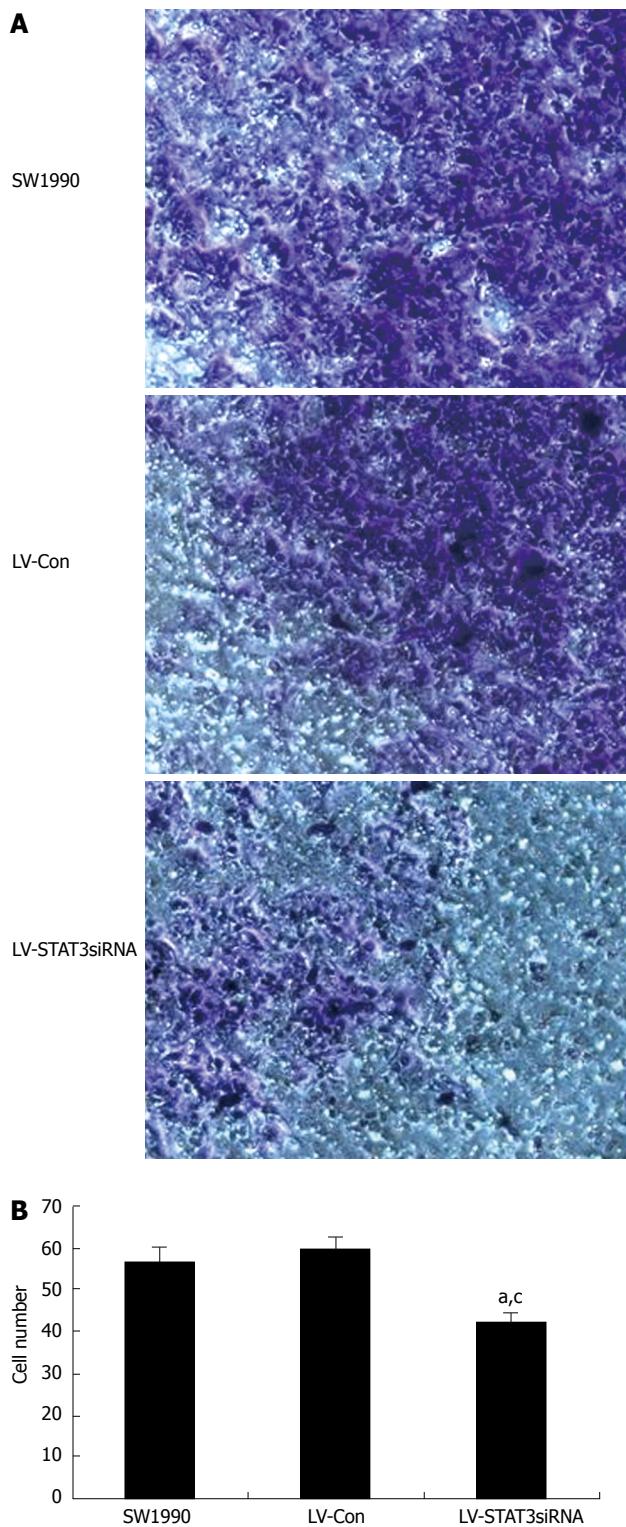


**Figure 5 The expression of MMP-2 and VEGF was analyzed by real-time PCR and Western blotting in the human pancreatic cancer cell lines.** LV-STAT3siRNA-2 significantly inhibited mRNA and protein expression of MMP-2 and VEGF in SW1990 cell. A: MMP-2 mRNA expression in every group. B: VEGF mRNA expression in every group. C: P-STAT3, MMP-2 and VEGF protein expression in every group. D: Densitometric analysis protein expression of P-STAT3, MMP-2 and VEGF in every group.

signaling pathway plays a significant role in various physiological processes, including immune function, cell growth, differentiation, and hematopoiesis<sup>[20]</sup>. However, STAT3 has been implicated in important roles for cell proliferation, invasion and metastasis in diverse human cancers, including pancreatic cancer. Recent studies have also revealed that the promotion of STAT3 activation can contribute to oncogenesis. For example, Huang<sup>[11,21]</sup> and coworkers reported that activation of the STAT3 signaling pathway plays an important role in the progression of pancreatic cancer. These published reports all demonstrated the crucial importance of the

JAK/STAT3 signaling pathway in tumorigenesis and progression. In this study, our aim was to determine the role of the JAK/STAT3 signaling pathway in pancreatic cancer progression and to test the hypothesis that the STAT3 gene could serve as a therapeutic target.

RNAi is the process by which double-stranded RNA induces potent and specific inhibition of eukaryotic gene expression through the degradation of complementary messenger RNA, and is functionally similar to the processes of post-transcriptional gene silencing<sup>[22,23]</sup>. In the past few years, RNAi has been widely used by researchers to silence the expression of many target



**Figure 6 Invasion assay was performed using a specialized invasion chamber.** A: The blue-stained cells are those that invaded the ECM matrix and migrated through the polycarbonate membrane to the lower surface of the membrane (original magnification  $\times 200$ ). B: Invasion assay indicated LV-STAT3siRNA significantly decreased the invasion ability of SW1990 cells. Bars indicate mean  $\pm$  SD. <sup>a</sup>P < 0.05 vs SW1990 group, <sup>c</sup>P < 0.05 vs LV-Con group.

genes because of their high specificity and apparent non-toxicity<sup>[24]</sup>. Furthermore, systems based on lentiviral vectors have provided new solutions to achieving stable shRNA-mediated knockdown<sup>[25]</sup>. In this research, we chose a lentivirus vector as our shRNA delivery vehicle

because they can infect both dividing and nondividing cells at a high efficiency and sustain long-term gene expression by integrating into the host genome. shRNA was proved to provide long-lasting silencing and maximal inhibition of gene expression at low concentration<sup>[26]</sup>. Since the potency of the inhibitory effect of shRNA is related to the specificity to its target sequence, we used RT-PCR and western blotting to confirm the efficacy of STAT3siRNA in SW1990 and 293T cells. The greatest STAT3 gene silencing effect was observed when LV-STAT3siRNA-2 was applied and the expression of STAT3 mRNA and protein were markedly inhibited. Given this result, we concluded that LV-STAT3siRNA-2 had a high specificity for STAT3 in SW1990 cells.

Constitutive activation of STAT3 is observed in many types of tumors and promotes cell proliferation and survival<sup>[27,28]</sup>. Inappropriate and constitutive activation of STAT3 may be responsible for pancreatic cancer progression by regulating the expression of target genes, such as c-Myc, Bcl-xL, p21WAF1 and cyclinD1. Moreover, functional inactivation of STAT3 by dominant-negative STAT3 or AG490 (a JAK-specific inhibitor) could inhibit proliferation and promote the apoptosis of pancreatic cancer cells<sup>[29,30]</sup>. In our study, cell cycle and proliferation assays revealed that LV-STAT3siRNA-2 markedly inhibited cell growth and proliferation.

An increasing number of studies suggest that the activation of STAT3 might play an important role in the invasion and metastasis of carcinomas<sup>[5]</sup>. Furthermore, disruption of the STAT3 signaling pathway has been reported to suppress cell invasion by decreasing cell-cell homotypic adhesions and increasing cell motility and scattering<sup>[31]</sup>. In the present study, the invasion ability of these cells with a cell invasion assay was examined and found that STAT3 silencing by RNAi in SW1990 cells resulted in a weak level of invasiveness. Therefore, there is a strong relationship between STAT3 and the invasive ability of human pancreatic cancer cells.

Tumor invasion and metastasis is dependent on angiogenesis, the formation of new blood vessels from a pre-existing network of capillaries. VEGF is known to be a potent angiogenic mitogen that plays an important role in tumor angiogenesis, invasion, and metastasis<sup>[32]</sup>. The role of STAT3 in angiogenesis was first shown when VEGF was found to be a direct target of STAT3 in mouse melanoma cells<sup>[6]</sup> and then confirmed by a study in a human pancreatic cancer system<sup>[33]</sup>. According to studies of clinical samples from pancreatic cancer and pancreatic cancer cell lines, MMPs play important roles in tumor cell invasion and metastasis by degrading components of the basement membranes and extracellular matrix<sup>[34-36]</sup>. Specifically, activated STAT3 regulates tumor invasion of melanoma cells by regulating the transcription of the MMP-2 gene<sup>[7,37]</sup>. In the present study, inhibition of the STAT3 gene by RNAi markedly decreased both MMP-2 and VEGF expression in the pancreatic cancer cell line, SW1990. This suggests that silencing of the STAT3 gene could suppress invasion ability based on the down-regulation MMP-2 and VEGF

gene expression in SW1990 cells.

Overall, the present study indicates that siRNA targeting of STAT3 mRNA via a lentivirus vector system effectively sustains knockdown of the STAT3 gene expression in SW1990 cells. Here we describe the successful construction of a lentivirus RNAi vector targeting STAT3 that will provide a useful tool to study the function of the STAT3 gene in pancreatic cancer cells. Our findings strongly suggest that the JAK/STAT3 pathway plays a significant role in pancreatic cancer cell invasion. Targeting of STAT3 activation may prove to be a more effective approach to controlling invasion than merely targeting individual molecules, such as VEGF and MMP-2, representing a novel approach to regulating pancreatic cancer invasion.

## COMMENTS

### Background

Signal transducer and activator of transcription 3 (STAT3) is a member of the JAK/STAT signaling pathway. Abnormal activation of STAT3 plays a critical role in metastasis and invasion in a variety of human tumors including pancreatic cancer. The authors aim was to study the effect of silencing of STAT3 on invasion in human pancreatic cancer cells.

### Research frontiers

Activated STAT3 has been shown to promote cell proliferation, metastasis, and angiogenesis, as well as protect tumor cells from apoptosis by regulating associated genes, such as Bcl-xL, Mcl-1, Bcl-2, Fas, cyclin D1, survivin, c-Myc, VEGF, MMP-2, and MMP-9. The authors sought to determine whether the STAT3 signaling pathway regulates the invasive potential of pancreatic cancer cells. The inhibition of STAT3 may offer a novel strategy for pancreatic cancer intervention.

### Innovations and breakthroughs

The authors successfully constructed the lentivirus LV-STAT3siRNA vector. Expression of LV-STAT3siRNA can suppress expression of STAT3 gene in SW1990 cells. Flow cytometry analysis showed that the cell cycle of SW1990 cells was inhibited by LV-STAT3siRNA compared with controls. Moreover, LV-STAT3siRNA significantly suppressed the invasion ability of SW1990 cells by down-regulating the VEGF and MMP-2 genes.

### Applications

Lentivirus vectors are safe for human use. Lentivirus vectors encoding the antisense targeting sequence have been used previously for treatment in clinical trials with no obvious side effects. Most recently, a lentivirus vector containing β-globin gene has been approved in phase I/II clinical trials for human β-thalassemia and sickle cell anemia gene therapy. Targeting of STAT3 activation may prove to be an effective approach to controlling invasion in pancreatic cancer cells.

### Peer review

This is an interesting manuscript. The authors utilized three different siRNAs against STAT3 and infected the pancreatic cell line SW1990. They show that siRNA is the most effective at suppressing protein levels of VEGF, MMP-9 downstream effector molecules of STAT3 signaling pathway as well as STAT3 itself. They also show siRNA suppressed the proliferative capacity of SW1990 cells and also its invasive ability.

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# Reinterpretation of histology of proximal colon polyps called hyperplastic in 2001

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## INTRODUCTION

Serrated colorectal polyps in the proximal colon are now recognized as precancerous lesions that should be removed during colonoscopy<sup>[1-3]</sup>. Previously, hyperplastic polyps have been considered innocuous when identified anywhere in the colon; however, some proximal colon serrated polyps are better classified as sessile serrated polyps (sessile serrated adenomas) and closer endoscopic follow-up may be appropriate<sup>[4-7]</sup>.

Much about hyperplastic polyps remains unknown. The extent to which they are recognized as polyps by endoscopists is uncertain, since miss rates for proximal colon hyperplastic polyps have not been reported<sup>[8]</sup>. Further, the extent to which they have been recognized endoscopically but not removed (their endoscopic appearance might initiate observation or sampling, rather than removal), or ineffectively removed, is uncertain and has not been reported. Finally, the extent to which proximal colon serrated lesions are distinguished as hyperplastic *versus* sessile serrated polyps (or sessile serrated adenomas) *versus* traditional serrated adenomas by pathologists is not known<sup>[9,10]</sup>.

In this report, we describe the results of a quality improvement project in which we sought to estimate the extent to which proximal colon polyps removed in prior years and designated as hyperplastic polyps by

## Abstract

**AIM:** To evaluate how proximal colon polyps interpreted as hyperplastic polyps in 2001 would be interpreted by expert pathologists in 2007.

**METHODS:** Forty consecutive proximal colon polyps ≥ 5 mm in size, removed in 2001, and originally interpreted as hyperplastic polyps by general pathologists at Indiana University, were reviewed in 2007 by 3 GI pathologists.

**RESULTS:** The gastrointestinal (GI) pathologists interpreted 85%, 43% and 30% of the polyps as sessile serrated polyps (sessile serrated adenomas). The overall Kappa was 0.16. When diagnoses were compared in pairs, Kappa values were 0.38 and 0.25 (fair agreement) and 0.14 (slight agreement).

**CONCLUSION:** Many polyps interpreted as hyperplastic in 2001 were considered sessile serrated lesions by GI pathologists in 2007, but there is substantial inter-observer variation amongst GI pathologists.

pathologists might now be considered more clinically significant lesions, such as sessile serrated polyps or serrated adenomas<sup>[11-13]</sup>. The issue was deemed pertinent to our colonoscopy callback process, since in prior years we had recommended routine colonoscopic follow up if only hyperplastic polyps were removed from the colon, including from the proximal colon. Recently, we began recommending follow up at shorter intervals if hyperplastic or other serrated polyps were removed from the proximal colon<sup>[14]</sup>. In order to assess whether we had patients who had serrated proximal colon polyps removed earlier and whom we should consider calling back earlier than we previously recommended, we evaluated histologic slides of 40 consecutive proximal colon "hyperplastic polyps" ≥ 5 mm in size removed at Indiana University Hospital in 2001 which were reviewed by three expert gastrointestinal (GI) pathologists.

## MATERIALS AND METHODS

We utilized an endoscopic database that includes histology of more than 10 000 consecutive colorectal polyps<sup>[15]</sup>. We selected 40 consecutive lesions originally diagnosed as hyperplastic polyps removed from the proximal to the sigmoid-descending junction that were at least 5 mm in size. The sample size was selected for feasibility of review in our quality improvement process and not on the basis of a power calculation. The original interpretation in each case was made by 1 of 8 attending pathologists at Indiana University Hospital, none of whom was a specialist in GI pathology. The review was performed by a specialist in GI pathology currently in GI practice at I.U. (OC) and two outside pathologists (J.G. and M.O.). Each of the reviewers was aware that the original slide had been interpreted as a hyperplastic polyp. Each pathologist performed their review independently without any knowledge of the other's findings. We did not supply the reviewing pathologists with standard terminologies to utilize in describing their findings. Permission to report the results of the quality study was granted by the Institutional Review Board of Clarian Health Partners. The Kappa statistic was used to determine agreement between the three specialists in interpretation of the polyps. For the purposes of the comparisons, we considered "sessile serrated polyp" and "sessile serrated adenoma" to be equivalent, but distinct from traditional serrated adenoma.

After the reviews, the pathologists were asked to provide the criteria they used to interpret the histology. Pathologist A indicated that he used the World Health Organization criteria<sup>[16]</sup> for serrated adenoma and published criteria from another source for sessile serrated adenoma and hyperplastic polyp<sup>[2]</sup>. Pathologist B utilized the criteria of Torlakovic *et al*<sup>[17]</sup> that described 3 variants of hyperplastic polyp (the microvesicular serrated polyp, the goblet cell serrated polyp, and the sessile serrated adenoma). The term serrated adenoma was used by pathologist B when some component of the polyp has serrated architecture and overt cytological dysplasia was present. Pathologist C provided two references that best described his criteria for diagnosis of serrated polyps<sup>[18,19]</sup>.

**Table 1 Pathologic interpretation of the 40 polyps by the 3 GI specialist pathologists**

Polyp <sup>1</sup>	Pathologist A	Pathologist B	Pathologist C
1	SSA	HP	SSP
2	HP	HP	SSP
3	HP	HP	SSP
4	SSA	SSA	SSP
5	HP	SSA	SSP
6	HP	HP	HP
7	HP	HP	SSP
8	SSA	HP	SSP
9	HP	HP	SSP
10	SSA	HP	SSP
11	SSA	SSA	SSP
12	SSA	SSA	SSP
13	HP	HP	SSP
14	SSA	SSA	SSP
15	SSA	HP	SSP
16	HP	HP	SSP
17	SSA	SSA	SSP
18	HP	HP	HP
19	SSA	HP	SSP
20	HP	HP	SSP
21	HP	HP	SSP
22	SSA	HP	SSP
23	HP	HP	SSP
24	HP	HP	SSP
25	SSA	SSA	SSP
26	HP	SSA	SSP
27	SA	HP	SSP
28	HP	HP	HP
29	HP	HP	HP
30	HP	HP	SSP
31	SSA	SSA	SSP
32	SSA	SSA	SSP
33	HP	HP	SSP
34	HP	HP	HP
35	HP	HP	SSP
36	SSA	HP	SSP
37	SSA	HP	SSP
38	HP	HP	HP
39	SSA	SSA	SSP
40	HP	SSA	SSP

<sup>1</sup>Polyp number (there are 40 polyps). HP: Hyperplastic polyp; SSP: Sessile serrated polyp; SSA: Sessile serrated adenoma; SA: Serrated adenoma; GI: Gastrointestinal.

## RESULTS

The three GI pathologists interpreted 43%, 30% and 85% of the (cases) as sessile serrated polyps or sessile serrated adenomas (Table 1). Only one polyp was called a traditional serrated adenoma, and by only one of the pathologists.

Based on diagnoses for all three pathologists, the overall Kappa value was 0.16, indicating only slight agreement. When the pathologists' diagnoses were compared in pairs, the Kappa values for the comparisons were 0.38 and 0.25 (fair agreement) and 0.14 (slight agreement).

## DISCUSSION

In this report, we describe a quality improvement project in which we explored issues relating to pathologic interpretation of proximal colon polyps with serrated

histology. Our results indicate that polyps interpreted as hyperplastic in 2001 by non-specialist pathologists at a university hospital were frequently called sessile serrated polyps or sessile serrated adenomas by GI pathologists in 2007. To the extent that “sessile serrated polyp” triggers a recommendation for earlier colonoscopic follow-up compared to “hyperplastic polyps,” this could affect clinical outcomes for patients. The extent to which earlier follow-up by clinicians would be undertaken is unknown, as current postpolypectomy surveillance colonoscopy guidelines indicate that shorter intervals are appropriate for these patients but these guidelines do not make distinct interval recommendations for these patients<sup>[14]</sup>.

We found that each of three expert pathologists used different published references to support their criteria for sessile serrated adenoma (polyp) and serrated adenoma. A review of the various references<sup>[2,16-19]</sup> cited by the pathologists indicated that they utilize substantially similar but not identical language to describe these pathologic entities. The extent to which only slight-to-fair interobserver agreement in this report is the result of variations in criteria *vs* interobserver variation with essentially equivalent criteria is uncertain.

After examining these results, we elected not to systematically call back patients for earlier surveillance colonoscopy if they had undergone colonoscopy less than 10 years ago and had proximal colon polyps originally interpreted as hyperplastic and no adenomas anywhere else in the colon. Our rationale for this decision included (1) lack of clear recommendations on appropriate follow up intervals for this population and (2) the marked interobserver variation in the interpretation of these polyps among experts in GI pathology, and (3) no specific observation that patients with these polyps are a source of interval cancers in our endoscopy unit, and (4) no clear evidence that patients would benefit from being called back early for “sessile serrated polyp” or “sessile serrated adenoma.” We were reassured that only one polyp was called a true serrated adenoma, and this by only one pathologist. We acknowledge uncertainty regarding the appropriateness of the decision to not call patients back earlier than originally planned.

Others have also noted interobserver variation in the pathologic interpretation of serrated polyps<sup>[9,10]</sup>, even among specialists in GI pathology<sup>[9]</sup>, as well as variation in the terminology used to describe these lesions<sup>[9]</sup>. Certainly, recent evidence suggesting that colonoscopy is more effective at reducing the risk of distal compared to proximal cancers<sup>[20]</sup> has raised concerns in our unit that these polyps be carefully looked for, removed completely, and interpreted correctly by pathologists.

In summary, our results suggest that as recently as 2001, non-GI pathologists at a university hospital were uniformly interpreting proximal colon polyps with serrated histology as hyperplastic polyps that GI pathologists would now interpret as sessile serrated polyps (sessile serrated adenomas). If this phenomenon has occurred on a widespread basis (which seems likely)

it might have a negative effect on colorectal cancer prevention, but this is very uncertain. We elected not to bring patients with only proximal colon hyperplastic polyps diagnosed in the period around 2001 back for follow-up colonoscopy sooner than originally planned. However, we admit uncertainty regarding the optimal management of these patients, and physicians might reasonably choose to recall these patients for colonoscopy at earlier intervals<sup>[14]</sup>. Clinicians should be aware that substantial percentages of patients with lesions diagnosed as proximal colon hyperplastic polyps may have had lesions which would now be called sessile serrated polyps or sessile serrated adenomas by expert pathologists.

## COMMENTS

### Background

Serrated polyps in the proximal colon share molecular features with a group of proximal colon cancers and maybe precursors of these cancers. Subtypes of serrated polyps include hyperplastic polyps, sessile serrated polyps (also called sessile serrated adenomas) and serrated adenomas.

### Research frontiers

The clinical relevance of the distinction between hyperplastic and sessile serrated polyps is unknown. Possibly the number, size, and location of serrated polyps has as much relevance as the pathologic distinction between hyperplastic polyps and sessile serrated polyps. True serrated adenomas, however, are likely more important than hyperplastic polyps and sessile serrated polyps, and also are much less common. The accuracy of the pathologic distinction of these polyp types in clinical practice is probably quite low.

### Innovations and breakthroughs

Serrated polyps in the proximal colon often have molecular features in common with a subgroup of colon cancers, including *BRAF* mutations, the CpG island methylator phenotype, and microsatellite instability. These common molecular features underlie the hypothesis that some hyperplastic polyps become serrated adenomas and then cancers.

### Applications

The results indicate that clinical decision making regarding serrated polyps is complicated by variations in pathology terms and substantial interobserver variation in pathologic interpretation of serrated polyps, even among experts.

### Terminology

The term “serrated polyps” describes a set of polyps divided into subgroups believed to have variable risk for short term transformation into cancer. Hyperplastic polyps are believed to have the most benign behavior, while true serrated adenomas (which are dysplastic) have the greatest risk. Sessile serrated polyp is a term often used for a set of polyps called sessile serrated adenoma, and these lesions may be intermediate in the transformation of hyperplastic polyp to serrated adenoma.

### Peer review

Original evaluation of histologic features of hyperplastic polyps in different periods, with experienced pathologists applying new concepts. Several problems are highlighted in this paper: Missing polyps during colonoscopy; Absence of unique terminology; Difficulties in interobserver agreement in histologic interpretation.

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BRIEF ARTICLES

## No association between phosphatase and tensin homolog genetic polymorphisms and colon cancer

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= 421) and controls ( $n = 483$ ) aged  $\geq 30$  years to conduct a comprehensive tagSNP association analysis of the PTEN gene.

**RESULTS:** None of the PTEN SNPs were statistically significantly associated with colon cancer when controlled for age, gender, and race, or when additionally adjusted for other known risk factors ( $P > 0.05$ ). Haplotype analyses similarly showed no association between the PTEN gene and colon cancer.

**CONCLUSION:** Our study does not support PTEN as a colon cancer susceptibility gene.

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**Key words:** Colon cancer; Phosphatase and tensin homolog; Candidate gene; Genetic polymorphisms; Single nucleotide polymorphism association

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## INTRODUCTION

The phosphatase and tensin homolog (PTEN) tumor suppressor gene is second only to p53 in mutation frequency in human cancers<sup>[1]</sup>. PTEN utilizes multiple mechanisms to control cellular growth, the most important of which is by inhibiting phosphoinositide 3-kinase (PI3K) activation<sup>[2]</sup>. The PI3K signaling cascade has been shown to play an important role in the development of colon tumors and other neoplasia<sup>[3-5]</sup>, suggesting that genetic variations in this pathway might confer susceptibility to colon cancer. PTEN is a likely candidate, because mutations<sup>[6-9]</sup>, deletions<sup>[10]</sup>, and loss of heterozygosity<sup>[11]</sup> in PTEN have been found in a variety of tumors. Although PTEN alterations were

## Abstract

**AIM:** To investigate the association between single nucleotide polymorphisms (SNPs) in the phosphatase and tensin homolog (PTEN) tumor suppressor gene and risk of colon cancer.

**METHODS:** We utilized a population-based case-control study of incident colon cancer individuals ( $n$

initially discovered in glioma, prostate, kidney, and breast carcinomas, more recent discoveries in gastrointestinal cancers suggest a likely association between PTEN and colorectal cancers<sup>[12-14]</sup>. PTEN expression is decreased in over 50% of colon tumors, and PTEN loss is associated with increased risk of local colon cancer recurrence<sup>[3,15]</sup>. In addition, PTEN missense and nonsense mutations occur in Cowden Disease, a hereditary cancer susceptibility syndrome involving multiple hamartomas of the gastrointestinal tract and other tissues<sup>[16,17]</sup>.

To date, three published studies have examined PTEN as a potential candidate susceptibility gene for four cancer types: breast and prostate cancer<sup>[18]</sup>, meningiomas<sup>[19]</sup>, and colorectal cancer<sup>[20]</sup>. These studies found no association with PTEN genetic polymorphisms. However, the colorectal cancer study included a relatively small number of predominantly Jewish patients and examined only two PTEN single nucleotide polymorphisms (SNPs) that did not cover the full PTEN gene region. Therefore, a more thorough investigation of PTEN and colon cancer is warranted. Here we carried out a comprehensive tagSNP association analysis to further clarify the relationship between PTEN genetic polymorphisms and the risk of colon cancer in a population-based case-control study.

## MATERIALS AND METHODS

### **Study design and data collection**

The study design, data collection and study population have been described in detail elsewhere<sup>[21]</sup>. Briefly, between 2003 and 2006, incident colon cancer cases identified from the Surveillance, Epidemiology, and End Results (SEER) Kentucky Cancer Registry (KCR) and population controls were recruited for an incident case-control of colon cancer. Histopathologically confirmed colon cancer cases were identified through regular queries of the KCR database. Random digit dialing using the same area codes and exchanges as cases was used to recruit population controls who were  $\geq 30$  years and had no personal history of cancer, with the exception of non-melanoma skin cancer. Those with known inflammatory bowel disease, a family history of familial adenomatous polyposis, or hereditary nonpolyposis colorectal cancer, were excluded. Participants provided a blood sample collected at approved medical facilities after an overnight fast. They also completed a self-administered questionnaire ([http://epi.grants.cancer.gov/CFR/about\\_questionnaires.html](http://epi.grants.cancer.gov/CFR/about_questionnaires.html)), which collected detailed information on personal and family history of colon (and other) cancers, lifestyle, and behavioral risk factors. Participation rates were 72.2% for cases and 62.5% for eligible controls. The study was approved by the Institutional Review Boards of Case Western Reserve University/University Hospitals of Cleveland, the University of Kentucky, Lexington, and the University of Southern California, Los Angeles.

### **Genotyping**

Fifty validated SNPs have been identified within the PTEN gene in HapMap (NCBI Build 36). Four tagging SNPs (rs2299939, rs12357281, rs2248293, and rs926091)

**Table 1 Descriptive characteristics of the CWRU/kentucky colon cancer genetic epidemiology study population**

	Cases (n = 421 )	Controls (n = 483)	P <sup>1</sup>
Age <sup>2</sup> (yr)	62.7 ± 10.6	57.6 ± 11.2	< 0.0001
Gender (%)			
Female	214 (50.8)	307 (63.6)	0.0001
Male	207 (49.2)	176 (36.4)	
Race (%)			
Caucasian	394 (93.6)	450 (93.2)	0.3
African-American	22 (5.2)	21 (4.4)	
Other	5 (1.2)	12 (2.5)	
BMI <sup>3,4</sup> (kg/m <sup>2</sup> )	29.2 ± 6.2	28.2 ± 6.0	< 0.0001
Family History <sup>3,5</sup>			
Yes	94 (26.8)	72 (17.1)	0.0015
No	257 (73.2)	349 (82.9)	
NSAID use <sup>3,6</sup> (%)			
Yes	235 (64.2)	306 (68.9)	0.18
No	131 (35.8)	138 (31.1)	
Physical activity <sup>3</sup> (%)			
Vigorous	165 (42.7)	247 (53.8)	0.006
Moderate	106 (27.5)	98 (21.4)	
Light	115 (29.8)	114 (24.8)	

<sup>1</sup>P-value of significance difference between cases and controls in a  $\chi^2$  test (discrete variables and genotypes) or t-test (continuous); <sup>2</sup>Age (mean ± SD) at diagnosis for cases and age at recruitment for controls; <sup>3</sup>Calculations based on cases and controls with available information; <sup>4</sup>BMI (mean ± SD); <sup>5</sup>Family history of first-degree relatives with colorectal cancer; <sup>6</sup>NSAID: Yes = either ibuprofen or aspirin use in the last 6 mo.

were selected for genotyping in our study. These tag SNPs were selected based on the following criteria: (1) minor allele frequency  $\geq 5\%$ ; (2) pair-wise  $r^2 \geq 0.8$ ; (3) spanning 5 kb upstream of the 5' end and 2 kb downstream of the 3'end of the PTEN gene. Genotyping was done using the TaqMan allelic discrimination assay with pre-designed primer/probe sets (Applied Biosystems). The failure rate for genotyping was less than 0.1%. For quality assurance, assays were repeated on 2% of random samples, with a concordance call rate of 100%.

In our analysis, in addition to age, gender, and race, we also included risk factors known to be associated with colon cancer. Body mass index (BMI) was computed by dividing self-reported weight in kilograms (kg) by height in meters squared (m<sup>2</sup>). A positive family history of colon cancer was defined as having at least one first-degree relative with colon cancer. Non-steroidal anti-inflammatory drug (NSAID) use was defined as using any NSAID at least twice a week for  $> 6$  mo. Physical activity was quantified using metabolic equivalents of energy expenditure units (METS), with light activity  $< 3.0$  METS, moderate 3-6 METS, and vigorous activity  $> 6$  METS.

### **Statistical analysis**

Unconditional logistic regression analyses were performed to test the association of each individual SNP with the risk of colon cancer. The higher frequency allele was considered the referent for each SNP. Association was assessed for dominant, additive, and recessive modes of inheritance by number of copies of the risk allele. For the dominant model, participants with at least one copy of the risk allele were coded as 1 and those with no risk allele

**Table 2** Associations of PTEN SNPs with colon cancer *n* (%)

SNP	Cases	Controls	Base model <sup>1</sup>			Full model <sup>2</sup>		
			OR	95% CI	P	OR	95% CI	P
<b>rs926091</b>								
CC	289 (69.1)	351 (72.8)	1.0			1.0		
CT	122 (29.2)	122 (25.3)	1.18	0.87-1.60	0.61	1.12	0.79-1.60	0.73
TT	7 (1.7)	9 (1.9)	1.12	0.40-3.13	0.71	0.99	0.29-3.41	0.92
<b>rs2299939</b>								
CC	270 (64.6)	325 (67.4)	1.0			1.0		
CA	134 (32.1)	147 (30.5)	1.15	0.85-1.55	0.56	1.01	0.72-1.42	0.18
AA	14 (3.3)	10 (2.1)	1.96	0.82-4.66	0.25	2.13	0.84-5.38	0.11
<b>rs2248293</b>								
TT	181 (43.3)	212 (44.0)	1.0			1.0		
TC	196 (46.9)	210 (43.6)	1.04	0.78-1.39	0.21	1.04	0.47-1.45	0.52
CC	41 (9.8)	60 (12.4)	0.77	0.48-1.22	0.23	0.86	0.51-1.46	0.51
<b>rs12357281</b>								
GG	353 (84.4)	408 (84.7)	1.0			1.0		
GC/CC <sup>3</sup>	65 (15.6)	74 (15.3)	0.93	0.64-1.37	0.98	0.99	0.65-1.53	0.98

<sup>1</sup>Base model adjusted for age, gender and race; <sup>2</sup>Full model further adjusted for BMI, family history of colorectal cancer, NSAID use, and physical activity based on 328 cases and 390 controls; <sup>3</sup>Only three cases and two controls were of genotype CC, so they were combined with the heterozygotes to ensure validity of model fit.

**Table 3** PTEN haplotype associations with colon cancer *n* (%)

Haplotype <sup>3</sup>	Frequency	Cases	Controls	Base model <sup>1</sup>			Full model <sup>2</sup>		
				OR	95% CI	P	OR	95% CI	P
A-G-T-C	0.180	146 (48.2)	157 (51.8)	1.16	0.865-1.550	0.32	1.05	0.751-1.456	0.79
C-C-T-C	0.079	53 (48.2)	57 (51.8)	0.98	0.643-1.485	0.91	1.04	0.642-1.669	0.89
C-G-C-C	0.184	88 (42.1)	121 (57.9)	0.76	0.548-1.060	0.11	0.94	0.645-1.356	0.72
C-G-C-T	0.151	71 (52.6)	64 (47.4)	1.44	0.979-2.106	0.06	1.31	0.849-2.029	0.22
C-G-T-C	0.398	58 (41.1)	83 (58.9)	0.75	0.510-1.089	0.13	0.70	0.449-1.082	0.11

<sup>1</sup>Base model adjusted for age, gender and race; <sup>2</sup>Full model further adjusted for BMI, family history of colorectal cancer, NSAID use, and physical activity based on 329 cases and 390 controls; <sup>3</sup>Five other haplotypes representing a total of six participants were removed due to rarity.

copies were coded as 0. Number of risk alleles present (0, 1, or 2) determined coding for the additive model. For the recessive model, participants with two risk alleles were coded as 1, and all others were 0.

Haplotypes and their frequencies were estimated using PROC HAPLOTYPE in SAS/Genetics version 9.1. Due to the high certainty of haplotype pairs for each individual (>98%), each haplotype was coded as 1 if estimated to be present in that individual (1 or 2 copies) and 0 otherwise. Haplotypes with a frequency < 5% in our study population were excluded from analyses due to small sample sizes. All univariate and multivariate analyses were conducted using SAS version 9.1 (SAS Institute, Cary, NC) with an  $\alpha = 0.05$  cutoff for statistical significance.

## RESULTS

Data from 904 participants (421 cases and 483 controls), of which 93% were Caucasian, were included in the analyses. Table 1 summarizes the descriptive characteristics of the study population. Cases were evenly split by gender, whereas controls were more likely to be female. A higher percentage of cases than controls reported a positive family history of colon cancer.

All SNPs were in Hardy-Weinberg equilibrium in both the case ( $P > 0.10$ ) and the control ( $P > 0.15$ )

groups. None of the four PTEN SNPs were statistically significantly associated with colon cancer when adjusted for age, gender, and race (Table 2). Further adjustment for family history, BMI, non-steroidal anti-inflammatory drug use, and physical activity did not alter the results. The additive model results are shown for the three SNPs that had sufficient numbers. The dominant model results are displayed for rs12357281, as only five participants had the rare genotype for this SNP.

Five haplotypes represented 99.2% of the variants for PTEN in this population (Table 3). Similar to the SNP results, none of the haplotypes were associated with colon cancer in the base model (adjusted for age, gender, and race) or the full model (further adjusted for BMI, family history, NSAID use, and physical activity). Odds ratios for the base model ranged from 0.75 (95% CI = 0.510-1.09) for the most frequent haplotype (C-G-T-C) to 1.44 (95% CI = 0.979-2.11) for the haplotype with the fourth highest frequency in our population (C-G-C-T).

When the above analyses were restricted to Caucasian only, the results did not substantially change (data not shown).

## DISCUSSION

Recent studies have shown changes in the PTEN

gene in colon cancer tumors, including mutations<sup>[6]</sup>, loss of heterozygosity<sup>[12]</sup>, and low or absent gene expression<sup>[15,22,23]</sup>, making it a strong candidate susceptibility gene for colon cancer. In the present study, we selected four tag SNPs covering the entire PTEN region to examine association between PTEN genetic variation and risk of colon cancer. We found no evidence for association for the individual SNPs or the haplotypes. Our results indicate that common inherited variations in PTEN are unlikely to predispose to colon cancer, despite the reported high frequency of somatic mutations of the PTEN gene in colon tumors<sup>[6,12]</sup>.

The importance of PTEN as a tumor suppressor and protector of chromosomal stability is well documented<sup>[24-28]</sup>. However, its particular function and mechanism in specific cancers is unknown. While PTEN might be necessary to prevent Akt from being phosphorylated in the PI3K pathway, its loss might not be sufficient for tumorigenesis. Recent studies suggest that mutations in other parts of the PI3K signaling pathway, such as PIK3CA and PIK3CB, might be more important in leading to tumor growth<sup>[21,23,29,30]</sup>. In addition, PTEN might be more influential in affecting local recurrence<sup>[15]</sup> or metastases<sup>[31]</sup> than primary tumors. These avenues warrant further investigation with regard to PTEN and colon cancers.

Although we only genotyped four tagging SNPs out of the total 61 (50 validated) possible SNPs on PTEN, they were spaced to cover the entire length of the gene, including both upstream and downstream regions, and the entire PTEN gene is in a single LD block for Caucasians. Analyses, excluding African Americans or other minorities, yielded similar results, indicating that population stratification is unlikely to have confounded our results. Our study has over 90% power to detect an odds ratio of 1.7 and > 80% power to detect an odds ratio of 1.5 with a type I error rate of 0.05, assuming a dominant model and a minor allele frequency of 16%.

To our knowledge, this is the first population-based study to examine PTEN genetic polymorphisms with risk of "sporadic" colon cancer. Taken together with other studies<sup>[18-20]</sup>, our results do not support PTEN as a colon cancer susceptibility gene.

## COMMENTS

### Background

Colon cancer is the third leading cause of cancer death in the United States and worldwide for men and women. Up to 30% of all colon cancer cases may be due to heritable factors, but only five percent are associated with known genes. The phosphatase and tensin homolog (PTEN) tumor suppressor gene is a likely candidate for association with colon cancer.

### Research frontiers

While the phosphoinositide 3-kinase (PI3K) signaling cascade has been shown to play an important role in the development of colon tumors, it is unclear which elements are controlling this association. PTEN controls cellular growth by inhibiting the PI3K pathway. In addition, PTEN expression is decreased in over 50% of colon tumors, and PTEN loss is associated with increased risk of local colon cancer recurrence. Therefore, PTEN is a promising candidate gene for colon cancer.

### Innovations and breakthroughs

This study has provided further insight into the role of the PTEN gene in colon cancer risk.

### Applications

In the process of identifying genetic causes of cancer, it is important to determine precisely which elements of a biologic pathway are responsible for affecting tumor suppression or development. Then, treatments and preventive measures can be tailored to those who would benefit most. The PTEN gene directly impacts a known signaling mechanism associated with colon cancer but has not been well-evaluated in a genetic association study. This study found that PTEN is not a likely colon cancer candidate gene, and future research should focus on other parts of the PI3K signaling pathway to understand its role in colon cancer risk.

### Terminology

PTEN is a gene located on chromosome 10 known to affect tumor cell growth.

### Peer review

Various genetic changes of PTEN gene in colon cancer have been described so far in the literature. In the present study, the authors examined the possibility of PTEN as susceptibility gene for sporadic colon cancer. However there was no association of individual single nucleotide polymorphisms or the haplotypes examined and therefore they concluded that common inherited variations in PTEN are unlikely to predispose to colon cancer. Although results of this study show no association of PTEN expression or susceptibility to sporadic colon cancer, and no-association results are not as attractive as positive associations, I feel they should be presented to the scientific audience in order not to create a literature bias towards publishing only studies with positive associations.

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BRIEF ARTICLES

## IL-10 and TNF- $\alpha$ promoter haplotypes are associated with childhood Crohn's disease location

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### Abstract

**AIM:** To determine the distribution and frequencies of the genotypes and haplotypes of the genes encoding for the glucocorticoid receptor (GR), the tumor necrosis factor (TNF)- $\alpha$  and the interleukin (IL)-10 in childhood Crohn's disease (CD) and to assess the impact of the corresponding DNA variants on clinical and disease phenotypes.

**METHODS:** Ten variants in GR, TNF- $\alpha$  and IL-10 were genotyped in 113 childhood CD cases and 95 healthy subjects, both of French-Canadian origin.

**RESULTS:** For the GR polymorphisms (R23K and N363S) and IL-10 variants in the 5'flanking region (-1082 G > A, -819 T > C and -592 A > C), no difference was observed in allele and genotype frequencies between CD patients and controls. At the haplotype level, we found three IL-10 haplotypes previously described in Caucasians (GCC, ACC and ATA) and three novel haplotypes only present in IBD patients. When we analyzed the haplotype distribution with the anatomical location of the disease, the GCC haplotype was associated with the colonic and the ACC haplotype with the terminal ileum location, respectively. The genotyping of five polymorphisms in the promoter

region of the TNF- $\alpha$  gene (-1031 T > C, -863 A > C, -857 T > C, -308 A > G and -238 A > G) revealed a significant overrepresentation of homozygous -1031 CC among CD patients (OR = 9.9) and an association with the colonic location. For TNF- $\alpha$ , eleven haplotypes were inferred, including two frequent ones, TCCGG and CACGG, which were significantly observed more frequently in controls and cases, respectively.

**CONCLUSION:** This is one of the first studies investigating the association between haplotype structure and disease location in a CD pediatric cohort. Our results will help to increase our understanding of the genetic determinants of childhood CD.

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**Key words:** Haplotype; Polymorphism; Crohn's disease; Glucocorticoid receptor; Interleukin-10; Tumor necrosis factor- $\alpha$

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### INTRODUCTION

Inflammatory bowel diseases (IBD) represent a complex genetic disease that is defined by at least two major disorders: Crohn's disease (CD) and ulcerative colitis. These diseases are distinct in terms of disease extension, localization, behaviour and the occurrence of extra-intestinal manifestations<sup>[1]</sup>. The incidence of IBD is bimodal, with the first peak occurring in the second and third decades of life and the second peak between the fifth and seventh decades. However, in North America, IBD also occurs during childhood or adolescence in up to 25% of patients<sup>[2]</sup>. Recent studies suggest that genetic predisposition may play an important role. For instance, the NOD2/CARD15 gene (chromosome 16)

and the IBD5 locus (chromosome 5) are associated with susceptibility to CD in adults<sup>[3]</sup>. Although initial studies suggested that the association of CARD15 with susceptibility to CD was higher for pediatric-onset disease, recent work with children suggests that this association in pediatric cases is roughly comparable to adult-onset disease. Furthermore, the association between the IBD5 locus and the risk of CD is weaker for children than for adults<sup>[3]</sup>. Despite extensive research, the etiology of childhood IBD is still unknown, but the immune-mediated chronic intestinal inflammation results from complex interactions between genes conferring susceptibility, exogenous or endogenous triggers, and environmental factors. In this study, we investigated the association between DNA variants in genes encoding for the glucocorticoid receptor (GR), the tumor necrosis factor (TNF)- $\alpha$  and the interleukin (IL)-10 and childhood CD phenotypic and clinical features. We found significant associations between IL-10 and TNF- $\alpha$  promoter haplotypes and disease location.

## MATERIALS AND METHODS

### Subjects

Incident cases of CD ( $n = 113$ ), ranging from 5 to 20 years old, were diagnosed in the Division of Gastroenterology of Ste-Justine Hospital, Montreal, Canada. The criteria for inclusion in this group were: (1) Complete clinical history; (2) Caucasians of French-Canadian origin residing in the Province of Quebec as judged by their names, languages and places of birth; (3) Availability of biological material. Patient characteristics are given in Table 1. The control group ( $n = 95$ ) was composed of unrelated healthy French-Canadian individuals who had agreed to donate their blood on an anonymous basis and to provide the information on their geographic origin, age, and gender. The targeted population was the one served by Ste-Justine Hospital, and our requests were addressed to parents and/or children that visited the Hospital for non-gastroenterological conditions.

The French-Canadian population represents a suitable model for genetic epidemiological studies because of its relative homogeneity in terms of genetics, socio-demographics and history<sup>[4,5]</sup>. The Ste-Justine's Institutional Review Board approved the research protocol and informed consent was obtained from all participating individuals and/or their parents.

### Genotyping

Genomic DNA was isolated from peripheral blood cells using standard methods (GENTRA Kit). Individuals were genotyped for TNF- $\alpha$  (-1031 C > T, -863 A > C, -857 T > C, -308 A > G, -238 A > G), IL-10 (-1082 G > A, -819 T > C, -592 A > C) and GR (R23K G > A and N363S A > G) polymorphisms by allele specific oligonucleotide (ASO) hybridization assay, as described in Labuda *et al*<sup>[6]</sup>. Briefly, primers flanking the polymorphic sites were used to amplify PCR products that were dot-blotted in duplicate on nylon membranes and assayed for the ab-

**Table 1 Characteristics of IBD patients**

Variables	CD ( $n = 94$ )
Boys	44
Girls	50
Age of onset (yr)	$11.3 \pm 3.7$
Location of disease <sup>1</sup>	
Terminal ileum	11
Ileocolonic	56
Colon	8
Upper digestive tract <sup>2</sup>	19
H-B index	$5.0 \pm 1.5$
ESR (mm/h)	$39.0 \pm 14$
Albumin (g/L)	$32.0 \pm 7$
WBC ( $\times 10^9$ /L)	$9.3 \pm 3.4$
HgB (g/L)	$110.0 \pm 14$
MCV (fL)	$76.3 \pm 7.2$
PLT ( $\times 10^9$ /L)	$468 \pm 146$
ABS polys (%)	$6.1 \pm 3$
ABS lymphs (%)	$2.0 \pm 2.1$
ALT (IU/L)	$13.0 \pm 8.9$
AST (IU/L)	$19.5 \pm 7.3$

<sup>1</sup>Vienna classification (1998); <sup>2</sup>Always associated with other location. H-B index: Harvey-Bradshaw index; ESR: Erythrocyte sedimentation rate; WBC: White blood cells; HgB: Haemoglobin; MCV: Mean corpuscular value; PLT: Platelet; ABS polys: Absolute value of neutrophils; ABS lymphs: Absolute value of lymphocytes; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; IBD: Inflammatory bowel disease; CD: Crohn's disease.

sence or presence of a specific mutation by hybridization with ASO for both alleles in parallel experiments. Samples with known genotypes were interspersed among study samples (positive controls) to ensure genotyping accuracy. For each experiment, the genotypes were read manually by two independent individuals and only concordant readings were accepted. To detect potential genotyping errors, Hardy-Weinberg equilibrium was tested. The oligonucleotides and assay conditions used for ASO are given in the supplementary materials (Table 2).

### Haplotype solving and linkage disequilibrium (LD) analysis

Haplotypes were inferred for all sites with minor allele frequency > 5% using the software PHASE, v. 2.1.1<sup>[7,8]</sup>. The LD statistics D and D' were both computed for each pair of single nucleotide polymorphisms (SNPs) with the software Arlequin v. 2.0<sup>[9]</sup>.

### Haplotype networks

The NETWORK 4.1.1.2 program was used to infer the likely genealogical history between the most frequent haplotypes using the Median-Joining (MJ) algorithm<sup>[10]</sup>. In an MJ network, circles represent distinct haplotypes and are scaled to reflect the frequency of these haplotypes. The branches connect the haplotypes and indicate the mutational steps between the haplotypes. The MJ network was generated only for IL-10 and TNF- $\alpha$  haplotypes.

### Statistical analysis

The chi-squared test was used to examine the differences in the distribution of genotypes between cases and controls.

**Table 2 Characteristic of the primers used to genotype the SNP in *TNF- $\alpha$* , *IL-10* and *GR* genes**

Locus	SNP	Amplimers	ASO probe <sup>1</sup>
TNF- $\alpha$		F 5'CTAAGGAATGGAGGGAGGGA3' R 5'CTTCTGTCTCGGTTCTCT3'	
	T-1031C		rs1799964
	C-863A		rs1800630
	C-857T		rs1799724
	G-308A		rs59729336
IL-10	G-238A		rs361525
		F 5'CACTACTAAGGCTTCTTGGG3' R 5'CTGTAGGAAGCCAGTCTCTG3'	
	G-1087A		rs1800896
	C-824T		rs1800871
GR	C-597A		rs1800872
	R23K	F 5'TGTAGGATTGATATTCACTGATG3'	rs6189
	G > A	R 5'CAAAAGTCTTCGCTGCTGG3'	
	N363S	F 5'TCCATGGTGTGAGTACCTCTGG3'	rs6195
	A > G	R 5'GACCAGGGAAGTCAGACTGCC3'	

ASO: Allele specific oligonucleotide. <sup>1</sup>Boldface characters indicate the polymorphic position.

**Table 3 Allele and genotype distribution of the GR polymorphism n (%)**

Position	Controls	CD
R23K G > A		
GG	79 (94)	106 (94)
GA	5 (6)	7 (6)
AA	-	-
G	163 (97)	219 (97)
A	5 (3)	7 (3)
N363S A > G		
AA	-	-
AG	5 (6)	4 (4)
GG	82 (94)	108 (94)
A	5 (3)	4 (2)
G	169 (97)	220 (98)

The level of significance was calculated by Fisher's exact test (two-sided). Odds ratios (OR) were given with 95% confidence intervals (CI). Log linear analysis was used to include potential confounding factors in the analysis (age and gender). All analyses were performed using the SPSS statistical package (version 11.0.1). A proportional test was done to compare the association of various genotypes and haplotypes with different anatomical disease locations.

## RESULTS

Using PCR ASO hybridization assays, we genotyped 10 SNPs in *GR*, *IL-10* and *TNF- $\alpha$*  genes in 113 CD children and 95 healthy subjects, all of French-Canadian origin. All SNPs tested were in Hardy-Weinberg equilibrium in the control group, although no homozygous genotypes were observed for the *GR* minor alleles.

**Table 4 Distribution of GR haplotypes in patients and control groups (%)**

Haplotype <sup>1</sup>	Controls (n = 174)	CD (n = 226)
GG	94	95
GA	3	2
AG	3	3

<sup>1</sup>The SNP positions within a haplotype are the following: R23K G > A, N363S A > G. n: Number of chromosomes.

For *GR*, we genotyped two SNPs in the exon 2, a G > A substitution, resulting in an amino acid change from arginine to lysine in the codon 23 (R23K), and an A > G substitution, resulting in an asparagine to serine change at codon 363 (N363S). The observed allele frequencies were consistent with those reported elsewhere for Caucasian populations<sup>[11-14]</sup>. The distribution of the alleles and genotypes did not differ between control and patient groups (supplementary materials, Table 3). Using the genotyping data, we were able to infer three haplotypes, including the major one GG (94%) and two minor ones, GA and AG, both at 3% (supplementary materials, Table 4). No significant differences were observed between the distribution of these haplotypes among cases and controls. No association was found between any *GR* genotypes and haplotypes and any clinical or phenotypic features (data not shown).

For *IL-10*, we genotyped three SNPs in the 5' flanking region of *IL-10*: -1082 G > A, -819 T > C and -592 A > C. The frequencies and the distribution of the alleles and genotypes were similar in CD patients and controls (Table 5) and similar to those reported in other Caucasian populations<sup>[15,16]</sup>. However, we found that the homozygous

**Table 5** Allele and genotype distribution of the IL-10 polymorphisms *n* (%)

Position	Controls	CD
-1087 G > A <sup>1</sup>		
GG	19 (20)	24 (22)
GA	42 (45)	50 (45)
AA	33 (35)	37 (33)
A	108 (57)	124 (56)
G	80 (43)	98 (44)
-824 T > C		
TT	7 (7)	14 (13)
TC	37 (39)	39 (35)
CC	50 (53)	64 (58)
T	51 (27)	55 (25)
C	137 (73)	167 (75)
-597 A > C		
AA	1 (1)	8 (7)
AC	37 (39)	40 (36)
CC	50 (53)	63 (57)
A	51 (27)	56 (25)
C	137 (73)	166 (75)

<sup>1</sup>The homozygous GG is significantly associated ( $P = 0.008$ ) with disease location in the colonic region.

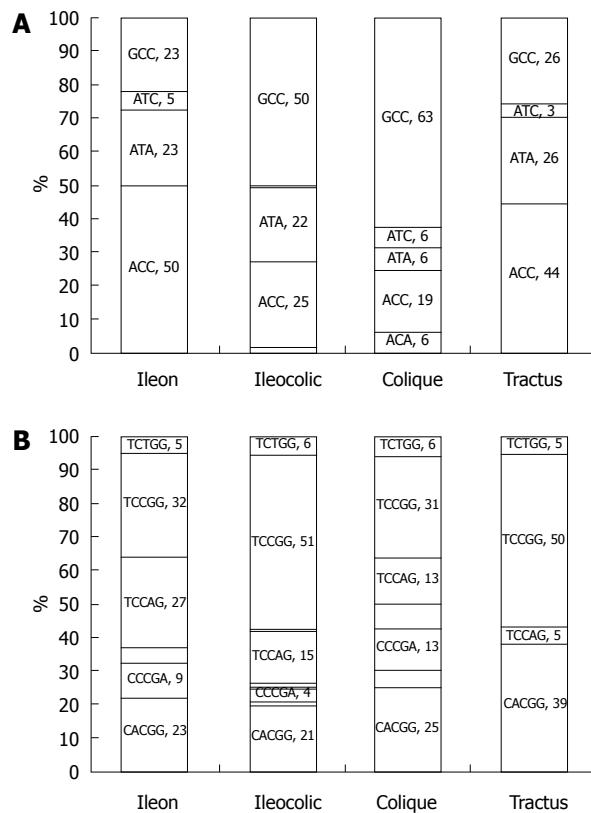
**Table 6** IL-10 haplotypes inferred in patients and control groups (%)

Haplotype <sup>1</sup>	Controls (n = 188)	CD (n = 222)
GCC	43	44
ACC	30	30
ACA	-	0.9
ATC	-	0.45
ATA	27	24

<sup>1</sup>The SNP positions within a haplotype are the following: -1082 G > A, -819 C > T, -592 C > A. *n*: Number of chromosomes.

GG genotype at the SNP -1082 G > A was significantly associated ( $P = 0.008$ ) with the colonic localization of the disease. No other significant associations were found at the genotype level. Using the genotyping data, we inferred 5 haplotypes, including 3 frequent ones and 2 rare ones, found only in CD patients (Table 6). The three most frequent haplotypes (GCC, ACC and ATA) were previously described in other Caucasian populations<sup>[15-18]</sup>. The haplotype GCC was statistically significantly associated with the ileo-colonic ( $P = 0.01$ ) and colonic ( $P = 0.01$ ) locations, whereas the haplotype ACC was significantly associated with the terminal ileum ( $P = 0.01$ ) and upper digestive tract ( $P = 0.032$ ) (Figure 1). We estimated pair-wise LD and found a strong LD ( $D' = 0.989$ ,  $r^2 = 0.93$ ) between -819 T > C and -592 C > A, but a weak LD between -1082 G > A and -819 T > C ( $D' = 1.0$ ,  $r^2 = 0.27$ ) (data not shown). These results confirmed the putative independent role for the -1082 G > A variant. Altogether, these results suggest an important role for the SNP -1082 G > A in determining disease location.

For TNF- $\alpha$  we genotyped five SNPs in the promoter region: -1031 T > C, -857 T > C, -863 A > C, -308 A > G and -238 A > G. The allelic and genotypic frequencies are shown in Table 7. The homozygous CC genotype for the



**Figure 1** Distribution of haplotypes by disease location. A: IL-10 haplotypes; B: TNF- $\alpha$  haplotypes. The percentage of each haplotype according to disease location is indicated at the right of the corresponding haplotype, as described in Tables 6 and 8.

SNP -1031 T > C was significantly overrepresented in CD patients (11%,  $P = 0.008$ ) when compared to controls (1%) suggesting an increased risk of disease (OR = 9.9, 95% CI: 1.1-78). Although not significant, we also observed differences for the SNP -863 A > C, with the allele A present in 28% of cases and 19% of controls. The -1031 CC homozygotes were associated ( $P = 0.02$ ) with the colonic region and the heterozygote CT with the terminal ileum location ( $P = 0.03$ ). For SNPs -308 A > G and -238 A > G, both homozygous GG genotypes were associated with the upper digestive tract ( $P = 0.008$  and  $P = 0.05$ , respectively), whereas the heterozygous GA was associated with the terminal ileum location ( $P = 0.008$  and  $P = 0.05$ ). Using the genotyping data, we inferred eleven haplotypes (Table 8), including 3 major ones (H1, H3 and H7). These results were similar to those reported by Bennet *et al*<sup>[19]</sup> in myocardial infarction (MI) patients. The haplotype H1 (TCCGG) was more frequent in the control group (56%), suggesting a protective effect (OR = 0.69, 95% CI: 0.49-0.99,  $P = 0.04$ ) when compared with patients (48%), whereas the haplotype H7 (CACGG) was more frequent in CD patients (25%), suggesting an increased risk effect (OR = 1.64, 95% CI: 1.05-2.56,  $P = 0.03$ ) when compared with the control group (17%). The SNPs -1031 T > C and -863 A > C might explain this effect because the other three SNPs are shared by both haplotypes. Furthermore, the haplotype H3 (TCCAG) was observed more frequently in cases with terminal

**Table 7** Allele and genotype distribution of the TNF- $\alpha$  polymorphisms  $n$  (%)

Position <sup>2</sup>	Controls	CD
-1031 C > T		
CC <sup>1</sup>	1 (1)	12 (11)
CT	39 (47)	45 (40)
TT	43 (52)	55 (49)
C	41 (25)	69 (31)
T	125 (75)	155 (69)
-863 A > C		
AA	4 (5)	10 (10)
AC	22 (28)	32 (32)
CC	52 (67)	58 (58)
A	30 (19)	52 (26)
C	126 (81)	148 (74)
-857 T > C		
TT	1 (1)	1 (1)
CT	14 (18)	17 (17)
CC	63 (80)	82 (82)
T	16 (10)	19 (10)
C	140 (90)	181 (90)
-308 A > G		
AA	1 (1)	-
AG	27 (29)	29 (26)
GG	66 (70)	83 (74)
A	29 (15)	29 (13)
G	159 (85)	195 (87)
-238 A > G		
AA	2 (2)	-
AG	7 (8)	11 (10)
GG	80 (90)	97 (90)
A	11 (6)	11 (5)
G	167 (94)	205 (95)

<sup>1</sup>The homozygous -1031 CC is associated with an increased risk of disease (OR = 9.9, 95% CI: 1.3-78,  $P$  = 0.008); <sup>2</sup>The homozygous -1031 CC is associated ( $P$  = 0.02) with the colonic region; the heterozygous -1031 CT is associated with the terminal ileum location ( $P$  = 0.03); the homozygous -308 GG ( $P$  = 0.008) and -238 GG ( $P$  = 0.05) with the upper digestive tract.

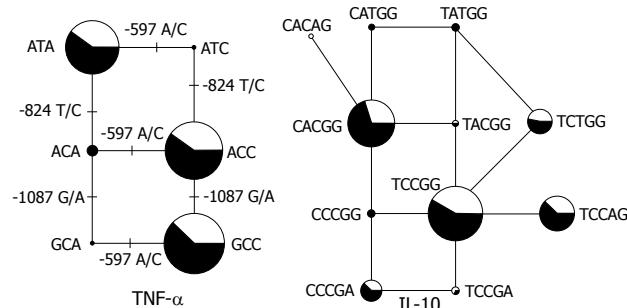
ileum localization ( $P$  = 0.015), whereas the haplotypes H6 (CCCGA) and H7 (CACGG) were overrepresented in the colonic region ( $P$  = 0.02) and in the upper digestive tract ( $P$  = 0.02), respectively (Figure 1).

To gain knowledge about the history of each SNP found in a given haplotypes and possibly about their functional impact, we built haplotype networks (Figure 2). For IL-10, the network suggests that the haplotype GCC was derived from the ancestral haplotype ACC (based on chimpanzee sequence), while the third major haplotype ATA was probably created through a recombination event involving one of the rare haplotypes (ATC or ACA) (Figure 2). The haplotype network for TNF- $\alpha$  was more complex because its promoter region of TNF- $\alpha$  underwent numerous recombination and mutation events. Indeed, five SNPs in a 1 kb region led to the inference of eleven distinct haplotypes: one major haplotype (H1) with a frequency of 56%, four intermediate ones (H3, H4, H6 and H7) with frequencies ranging from 4% to 17%, and six rare ones, with frequencies below 1% (Figure 2). The ancestral haplotype, ACCGG (based on the chimpanzee sequence), was not found, suggesting that the major haplotype (TCCGG) appeared very soon in human history. This could suggest a positive selection supporting a key role for the SNP-1031 T > C.

**Table 8** TNF- $\alpha$  haplotypes inferred in patients and control groups (%)

Haplotype <sup>1</sup>	Controls (n = 202)	CD (n = 228)
H1 TCCGG <sup>2</sup>	56	48
H2 TCCGA	1	-
H3 TCCAG	12	13
H4 TCTGG	8	7
H5 TACGG	0.5	-
H6 CCCGA	4	5
H7 CACGG <sup>3</sup>	17	25
H8 CACAG	0.5	-
H9 TATGG	-	1
H10 CCCGG	-	0.9
H11 CATGG	-	0.4

<sup>1</sup>SNP positions within a haplotype are the following: -1031 T > C, -863 C > A, -857 C > T, -308 G > A, -238 G > A; <sup>2</sup>OR = 0.69, 95% CI: 0.49-0.99; <sup>3</sup>OR = 1.64, 95% CI: 1.05-2.56. n: Number of chromosomes.



**Figure 2** TNF- $\alpha$  and IL-10 haplotype networks. The haplotype networks were drawn using the Phylogenetic Network Analysis Software NETWORK 4.1.1.2<sup>[10]</sup>. Left: IL-10 haplotypes are named as listed in Table 6; Right: TNF- $\alpha$  haplotypes are named as listed in Table 8. The area of the circle is proportional to the overall haplotype frequency, while colors indicate the distribution among CD patients (black) and controls (white). The solid lines connecting each haplotype represent single mutations occurring without recombination, and correspond to the maximum parsimony tree for this network. "ANC" designates the ancestral promoter haplotype derived from chimpanzee DNA analysis.

## DISCUSSION

In the present study, we determined the allelic and genotypic distribution of SNPs and the corresponding haplotypes in the GR, TNF- $\alpha$  and IL-10 genes among CD children and healthy controls, all of French-Canadian origin.

Genes encoding for immunoregulatory molecules clearly constitute important candidate susceptibility loci for IBD, and a number of recent studies have highlighted the central roles played by IL-10 in orchestrating the immune response. With regard to the IL-10 promoter SNPs (-1082 G > A, -819 C > T, -592 C > A), we found three promoter haplotypes (GCC, ACC and ATA), previously described in Caucasians, and two haplotypes (ACA, ATC) only present in CD children. We found that children carrying the haplotypes GCC (OR = 2.0, CI = 1.08-3.69) and ACC (OR = 2.5, CI = 1.02-6.2) were at increased risk of developing the disease at the ileo-colonic and terminal ileum locations, respectively. Conversely, a protective effect was noted when carrying haplotype GCC against the terminal ileum (OR = 0.34, CI = 0.12-0.96)

and the upper digestive tract ( $OR = 0.4$ ,  $CI = 0.17\text{--}0.91$ ) locations, while the ACC haplotype protected against the ileo-colonic location ( $OR = 0.49$ ,  $CI = 0.26\text{--}0.93$ ). These observations suggest that the SNP -1082 G > A plays an important role in determining disease location. The functional impact of this SNP has been shown in other studies: the -1082 GG genotype was the most important genetic factor in the regulation of high constitutive IL-10 expression (mRNA levels) and high IL-10 serum levels (protein basal expression)<sup>[18]</sup>; the homozygous -1082 AA was associated with decreased IL-10 production in CD patients and controls<sup>[17]</sup>; and the -1082 G > A was implicated in the production of IL-10 in whole blood samples stimulated with LPS for 24 h<sup>[18]</sup>.

TNF- $\alpha$  is a multifunctional cytokine involved in the promotion of inflammatory responses and plays a critical role in the pathogenesis of inflammatory, autoimmune and malignant diseases<sup>[20]</sup>. The functional changes in intestinal mucosa of patients with IBD lead to an increased secretion of proinflammatory cytokines in small and large bowel lamina propria. In fact, TNF- $\alpha$  levels are elevated in serum, mucosa and stool of patients with IBD and the infusion of monoclonal anti-TNF- $\alpha$  antibodies is a highly effective treatment against IBD<sup>[21]</sup>. Intestinal phagocytes or activated macrophages secrete ample amounts of TNF- $\alpha$ , which play an essential role in the pathogenesis of IBD. Variability in the capacity to produce TNF- $\alpha$  seems to be genetically determined<sup>[22]</sup>. For the TNF- $\alpha$  promoter SNPs (-1031 T > C, -863 A > C, -857 T > C, -308 A > G and -238 A > G), we found that children carrying at least one -1031 C allele were associated with the disease in certain locations, i.e. the colon in the case of the homozygous -1031 CC ( $P = 0.02$ ), and the terminal ileum in the case of the heterozygous -1031 TC ( $P = 0.03$ ). For SNPs -308 A/G and -238 A/G, both homozygous GG genotypes were associated with the upper digestive tract ( $P = 0.008$  and  $P = 0.05$ , respectively), whereas the heterozygous GA ( $P = 0.008$  and  $P = 0.05$ , respectively) was associated with the terminal ileum location. The promoter allele -308 A was reported to be associated with greater TNF- $\alpha$  transcription *in vitro*<sup>[23]</sup>. There are some studies confirming the association between the SNP -308 G > A and susceptibility to IBD<sup>[24,25]</sup> and others showing no association between any of the SNPs tested (-308 A > G, -857 T > C and -238 A > G) and IBD<sup>[16,26,27]</sup>. Furthermore, among the eleven TNF- $\alpha$  promoter haplotypes resolved, TCCGG was more frequent in the control group whereas CACGG was overrepresented in CD children. Also the CACGG haplotype increased the risk of developing the disease in the upper digestive tract ( $OR = 2.4$ ,  $CI = 1.12\text{--}5.1$ ).

In several inflammatory diseases, variations in glucocorticoid sensitivity have been reported to be associated with SNPs in the GR gene<sup>[13,28-30]</sup>. In this study we found no evidence of independent involvement of GR allelic variants (R23K and N363S) and CD phenotypic and clinical parameters, despite the reported correlations of the variants N363S with glucocorticoid hypersensitivity<sup>[13]</sup> and R23K with decreased response to

dexamethasone<sup>[29]</sup>.

In conclusion, this is one of the first studies assessing the impact of candidate gene haplotypes and disease location in childhood CD. The fact that certain associations were observed for given haplotypes rather than at individual SNPs reflects the benefit of haplotype-based analysis. Our results highlight the importance of knowing the haplotype structure because haplotypes potentially capture more genetic diversity than single-locus genotypes and therefore serve as better hallmarks to understand complex traits. Finally, one of the limitations of studying pediatric CD is that a relative small number of patients are available for study. Because of the small sample size, this report should be considered as exploratory and further studies are required to confirm these genetic associations with childhood CD.

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## COMMENTS

### Background

Crohn's disease (CD) is a chronic inflammatory bowel disease that affects more than half a million North Americans. The deregulated expression of the glucocorticoid receptor (GR) and the cytokines interleukin (IL)-10 and tumor necrosis factor (TNF)- $\alpha$  plays a crucial role in the pathogenesis of inflammatory diseases. Single nucleotide polymorphisms (SNPs) in the promoter regions of TNF- $\alpha$ , IL-10 and GR genes may influence the expression of these genes, thereby modulating the susceptibility to CD. This study assesses the impact of specific SNPs and haplotypes on the risk of developing childhood CD.

### Research frontiers

Some variants in the promoter region of genes may affect either the expression or activity levels of proteins and therefore may be mechanistically associated with CD risk. Although several gene loci have been associated with susceptibility to CD in adults, the etiology of childhood CD is still unknown. The current study is one of the first studies assessing the impact of candidate gene's haplotypes and disease location in childhood CD. Furthermore, it highlights the importance of haplotype structures as better hallmarks than single-locus phenotypes to understand the genetics underlying complex traits.

### Innovations and breakthroughs

It is important to investigate the genetic variation in susceptibility to CD and identify markers that will facilitate identification of individuals at risk of developing this disease. Although no significant association was found between GR (R23K and N363S) polymorphisms and risk of CD, TNF- $\alpha$  promoter SNPs and IL-10 and TNF- $\alpha$  promoter haplotypes were overrepresented in children with CD and were associated with specific disease location.

### Applications

This is one of the first studies investigating the association between haplotype structure and disease location in a CD pediatric cohort. The results of this study will help us to further understand the genetic determinants of childhood CD. Future studies in larger pediatric cohorts and further analysis of the biological function of the identified variants are required to understand the role of TNF- $\alpha$  and IL-10 polymorphisms and related haplotypes in determining the risk of CD.

### Peer review

The distribution and frequencies of the genotypes and haplotypes of the genes encoding for TNF- $\alpha$ , IL-10 and GR and the impact of the corresponding variants on risk of CD in a childhood French-Canadian cohort was studied. The results show that polymorphisms in the TNF- $\alpha$  promoter region as well as specific TNF- $\alpha$  and IL-10 haplotypes are associated with the risk of CD, at least in this study population.

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# Superiority of split dose midazolam as conscious sedation for outpatient colonoscopy

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reduction in procedure-related pain and memory, but resulted in longer recovery time.

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**Key words:** Amnesia; Colonoscopy; Conscious sedation; Midazolam

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Lee H, Kim JH. Superiority of split dose midazolam as conscious sedation for outpatient colonoscopy. *World J Gastroenterol* 2009; 15(30): 3783-3787 Available from: URL: <http://www.wjgnet.com/1007-9327/15/3783.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.3783>

## Abstract

**AIM:** To elucidate the efficacy and safety of a split dose of midazolam in combination with meperidine for colonoscopy.

**METHODS:** Eighty subjects undergoing outpatient colonoscopy were randomly assigned to group A or B. Group A ( $n = 40$ ) received a split dose of midazolam in combination with meperidine. Group B ( $n = 40$ ) received a single dose of midazolam in combination with meperidine. Outcome measurements were level of sedation, duration of sedation and recovery, degree of pain and satisfaction, procedure-related memory, controllability, and adverse events.

**RESULTS:** Group A had a lower frequency of significant hypoxemia ( $P = 0.043$ ) and a higher sedation score on withdrawal of the endoscope from the descending colon than group B ( $P = 0.043$ ). Group B recovered from sedation slightly sooner than group A ( $P < 0.002$ ). Scores for pain and memory, except insertion-related memory, were lower in group A one week after colonoscopic examination ( $P = 0.018$  and  $P < 0.030$ , respectively). Poor patient controllability was noted by the endoscopist and nurse in group B ( $P = 0.038$  and  $P = 0.032$ , respectively).

**CONCLUSION:** Split dose midazolam in combination with meperidine resulted in a safer, more equitable sedation status during colonoscopic examination and a

## INTRODUCTION

Colonoscopy is one of the most commonly performed medical procedures worldwide. Although recent advances in endoscopy have improved endoscope imagery and flexibility, colonoscopy remains a difficult and lengthy endoscopic procedure that causes the patient considerable discomfort or pain. Most gastroenterologists use moderate sedation for colonoscopy. Under deep sedation, patients may develop inadequate spontaneous ventilation and may require assistance to maintain a patent airway. In addition, constant repositioning of such patients because of their poor cooperation may exhaust nurses or assistants<sup>[1-7]</sup>.

Although several agents are available for inducing moderate sedation, the combination of a benzodiazepine and an opiate (midazolam and meperidine) is most commonly used<sup>[3,7-9]</sup>. The use of propofol is increasing in clinical practices because practitioners believe that it shortens the duration of sedation and recovery<sup>[10-15]</sup>. However, many endoscopic units, especially in Korea, are hesitant to use this agent because it frequently results in deep sedation. Moreover, it is doubtful whether it is suitable for colonoscopic procedures because of poor patient control<sup>[2,4,16]</sup>. A modified administration of a conventional combination of sedatives such as midazolam and meperidine may be safer and have a more positive outcome than propofol, especially for colonoscopic procedures, the duration of which

is greater and more variable than that of upper gastrointestinal endoscopy.

The objective of this study was to compare the efficacy and safety of split dose midazolam in combination with meperidine with conventional single dose midazolam in combination with meperidine.

## MATERIALS AND METHODS

### **Patients**

This study was a randomized, controlled clinical trial and involved 80 consecutive outpatients who presented for colonoscopy at the Armed Forces Capital Hospital in Korea. Patients were considered eligible to participate if they were 18 years of age or older and were scheduled for colonoscopy only. Associated medical illnesses were graded according to the American Society of Anesthesiologists' Physical Status Classification (ASA grade). Exclusion criteria included ASA risk Class 3 or higher, history of a colonic surgical procedure, inpatient status, chronic use of benzodiazepines or opiates, sleep apnea, liver cirrhosis, pregnancy, willingness to undergo unsedated colonoscopy, and allergies to soybeans or eggs. A total of 86 patients were invited to participate. Six of these patients were excluded because the colonoscopy was aborted due to poor preparation. Thus, 80 patients gave their informed consent and completed the study. All colonoscopies were performed by one gastroenterologist who had 6 years of colonoscopy experience. The sedative agents were administered by one nurse. The study was blinded to the endoscopist and recovery nurse, but not to the sedating nurse. Sedation endpoints were drowsiness, facial relaxation, slurred speech, and tolerance to insertion of the colonoscope. Patients were randomly assigned to one of the two groups for sedation according to a table of random numbers. All patients received 50 mg of meperidine intravenously prior to administration of midazolam. Patients in group A (split dose midazolam and meperidine) initially received 2.5 mg (< 70 kg b.w.) or 3 mg (> 70 kg b.w.) of midazolam intravenously. A second dose of midazolam (< 70 kg b.w., 1.5 mg; > 70 kg b.w., 2 mg) was administered immediately after ileal intubation. Patients in group B (single dose midazolam and meperidine) initially received 4 mg (< 70 kg b.w.) or 5 mg (> 70 kg b.w.) of midazolam intravenously. Placebo administration of saline was performed after ileal intubation in group B. Supplemental doses of midazolam or meperidine were not administered to ensure that consistent doses were used during the trial. The times at which sedation and colonoscopic insertion were initiated, the distal ileum was intubated, and the colonoscope was withdrawn from the anus were recorded.

### **Assessment of safety**

The endoscopist and registered nurse who administered the sedative agent were certified in advanced cardiac life support. An endoscopy technician was present to assist the endoscopist with technical maneuvers. All patients were continuously monitored for heart rate

(three-lead electrocardiogram), oxygen saturation (pulse oximetry), and mean arterial blood pressure (serial blood pressure measurements every 5 min). Significant oxygen desaturation was defined as an oxygen saturation of less than 90% for more than 15 s. Supplemental oxygen (2 L/min via a nasal cannula) was administered for any episode in which  $\text{SaO}_2$  was less than 85% or oxygen saturation was less than 90% on three separate occasions. Oxygen desaturation and the need for supplemental oxygen were recorded as significant outcomes. Heart rate was monitored continuously using pulse oximetry and bradycardia was defined as a heart rate of less than 60 beats per minute or a heart rate 25% below baseline. Blood pressure was measured before and after the procedure.

### **Assessment of sedative efficacy and recovery**

After sedation was initiated, the assistant responsible for measuring sedation level was brought into the room. The assistant was unaware of the protocol concerning delivery of the sedative. The degree of sedation was assessed using a sedation score (5 = not arousable, 4 = arousable to stimuli, 3 = arousable to command, 2 = drowsy, 1 = awake). The sedation score was recorded twice for each patient: on advancing the endoscope into the descending colon and on withdrawing it from the descending colon. Patients were transferred to the recovery area immediately after the procedure if their vital signs were stable. The time taken to recover from sedation was assessed using a modified Aldrete score at 5, 10, and 30 min after the procedure. The modified Aldrete score is an established postanesthetic recovery score that takes into account respiration and circulation parameters, and patient consciousness and activity<sup>[17]</sup>. The score ranges from 0 to 10, the latter indicating that the patient is fit for discharge.

### **Patient assessment**

When the patients were contacted by phone or visited one week after the procedure, they were asked to fill out a questionnaire using a 10-point visual analog scale to grade the amount of pain, overall satisfaction, and memory of specific aspects of the procedure (insertion of the colonoscope, withdrawal of the colonoscope, and intentional position changes on ileal intubation). This was conducted by an investigator blinded to the protocol of study.

### **Endoscopist and assistant nurse assessment**

The endoscopist's and assistant nurse's evaluations of the patient's controllability were obtained immediately after the procedure using a 10-point visual analog scale.

### **Statistical analysis**

On the basis of a previous study<sup>[18]</sup>, assuming that the split dose group will have a mean procedure-related pain score of 8.0/10.0 with a 1% reduction in pain, a sample size of 37 per group was required to detect the difference with a power of 90% and a type I error of 0.05 (PS Power and Sample Size Calculations, Version

**Table 1** Clinical characteristics of patients (mean  $\pm$  SD)

	<b>Group A (n = 40)</b>	<b>Group B (n = 40)</b>	<b>P value</b>
Age (yr)	31.33 $\pm$ 9.76	31.23 $\pm$ 9.13	0.962
Gender (M/F)	34/6	36/4	0.737
Body mass index	23.80 $\pm$ 3.40	23.43 $\pm$ 3.78	0.642
No. of patients with previous colonoscopy (%)	11 (27.5%)	10 (25.0%)	0.779
Indication for colonoscopy n (%)			
Change in bowel habit	9 (22.5)	10 (25.0)	
Abdominal pain	10 (25.0)	12 (30.0)	
Rectal bleeding	6 (15.0)	7 (17.5)	0.527
Polyp surveillance	13 (32.5)	9 (22.5)	
Anemia	2 (5.0)	2 (5.0)	
Time from onset of sedation to scope (min)	1.18 $\pm$ 0.50	0.89 $\pm$ 0.31	0.003
Total procedure time (min)	14.80 $\pm$ 3.80	14.61 $\pm$ 3.83	0.824
Time to ileal intubation (min)	7.49 $\pm$ 3.24	8.21 $\pm$ 3.37	0.328

**Table 3** Recovery rate from sedation state (mean  $\pm$  SD)

	<b>Group A (n = 40)</b>	<b>Group B (n = 40)</b>	<b>P value</b>
Aldrete score (5 min)	4.28 $\pm$ 1.06	5.75 $\pm$ 0.95	< 0.001
Aldrete score (10 min)	6.55 $\pm$ 0.50	7.00 $\pm$ 0.68	0.001
Aldrete score (30 min)	8.73 $\pm$ 0.85	9.32 $\pm$ 0.69	0.001
Time from scope out to discharge	37.05 $\pm$ 6.87	33.75 $\pm$ 5.75	0.022

3.0; Biostatistics Department, Vanderbilt University, Nashville, TN). Descriptive data are presented as the number of patients (%) or as the mean  $\pm$  SD. Continuous data were compared using the unpaired Student's *t* test, and categorical variables were tested using the corrected chi-square method. The criterion for statistical significance was  $P < 0.05$ .

## RESULTS

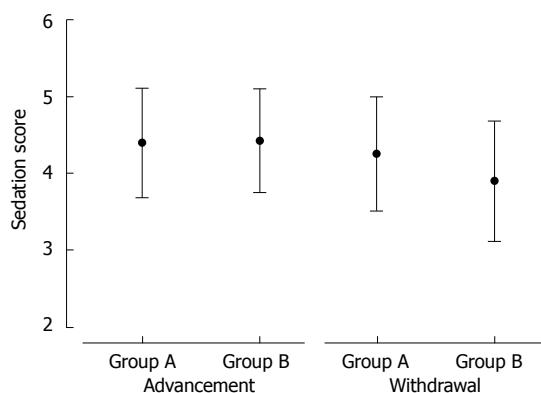
Demographic data and procedure indications for the subjects are presented in Table 1. There were no significant differences among the groups in age, gender, body mass index, or procedure indication. Patients who received single dose midazolam had a significantly shorter time from onset of sedation to initiation of endoscopy than those who received split dose midazolam ( $P = 0.003$ ,  $0.89 \pm 0.31$  vs  $1.18 \pm 0.49$ ). There was no difference in the total duration of the procedure and the time of ileal intubation between patients in both groups. The frequency of a significant drop in oxygen saturation was higher in group B than in group A ( $P = 0.043$ , 20% vs 5%). There was no difference between the groups in terms of a significant drop in mean arterial blood pressure or a significant alteration in heart rate (Table 2). Reversal agents were needed in two cases. However, no patient experienced severe cardiopulmonary complications. There was a significant difference between the sedation scores in group A and B at the time at which the scope was advanced into the descending colon. However, the sedation score in group A was significantly higher than that in group B on withdrawal of the endoscope from the

**Table 2** Safety of sedation with split dose midazolam and meperidine vs single dose midazolam and meperidine (mean  $\pm$  SD)

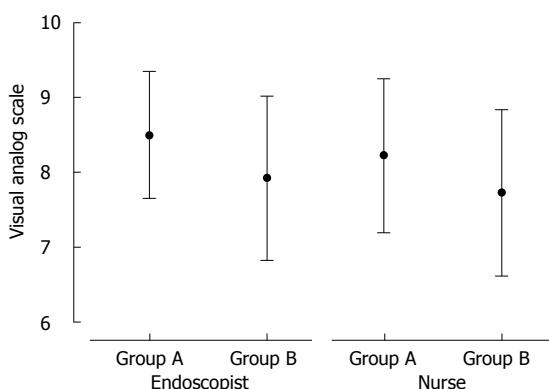
	<b>Group A (n = 40)</b>	<b>Group B (n = 40)</b>	<b>P value</b>
Baseline systolic BP (mmHg)	125.20 $\pm$ 9.76	125.40 $\pm$ 10.15	0.929
Baseline pulse rate (/min)	71.90 $\pm$ 9.71	72.63 $\pm$ 8.99	0.730
Baseline oxygen saturation (%)	97.88 $\pm$ 1.98	97.95 $\pm$ 2.04	0.868
Drop in blood pressure	1 (2.5%)	2 (5.0%)	0.556
Alteration in heart rate	3 (7.5%)	2 (5.0%)	0.745
Drop in oxygen saturation	2 (5.0%)	8 (20.0%)	0.043

**Table 4** Visual analog scale for pain, memory, and satisfaction reported by patients

	<b>Group A (n = 40)</b>	<b>Group B (n = 40)</b>	<b>P value</b>
Pain	0.95 $\pm$ 1.15	1.58 $\pm$ 1.15	0.018
Memory			
On insertion	0.80 $\pm$ 0.82	1.08 $\pm$ 0.89	0.155
On ileal intubation	1.38 $\pm$ 1.21	1.93 $\pm$ 0.94	0.026
On scope out	0.93 $\pm$ 1.02	1.58 $\pm$ 1.38	0.019
Satisfaction	8.93 $\pm$ 1.07	8.68 $\pm$ 1.09	0.305

**Figure 1** Comparison of sedation score on the advancement or withdrawal of the colonoscope.

descending colon ( $4.25 \pm 0.74$  vs  $3.9 \pm 0.78$ ,  $P = 0.043$ ) (Figure 1). Table 3 shows recovery status according to the modified Aldrete score. Recovery from sedation was slightly sooner for group B than for group A, 5 min ( $4.28 \pm 1.06$  vs  $5.75 \pm 0.95$ , respectively,  $P < 0.001$ ), 10 min ( $6.55 \pm 0.50$  vs  $7.00 \pm 0.68$ , respectively,  $P < 0.001$ ), and 30 min ( $8.73 \pm 0.85$  vs  $9.32 \pm 0.69$ , respectively,  $P < 0.001$ ) after the procedure. Moreover, the interval from removal of the endoscope to discharge was longer for group A than group B ( $37.1 \pm 6.87$  min vs  $33.8 \pm 5.75$  min, respectively,  $P = 0.022$ ). The patients' recollection of pain during colonoscopy and satisfaction with the procedure at one week after the procedure are shown in Table 4. One week after the colonoscopic examination, all scores for pain and memory except for insertion-related memory were lower for group A than for group B. The endoscopist and the assistant nurse reported that group B was more difficult to control during the procedure than group A ( $P = 0.038$  and  $P = 0.032$ , respectively) (Figure 2).



**Figure 2** Visual analog scale on patient controllability reported by the endoscopist and assistant nurse.

## DISCUSSION

This study is the first to compare the safety and efficacy of split dose midazolam in combination with meperidine with that of single dose midazolam in combination with meperidine for conscious sedation during colonoscopy. Although propofol is used increasingly during routine colonoscopy, nonanesthesiologists are hesitant to use propofol because of its known potential to cause transient apnea or general anesthesia, for which there is no reversal agent<sup>[2,4,19]</sup>.

There is a widespread belief among endoscopists that, for colonoscopy, sedation with the combination of a benzodiazepine and a narcotic is superior to sedation with either agent alone. A recent uncontrolled prospective study of a cohort of patients sedated using a combination of midazolam and meperidine reported that unintended deep sedation occurred in 11% of colonoscopies<sup>[11]</sup>. Our aim was to identify a practical and alternative sedation method based on the combination of a benzodiazepine and meperidine. Most endoscopists prescribe single dose midazolam at the time of initiating the colonoscopy without adequately monitoring the patient's sedative status or the amount of additional sedative administered. Endoscopists routinely prescribe additional administration of a sedative when patients are severely agitated or experience pain, but there are no coherent guidelines for this practice. Moreover, the small painful response of patients during the procedure tends to be neglected by endoscopic unit staff. This randomized study was designed to evaluate the promising effect of a divided dose of midazolam on sedation status.

Midazolam is known to produce retrograde amnesia as well as anterograde amnesia<sup>[20]</sup>. Because of the long duration of colonoscopic procedures, it is thought that single dose midazolam administration results in uneven distribution of the sedative effect during the procedure. Moreover, administration of a single full dose of midazolam is associated with the risk of hypoxemia and paradoxical reactions such as hostility, rage, and physical violence, especially during the initial part of the procedure. For these reasons, an alternative method is required to ensure adequate

moderate sedation for the duration of colonoscopy. Our study showed that the sedative efficacy of the split dose was superior to that of the single dose at the time of withdrawal of the endoscope. Although there was no serious cardiopulmonary depression in either group, the split dose reduced the frequency of a drop in oxygen saturation, unlike the single dose. Split dose midazolam treatment is a stable, sustainable, and safe sedation method for colonoscopy. Unfortunately, the post endoscopic sedation-related recovery of the split dose group was delayed according to the Aldrete score. It seems inevitable that additional administration of sedative will affect the recovery of patients.

The split dose method induced sleep by the end of the colonoscopic procedure and was associated with loss of memory of the events that occurred during colonoscopy 1 wk later. It seems that split dose midazolam administration and the subsequent post endoscopic sleep played a role in the amnesia. This should be considered for moderate sedation even if the main concern is to facilitate rapid recovery to ensure fast turnover at the endoscopic unit.

Initial excessive sedation to ensure easy patient control during colonoscopy seems to be undesirable at the time of advancement of the endoscope. Our study suggests that less sedation should be administered during advancement of the endoscope and more sedation with the moderate agent should be administered during endoscope withdrawal. Regrettably, the satisfaction score in our study was not different between the groups. This seems to have been caused by an inadequate number of subjects because the sampling size was calculated on the basis of pain score in a previous report. Hence, further study with a larger number of subjects should be considered.

In summary, our study shows that a split dose of midazolam in combination with meperidine is superior to a single dose of midazolam in combination with meperidine with respect to safety, equable sedation status, procedure-related pain, and unpleasant memory, but results in a longer recovery time.

## COMMENTS

### Background

For conscious sedation during colonoscopy, the modified administration of a conventional sedative combination such as midazolam and meperidine may be safer and have a more positive outcome than propofol.

### Research frontiers

Researchers assessed the efficacy of various premedications such as propofol, midazolam, meperidine or fentanyl on moderate sedation during the colonoscopic procedure.

### Innovations and breakthroughs

Split dose midazolam in combination with meperidine led to a lower frequency of significant hypoxemia and a higher sedation score on withdrawal of the colonoscope. One week after colonoscopic examination, scores for pain and memory were lower in patients who received split dose midazolam compared to those who received single dose midazolam.

### Applications

A split dose of midazolam in combination with meperidine is superior to a single dose of midazolam in combination with meperidine with respect to safety, equable sedation status, procedure-related pain, and unpleasant memory.

**Peer review**

This is a randomized study to compare the safety and efficacy of split dose midazolam in combination with meperidine with that of single dose midazolam in combination with meperidine for conscious sedation during colonoscopy. This manuscript suggested that split dose midazolam treatment is a stable, sustainable, and safe sedation method for colonoscopy compared with the administration of single dose midazolam.

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S-Editor Tian L L-Editor Webster JR E-Editor Yin DH



BRIEF ARTICLES

## Acute transient hepatocellular injury in cholelithiasis and cholecystitis without evidence of choledocholithiasis

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in the group undergoing urgent surgery (total 74 patients), the 15 patients with a gangrenous gallbladder had a higher mean level of total bilirubin ( $1.14 \pm 1.27 \text{ mg/dL}$  vs  $2.66 \pm 1.97 \text{ mg/dL}$ ,  $P < 0.001$ ) and white cell count ( $9480 \pm 4681/\mu\text{L}$  vs  $12840 \pm 5273/\mu\text{L}$ ,  $P = 0.018$ ).

**CONCLUSION:** Acute hepatocellular injury in cholelithiasis and cholecystitis without choledocholithiasis is mild and transient. Hyperbilirubinemia and leukocytosis may predict severe inflammatory changes in the gallbladder.

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**Key words:** Acute transient hepatitis; Cholelithiasis; Cholecystitis; Hyperbilirubinemia; Leukocytosis

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Chang CW, Chang WH, Lin CC, Chu CH, Wang TE, Shih SC. Acute transient hepatocellular injury in cholelithiasis and cholecystitis without evidence of choledocholithiasis. *World J Gastroenterol* 2009; 15(30): 3788-3792 Available from: URL: <http://www.wjgnet.com/1007-9327/15/3788.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.3788>

### Abstract

**AIM:** To investigate acute transient hepatocellular injury in patients with cholelithiasis and cholecystitis but no evidence of choledocholithiasis.

**METHODS:** The medical records of patients with cholelithiasis who underwent cholecystectomy between July 2003 and June 2007 were retrospectively reviewed. Imaging studies to detect common bile duct (CBD) stones were performed in 186 patients, who constituted the study population. Biochemical liver tests before and after surgery, and with the presence or absence of CBD stones were analyzed.

**RESULTS:** In 96 patients with cholelithiasis and cholecystitis without evidence of CBD stones, 49 (51.0%) had an alanine aminotransferase level elevated to 2-3 times the upper limit of normal, and 40 (41.2%) had an elevated aspartate aminotransferase level. Similar manifestations of hepatocellular injury were, as would be expected, even more obvious in the 90 patients with CBD stones. These markers of hepatocellular injury resolved almost completely within 2 wk to 1 mo after cholecystectomy. Compared to 59 patients with histologically less severe cholecystitis

### INTRODUCTION

Although viral hepatitis is quite prevalent in Taiwan, viral infection is by no means the only cause of acute hepatitis<sup>[1]</sup>. One setting in which there may be acute inflammation of the liver parenchyma is the presence of common bile duct (CBD) stones and cholangitis, situations in which liver tests have been widely studied<sup>[2-4]</sup>. We have found few investigations, however, of biochemical evidence of hepatocellular injury (hepatitis) in patients with cholelithiasis and cholecystitis who do not have CBD stones.

Hepatocellular injury is indicated by increases in serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT). The severity of such injury may be reflected in decreased hepatic production of

certain products such as albumin and clotting factors. Inflammation or damage to the biliary tract is signaled by an increase in alkaline phosphatase (ALK-P), which is sometimes confirmed by measuring gamma-glutamyltransferase. Serum bilirubin levels may be elevated in both biliary tract and hepatocellular diseases. Discussion of this topic is therefore often confusing when the general term is used rather than the names of particular biochemical markers of interest.

We designed this study to address the specific question of how often hepatocellular injury occurs in patients with cholelithiasis and cholecystitis in the absence of CBD stones, as opposed to cases where CBD stones/obstruction were found. We also wanted to see if there was any correlation between the degree of hepatocellular injury and the severity of the cholecystitis.

## MATERIALS AND METHODS

We retrospectively reviewed the medical records of patients admitted to Mackay Memorial Hospital, Taipei, from July 2003 to June 2007 who underwent cholecystectomy for cholelithiasis associated with cholecystitis (acute or chronic). Of 1168 such records, we retrieved those of patients who had undergone either preoperative endoscopic retrograde cholangiopancreatography (ERCP) or magnetic resonance cholangiopancreatography (MRCP) or intra-operative cholangiography. The imaging procedures were done to determine whether CBD stones were present in patients who had either sonographic evidence of a dilated CBD and/or biochemical evidence suggesting biliary tract obstruction. The cholecystectomized patients who had biochemical or sonographic evidence suspicion of a CBD disorder but refused to undergo invasive imaging such as ERCP, were not included in the study. Patients with viral hepatitis (that is, hepatitis A, hepatitis B or C virus infection), alcoholic liver disease, drug-related hepatitis, metabolic liver disease, autoimmune hepatitis, pancreatitis, or a previous history of ERCP with endoscopic sphincterotomy for removal of CBD stones were excluded. The liver tests analyzed for this study included AST, ALT, ALK-P, and total and direct bilirubin. The peripheral leukocyte count was also recorded. All the patients had stones in the gallbladder as demonstrated by sonography, and also in the operative specimens. Cholecystitis was diagnosed and its severity graded on the basis of the pathology examination as either gangrenous or simple cholecystitis.

### Statistical analysis

The Student *t*-test was used to analyze differences between groups. We compared the liver tests (including AST, ALT, ALK-P, direct bilirubin and total bilirubin) in patients with cholelithiasis or cholecystitis who underwent cholecystectomy with or without CBD stones. We also compared the liver tests in patients with a histological diagnosis of gangrenous vs simple cholecystitis, but only in those without evidence of CBD stones.  $P < 0.05$  was considered significant.

## RESULTS

Of all 1168 records, 186 patients (92 males, 94 females) met the study inclusion criteria. The number of patients with cholelithiasis and abnormal liver function tests was more than 186. However, as the aim of our study was to ensure the status of the CBD, we only included patients whose CBDs were evaluated using imaging procedures. In the included 186 patients, a total of 226 imaging procedures for evaluation of CBD were performed, with 114 instances of ERCP, three of MRCP, and 109 of intra-operative cholangiography. CBD stones were found in 90 patients. It was very remarkable that few MRCPs were done in comparison to ERCPs and intra-operative cholangiographies. It was because of a limitation of the medical insurance system in Taiwan. Cholecystectomy was performed urgently in 154 patients and electively in 32 (Table 1). The method of cholecystectomy was laparoscopic in 142 and conventional in 44. In patients who underwent an urgent procedure, acute right upper quadrant or epigastric pain had been present for no more than 2 d prior to surgery. Those undergoing elective surgery had had at least one recent episode of pain typical of biliary tract disease and had been deemed to have symptomatic cholelithiasis.

Most of the liver tests were abnormal, not only in patients with stones both in the gallbladder and the CBD, but also in patients with stones limited to the gallbladder. However, the mean values in patients with CBD stones were significantly higher than those in patients without evidence of CBD stones. Of the 96 patients without evidence CBD stones, 49 (51.04%) had an elevated ALT, averaging 2-3 times the upper limit of normal. A slightly lower proportion (41.67%) also had an elevated AST. The highest AST was 670 IU/dL and the highest was ALT 566 IU/dL. Mild elevations of serum total bilirubin and ALK-P were also present in 27% and 23%, respectively, of these patients (Table 2). Among these 96 patients, 82 patients were seen for follow-up 2 wk to 1 mo after cholecystectomy, by which time the mean values of the liver tests had almost returned to normal, with only the mean ALT still slightly elevated, but already much lower than the preoperative level (Table 3).

There were 74 patients who had cholelithiasis and cholecystitis without evidence of CBD stones and who underwent urgent cholecystectomy. The gallbladder histology revealed gangrenous cholecystitis in 15 patients. The only significant differences between that group and the 59 patients with simple cholecystitis were a higher bilirubin level ( $1.14 \pm 1.27$  mg/dL vs  $2.66 \pm 1.97$  mg/dL,  $P < 0.001$ ) and white cell count ( $9480 \pm 4681/\mu\text{L}$  vs  $12840 \pm 5273/\mu\text{L}$ ,  $P = 0.018$ ). The aminotransferase and ALK-P levels did not differ significantly between the two groups (Table 4).

## DISCUSSION

Hepatocellular injury in patients with cholecystitis is usually attributed to injury related to CBD stones. However,

**Table 1 Basic data of 186 patients with cholelithiasis and cholecystitis who underwent cholecystectomy (mean  $\pm$  SD)**

<b>Cholecystectomy</b>	<b>CBD stones</b>	<b>Patients (M/F)</b>	<b>Age (yr)</b>	<b>ERCP</b>	<b>MRCP</b>	<b>IOC</b>
Urgent	Yes	80 (43/37)	63.8 $\pm$ 16.2	47	3	61
	No	74 (33/41)	57.9 $\pm$ 15.9	42	0	34
Elective	Yes	10 (7/3)	59.8 $\pm$ 11.1	9	0	6
	No	22 (9/13)	58.2 $\pm$ 15.4	16	0	8
Total		186	60.6 $\pm$ 15.9	114	3	109

CBD: Common bile duct; ERCP: Endoscopic retrograde cholangiopancreatography; MRCP: Magnetic resonance cholangiopancreatography; IOC: Intra-operative cholangiography.

**Table 2 Liver tests in patients with cholelithiasis and cholecystitis who underwent cholecystectomy**

<b>Variable (normal range)</b>	<b>CBD stones (90)</b>		<b>No CBD stones (96)</b>		<b>P-value<sup>1</sup></b>
	<b>mean <math>\pm</math> SD (range)</b>	<b>Incidence of abnormal results</b>	<b>mean <math>\pm</math> SD (range)</b>	<b>Incidence of abnormal results</b>	
AST (5-35 IU/L)	137.2 $\pm$ 131.8 (19-693)	84.40% (76/90)	62.9 $\pm$ 96.9 (10-670)	41.67% (40/96)	< 0.001
ALT (5-30 IU/L)	178.7 $\pm$ 159.0 (17-696)	95.55% (86/90)	83.8 $\pm$ 109.5 (12-566)	51.04% (49/96)	< 0.001
ALK-P (40-133 IU/L)	204.7 $\pm$ 122.7 (14-672)	71.11% (64/90)	118.8 $\pm$ 92.5 (38-615)	22.92% (22/96)	< 0.001
D. Bilirubin (0.0-0.4 mg/dL)	2.16 $\pm$ 2.08 (0.1-12)	88.89% (80/90)	0.64 $\pm$ 1.01 (0-5.0)	32.29% (31/96)	< 0.001
T. Bilirubin (0.2-1.3 mg/dL)	3.81 $\pm$ 3.27 (0.3-18.6)	76.67% (69/90)	1.50 $\pm$ 1.68 (0.2-9.3)	27.08% (26/96)	< 0.001

<sup>1</sup>Statistical analysis using the Student's *t*-test. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALK-P: Alkaline phosphatase; D.: Direct; T.: Total.

**Table 3 Liver tests before and 2 wk to 1 mo after cholecystectomy in 82 patients without CBD stones (mean  $\pm$  SD)**

<b>Value (normal range)</b>	<b>Before</b>	<b>After</b>
AST (5-35 IU/L)	62.89 $\pm$ 96.85	30.95 $\pm$ 15.14
ALT (5-30 IU/L)	83.80 $\pm$ 109.50	35.95 $\pm$ 26.05
ALK-P (40-120 IU/L)	118.83 $\pm$ 92.53	118.79 $\pm$ 78.05
D. Bilirubin (0.0-0.4 mg/dL)	0.64 $\pm$ 1.00	0.37 $\pm$ 0.31
T. Bilirubin (0.2-1.3 mg/dL)	1.50 $\pm$ 1.68	0.90 $\pm$ 0.58

we have demonstrated that patients with cholecystitis and cholelithiasis but without evidence of CBD stones may also have elevated serum aminotransferase levels. Although the liver test abnormalities in such patients in our study were milder on average than in those with CBD stones, the highest ALT (566 IU/L) and total bilirubin (9.3 mg/dL) levels were still quite striking.

Pathologically, liver changes such as chronic cholestasis or portal-portal linking fibrosis are a common finding in patients with cholelithiasis with CBD stones. However, other non-specific changes in liver histology are also found in patients without CBD stones<sup>[5]</sup>. In our study, hepatocellular injury (diagnosed clinically by elevation of aminotransferases and bilirubin levels) was common regardless of whether or not CBD stones were present. The incidence was higher and the aminotransferase elevations were more marked in those with stones<sup>[6]</sup>, but a substantial proportion of patients without CBD stones also had some degree of hepatocellular injury. In most patients, the AST, ALT, and bilirubin levels had almost returned to normal 2 wk to 1 mo after cholecystectomy (Table 3). This hepatocellular injury appeared to be a transient, reactive phenomenon secondary to cholecystitis associated with cholelithiasis, whether or not there had been stones in the CBD. This kind of hepatocellular

injury, even showing relatively high serum levels of aminotransferases and bilirubin, should not be an obstacle to preparation of these patients for surgical intervention. Its activity is transient and mild. It needs no further management and will resolve spontaneously as expected, assuming the diseased gallbladder is removed.

There are two main origins of ALK-P: the liver and bone. An elevated serum ALK-P found to originate from the liver, particularly if it persists for a period of time, suggests prolonged cholestasis<sup>[7]</sup>. In our patients in whom no CBD stones were identified, the mean serum ALK-P was not significantly elevated (Table 2), implying that they had no obvious biliary obstruction before operation. In general, there are two distinct patterns of liver injury: one is hepatocellular damage demonstrated clinically by elevated levels of the aminotransferases but with a relatively normal ALK-P; the other is that of cholestasis with an ALK-P elevated out of proportion to the aminotransferases. It is not difficult to understand why hepatocellular damage, and hence elevations of the aminotransferase levels, would occur in the presence of CBD stones. Higher biliary tree pressures can lead to impaired bile secretion, retention of bile acids, and consequent apoptosis or necrosis of hepatocytes<sup>[7]</sup>. However, the exact mechanism of hepatocellular damage in cholecystitis associated with cholelithiasis but without choledocholithiasis is not fully understood. Presumably such patients have normal biliary pressure. Free radical reactions in the human gallbladder have been demonstrated to occur in cholecystitis, suggesting that oxidative stress may partially account for liver injury<sup>[8,9]</sup>. It may also be that inflammation in the gallbladder induces inflammation in the closely apposed neighboring liver tissue. However, elevation of aminotransferases could not be explained based on the proximity effect

**Table 4 Comparison of basic data between gangrenous cholecystitis and simple cholecystitis in patients who underwent urgent surgery (total 74 patients) (mean  $\pm$  SD)**

Variable	Gangrenous cholecystitis (15)	Simple cholecystitis (59)	P-value	95% CI	
				Lower	Upper
Age	64.9 $\pm$ 11.8	56.1 $\pm$ 16.4	0.053	-17.83	0.13
Hospital stay (d)	13.3 $\pm$ 5.6	11.0 $\pm$ 8.7	0.334	-7.01	2.41
AST (5-35 IU/L)	61.5 $\pm$ 74.4	72.8 $\pm$ 116.3	0.721	-51.76	74.42
ALT (5-30 IU/L)	72.3 $\pm$ 66.8	110.8 $\pm$ 187.1	0.438	-59.78	136.74
ALK-P (40-120 IU/L)	128.1 $\pm$ 68.9	103.7 $\pm$ 48.5	0.116	-55	6.19
D. Bilirubin (0.0-0.4 mg/dL)	1.23 $\pm$ 1.31	0.41 $\pm$ 0.70	0.001	-1.31	-0.33
T. Bilirubin (0.2-1.3 mg/dL)	2.66 $\pm$ 1.97	1.14 $\pm$ 1.27	< 0.001	-2.35	-0.69
WBC (3400-10000/ $\mu$ L)	12840.0 $\pm$ 5273.9	9480.2 $\pm$ 4681.3	0.018	-6128	-591.6

alone since it bears no relation to prognosis of severe acute cholecystitis<sup>[10]</sup>. On the other hand, severity of acute cholecystitis also had no significant influence on the level of serum aminotransferases<sup>[11]</sup>.

Jaundice is a common feature of patients with CBD stones<sup>[12]</sup>. In fact, it is not a rare manifestation of gallbladder disease without evidence of CBD stones; the difference is that in the latter, the serum total bilirubin level is usually less than 4 mg/dL<sup>[13,14]</sup>. In our patients without evidence of CBD stones, the mean total bilirubin level of 1.5 mg/dL was comparable with reports in the literature. However, the highest value of up to 9.3 mg/dL means that hyperbilirubinemia beyond the so-called 'mild degree' will not always suggest a complicated condition of gallstone disease.

Peng *et al*<sup>[11]</sup> documented that the severity of inflammation in cholecystitis with CBD stones did not influence the level of aminotransferases and bilirubin. However, the severity of inflammation was defined as acute attack or not. In our study, the definition of severity is based on the gallbladder histology (simple or gangrenous cholecystitis). In patients with gangrenous cholecystitis, while aminotransferase levels were not particularly elevated, the mean serum bilirubin was significantly higher than that in patients with simple cholecystitis (Table 4). Diabetes mellitus and leukocytosis have been associated in some studies with gangrenous cholecystitis, but hyperbilirubinemia has not<sup>[15-17]</sup>. However, these studies did not exclude patients with CBD stones, which may have skewed the comparison between those with and without a gangrenous gallbladder. For this comparison, we also analyzed only patients without evidence of CBD stones and found that leukocytosis as well as hyperbilirubinemia were associated with more severe, i.e. gangrenous, cholecystitis.

There could be the possibility that small stones passed through the duct during biliary colic so as to result in biochemical liver function abnormalities. Our results seemed not to favor this proposal. Firstly, ERCP (most of our patients underwent ERCP examination) is so far the most reliable (both sensitivity and specificity are over 95%) and widely applicable imaging procedure to evaluate the CBD, even in the elderly<sup>[18,19]</sup>. Our judgment of further management for the patients mainly depended on these evaluations. Secondly, small stones

that pass through the duct, especially stones less than 0.5 cm in diameter, may induce pancreatitis<sup>[20]</sup>. We excluded patients with pancreatitis to reduce this possible confounding factor. Finally, the averaged normal ALK-P levels in the group without evidence of CBD stones also argues against transient stone passage as a cause of the hepatocellular injury.

The limitation of our study is that it was a retrospective observation. We selected patients who underwent image evaluation of the CBD because of suspicion of biliary obstruction from abnormalities in biochemical liver function and/or sonography. The elevated aminotransferase levels in the group of patients may have resulted in a falsely high incidence of hepatocellular injury induced by gallbladder disease without CBD stones. However, regardless of the pathophysiology and true occurrence rate, the abnormal biochemical liver function of almost all the patients resolved rapidly and spontaneously after cholecystectomy. This phenomenon implied that the transient hepatocellular injury was brought about by symptomatic gallstones with cholecystitis even in the absence of CBD stones.

We observed persistently high liver enzymes in one patient who was subsequently found to have cancer of the Ampulla of Vater. This suggests that if there is incomplete postoperative resolution of the abnormal tests, residual or additional pathology should be investigated. However, in the absence of other symptoms, it would be reasonable to wait for 1 mo to see if the hepatocellular injury resolves before initiating more invasive studies such as ERCP.

## COMMENTS

### Background

Acute hepatocellular injury is a commonly encountered phenomenon in patients with cholelithiasis and concomitant common bile duct (CBD) stones. However, in clinical practice, it seemed to occur also in cholelithiasis patients without evidence of CBD stones. Its incidence and final outcome necessitated clarification.

### Research frontiers

Abnormal liver function tests for predicting CBD stones have been well discussed, however, the relationship between liver function and cholelithiasis/cholecystitis alone is not well understood.

### Innovations and breakthroughs

In this study, the authors studied a phenomenon of hepatocellular injury in patients with cholelithiasis without evidence of concomitant stones in the CBD. It was transient and resolved spontaneously after cholecystectomy.

## Applications

This kind of hepatocellular injury, even showing relatively high serum levels of aminotransferases and bilirubin, should not be an obstacle to preparation of such patients for surgical intervention. The abnormalities will be expected to recover spontaneously and need no further investigation or treatment unless they persist for more than 1 mo.

## Peer review

This is an interesting paper addressing an acute transient hepatocellular injury which is often presented but ignored. The article is well balanced and the discussion is clear and exhaustive.

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# Lymphovascular invasion in rectal cancer following neoadjuvant radiotherapy: A retrospective cohort study

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(42.5 mo vs 26.5 mo,  $P = 0.035$ ) compared to those in the control group, despite the fact that no significant difference in 5-year OS rate was observed (54.4% vs 48.3%,  $P = 0.137$ ). Multivariate analysis showed the distance of tumor from the anal verge, pretreatment serum carcinoembryonic antigen level, pathologic TNM stage and LVI were the major factors affecting OS.

**CONCLUSION:** Neoadjuvant radiotherapy does not reduce LVI significantly; however, the prognostic meaning of LVI has changed. Patients with LVI may benefit from neoadjuvant radiotherapy.

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**Key words:** Lymphovascular invasion; Rectal cancer; Neoadjuvant radiotherapy; Total mesorectal excision; Pathology; Prognosis

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## Abstract

**AIM:** To investigate the meaning of lymphovascular invasion (LVI) in rectal cancer after neoadjuvant radiotherapy.

**METHODS:** A total of 325 patients who underwent radical resection using total mesorectal excision (TME) from January 2000 to January 2005 in Beijing cancer hospital were included retrospectively, divided into a preoperative radiotherapy (PRT) group and a control group, according to whether or not they underwent preoperative radiation. Histological assessments of tumor specimens were made and the correlation of LVI and prognosis were evaluated by univariate and multivariate analysis.

**RESULTS:** The occurrence of LVI in the PRT and control groups was 21.4% and 26.1% respectively. In the control group, LVI was significantly associated with histological differentiation and pathologic TNM stage, whereas these associations were not observed in the PRT group. LVI was closely correlated to disease progression and 5-year overall survival (OS) in both groups. Among the patients with disease progression, LVI positive patients in the PRT group had a significantly longer median disease-free period (22.5 mo vs 11.5 mo,  $P = 0.023$ ) and overall survival time

## INTRODUCTION

Currently, the treatment of rectal cancer has stepped into a new era of multimodality therapy<sup>[1]</sup>. Neoadjuvant therapy, including preoperative radiotherapy (RT) or radiochemotherapy (RCT), has become a standard regimen for locally advanced rectal cancer<sup>[2]</sup>. There have been growing concerns in recent years about the pathologic evaluation of rectal cancer after neoadjuvant therapy, since the pathologic stage (ypTNM) is now significantly different from its original meaning<sup>[3,4]</sup>. Due to neoadjuvant therapy, a considerable number of patients experience tumor regression or downstaging, and a minority of patients experience a complete pathologic response (CPR)<sup>[5]</sup>. However, the problem is that there are still few favorable pathological indicators to reflect and predict the clinical consequence of rectal cancer after neoadjuvant therapy.

Lymphovascular invasion (LVI) has been widely acknowledged as a useful independent pathological indicator for predicting prognosis, as well as a good index to guide postoperative therapy in colorectal cancer. Patients with LVI usually have a higher chance of disease progression and poorer prognosis<sup>[6-8]</sup>. The NCCN (National Comprehensive Cancer Network) guideline recommended LVI as a high risk factor of disease advance for colon cancer after surgery<sup>[9]</sup>. For rectal cancer, LVI is also a crucial high risk factor for recurrence post transanal local resection<sup>[10]</sup>. However, it has still to be established whether LVI has the same predictive meaning and clinical significance for patients with rectal cancer undergoing preoperative radiation. Does the biological behavior of the cancer cells involved in the blood or lymphatic vessels change after radiation? This is the issue we focus on.

## MATERIALS AND METHODS

### Clinical data

Data from all consecutive patients with resectable rectal carcinoma treated in our hospital from January 2000 to January 2005 were collected retrospectively. Among them, we selected eligible patients according to the following criteria: (1) resectable rectal cancer 12 cm or less from the anal verge; (2) evaluated by endorectal ultrasound (ERUS) or magnetic resonance imaging (MRI) before treatment; (3) histologically identified primary carcinoma of the rectum; (4) no clinical evidence of distant metastases; (5) transabdominal radical resection based on the principle of total mesorectal excision (TME); (6) R0 resection.

Exclusion criteria were: (1) patients who underwent concurrent RCT; (2) patients with CPR after neoadjuvant radiotherapy; (3) patients with synchronous tumors or history of other malignant tumors within 5 years; (4) familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal carcinoma (HNPCC); (5) died of complications or other non-cancer related reasons.

In total 325 patients were included (Table 1). All included patients were divided into a preoperative radiotherapy (PRT) group ( $n = 103$ ) and a control group ( $n = 222$ ), according to whether or not they underwent neoadjuvant radiation. There was no statistically significant difference in the gender, age, tumor location, preoperative serum carcinoembryonic antigen (CEA) level, pathologic stage and LVI between the two groups (Table 1). The conditions of histological differentiation and pretreatment stage (by imaging) were better in the control group, which implied a potentially better prognosis of patients in the control group. But the following multivariate analysis demonstrated that these two factors were not the major factors affecting the clinical consequence, so we considered the patients in the two groups to be comparable. Furthermore, we believe it more reasonable to investigate the influence of LVI on clinical consequence under the same pathologic stage rather than the same pretreatment stage in the

**Table 1 Demographic details of patients and tumor characteristics**

	PRT $n = 103$	Control $n = 222$	P value
Sex			
Male	62	130	0.875
Female	41	92	
Age (yr) <sup>1</sup>	56 (52-61)	57 (53-64)	0.232
Distance of tumor from anal verge			
< 5 cm	35	53	0.061
5-12 cm	68	169	
Surgery			
APR	27	55	0.564
LAR	71	161	
CR	5	6	
Preoperative serum CEA level			
Normal	52	121	0.691
Abnormal	35	65	
Unknown	16	36	
Pretreatment TNM stage (%)			
I (T1-2 N0)	0 (0)	43 (19.4)	< 0.01
II A (T3 N0)	25 (24.3)	63 (28.4)	
II B (T4 N0)	4 (3.9)	5 (2.3)	
III A (T1-2 N1)	3 (2.9)	8 (3.6)	
III B (T3-4 N1)	33 (32.0)	43 (19.4)	
III C (AnyT N2)	38 (36.9)	60 (27.0)	
Pathologic TNM stage (%)			
I (T1-2 N0)	35 (34.0)	55 (24.8)	0.377
II A (T3 N0)	27 (26.2)	62 (27.9)	
II B (T4 N0)	1 (1.0)	1 (5.0)	
III A (T1-2 N1)	6 (5.8)	7 (3.2)	
III B (T3-4 N1)	18 (17.5)	45 (20.3)	
III C (AnyT N2)	16 (15.5)	52 (23.4)	
Histological differentiation (%)			
High	4 (3.9)	29 (13.1)	< 0.01
Moderate	70 (68.0)	156 (70.3)	
Poor	24 (23.3)	27 (12.2)	
Mucinous and signet	5 (4.9)	10 (4.5)	
LVI (%)			
Present	22 (21.4)	58 (26.1)	0.353
Absent	81 (78.6)	164 (73.9)	
Disease progression (%)			
Local Recurrence	6 (5.8)	32 (14.4)	0.025
Distant Metastasis	22 (21.4)	47 (21.2)	0.969
Death	24 (23.3)	66 (29.7)	0.228

<sup>1</sup>Values are medians (interquartile ranges). PRT: Preoperative radiotherapy.

two groups, so it was inevitable that the pretreatment stage of the PRT group was later because of tumor-downstaging after neoadjuvant radiotherapy.

### Pretreatment evaluation

All included patients underwent ERUS or MRI to evaluate the tumor size, invasion depth and extent, and the involvement of pararectal lymph nodes. In total 280 patients (86.2%) were evaluated by ERUS and 45 patients (13.8%) by MRI. Serum CEA was measured and abdominal CT and chest radiography were also routinely performed before treatment.

### Neoadjuvant therapy

We adopted neoadjuvant radiation with a total dose of 30 Gy (30 Gy/10 fractions), recommended by the

Chinese Anti-Cancer Association (CACA)<sup>[11]</sup>, based on some high-level clinical evidence<sup>[12,13]</sup>. Surgery was performed 2-3 weeks after full dose radiation.

### Surgery

All included patients underwent radical resection strictly according to the principles of TME<sup>[14]</sup>, regardless of abdominoperineal resection (APR) or low anterior resection (LAR). In addition, 11 patients underwent combined resection (CR) involving partial or total resections of some pelvic organs; all resection margins were identified as negative by pathologic examination.

### Pathologic evaluation

All slides of postoperative specimens were stained with hematoxylin and eosin (HE) and were reviewed by one senior pathologist who was blind to the prognosis of patients. The available criteria for the histologic diagnosis of LVI included<sup>[15]</sup>: presence of tumor cells within lymphatic or vascular space; identification of endothelial cells lining the space; the presence of an elastic lamina surrounding the tumor; and attachment of tumor cells to the vascular wall.

Tumor regression was mostly in the form of fibro-inflammatory changes or necrosis replacing neoplastic glands. Mucin pools were also seen sometimes, as another type of degeneration post radiotherapy. Comparison between the pathologic T stage and clinical T stage (by imaging) was made to identify tumor-downstaging in the PRT group<sup>[16]</sup>.

### Postoperative therapy

All patients in the PRT group were given postoperative chemotherapy for 6-8 cycles, using the standard regimens based on 5-FU or capecitabine, such as FOLFOX, CapeOX or capecitabine alone. In the control group, only patients with lymph node involvement or with the pathologic T3 or T4 stage were given adjuvant chemotherapy, with the same regimens as were used in the PRT group. Notably, 95% (76/80) of LVI positive patients underwent postoperative adjuvant chemotherapy, while only 4/58 LVI positive patients in the control group were not given postoperative chemotherapy due to the early TNM stage. Therefore, our results concerning prognosis involved the influence of postoperative chemotherapy.

For the patients with disease progression, three patients with resectable liver metastasis underwent partial liver resection and one patient with solitary lung metastasis underwent lung wedge resection. Two patients with resectable local recurrence underwent APR. Other patients with disease progression underwent systematic chemotherapy or support therapy.

### Follow up

Patients were followed at 3 mo intervals for the first two years and then at 6 mo intervals for the next three years. Evaluations consisted of physical examination, serum CEA, a complete blood count, and blood chemical analysis. Proctoscopy, abdominal ultrasonography, CT of

the abdomen and pelvis, and chest radiography were also routinely used every 6-12 mo, according to the NCCN guideline<sup>[19]</sup>. Follow up time ranged from 3 to 96 mo, and the median follow up time was 72 mo. We chose 5 years as a time terminal for evaluation of outcomes. The follow up rate was 86.5% (281/325), with 44 inconclusive patients.

### Statistical analysis

Statistical analyses were performed using SPSS 16.0 statistical software. The categorical variables were analyzed with the Pearson chi-squared or Fisher's exact test, as appropriate. The Kaplan-Meier survival curve was used to estimate the proportion of patients surviving or remaining disease-free at each time interval. Disease free survival (DFS) and overall survival (OS) curves were compared between groups using the Wilcoxon's test for time-to-event parameters. Disease-free periods were compared using a log-rank test. Multivariate Cox proportional hazards regression was used to analyze the major factors affecting overall survival. All statistical tests were 2-sided, and the level of significance set at 5%.

## RESULTS

### The distribution and related factors of LVI

The overall positive rate of LVI was 24.6% (80/325), with no statistically significant difference in distribution between the PRT and control groups (21.4% and 26.1% respectively,  $P = 0.353$ ) (Table 1). Within the PRT group, LVI was not significantly reduced in patients with tumor-downstaging (Table 2). In the control group, LVI strongly correlated with histological differentiation and pathologic T and N stages whereas these associations were not observed in the PRT group (Table 2).

### The influence of LVI to clinical consequence

To get credible statistical results, we merged the local recurrence and distant metastasis to disease progression data because of the limited number of local recurrences in the PRT group. LVI was significantly associated with disease progression in both groups (Table 3): 38.5%-43.2% of LVI positive patients developed recurrence or metastasis, whereas 15.6%-17.6% of LVI negative patients progressed finally ( $P < 0.05$ ). LVI was also strongly correlated with DFS and OS: patients with LVI had lower rates of 5 year DFS and OS in both groups (Table 3, Figure 1A and B).

However, the influence of LVI on prognosis was not same in the two groups (Figure 2A and B). Among the patients with disease progression, LVI positive patients in the PRT group had a longer disease-free period and survival time than those in the control group (the median DFS time was 22.5 and 11.5 mo respectively,  $P = 0.023$ ; the median OS time was 42.5 and 26.5 mo respectively,  $P = 0.035$ ). There were no statistically significant differences in the 5 year DFS rate (54.4% vs 44.8%,  $P = 0.099$ ) and OS rate (54.4% vs 48.3%,  $P = 0.137$ ). For LVI negative patients, neither DFS nor OS showed significant differences between the two groups

**Table 2** The relevance of LVI and pathologic factors

		LVI		Positive rate (%)	P value
		Present	Absent		
PRT	ypT	T1	1	5	16.7
		T2	6	31	16.2
		T3	14	44	24.1
		T4	1	1	50.0
ypN	N0	9	54	14.3	0.057
	N1	9	15	37.5	
	N2	4	12	25.0	
Histological differentiation	High	1	3	25.0	0.998
	Moderate	15	55	21.4	
	Poor	5	19	20.8	
	Mucinous and signet	1	4	21.0	
Downstaging	Yes	7	34	17.1	0.388
	No	15	47	24.2	
Control	pT	T1	0	11	0
		T2	8	50	13.8
		T3	49	98	33.3
		T4	1	5	16.7
	pN	N0	15	103	12.7
		N1	15	37	28.8
		N2	28	24	53.8
Histological differentiation	High	7	22	24.1	0.015
	Moderate	34	122	21.8	
	Poor	11	16	40.7	
	Mucinous and signet	6	4	60.0	

(the median DFS time was 14.0 and 15.0 mo respectively,  $P = 0.980$ ; the median OS time was 29.8 and 34.0 mo respectively,  $P = 0.247$ ).

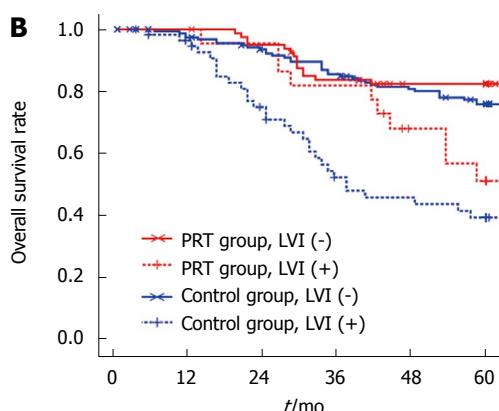
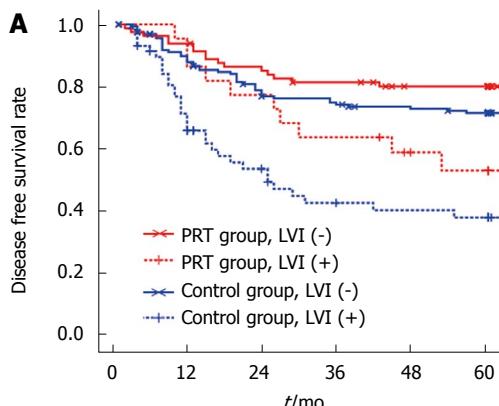
### The factors influencing prognosis

Multivariate analysis demonstrated that the distance of the tumor from the anal verge, pretreatment serum CEA level, pathologic TNM stage and LVI were the major factors affecting the 5 year OS (Table 4) whereas gender, age, neoadjuvant radiotherapy, tumor-downstaging, histological differentiation and pretreatment stage were not significantly associated with long-term survival.

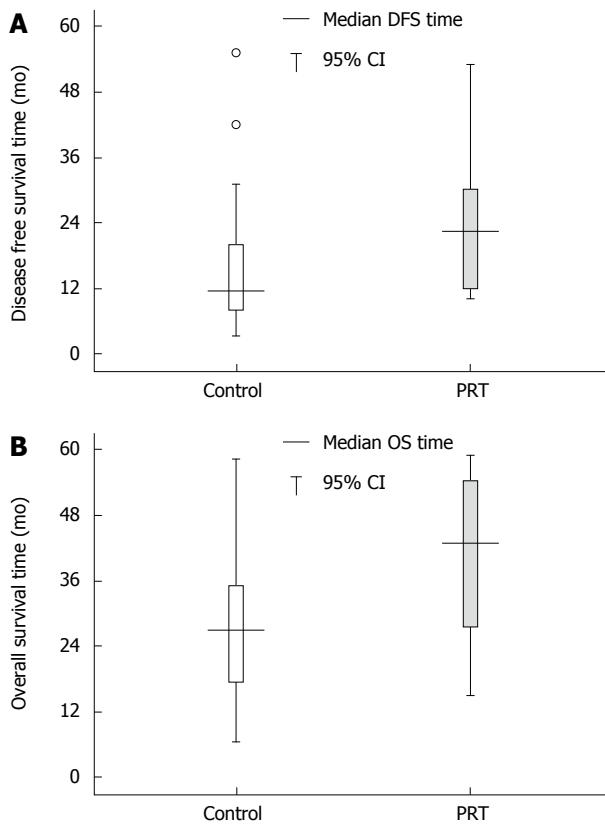
## DISCUSSION

Strictly speaking, lymphovascular invasion implies involvement of vascular and lymphatic vessels. However, histological distinction between larger lymphatic and smaller venous channels may not always be possible. Therefore, the term lymphovascular is used to refer to any or all of these structures<sup>[17]</sup>. LVI could be recognized clearly on HE-stained slides, despite the fact that some reports indicate using immunohistochemical stains with CD31 and D2-40 may improve the diagnosis<sup>[18,19]</sup>.

LVI has long been recognized as a favorable independent pathological indicator for predicting the prognosis of patients with colorectal cancer, which is usually associated with a poor consequence<sup>[6-8]</sup>. However, most studies concerning LVI were made in patients not given neoadjuvant therapy. Although several authors mentioned



**Figure 1** K-M plot of DFS (A) and OS (B) for LVI between the two groups. The LVI negative patients had a significantly higher 5-year DFS rate ( $P < 0.05$ ) and OS rate ( $P < 0.01$ ) than LVI positive patients in both groups ( $P < 0.05$ ).



**Figure 2** Stem and Leaf plot for comparison of DFS time (A) and OS time (B) for LVI positive patients between the two groups. Among the 42 (A) and 40 (B) LVI positive patients with disease progression, the patients in the PRT group had a longer median DFS time ( $P < 0.05$ ).

**Table 3** The association of LVI and prognosis

	PRT		P	Control		P
	Present	Absent		Present	Absent	
Disease progression (%)	Yes	10 (38.5)	16 (61.5)	0.014	32 (43.2)	42 (56.8) < 0.01
	No	12 (15.6)	65 (84.4)		26 (17.6)	122 (82.4)
5-year DFS rate	%	54.5 (12/22)	80.2 (65/81)	0.020	44.8 (26/58)	73.2 (120/164) < 0.01
5-year OS rate	%	54.5 (12/22)	82.7 (67/81)	< 0.010	48.3 (28/58)	78.0 (128/164) < 0.01

**Table 4** Multivariate analysis of OS by COX model (backward elimination)

Variable	Hazard ratio	95% CI	P value
Distance from anal verge	0.540	0.346-0.845	< 0.01
Serum CEA level	1.334	1.027-1.732	0.031
pTNM stage	1.347	1.198-1.514	< 0.01
LVI	0.450	0.292-0.692	< 0.01

in their studies that LVI correlated with awful prognosis in patients undergoing neoadjuvant therapy<sup>[20,21]</sup>, there have been very few studies that specifically investigated the difference in LVI after neoadjuvant therapy compared to LVI after surgery directly. Our study was undertaken to illuminate such differences.

Despite the fact that preoperative radiation may lead to some histological changes, such as tumor regression or even a complete response<sup>[3,22,23]</sup>, our study found that preoperative radiotherapy alone did not significantly reduce the occurrence of LVI: at the same pathologic stage, the positive rates of LVI in the two groups were not significantly different (21.4% vs 26.1%, P = 0.353). Even for patients with tumor-downstaging, who could be considered sensitive to radiation, the LVI positive rate was not significantly reduced which implied the killing effect of X-rays to cancer cells involved in the vessels may be limited. Perhaps the addition of concurrent chemotherapy may work on LVI more effectively, which will be our continuing work based on the results from the current study. However, the influence of radiochemotherapy on LVI is more complicated, so we chose radiotherapy alone as the first step.

However, the behavior of tumor cells involved in the vessels somewhat changed after radiation. Our results demonstrated that the disease progression of patients with LVI in the PRT group was significantly delayed, which suggested that the aggression of those tumor cells in the blood or lymphatic vessels may have been significantly weakened by X-ray, though they were not completely eliminated. Currently, it is generally believed that radiation can cause DNA damage and chromosome aberrations, leading to an abortion of cell mitosis and proliferation, as well as inducing cell apoptosis<sup>[24,25]</sup>. Therefore, we inferred that the tumor cells involved in the vessels may be partly killed or inhibited by neoadjuvant radiotherapy so that disease progression was delayed and the survival time was prolonged.

At present, the issue about who would benefit from

neoadjuvant therapy is still being debated. Some authors believe that only patients with a good response to radiation could benefit from neoadjuvant therapy<sup>[3,16,26,27]</sup>. Our study demonstrated patients with LVI could gain a prolonged disease-free period and survival time from neoadjuvant radiotherapy. Thus, LVI positive patients may also benefit from neoadjuvant radiotherapy in a sense.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Currently, neoadjuvant therapy has become a standard protocol for locally advanced rectal cancer. Although some studies have demonstrated that lymphovascular invasion (LVI) after neoadjuvant therapy is a high risk factor for recurrence, there have been very few studies that specially addressed the meaning of LVI after neoadjuvant therapy. The study is designed to compare the difference in LVI between patients undergoing neoadjuvant radiotherapy and those undergoing surgery directly.

### Research frontiers

The pathologic evaluation of the rectal cancer after neoadjuvant therapy is the hotspot most oncologists and scholars focus on.

### Innovations and breakthroughs

The study specifically addresses the meaning of LVI in rectal cancer after neoadjuvant radiotherapy; furthermore, they are first to report the difference in LVI after neoadjuvant radiotherapy.

### Applications

They demonstrated that patients with LVI could gain a prolonged disease-free period and survival time from neoadjuvant radiotherapy which provides significant evidence and reference for the application of neoadjuvant radiotherapy.

### Terminology

Lymphovascular invasion: a morphological concept which refers to the presence of tumor cells within lymphatic or vascular vessels, representing strong aggression of tumor cells.

### Peer review

The manuscript, reported by Du et al, investigates, in a retrospective cohort study, the difference in LVI after neoadjuvant therapy compared to LVI after surgery directly. This manuscript is well presented and gives us an interesting result. The patient's cohort is important.

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## Methylation of *PTCH1a* gene in a subset of gastric cancers

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**Author contributions:** Du P and Ye HR performed the majority of the experiments; Ye HR and Xu J were involved in editing the manuscript; Chen W, Wang ZC, Jiang HH and Zhang JW collected all the human material; Zhang JC performed the methylation detection; Gao J and Cui L designed the study and wrote the manuscript; Du P, Ye HR and Gao J contributed equally to this work.

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The prevalence of *PTCH1a* TRR methylation was investigated in 170 gastric cancer tissue samples and the adjacent normal tissues by MSP. The correlation of *PTCH1a* TRR methylation with *PTCH1* expression or with patients' clinical features was analyzed.

**RESULTS:** Methylation of *PTCH1a* TRR was observed in AGS cells and a subset of gastric cancer tissues (32%, 55/170), while no methylation amplification products were observed in any normal tissues by MSP. The methylation of *PTCH1a* TRR was correlated negatively with *PTCH1* expression (Spearman's  $r = -0.380$ ,  $P = 0.000$ ). However, methylation of *PTCH1a* TRR was not related to the gastric cancer patients' clinical features, such as sex, age of onset, clinical stage, lymph node metastasis or histological grade. The methylation of *PTCH1a* TRR in AGS cells was almost converted to non-methylation after 5-Aza-dC treatment, which increased *PTCH1* expression ( $5.3 \pm 2.5$  times;  $n = 3$ ) and apoptosis rate ( $3.0 \pm 0.26$  times;  $P < 0.05$ ;  $n = 3$ ).

**CONCLUSION:** Methylation of *PTCH1a* TRR is present in a subset of gastric cancers and correlated negatively with *PTCH1* expression. This may be an early event in gastric tumorigenesis and a new treatment target.

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**Key words:** Carcinogenesis; Methylation; Hedgehog signaling pathway; Methylation; *PTCH1*; Stomach neoplasms

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## INTRODUCTION

The hedgehog (HH) pathway plays a critical role in embryonic development, tissue polarity and

carcinogenesis. In the HH pathway, Sonic HH binds to the receptor PTCH1, which is encoded by the *PTCH1* gene. This liberates the Smoothened protein, which allows glioma-associated oncogene homolog 1 zinc finger protein (GLI) and MYCN transcription factors to turn on target genes, including the *PTCH1* gene itself, in a negative feedback loop as a tumor suppressor gene. More recently, abnormal activation of the HH pathway has been reported in subsets of human basal cell carcinoma<sup>[1]</sup>, medulloblastoma<sup>[2]</sup>, pancreatic cancer<sup>[3-5]</sup>, lung cancer<sup>[6]</sup>, prostate cancer<sup>[7]</sup> and gastrointestinal cancer<sup>[8-10]</sup>.

Gastric cancer is one of the most common cancers worldwide, and has high mortality. Patients with gastric cancer usually present at late stages and have a poor prognosis. Loss-of-function mutation of *PTCH1* gene participates in the abnormal activation of the HH pathway, which occurs frequently in some cases of human basal cell carcinoma<sup>[11]</sup> and medulloblastoma<sup>[12]</sup>, but it has never been observed in gastric cancer<sup>[13]</sup>. Loss-of-function of tumor suppressor gene is also known to result from methylation of the transcriptional regulation region (TRR). Recently, several studies have argued that *PTCH1* TRR methylation is involved in tumorigenesis<sup>[14-17]</sup>; however, none has been reported in gastric cancer. Previous studies have shown that the *PTCH1* gene has three major isoforms in the first exon, *PTCH1a*, *PTCH1b* and *PTCH1c* that code for different N-sequence *PTCH1* proteins, *PTCH1-l*, *PTCH1-m* and *PTCH1-s*, respectively, and expression of each is regulated by its own independent TRR<sup>[18]</sup>.

The present study analyzed the methylation of *PTCH1a* TRR in gastric cancer cell line AGS and some gastric cancer tissue samples. We showed that methylation of *PTCH1a* TRR took place in a subset of gastric cancers, and was correlated negatively with *PTCH1* gene expression. It was not related to the patients' clinical features of gastric cancer, which suggested that the methylation of *PTCH1a* TRR might be an early event in gastric tumorigenesis.

## MATERIALS AND METHODS

### Gastric cancer patients' tissue samples and cell line

All the tissue samples were obtained from Shanghai Xinhua Hospital with hospital ethics board approval. One hundred and seventy gastric cancer tissue samples were collected from radical gastrectomy, to analyze the methylation of the *PTCH1* gene, and its expression. All patients gave informed consent for their specimens to be studied. The tumor and adjacent macroscopically normal tissue samples were preserved in liquid nitrogen immediately after being resected. Only the samples in which the proportion of tumor cells was > 70% and adjacent normal tissues with no inflammation or tumor infiltration were selected. Patients' clinical features were recorded, including sex, age of onset, clinical stage, lymph node metastasis, and histological grade. Gastric cancer cell line AGS was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured under recommended conditions.

### DNA/RNA isolation

Frozen tissue in liquid nitrogen was pulverized for subsequent DNA isolation using the Blood and Cell Culture DNA kit (Qiagen, Hilden, Germany) or RNA isolation with TRIzol (Gibco-BRL, Glasgow, UK) according to the protocols of the manufacturers.

### Relative quantitative (RQ) RT-PCR

Total RNA (2.5 µg) was treated with DNase RQ1 (Promega, Madison, WI, USA) to remove trace amounts of genomic DNA contamination, and converted to cDNA using the oligo (dT) primer system (TaKaRa, Dalian, China), in a total volume of 50 µL. Aliquots of the reaction mixture were used for quantitative PCR amplification with ABI7500 (Applied Biosystems, Foster City, CA, USA) using SYBR Premix EX TagTM (TakaRa). PCR was run for 30 cycles of denaturation at 95°C for 5 s, annealing at 55°C for 20 s, and elongation at 72°C for 20 s. Gene expression was quantified by the comparative CT method, with normalizing CT values to the housekeeping gene β-actin. After amplification, melting curve analysis was performed to ensure the products' specificity. The RQ value of *PTCH1* expression in the samples was calculated in comparison with a calibrator (the expression level of pooled adjacent normal tissue samples). To ensure experimental accuracy, all reactions were performed in triplicate. The primer sequences for the gene amplification are shown in Table 1.

### Methylation-specific PCR (MSP) and bisulfite sequencing PCR (BSP)

Bisulfite modification, MSP and BSP were performed as described before<sup>[19,20]</sup>. The primers of MSP and BSP are shown in Table 1. One microgram of genomic DNA was treated using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. For MSP, 1 µL modified DNA was amplified using MSP primers that specifically recognized the methylated or unmethylated DNA after bisulfite conversion. CpGenome Universal Methylated DNA (S7821) and CpGenome Universal Unmethylated DNA (S7822) (Chemicon Company, Temecula, CA, USA) were used as control DNA for methylated and unmethylated detection, respectively. Amplification products were visualized by UV illumination on 3% low-range ultra-agarose gel (Bio-Rad Laboratories, Hercules, CA, USA) that contained ethidium bromide. For BSP clone sequence analysis, the PCR products were subcloned into a pMD-18-T vector (TaKaRa). Ten clones were sequenced for cell line AGS and some gastric cancer tissues.

### 5-Aza-2'-deoxycytidine (5-Aza-dC) treatment

Cells were plated at a density of  $3 \times 10^4$  cells/cm<sup>2</sup> in a six-well plate on day 0. The demethylating agent 5-Aza-dC (Sigma-Aldrich, Deisenheim, Germany) was added on days 1, 2 and 3 to maintain its concentration as 1 µmol/L in fresh medium. Cells were harvested on

**Table 1** Primers and size of PCR products

Methods	Primers	Sequence	Length (bp)
QRT-PCR	PTCH1	5'-TGTGCGCTGTCCTCCCTG-3' 5'-ACGGCACTGAGCTTGATTG-3'	119
	β-actin	5'-GCCATCCTCGCTCG-3' 5'-TGGGCACCGGAACCGCT-3'	260
BSP		5'-GGGAGTATTGGGTGGTATAATT-3'	351
MSP	Methylation	5'-AAAAAAACTACAAAAAACACCCTITC-3'	143
	Non-methylation	5'-GAGGGATGATGATGAATT-3' 5'-AAAAACACAAAAAACTAAA-3'	143

QRT-PCR: Quantitative PCR.

day 4 for RNA and DNA extraction. Control cells were incubated without the addition of 5-Aza-dC.

#### Analysis of cell cycle and apoptosis by flow cytometry

About  $1 \times 10^6$  AGS cells were centrifuged at 1000 r/min for 5 min to remove the culture solution. Cell cycle was measured by propidium iodide (PI) staining (final concentration 100 µg/mL, 0.01 mol/L PBS, pH 7.4; R&D System, Abingdon, UK) and flow cytometry (Becton Dickinson, Fullerton, CA, USA). Meanwhile, cell apoptosis rate was measured by annexin V/PI double staining (R&D Systems) and flow cytometry.

#### Immunohistochemistry

Sections of 3 µm were dried for 30 min at 72°C, deparaffinized in xylene, and rehydrated in a decreasing ethanol series. Endogenous peroxidase activity was blocked with 3% H<sub>2</sub>O<sub>2</sub> in methanol for 30 min and endogenous biotin with a blocking kit (SP-2001; Vector Laboratories, Burlingame, CA, USA). Antigen retrieval was performed by autoclaving for 10 min at 120°C in 10 mmol/L citrate buffer, pH 6.0. Sections were blocked for 30 min in a protein block (X0909; Dako, Carpinteria, CA, USA), and incubated overnight at 4°C with diluted goat polyclonal antibody directed against human PTCH1 protein (sc-6149, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing the sections with PBS that contained 0.1% Tween 20, biotinylated secondary antibodies were added for 30 min at room temperature. After extensive rinsing and incubation with avidin-biotin, immunoperoxidase antibody staining was visualized with the 3,3' diaminobenzidine system (Nichirei, Tokyo, Japan), and the sections were counterstained with Mayer's hematoxylin. The application of primary antibody to tissue sections was omitted in negative controls.

#### Statistical analysis

Statistical analysis was carried out using SPSS version 14.0 (SPSS, Chicago, IL, USA). Differences were considered statistically significant at  $P < 0.05$ . The nonparametric correlations of PTCH1 expression with methylation were analyzed with Spearman's test. Differences in the clinicopathological parameters between positive and negative PTCH1a TRR methylation were determined with the  $\chi^2$  test.

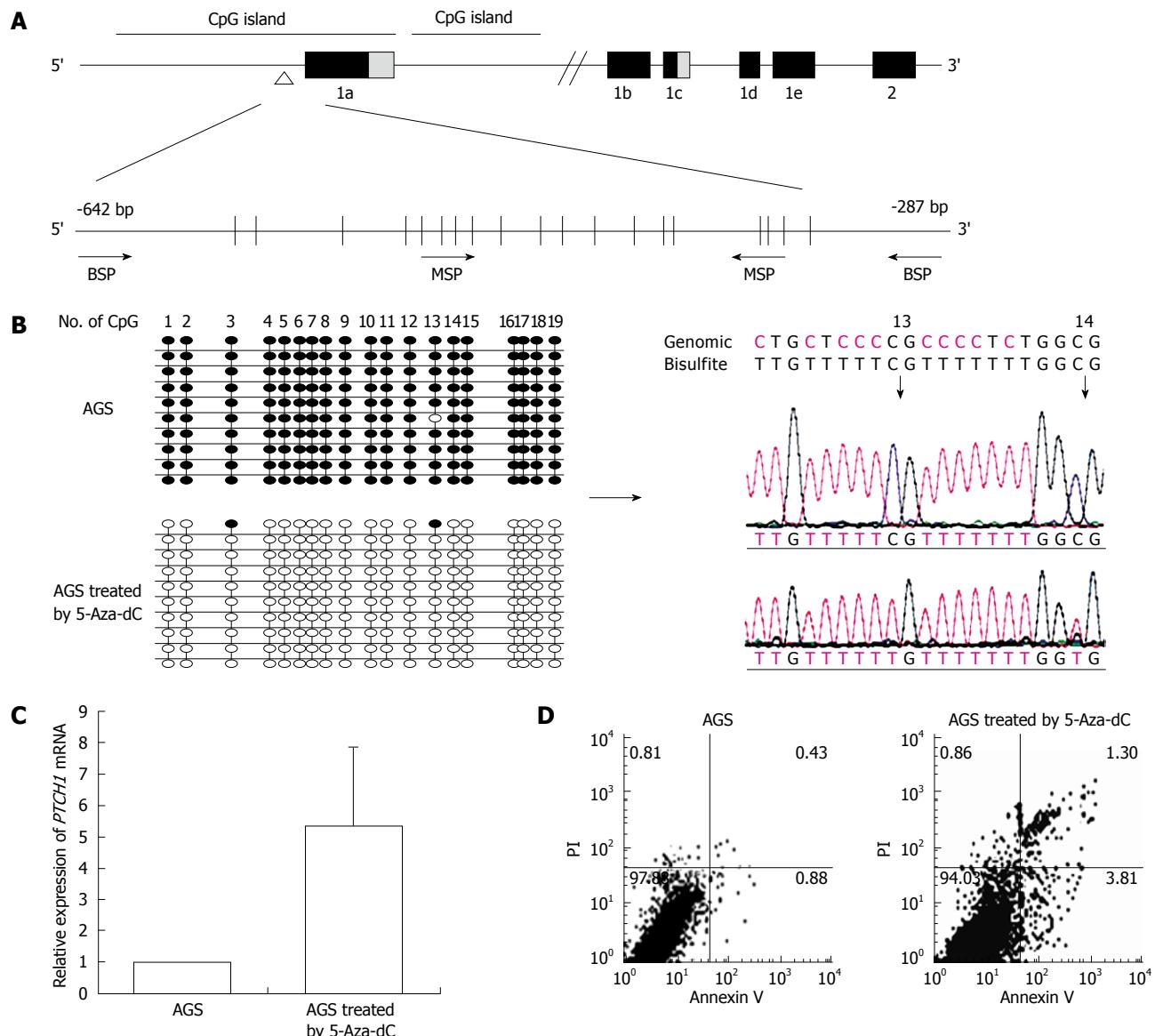
## RESULTS

#### PTCH1a TRR methylation and PTCH1 gene expression in gastric cancer cell line AGS

According to National Center for Biotechnology Information, PTCH1 gene has three mRNA transcripts. We analyzed the CpG island at -3950 bp upstream and +2050 bp downstream from the transcription initiation site of PTCH1a (designated as 0) by the methylation analysis software Methyl Primer Express v1.0 (Applied Biosystems). As shown in Figure 1A, two CpG islands exist in this region. One is -1139 to +860 bp and the other is +875 to +1692 bp. The region from -643 to -355 bp in the first CpG island that contained 19 CpG sites was chosen for the BSP and MSP primer amplifications. The MSP up-primer contained the fifth to eighth CpG sites, while the down-primer contained the sixteenth to eighteenth CpG sites. The methylation level of PTCH1a TRR in gastric cancer cell line AGS treated with 5-Aza-dC after 72 h was measured through BSP clone sequences. As shown in Figure 1B, almost all the CpG sites were methylated in the untreated cells, while almost all of them were converted to unmethylated after treatment with 5-Aza-dC. The RQ value of PTCH1 expression increased by  $5.3 \pm 2.5$  times ( $P < 0.05$ ,  $n = 3$ ) (Figure 1C). The cell cycle had no significant alteration after treatment (data not shown), by PI staining. However, as shown in Figure 1D, the apoptosis rate increased significantly by  $3.0 \pm 0.26$  times ( $P < 0.05$ ,  $n = 3$ ) by annexin V/PI double staining. These results indicated that the PTCH1a TRR was highly methylated in AGS cells, and became unmethylated after 5-Aza-dC treatment, which substantially increased PTCH1 expression and induced more apoptosis.

#### PTCH1a TRR methylation in gastric cancer tissues

In order to investigate the prevalence of PTCH1 TRR methylation in gastric cancer tissues, detection of PTCH1a TRR methylation in 170 gastric cancer tissues was performed by MSP. If the methylation amplification products appeared after electrophoresis in the investigated sample, PTCH1 TRR was predicted to be methylated. The prevalence of PTCH1a TRR methylation was 32% (55/170) in gastric cancer tissues, while no methylation amplification products were observed in any normal tissues (data not shown). Part

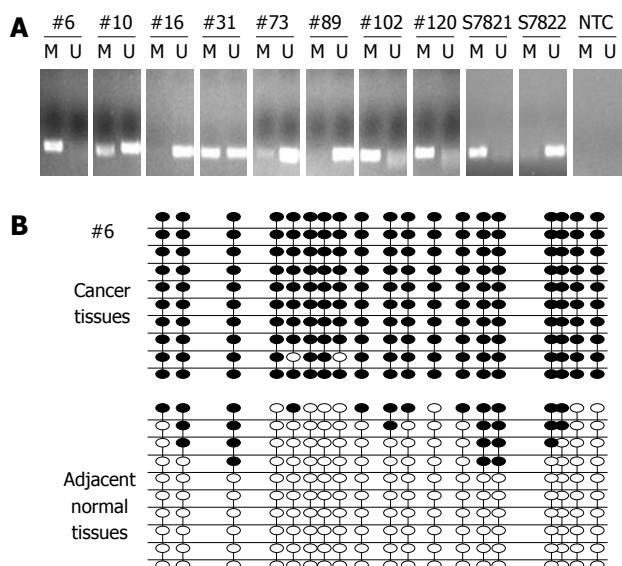


**Figure 1 Analysis of methylation and expression of *PTCH1a* in gastric cancer cell line AGS.** A: Illustration of *PTCH1a* TRR and topology of MSP and BSP primers. BSP detection region contained 19 CpG sites, and MSP up-primer and down-primer contained four and three CpG sites, respectively. The detection amplicon is indicated by the empty triangle; B: Alteration of *PTCH1a* TRR methylation in gastric cancer cell line AGS treated with 5-Aza-dC. Genomic DNA from untreated AGS cells and those treated with 5-Aza-dC (1  $\mu$ mol/L) were analyzed by BSP at day 4. The left column indicates alterations of the 19 CpG sites contained in the BSP amplicon through 10 cloned sequences after 5-Aza-dC treatment. The right column displays part of the sequence of the methylated and unmethylated clones. Black dot, methylated; white dot, unmethylated; C: Alteration of *PTCH1* expression in gastric cancer cell line AGS treated with 5-Aza-dC. *PTCH1* gene expression in AGS cells treated with 5-Aza-dC (1  $\mu$ mol/L) at day 4 was detected by real-time PCR relative to untreated AGS cells. Expression of *PTCH1* gene was enhanced significantly in the treated AGS cells compared with untreated ( $P < 0.01$ ,  $n = 3$ , independent tests). Box, mean; bar, SD; D: Analysis of apoptosis of AGS cells treated with 5-Aza-dC. The rate of apoptosis in AGS cells treated with 5-Aza-dC (1  $\mu$ mol/L) at day 4 was significantly higher compared to untreated cells ( $P < 0.01$ ,  $n = 3$ ). The representative analysis of AGS cell apoptosis by annexin V/PI method is shown.

of the representative MSP amplification products electrophoretogram is shown in Figure 2A. To further confirm the fact of *PTCH1a* TRR methylation, we chose gastric cancer tissue sample #6 with a positive methylation amplification product, and the pool of adjacent normal tissues ( $n = 12$ ) for BSP clone sequencing. As shown in Figure 2B, almost all of the 19 CpG sites in 10 clones exhibited methylation in cancer tissues, while very few CpG sites in adjacent normal tissues did. These results demonstrated that the methylation of *PTCH1a* TRR did exist in a subset of gastric cancer tissues.

#### Correlation between methylation of *PTCH1a* TRR and *PTCH1* expression in gastric cancer tissues

We analyzed the correlation between methylation of *PTCH1a* TRR and *PTCH1* expression. As shown in Figure 3, there was a significant difference in *PTCH1* mRNA expression between methylated and unmethylated gastric cancer tissues. High expression had a negative correlation with high methylation (Spearman's  $r = -0.380$ ;  $P = 0.000$ ). To further determine this negative correlation, the *PTCH1* protein was examined in four representative samples by immunohistochemistry. As shown in Figure 4, two samples (#3 and #5) with



**Figure 2** Methylation of *PTCH1a* TRR in gastric cancer tissues. A: Methylation of *PTCH1a* TRR in gastric cancer tissues ( $n = 170$ ) using MSP. MSP results from eight representative patients (#) are shown. The DNA bands in lanes labeled with M represent the products amplified with the methylation-specific primers, while DNA bands labeled with U represent the products amplified with the non-methylation-specific primers. CpGenome Universal Methylated DNA (S7821) and the CpGenome Universal Unmethylated DNA (s7822) were used as controls for methylation and non-methylation. Water was used as non-template control (NTC); B: Genomic DNA of gastric cancer tissues and its corresponding normal tissues from a representative patient (#6) was analyzed by BSP. Methylation patterns of the 19 CpG sites contained in the BSP amplicon through 10 clone sequence analyses in cancer tissues, and corresponding normal tissues are shown. Black dot, methylation; white dot, non-methylation.

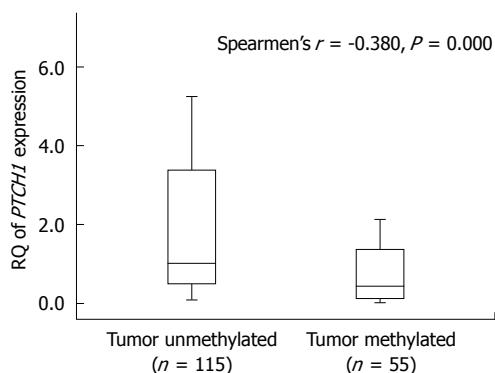
visible unmethylated products by MSP were positive for *PTCH1* protein and had higher RQ value of *PTCH1* mRNA expression, while another two samples (#7 and #10) with visible methylated products by MSP were negative for *PTCH1* protein and had lower RQ value of *PTCH1* mRNA expression. Samples #3 and #7 were well-differentiated, while #5 and #10 were poorly differentiated. Notably, this was further proof that a subset of gastric cancer tissues were characterized by methylation of *PTCH1a* TRR, along with lower expression of the *PTCH1* gene.

#### Relationship between *PTCH1a* TRR methylation in gastric cancer tissues and clinical features

We analyzed statistically the relationship between the methylation of *PTCH1a* TRR in gastric cancer tissues and clinical features. As shown in Table 2, there was no correlation between the methylation of *PTCH1a* TRR and clinical features, including sex, age of onset, clinical stage, lymph node metastasis, and histological grade. These data suggest that methylation of *PTCH1a* TRR is an early event in gastric tumorigenesis.

## DISCUSSION

The methylation of tumor suppressor gene plays an important role in the tumorigenesis of gastric cancer. *PTCH1* gene is a known tumor suppressor gene in



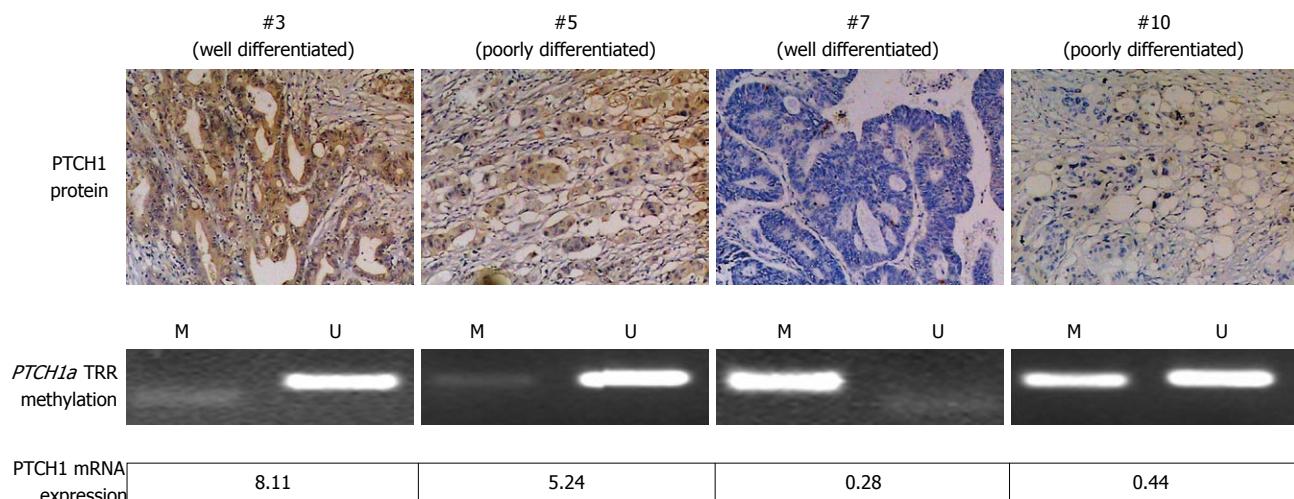
**Figure 3** Correlation between methylation of *PTCH1a* gene TRR and expression of *PTCH1* in gastric cancer tissues. Box plot illustrating the loss of *PTCH1* gene expression in relation to the methylation of *PTCH1a* gene TRR in human gastric cancer tissues ( $n = 170$ ). The Y axis indicates the RQ value of *PTCH1* gene mRNA expression was calculated in comparison with a calibrator (the expression level of pooled adjacent normal tissue samples). Horizontal lines: Group medians; Boxes: 25%-75% quartiles; Vertical lines: Range, peak and minimum.

**Table 2** Clinical features in relation to methylation of *PTCH1a* TRR in gastric cancer

Variable	<i>PTCH1</i> methylation			<i>P</i> value
	<i>n</i>	Positive	Negative	
Total	170	55	115	
Clinicopathological parameters				
Sex				
Male	89	26	63	0.359
Female	81	29	52	
Age of onset (yr)				
< 50	68	22	46	1.000
≥ 50	102	33	69	
Clinical stage				
pT1	75	25	50	0.808
pT2-4	95	30	65	
Lymph node metastasis				
pN0	55	16	39	0.530
pN1-3	115	39	76	
Histological grade				
Well and moderately differentiated	63	19	44	0.735
Poorly differentiated	107	36	71	

the HH pathway. Loss of function mutation and epigenetic regulation has been found in many kinds of tumors<sup>[3,6,13,14]</sup>.

*PTCH1* gene has three main isoforms of the first exon, *PTCH1a*, *PTCH1b*, and *PTCH1c*, which code for different N-sequence *PTCH1* proteins PTCH1-l, PTCH1-m and PTCH1-s, respectively<sup>[18]</sup>. Each expression is regulated by its own independent TRR. Although it has been reported that PTCH1-l and PTCH1-m have the same effect on inducing apoptosis and suppressing GLI-mediated transcription, only the methylation analysis of *PTCH1b* TRR has been reported by some research groups. Cretnik *et al*<sup>[17]</sup> have reported that methylation of *PTCH1b* TRR (-1593 bp, transcription initiation site of *PTCH1b* as 0) occurs in ovarian tumors (dermoids and fibromas) compared to healthy controls, but not in basal cell carcinoma. Wolf *et al*<sup>[14]</sup> have demonstrated methylation



**Figure 4 Correlation of PTCH1 mRNA and protein expression with methylation of PTCH1a TSS.** Expression of PTCH1 genes, as well as methylation of PTCH1a TSS are displayed in four representative gastric cancer tissue samples (#3, #5, #7 and #10). PTCH1 protein expression was detected by immunohistochemical staining (original magnification,  $\times 100$ ). Methylation of PTCH1a TSS was detected by MSP. The RQ value of PTCH1 gene mRNA expression was calculated in comparison with a calibrator (the expression level of pooled adjacent normal tissue samples). The well-differentiated tissue sample #3 and the poorly differentiated tissue sample #5 showed non-methylated products by MSP, positive expression of PTCH1 protein, and higher RQ value of PTCH1 mRNA expression. The well-differentiated tissue sample #7 and poorly differentiated tissue sample #10 showed methylated products by MSP, negative expression of PTCH1 protein, and lower RQ value of PTCH1 mRNA expression.

of PTCH1b TSS (-776 to +1238 bp, transcription initiation site of PTCH1b as 0) in breast cancer cell lines and tissues, which has a negative correlation with PTCH1 expression. However, Pritchard *et al*<sup>[15]</sup> have found that there is no methylation of PTCH1b TSS (-983 bp, transcription initiation site of mRNA1b as 0) in primary medulloblastoma.

In the present study, we analyzed the methylation status of PTCH1a TSS. We selected the upstream regulation region of PTCH1a (-643 to +355 bp, transcription initiation site of mRNA1b as 0) as the target region to be analyzed because this region was in the CpG island that appeared nearest to the transcription initiation site of PTCH1a gene, according to the software analysis. In order to investigate the methylation status in a number of gastric cancer tissues, the BSP colon sequence method was used to identify the suitable CpG sites for the MSP primer design. We found that the upstream regulatory sequence of PTCH1a gene was methylated in the gastric cancer cell line and a subset of gastric cancer tissues, and this methylation correlated with low expression of the PTCH1 gene. Nagao *et al*<sup>[18]</sup> have reported that the expression of these three isoforms is regulated by GLI transcription factors, one in exon 1a and the other between exons 1a and 1b, in the vicinity of which methylation is found in ovarian tumors<sup>[17]</sup>. However, we found methylation at -441 bp upstream of the GLI binding site in exon 1, and the methylation of this target region was correlated negatively with PTCH1 gene expression. These results suggest that CpG island methylation in the TSS of PTCH1a gene plays a role in the regulation of not only PTCH1a transcription, but also downstream PTCH1b and PTCH1 transcription.

Several recent studies have demonstrated that activation of the HH signaling pathway is involved in gastric

tumorigenesis<sup>[21-25]</sup>. However, PTCH1 gene expression has not been investigated extensively, especially in normal gastric tissues. Ma *et al*<sup>[26]</sup> have reported that PTCH1 mRNA expression was detected by hybridization in about 64% (63/99) of gastric cancer tissues but not in normal gastric tissues (0/18). Many other studies have shown by immunohistochemistry that PTCH1 gene expression was present in the fundic glandular epithelium of the stomach<sup>[8,24]</sup>. We found that adjacent normal tissues expressed PTCH1 gene, along with being unmethylated, which confirms that PTCH1 gene expression is present in normal gastric tissues.

Berman *et al*<sup>[13]</sup> have found that the PTCH1 gene was expressed in six human gastric cancer cell lines including AGS, which indicates that the Hedgehog signaling pathway is activated not by mutation, but by ligand expression. The expression of PTCH1a and PTCH1b genes was equally active in terms of suppressing GLI-mediated transcription, as a negative feedback for regulation of the HH signaling pathway, and induction of apoptosis<sup>[18]</sup>. We found that the demethylation reagent 5-Aza-dC reversed the methylation of PTCH1a gene, enhanced PTCH1 gene expression, and induced apoptosis. Our results implied that the enhanced expression of PTCH1 gene that resulted from demethylation strengthened the negative feedback function of PTCH1, which provided a new target for treating gastric cancer.

Previous studies have found that a high level of aberrant DNA methylation exists in *Helicobacter pylori* (*H. pylori*)-infected gastric mucosa and is possibly associated with gastric cancer risk<sup>[27,28]</sup>. Others have shown that *H. pylori* infection might affect the HH pathway that is involved in gastric carcinogenesis<sup>[29,30]</sup>. These results suggest that the methylation of PTCH1a TSS in gastric cancer may be triggered by *H. pylori* infection in the early

course of carcinogenesis. This will be studied in our laboratory in the future.

We demonstrated that methylation of *PTCH1a* TRR was present in a subset of gastric cancers. To the best of our knowledge, this phenomenon has not been observed or reported by any research group to date. Methylation of *PTCH1* was correlated negatively with *PTCH1* gene expression and was not related to clinical features of gastric cancer, which suggested that the methylation of *PTCH1a* TRR is an early event in gastric tumorigenesis. Downregulation of *PTCH1a* gene methylation may provide a new therapy for gastric cancer characterized by *PTCH1a* TRR methylation.

## COMMENTS

### Background

Abnormal activation of the hedgehog (HH) pathway has been reported in subsets of human basal cell carcinoma, medulloblastoma, pancreatic cancer, lung cancer, prostate cancer and gastrointestinal cancer. Although loss-of-function mutation in the *PTCH1* gene participates in the abnormal activation of the HH pathway, several studies have argued that the *PTCH1* transcriptional regulation region (TRR) methylation is involved in tumorigenesis. However, none has been reported in gastric cancer.

### Research frontiers

Previous studies have shown that the *PTCH1* gene has three major isoforms in the first exon, *PTCH1a*, *PTCH1b* and *PTCH1c* that code for different N-sequence *PTCH1* proteins *PTCH1-I*, *PTCH1-m* and *PTCH1-s*, respectively. Expression of each is regulated by its own independent TRR. The present study analyzed the methylation of *PTCH1a* TRR in gastric cancer.

### Innovations and breakthroughs

The present study showed that methylation of *PTCH1a* TRR is present in a subset of gastric cancers, and correlated negatively with *PTCH1* gene expression. It was not related to the clinical features of gastric cancer, which suggests that methylation of *PTCH1a* TRR is an early event in gastric tumorigenesis. To the best of our knowledge, these phenomena have never been observed or reported by any research group to date.

### Applications

By understanding that the methylation of *PTCH1a* TRR might be an early event in gastric tumorigenesis, this study may provide a new therapy for gastric cancer that is characterized by *PTCH1a* TRR methylation.

### Terminology

The HH pathway plays a critical role in embryonic development, tissue polarity and carcinogenesis. In the HH pathway, the receptor *PTCH1* is encoded by the *PTCH1* gene as a tumor suppressor gene, and plays a role in negative feedback regulation of activation of the HH pathway. Methylation of TRR can induce the loss of gene expression. Thus, methylation of *PTCH1a* TRR might be an early event in gastric tumorigenesis.

### Peer review

The authors detected the methylation of *PTCH1a* TRR in gastric cancer cell line AGS and 170 gastric cancer tissue samples and adjacent normal tissues, and analyzed the correlation of *PTCH1a* TRR methylation with *PTCH1* expression and clinical features. They revealed that the methylation of *PTCH1a* TRR correlated negatively with *PTCH1* expression. However, the methylation of *PTCH1a* TRR was not related to the clinical features of gastric cancer, such as sex, age of onset, clinical stage, lymph node metastasis, and histological grade. The results are interesting and may represent a molecular mechanism of gastric carcinogenesis.

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# Connective tissue growth factor hammerhead ribozyme attenuates human hepatic stellate cell function

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## Abstract

**AIM:** To determine the effect of hammerhead ribozyme targeting connective tissue growth factor (CCN2) on human hepatic stellate cell (HSC) function.

**METHODS:** CCN2 hammerhead ribozyme cDNA plus two self-cleaving sequences were inserted into pTriEx2 to produce pTriCCN2-Rz. Each vector was individually transfected into cultured LX-2 human HSCs, which were then stimulated by addition of transforming growth factor (TGF)- $\beta$ 1 to the culture medium. Semi-quantitative RT-PCR was used to determine mRNA levels for CCN2 or collagen I, while protein levels of each molecule in cell lysates and conditioned medium were measured by ELISA. Cell-cycle progression of the transfected cells was assessed by flow cytometry.

**RESULTS:** In pTriEx2-transfected LX-2 cells, TGF- $\beta$ 1 treatment caused an increase in the mRNA level for CCN2 or collagen I, and an increase in produced and secreted CCN2 or extracellular collagen I protein levels. pTriCCN2-Rz-transfected LX-2 cells showed decreased basal CCN2 or collagen mRNA levels, as well as produced and secreted CCN2 or collagen I protein. Furthermore, the TGF- $\beta$ 1-induced increase in mRNA or protein for CCN2 or collagen I was inhibited partially in pTriCCN2-Rz-transfected LX-2 cells. Inhibition of

CCN2 using hammerhead ribozyme cDNA resulted in fewer of the cells transitioning into S phase.

**CONCLUSION:** Endogenous CCN2 is a mediator of basal or TGF- $\beta$ 1-induced collagen I production in human HSCs and regulates entry of the cells into S phase.

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**Key words:** Connective tissue growth factor; Fibrosis; Hepatic stellate cell; Transforming growth factor- $\beta$ 1

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## INTRODUCTION

Activation of hepatic stellate cells (HSCs) is a central event in the pathobiology of hepatic fibrosis<sup>[1,2]</sup>. In response to chronic liver injury, quiescent HSCs undergo gradual phenotypic changes that result in their trans-differentiation into  $\alpha$ -smooth muscle actin (SMA)-positive, proliferative, myofibroblast-like cells. In the injured liver, activated HSCs are responsible for producing much of the excess extracellular matrix, including fibrillar collagen<sup>[1,2]</sup>. At the molecular level, this process is driven by a variety of growth factors, cytokines and matricellular proteins. While transforming growth factor (TGF)- $\beta$  has received special attention because of its potent fibrogenic effects *in vivo* and its ability to stimulate fibrogenic pathways in HSCs<sup>[3]</sup>, targeting of this molecule for therapeutic intervention is problematic because of its other important actions in the liver, including tumor suppression and immune modulation. However, a potentially more attractive target is connective tissue growth factor (CCN2), which appears to mediate many of the fibrogenic properties of TGF- $\beta$ <sup>[4]</sup>.

CCN2 was discovered as a TGF- $\beta$ -immediate early gene in 1989<sup>[5]</sup> and numerous studies since then have described an intimate relationship between these molecules in many fibrotic diseases, including those of the liver<sup>[6]</sup>. CCN2 is recognized increasingly as a central player in hepatic fibrosis and may offer new options for prognosis, diagnosis and therapy<sup>[7]</sup>. Previously, we have shown that exposure of HSCs to CCN2 induces cell adhesion, migration and proliferation, the latter of which is associated with transient induction of c-fos and activation of the extracellular signal-regulated kinase 1/2 signaling pathway<sup>[8-10]</sup>. In addition, CCN2 induces expression of  $\alpha$ -SMA and type I collagen in HSCs, consistent with a role in activation and fibrogenesis<sup>[8]</sup>. We have shown that adhesive signaling by CCN2 in HSCs increases expression of collagen type I, fibronectin, and tissue inhibitor of metalloproteinase (TIMP)-1, and decreases expression of caspase 8 and hepatocyte growth factor<sup>[11]</sup>. CCN2 also stimulates survival pathways in activated HSCs<sup>[12]</sup>. These data suggest that CCN2 drives both fibrogenic and anti-apoptotic pathways in activated HSCs and reinforce the notion that CCN2 is a potential novel therapeutic target in liver fibrosis.

We and others have shown that CCN2 mRNA and protein are expressed increasingly during progressive activation of cultured primary rat HSCs, or in response of the cells to stimulation by TGF- $\beta$ , vascular endothelial growth factor, lipid peroxidation products, acetaldehyde or platelet-derived growth factor-BB<sup>[8,13-16]</sup>. We have shown further that CCN2 promoter activity is enhanced in a Smad7-dependent fashion by TGF- $\beta$  in primary rat HSCs transfected with the CCN2 promoter, luciferase reporter construct<sup>[10]</sup>, and that CCN2 production in the HSCs is stimulated by TGF- $\beta$ <sup>[6]</sup>. We recently showed that TGF- $\beta$ -induced CCN2 promoter activity in activated mouse HSCs requires Smad and Ets-1 elements in the CCN2 promoter<sup>[16]</sup>, as described for normal fibroblasts or mesangial cells<sup>[17-20]</sup>. However, CCN2 promoter activity in activated HSCs is uniquely antagonized by ALK4/5/7 inhibition<sup>[16]</sup>. CCN2 gene regulation in activated HSCs is distinct from that of scleroderma, which is independent of Smad/ALK5-mediated TGF- $\beta$  signaling, but is dependent on endothelin-1<sup>[17,21,22]</sup>, or pancreatic cancer, which depends on activated ras/MEK/ERK rather than TGF- $\beta$  or endothelin-1<sup>[23]</sup>.

Surprisingly, there is no information regarding the role of basal or TGF- $\beta$ -induced CCN2 in the function of human HSCs. In the present study, we investigated the effect of a hammerhead ribozyme that targets CCN2 on TGF- $\beta$ 1-induced collagen I synthesis or cell-cycle progression in the LX-2 human HSC line, which was generated originally by spontaneous immortalization of primary human HSCs in low-serum conditions<sup>[24]</sup>.

## MATERIALS AND METHODS

### Reagents

Dulbecco's Modified Eagle's Medium (DMEM),

Lipofectamine<sup>TM</sup> 2000 and TRIzol were from Life Technologies (Carlsbad, CA, USA). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT, USA). Human TGF- $\beta$ 1 was purchased from PeproTech (Rocky Hill, NJ, USA). The human HSC line, LX-2, was a kind gift of Dr. Scott Friedman (Mount Sinai Hospital, New York). pTriEx2 vector was obtained from Novagen (Gibbstown, NJ, USA). *Xba*I and *Nco*I were from Toyobo (Osaka, Japan). Avian myeloblastosis virus and Taq DNA polymerase were from Promega (Madison, WI, USA). ELISA kits for human CCN2 or collagen I were from, respectively, USCN Life Science and Technology Co. (TX, USA) or Cosmo Bio (Tokyo, Japan).

### Ribozyme design and recombinant plasmid construction

The mRNA sequence of human CCN2 (NCBI, gi: 98986335) was scanned for potential hammerhead ribozyme cleavage sites using proprietary design software developed by the Shanghai Institute of Biological Chemistry of the Chinese Academy of Sciences. Of the five potential hammerhead ribozyme cleavage sites, one site located at 896 of CCN2 mRNA was identified with optimal secondary folding. A 34-mer hammerhead ribozyme cDNA that targeted at the C-U-A at position 896 of CCN2 mRNA (sense: 5'-CTTCTCCTGATGAGTCCTGAGGACG AAAGCCTG-3'; antisense: 5'-CAGGCTTCGTCCTC ACGGACTCATCAGGAGAAG-3'), plus two self-cleaving sequences, were synthesized chemically and inserted into pBluescript II SK9(-) cloning vector. Both pTriEx2 and pBluescript II SK9(-) were linearized with *Xba*I and *Nco*I individually, and the digested products were ligated using T4 DNA ligase. The resulting vector, termed pTriCCN2-Rz, (in which synthesis of the ribozyme was driven by the chicken  $\beta$ -actin promoter and the cytomegalovirus enhancer) was confirmed by DNA sequencing.

### Cell culture and transfection

LX-2 cells were allowed to grow at 37°C for 2 d in DMEM that contained 10% FBS, 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin. The cells were digested with 0.25% trypsin, and washed with Hank's Balanced Salt Solution (without  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ ). The cells were then cultured in either 20  $\times$  100-mm cell culture dishes for RNA isolation and determination of cell-cycle progression, or 12-well plates for measurement of production and secretion of CCN2 or collagen I. Prior to transfection, the cells were grown in DMEM that contained 2.5% FBS for 24 h, followed by DMEM that contained 0.1% FBS for another 24 h. Cells were transfected with vectors using Lipofectamine<sup>TM</sup> 2000 reagent under serum-free conditions for 4 h. The transfected cells were incubated for another 24 h in the presence or absence of 20 ng/mL TGF- $\beta$ 1.

### Semi-RT-PCR

Total RNA was extracted from cultured LX-2 cells using TRIzol reagent. An optimal RNA template (0.8  $\mu$ g)

was generated by semi-quantitative RT-PCR using a UNOII thermocycler (Biometra, Germany). The expression of mRNA for human CCN2, collagen  $\alpha$ 1 (I), or  $\beta$ -actin was examined by RT-PCR using the following forward and reverse primers: CCN2 forward, 5'-CCTGGTCCAGACCACAGAGT-3'; CCN2 reverse, 5'-ATGTCTTCATGCTGGTGCAG-3'; collagen  $\alpha$ 1 (I) forward, 5'-CCTCAAGGGCTCCAACGAG-3'; collagen  $\alpha$ 1 (I) reverse, 5'-TCAATCACTGTCTGCCCA-3';  $\beta$ -actin forward, 5'-GTCCTCTCCAAAGTCCACAC-3';  $\beta$ -actin reverse, 5'-GGGAGACCAAAAGCCTTCAT-3'.

Briefly, 0.8  $\mu$ g RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase in the presence of oligo(dT) and dNTP, followed by amplification of cDNA (1  $\mu$ L), using Taq DNA polymerase in a final volume of 50  $\mu$ L that contained 1 mmol/L MgCl<sub>2</sub>, 0.2  $\mu$ mol/L of each dNTP, and 0.5  $\mu$ mol/L of each primer. Amplification conditions were 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, over 32 cycles. Amplification products were visualized on ethidium-bromide-stained agarose gels. Image analysis of the individual transcripts was carried out using Gel-Pro 3.2 software (Media Cybernetics, Bethesda, MD, USA). Data were expressed as the gray-scale ratio of either CCN2 mRNA or collagen  $\alpha$ 1 (I) mRNA relative to that of  $\beta$ -actin mRNA.

### **CCN2 or collagen I ELISA**

CCN2 or collagen I levels were measured in cell lysates or conditioned medium of serum-starved, cultured LX-2 cells in the absence or presence of 20 ng/mL TGF- $\beta$ 1 for 24 h using a sandwich ELISA following the manufacturer's instructions for each kit. Briefly, microtiter wells were pre-coated for 2 h (CCN2) or 30 min (collagen I) at 37°C, with 100  $\mu$ L of each standard, or 1:20 dilutions of LX-2 cell lysates or conditioned medium supernatant. The plates were then developed by addition of biotinylated anti-CCN2 antibody or anti-collagen I antibody, followed by avidin-conjugated horseradish peroxidase. The color reaction was developed using tetramethylbenzidine substrate solution and measured by its absorbance at 450 nm using a Model 550 plate reader (BioRad, Hercules, CA, USA).

### **Flow cytometry**

LX-2 cell-cycle progression was determined by resuspending the cells at 1  $\times$  10<sup>6</sup> cells/mL in PBS, fixing the cells with 75% ethanol overnight, and then staining the cells with 0.1  $\mu$ g/mL propidium iodide in a 0.1% sodium citrate/0.1% Triton X-100 solution for 30 min at room temperature in the dark, in the presence of 0.2 mg/mL RNase. Analysis of cellular DNA content after cell staining with propidium iodide was performed by flow cytometry at an excitation wavelength of 488 nm. The distribution of cells in three major phases of the cycle (G0/G1, S, G2/M) was analyzed using CellQuest software (BD Biosciences, San Jose, CA, USA).

### **Statistical analysis**

The values reported represent the mean  $\pm$  SD of the

measurements of at least four different experiments. Photographs of RT-PCR products are representative of at least three independent experiments. Statistical analysis of the data was performed using SPSS for Windows version 11 (SPSS, Chicago, IL, USA). The Student *t* test was used for paired data that were normally distributed, and *P* < 0.05 was considered significant.

## **RESULTS**

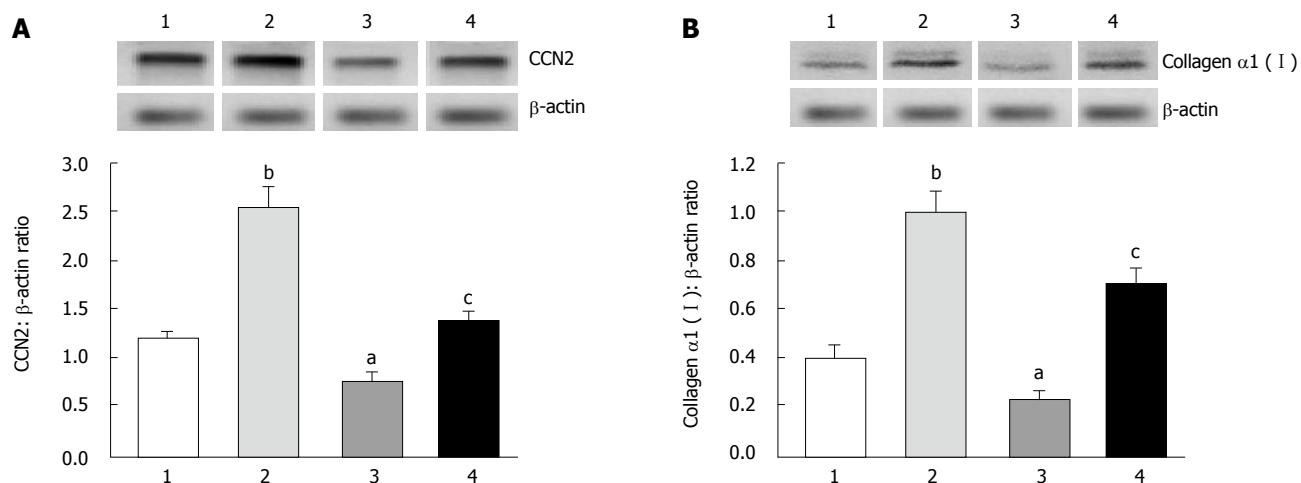
### **Effects of hammerhead ribozyme on CCN2 mRNA and protein expression in cultured LX-2 cells**

Previous studies have demonstrated that the LX-2 cell line contains CCN2 mRNA<sup>[25]</sup>, a feature that is characteristic of the activated HSC phenotype<sup>[6]</sup>. Since many TGF- $\beta$  pro-fibrogenic activities are mediated by CCN2<sup>[26]</sup> and TGF- $\beta$  stimulates collagen production in LX-2 cells<sup>[24]</sup>, we examined the functional relationship between TGF- $\beta$  and CCN2, utilizing hammerhead ribozymes designed to cleave CCN2 mRNA in the cells. Initial experiments were performed in which the LacZ reporter gene was inserted downstream of CCN2 hammerhead ribozyme cDNA in pTriCCN2-Rz, and into the downstream multiple cloning site of pTriEx2, to investigate the transcriptional activity of both vectors. When histochemical X-gal staining was performed on cell cultures transfected with either pTriEx2 or pTriCCN2-Rz individually, about 30% of the cells were positive for each vector (data not shown), and this transfection efficiency was sufficient to assess subsequently the functional effect of the hammerhead ribozyme that targeted CCN2.

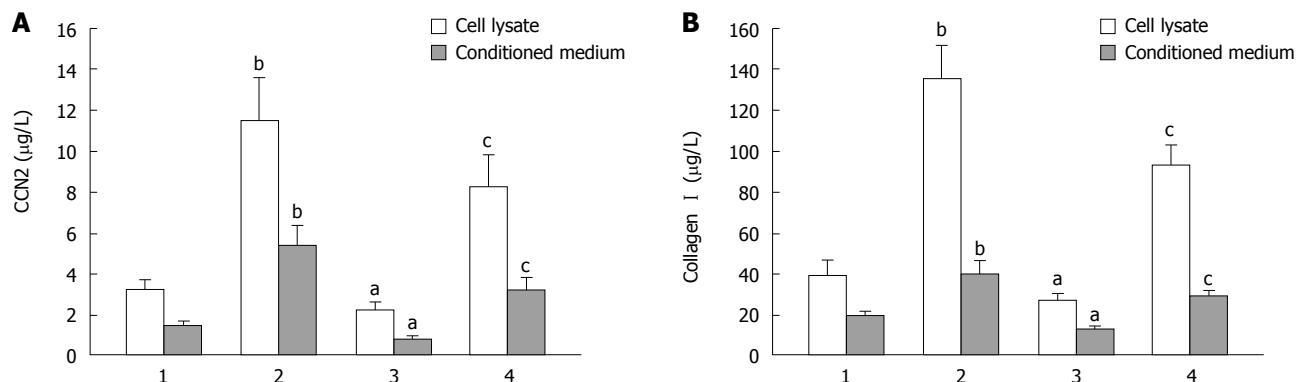
CCN2 mRNA and protein in pTriEX-2-transfected LX-2 cells were present under basal growth conditions and there was an approximately 2.5- and 3.5-fold increase, respectively, in CCN2 transcript and protein levels (*P* < 0.01, paired *t* test) after stimulation of the cells with TGF- $\beta$  for 24 h (Figures 1A and 2A). In contrast, after transfection with the pTriCCN2-Rz vector, the LX-2 cells demonstrated reduced constitutive expression of CCN2 mRNA and protein (*P* < 0.05) under baseline conditions and moreover, the stimulation of CCN2 mRNA and protein (*P* < 0.05) in response to TGF- $\beta$  was attenuated substantially (*P* < 0.05). These data confirmed the ability of the hammerhead ribozymes to target CCN2 mRNA and to block CCN2 production and secretion, thus allowing for its function in LX-2 cells to be further explored.

### **Effects of hammerhead ribozyme on TGF- $\beta$ 1-induced collagen I mRNA and protein expression in cultured LX-2 cells**

Under basal conditions, pTriEx-2-transfected LX-2 cells produced collagen  $\alpha$ 1 (I) mRNA and the amount of this transcript was enhanced two-fold by TGF- $\beta$  treatment (*P* < 0.01; Figure 1B). On the other hand, under basal conditions, pTriCCN2-Rz-transfected LX-2 cells produced approximately 60% of the level of collagen  $\alpha$ 1 (I) mRNA as control cells (*P* < 0.05). Whereas TGF- $\beta$  treatment of pTriCCN2-Rz-transfected



**Figure 1** Effect of CCN2 hammerhead ribozyme on basal or TGF-β-induced mRNA expression of CCN2 or collagen α1 (I). The figure shows the RT-PCR reaction products (upper panel) and the densitometric scans (lower panel) for (A) CCN2 mRNA or (B) collagen α1 (I) after transfection of LX-2 cells with pTriEx2 (lanes 1 and 2) or pTriCCN2F-Rz (lanes 3 and 4) under basal conditions (lanes 1 and 3), or after stimulation with 20 ng/mL TGF-β1 for 24 h (lanes 2 and 4). <sup>a</sup>P < 0.05 vs lane 1; <sup>b</sup>P < 0.01 vs lane 1; <sup>c</sup>P < 0.05 vs lane 2.



**Figure 2** Effect of CCN2 hammerhead ribozyme on basal or TGF-β-induced protein production or secretion of CCN2 or collagen I. The figure shows the protein levels of CCN2 (A) or collagen I (B) in cell lysates or conditioned medium measured by ELISA after transfection of the cells with pTriEx2 (lanes 1 and 2) or pTriCCN2F-Rz (lanes 3 and 4) under basal conditions (lanes 1 and 3), or after stimulation with 20 ng/mL TGF-β1 for 24 h (lanes 2 and 4). <sup>a</sup>P < 0.05 vs lane 1; <sup>b</sup>P < 0.01 vs lane 1; <sup>c</sup>P < 0.05 vs lane 2.

LX-2 cells resulted in stimulation of the amount of collagen α1 (I) mRNA, the level attained was only 70% of that seen in TGF-β-stimulated control cells ( $P < 0.05$ ; Figure 1B). Essentially identical results were obtained when cell lysates or conditioned medium from the cells were tested by ELISA, in that basal and TGF-β-stimulated collagen I protein production and secretion was attenuated significantly in pTriCCN2F-Rz-transfected LX-2 cells as compared to those transfected with pTriEx-2 ( $P < 0.05$ ; Figure 2).

### Effects of hammerhead ribozyme on LX-2 cell-cycle progression

We next used flow cytometry to assess the effect of basal CCN2 expression on LX-2 cell-cycle progression. As shown in Table 1, after pTriCCN2F-Rz transfection, a relatively higher proportion of cells were in the G0/G1 phase ( $P < 0.05$ ) and a lower proportion were in S phase ( $P < 0.05$ ), as compared to pTriEx2-transfected cells. This showed that endogenous CCN2 played a role in the G0/G1 → S transition in LX-2 cells. To verify that

this difference was not caused by greater cytotoxicity of pTriCCN2F-Rz than pTriEx2, the viability of transfected LX-2 cells was verified by trypan blue exclusion assay. Less than 3% of the pTriEx2- or pTriCCN2F-Rz-transfected cells stained blue, and there was no difference in the frequency of trypan-blue-positive cells between pTriEx2 and pTriCCN2F-Rz transfection (data not shown). Moreover, comparable amounts of β-actin mRNA were amplified from pTriEx2- or pTriCCN2F-Rz-transfected cells (Figure 1).

### DISCUSSION

In the present study, a recombinant vector, pTriCCN2F-Rz, bearing hammerhead ribozyme cDNA plus two self-cleaving sequences that targeted the 896 site of CCN2 mRNA, was constructed and transfected into the LX-2 human HSC line. The hammerhead ribozyme blocked CCN2 mRNA transcription and protein production in LX-2 cells and inhibited basal or TGF-β1-induced transcription and production of collagen I in LX-2 cells.

**Table 1 Distribution of cell-cycle phases in pTRIEx2- or pTriCCN2-Rz-transfected LX-2 cells**

Vector	G0G1 (%)	S (%)	G2M (%)
pTRIEx2	60.64 ± 2.26	27.53 ± 1.76	11.83 ± 1.36
pTriCCN2-Rz	80.45 ± 3.12 <sup>a</sup>	10.83 ± 1.02 <sup>b</sup>	8.27 ± 0.68

<sup>a</sup>P < 0.05, <sup>b</sup>P < 0.01 compared with pTRIEx2.

That the knockdown achieved in these experiments was only partial was likely attributable to the transfection efficiency of 30%, but even so, the data clearly showed a CCN2-dependency of basal or TGF-β-stimulated collagen I production in activated human HSCs. This finding, coupled with the ability of hammerhead ribozyme targeting of CCN2 to attenuate the rate of HSC proliferation, suggest that it is a useful therapeutic strategy in fibrosis of the liver and possibly, of other organ systems.

In recent years, the relationship between CCN2 and TGF-β in fibrogenic pathways has been explored using blocking strategies that exploit the targeting of CCN2 mRNA with antisense oligonucleotides or small interfering RNA (siRNA)<sup>[27]</sup>. The use of CCN2 antisense oligonucleotides has helped to establish the importance of CCN2 for TGF-β-induced collagen production in a variety of cell types including kidney mesangial cells, NRK cells, corneal fibroblasts, and conjunctival fibroblasts<sup>[28-33]</sup>. In *in vivo* animal models, CCN2 antisense oligonucleotides have proven effective in reducing matrix expansion or fibrosis in the skin or kidney<sup>[34-36]</sup>, and scarring around breast implants<sup>[37]</sup>. However, while CCN2 antisense therapy in CCl<sub>4</sub>-induced liver fibrosis is associated with reduced expression of mRNA for CCN2 or collagen, fibrous deposits are not reduced, an effect that has been attributed to high expression of TIMP-1<sup>[38]</sup>.

Insight into fibrogenic mechanisms has also been obtained from experiments that employ CCN2 siRNA technology that relies on short dsRNA for gene silencing, and shows improved potency and specificity as compared to antisense oligonucleotides<sup>[27]</sup>. CCN2 siRNA was effective in blocking collagen synthesis in scleroderma fibroblasts<sup>[39]</sup>, glucose-induced matrix production in vascular smooth muscle cells<sup>[40]</sup>, or TGF-β-stimulated collagen production in dermal fibroblasts, osteoblasts or rat HSCs<sup>[41-43]</sup>. Plasmids expressing short hairpin RNA, which are easier to produce and more effective than some traditional siRNA approaches, have been shown to disrupt CCN2 gene expression in cultured rat HSCs, and to cause marked attenuation in the production of collagen III and IV, laminin, and hyaluronic acid<sup>[44]</sup>. Finally, *in vivo* studies have shown that CCN2 siRNA delivery to rats is an effective anti-fibrotic therapy in renal allografts after transplantation or in livers after exposure to N-nitrosodimethylamine<sup>[45,46]</sup>. In mice, an HSC-specific targeting strategy has been employed for CCN2 siRNA therapy in CCl<sub>4</sub>-induced hepatic fibrosis<sup>[27]</sup>.

Additional approaches for antagonizing the

production or action of CCN2 have been described, such as those employing pharmacological inhibitors or neutralizing antibodies<sup>[27]</sup>. However, pharmacological inhibitors are not necessarily specific to CCN2, while the use of neutralizing antibodies or antisense oligonucleotides relies on a stoichiometric and relatively inefficient 1:1 complex formation with their respective CCN2 protein or RNA target. Since, in our studies, the CCN2 ribozyme acted catalytically by binding and cleaving its target RNA, whereupon the fragments were released and the cycle was repeated numerous times, this mode of CCN2 antagonism was considerably more efficient than other methods. On the other hand, since the hammerhead ribozyme motif cleaves the phosphodiester bond downstream of a NUY triplet (where N is any base and Y is any base except G), a potential drawback to the use of ribozymes in some applications is the availability of this required NUY cleavage site in the target RNA molecule.

We conducted these studies with LX-2 cells because they are viable in serum-free media, demonstrate many of the hallmarks of activated HSCs, and because human HSCs have not been studied previously in the context of CCN2 mRNA inhibition. Our data support the potential use of hammerhead ribozyme in targeting CCN2 in fibrotic livers since, in the presence or absence of added TGF-β, it reduced collagen synthesis and secretion by the cells, and reduced the frequency of S-phase cells, even though the cells were constitutively activated and had a high basal proliferative rate. This latter observation is of interest because we are aware of only one other report in which a CCN2 hammerhead ribozyme has been developed, and this has been shown to block TGF-β-mediated proliferation of dermal fibroblasts<sup>[47]</sup>. Thus, our data suggest that targeting CCN2 mRNA will likely cause anti-fibrogenic and anti-proliferative effects within the activated HSC population in fibrotic liver.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Fibrosis is a debilitating pathology that can occur in many different organs and affects millions of people around the world. It is characterized by excessive collagen deposition that can prevent essential organs from functioning properly, and can ultimately lead to organ failure. In the liver, a cell type called the hepatic stellate cell (HSC) is responsible for producing much of the collagen that contributes to hepatic fibrosis, and recent evidence suggests that connective tissue growth factor (CCN2) is an important molecule that drives collagen production in these cells.

### Research frontiers

There is much optimism that antagonism of CCN2 may be a novel and effective means of preventing or reversing fibrosis. Methods for preventing the production or action of CCN2 are at the forefront of research in this area, and it is important to examine the various options available so that rational therapeutic approaches can be developed.

### Innovations and breakthroughs

Previously, it has been shown that transforming growth factor (TGF)-β is potent

fibrogenic molecule *in vivo* and can stimulate fibrogenic pathways in HSCs. However, TGF- $\beta$  plays essential roles in the liver, such as tumor suppression and immune modulation, such that targeting of this molecule for therapeutic intervention is problematic. Since CCN2 acts downstream of TGF- $\beta$  in fibrogenic cascades and mediates many of the fibrogenic properties of TGF- $\beta$ , it is becoming accepted that CCN2 is a more appropriate molecule to target than TGF- $\beta$ . Recently, methods have begun to emerge that achieve antagonism of CCN2 mRNA through the use of blocking cDNA or RNA sequences. In the present study, the authors used this type of blocking approach in a human HSC line, LX-2, to prevent CCN2 mRNA transcription and protein production, to inhibit basal or TGF- $\beta$ 1-induced transcription and production of collagen I, and to reduce the number of actively dividing cells. The innovative features of these studies involve the use of human HSCs and the delivery of the antisense molecule in the form of a hammerhead ribozyme, which has a more efficient blocking action than some other conventional antisense methods. Their data show that the anti-fibrotic properties of CCN2 hammerhead ribozyme are caused by a reduction of collagen production and cell proliferation.

### Applications

The study results suggest that CCN2 hammerhead ribozyme may have utility as a therapeutic agent for treating hepatic fibrosis *in vivo*.

### Terminology

HSCs are a minor and quiescent cell type in the liver that usually reside in the space of Disse, but which undergo activation after hepatic injury to produce large quantities of fibrillar collagens, which are deposited as insoluble fibrous matrix. TGF- $\beta$  is a multifunctional molecule that has immunomodulatory and tumor-suppressive actions in the liver, but which also is fibrogenic through its stimulation of CCN2 transcription.

### Peer review

In this study Gao and Brigstock have focused on CCN2 that is considered to mediate many TGF- $\beta$ -induced fibrotic reactions, to develop potential treatment of liver fibrosis, because TGF- $\beta$ -targeting therapeutic intervention has been found to be problematic because of side effects in the liver. The authors examined the effects of CCN2 gene-targeting blocking on the TGF- $\beta$ -dependent activation of LX-2 cells, utilizing the hammerhead ribozyme method to suppress CCN2 gene expression. Recombinant vector pTriCCN2-Rz was prepared by inserting CCN2 hammerhead ribozyme cDNA plus two self-cleaving sequences into pTriEx2 vector, and transfected LX-2 cells together with a null vector, pTriEx2. The collagen type I synthetic and proliferative activities were assessed at the gene and protein levels, and compared between the cells transfected with pTriCCN2-Rz and pTriEx2 vectors. As a result, the authors were able to demonstrate the expected attenuation of collagen synthesis activity by CCN2-Rz transfection not only in the TGF- $\beta$ -treated cells, but also in the control cells. Introduction of pTriCCN2-Rz gene also suppressed the entry of the cells into the S phase.

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CASE REPORT

## Adalimumab in ulcerative colitis: Two cases of mucosal healing and clinical response at two years

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cytokine that plays a role in the pathogenesis of inflammatory bowel disease. The introduction of anti-TNF- $\alpha$  agents for the treatment of ulcerative colitis (UC) has been delayed because contrary to Crohn's disease (CD), UC has classically been considered a T helper cell type 2 (Th2) disease, for which the role of TNF- $\alpha$  is less relevant than that of other cytokines such as interleukin (IL)-10 and IL-5<sup>[1]</sup>. However, other more recent studies have shown that TNF- $\alpha$  may play a major role in the etiopathogenesis of UC, justifying the use of anti-TNF- $\alpha$  therapies<sup>[2]</sup>.

Infliximab (IFX) is presently the only biologic therapy employed in the treatment of moderate-to-severe UC<sup>[3]</sup>. With increased use of IFX in UC, cases of intolerance or adverse reaction to this drug are expected. In these clinical situations and based on the data and experience from CD, adalimumab (ADA) is likely to be the next biologic drug to be used in UC. There are currently very few data published regarding the efficacy of ADA in UC. Moreover, the data are short-to-medium term, and there is no evidence of its long-term efficacy.

We report on two patients with moderate-to-severe UC who, due to adverse reactions to IFX, have been under therapy with ADA for two years. Clinical remission and mucosal healing are described.

### Abstract

Infliximab (IFX) is currently the only biologic therapy used in the treatment of moderate-to-severe ulcerative colitis (UC). In the years to come, more biologic therapies will have a role in the management of moderate-to-severe UC. We report on two patients with steroid-dependent UC who, due to adverse reactions to IFX, have been under therapy with adalimumab for two years. Both patients received concomitant immunosuppressive treatment. Long term clinical remission and mucosal healing are described.

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**Key words:** Adalimumab; Ulcerative colitis; Mucosal healing; Maintenance treatment

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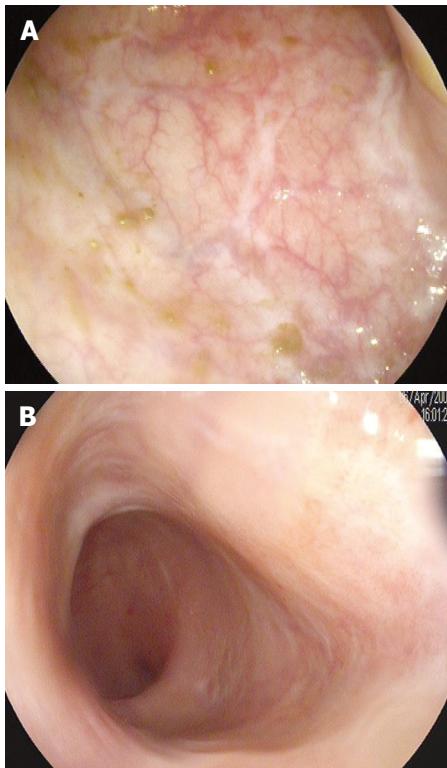
### INTRODUCTION

Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a proinflammatory

### CASE REPORT

#### Case 1

A 55-year-old male was diagnosed with ulcerative pancolitis in 2003. In 2005 he developed steroid dependence and treatment with azathioprine 2.5 mg/kg was started with clinical response for 1 year. In October 2006, the patient presented both clinical (8 bloody stools per day) and endoscopic relapse (3 points on the Mayo endoscopic subscore). After performing viral serologies, chest X-ray and Mantoux test, treatment with IFX 5 mg/kg was started. At the second dose, he developed a generalized skin rash on both trunk and extremities requiring drug discontinuation. On this basis and after requesting compassionate use, ADA treatment was started with an induction dose of 160 mg at week 0 followed by 80 mg at week 2 and then 40 mg every 2 wk thereafter as maintenance therapy. The patient had a very good clinical response, with normalization of stools after 2 mo. From the onset of treatment and over the subsequent two year period the patient has experienced no new flare and remains in clinical remission. A



**Figure 1 Colonoscopy.** A: Case 1 at 2 years of adalimumab treatment; B: Case 2 at 2 years of adalimumab treatment.

colonoscopy performed at 2 years from onset of ADA therapy showed no evidence of disease activity and complete mucosal healing (Figure 1A).

### Case 2

A 29-year-old woman with a previous diagnosis of rheumatoid arthritis was diagnosed with ulcerative pancolitis in 2004. After one year she developed steroid-dependency and treatment with azathioprine 2.5 mg/kg was started. Despite this treatment, she was readmitted to the hospital in March 2006 for a severe relapse. A colonoscopy was performed showing diffuse continuous mucosal disease and multiple erosions with signs of bleeding (2 points on the Mayo endoscopic subscore). Given these findings, it was decided to start treatment with IFX 5 mg/kg after performing the standard pretreatment evaluation for biologic therapies (viral serologies, chest X-ray and Mantoux test). On the second dose of IFX, the patient experienced an adverse drug reaction with difficulty breathing and edema of the face and glottis requiring drug discontinuation. Treatment with ADA was initiated with an induction dose of 160 mg at week 0, followed by 80 mg at week 2 and then 40 mg every 2 wk thereafter as maintenance therapy. The patient did not have any adverse drug reaction, corticosteroids were completely withdrawn and a marked improvement was observed in intestinal and articular symptoms. A colonoscopy performed at 2 years after starting ADA treatment showed absence of acute disease, no friability, and signs of healing, with findings compatible with 0 points on the Mayo endoscopic subscore (Figure 1B).

### DISCUSSION

Currently, IFX is the only biologic therapy approved for UC treatment, although in the not very distant future other biologic agents will be employed for treatment of this disease. As occurred in CD, ADA is likely to be the next biologic drug that will be used in UC, although only case reports<sup>[4]</sup> or very limited studies have been published to date. These studies only included patients with loss of response or intolerance to IFX. The few data published are from a French group who, in a first study, reported their short-term experience in 10 patients with UC who had loss of response or intolerance to IFX<sup>[5]</sup>. They evaluated the efficacy of ADA at 4 wk after two doses (160 mg at week 0 and 80 mg at week 2) and observed that patients showed improvement, and that among patients with no response two subsequently underwent colectomy. This same group has recently published medium-term data (median follow-up 42 wk) from 13 ADA-treated patients who had loss of response or intolerance to IFX<sup>[6]</sup>. During this period, 46% of patients underwent colectomy, with no differences being observed between the group of patients with loss of response or intolerance. Recently another open-label study has shown 50% response at week 24 in UC patients treated with AD<sup>[7]</sup>.

Our aim in reporting these two cases is to provide information on the clinical and mainly endoscopic long-term efficacy of ADA. Mucosal healing seems to be an important new therapeutic target in inflammatory bowel disease and the complete mucosal healing reported in our two cases seems to confirm the expectations created for ADA in UC, in that it seems to be a reasonable alternative in cases of intolerance or adverse reaction to IFX<sup>[8]</sup>.

Both patients received concomitant treatment with thiopurinic immunosuppressants. The studies conducted in CD do not provide sufficient evidence to recommend the routine long-term use of immunosuppressants in all patients treated with ADA, but concomitant use of immunosuppressant and ADA in UC should be evaluated in depth in controlled clinical trials.

The present study is a series of only two cases and solid conclusions cannot be drawn from its findings. We only aim to show that in the presence of severe UC, in patients with an adverse reaction to IFX, ADA treatment is a possible and safe alternative that can maintain long-term clinical and endoscopic remission.

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# Sepsis caused by endoscopic clipping for colonic diverticular bleeding: A rare complication

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## INTRODUCTION

Diverticular bleeding is one of the most common causes of lower-GI bleeding. It has been reported that endoscopic clipping is effective in diverticular bleeding<sup>[1-3]</sup>. However, we wish to report the rare complication of sepsis caused by endoscopic clipping for colonic diverticular bleeding.

## CASE REPORT

We herein report the rare complication of sepsis caused by endoscopic clipping for colonic diverticular bleeding. A 78-year-old man with a 12-h history of near syncope and painless hematochezia was admitted to our hospital. Following the transfusion of 4 U of blood and continued hematochezia, a colonoscopy was performed. Active bleeding was seen as continuous arterial spurting from a single diverticulum located in the middle ascending colon. This diverticulum was seamed by four endoclips. The next day, the patient became febrile with a temperature of 39.2°C. Laboratory data included a white blood cell count of 18100/mm<sup>3</sup> and a C-reactive protein level of 3.4 mg/dL. He was diagnosed with sepsis since *Escherichia coli* was detected in the blood culture. Antibiotics were started. Four days later his fever had improved and laboratory data improved 9 d later.

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**Key words:** Colonic diverticular bleeding; Endoscopic clipping; Rare complication; Endoscopic hemostasis; Sepsis

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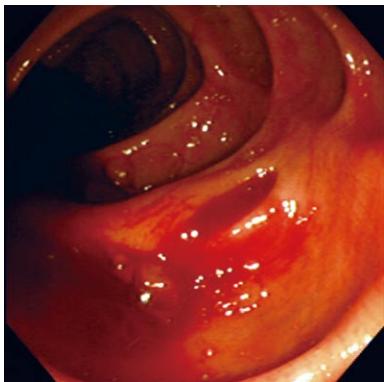
Kume K, Yamasaki M, Yoshikawa I. Sepsis caused by

The next day, the patient became febrile with a temperature of 39.2°C. Laboratory data included a white blood cell count of 18100/mm<sup>3</sup> and a C-reactive protein level of 3.4 mg/dL. He was diagnosed with sepsis since *Escherichia coli* (*E. coli*) was detected in the blood culture. Antibiotics were started. Four days later his fever improved and his laboratory data improved 9 d later.

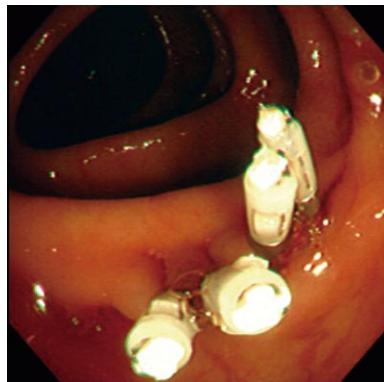
## DISCUSSION

Although diverticular bleeding episodes are often self-limiting, massive hemorrhage requiring therapeutic intervention occurs in a significant number of patients. Endoscopic clipping and other endoscopic therapies have been the treatment of choice in such cases<sup>[1-4]</sup>.

Normally, hemoclips are placed on and around the



**Figure 1** Continuous arterial spurting from a single diverticulum located in the middle ascending colon.



**Figure 2** The diverticulum was seamed by four endoclips.

visible vessel and immediate hemostasis is completed<sup>[1-4]</sup>. However, we seamed the diverticulum itself using endoclips because of difficulty in recognizing the visible vessel due to massive arterial spurting. The next day, the patient developed sepsis. This was probably because *E. coli* was present in the diverticulum which then invaded blood vessels.

It is important that hemoclips are placed on and around the visible vessel if possible, because it can alert the endoscopist to be mindful of potential hazards such as the one we encountered. However, hemoclips would appear to pose a risk of perforation when applied to the thin dome of a diverticulum.

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## Duodenal obstruction after successful embolization for duodenal diverticular hemorrhage: A case report

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### INTRODUCTION

Duodenal diverticulum is a disease entity that was first described in 1710 by Chromel. Most duodenal diverticula are diagnosed incidentally, 2% to 5% at barium study of gastrointestinal tract, 12% to 27% at endoscopy, and 22% in autopsy cases. Only 5% to 10% of patients with duodenal diverticula suffer from clinical symptoms. However, less than 1% of patients require treatment for various complications such as perforation, hemorrhage, and biliary/pancreatic/gastrointestinal obstruction.

Here, we report a patient who presented with duodenal obstruction after successful selective transcatheter arterial embolization (TAE) for a duodenal diverticular hemorrhage.

### CASE REPORT

A 69-year-old woman was admitted with abrupt onset of dull abdominal pain and vomiting. She had hypertension, did not drink alcohol, smoke, or use non-steroidal anti-inflammatory drugs. She had visited the department of internal medicine complaining principally of melena two years prior to admission. At that time, initial serum hemoglobin was 12.2 g/dL, and endoscopic evaluation, including gastroscopy and colonoscopy, showed no specific findings except for erosive gastritis. Computed tomography (CT) images showed a large duodenal diverticulum, however it was decided to merely observe the diverticulum because it did not cause any symptoms (Figure 1).

When she arrived at the emergency room this time, an intraabdominal bulging mass was palpated in the right upper quadrant of her abdomen. Her hemoglobin level was 7.9 g/dL and a CT scan showed an acute duodenal diverticular hemorrhage, large surrounding retroperitoneal hematoma, and an arterial pseudoaneurysm. The arterial pseudoaneurysm was thought to be the cause of the acute hemorrhage (Figure 2A). A superior mesenteric arteriogram

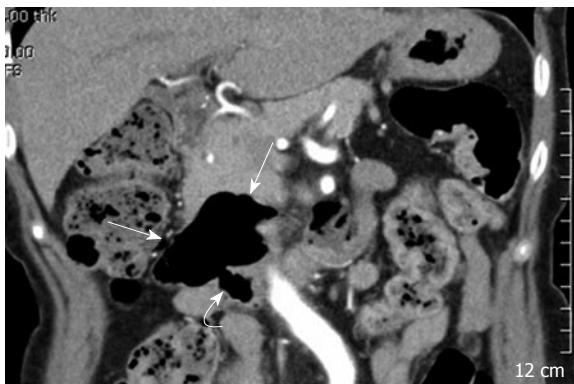
### Abstract

We present a 69-year-old woman with a duodenal obstruction after successful selective transcatheter arterial embolization (TAE) for a duodenal diverticular hemorrhage. Two weeks after TAE, the patient showed abrupt symptoms of duodenal obstruction. Resolving hematomas after successful selective transcatheter arterial embolization should be thoroughly observed because they might result in duodenal fibrotic encasement featuring inflammatory duodenal wall thickening, duodenal deformity, dysmotility, and finally obstruction.

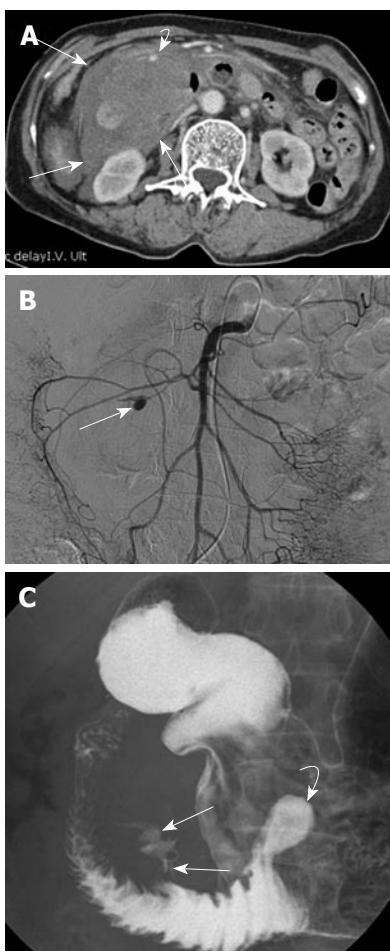
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**Key words:** Diverticulum; Gastrointestinal hemorrhage; Therapeutic embolization; Duodenal obstruction

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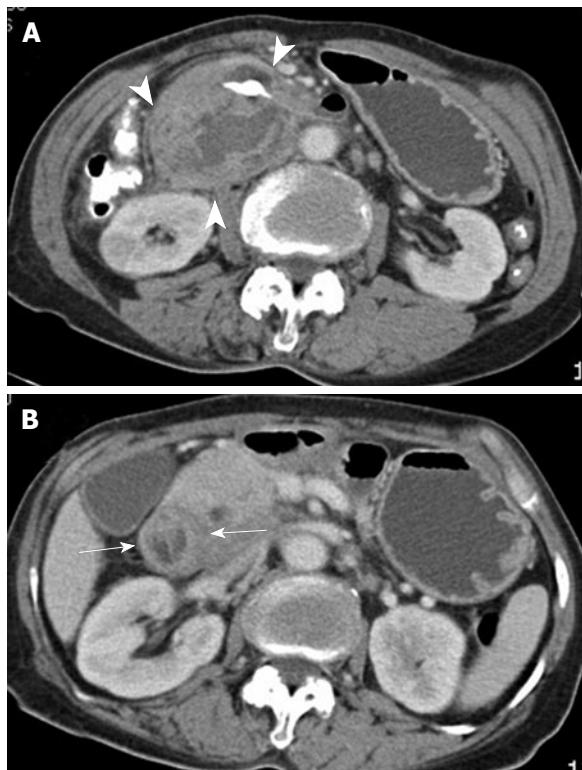


**Figure 1** Computed tomography (CT) image two years before the admission shows an approximately 6 cm sized silent diverticulum (arrows) originating from the proximal third portion of the duodenum (curved arrow).



**Figure 2** Diagnostic images on admission. A: An approximately 7 cm sized acute duodenal diverticular hemorrhage (curved arrow) and surrounding large retroperitoneal hematoma (arrows) around the duodenum on contrast enhanced CT image; B: The causative arterial pseudoaneurysm (arrow) on superior mesenteric arteriogram; C: Barium leakage into the large hemorrhagic duodenal diverticulum (arrows) and another small silent diverticulum (curved arrow) in the duodenal third portion on upper gastrointestinal barium study.

(Figure 2B), showed that the arterial pseudoaneurysm was in a duodenal branch of the anteroinferior pancreaticoduodenal artery and transcatheter glue embolization was performed in a superselective way using a Histoacryl (N-butyl-2-cyanoacrylate)-Lipiodol

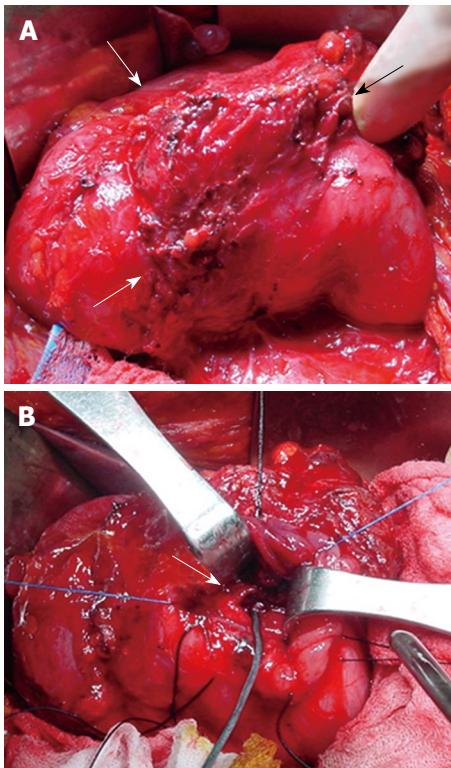


**Figure 3** CT images when the patient complained of gastrointestinal obstruction 2 wk after admission. A: Previously noted large duodenal diverticular hemorrhage and retroperitoneal hematoma were decreased in size (arrowheads); B: However, marked edematous bowel wall thickening is newly visualized in the duodenal second and third portions (arrows).

mixture. A post-embolization arteriogram showed successful embolization of the pseudoaneurysm.

Gastroscopy and an upper gastrointestinal barium study performed on the seventh day after the embolization revealed a huge diverticulum at the mesenteric border of the duodenal third portion, about 10 cm distal from the ampulla. The duodenal lumen was compressed by the duodenal diverticulum and surrounding hematoma. Another small diverticulum was identified at about 10 cm distal to the complicated diverticulum. There was no evidence of further active bleeding from the upper gastrointestinal tract or any ischemic changes to the duodenum. The duodenal passage was intact (Figure 2C).

Two weeks after admission, the patient showed gastrointestinal symptoms of obstruction (epigastric distension and postprandial bilious vomiting). A follow-up CT scan showed a decrease in the duodenal diverticular and the retroperitoneal hematoma; however, marked edematous duodenal wall thickening and luminal narrowing had developed in the duodenal second and third portions, suggesting duodenal deformity resulting from peridiverticular fibrosis (Figure 3). Despite the conservative treatment with total parenteral nutrition (TPN) and Levin tube drainage, the patient's symptoms of obstruction did not improve. Furthermore, TPN was no longer possible because of repeated central line sepsis. Therefore, a laparotomy was performed 24 d after the admission.



**Figure 4 Intra-operative findings.** A: A hard duodenal diverticulum filled with old hematoma, which was covering about 10 cm of the duodenal second portion, with severe peridiverticular fibrosis (arrows); B: The diverticular opening at the 3rd portion of the duodenum (arrow).

During the laparotomy, we found a hard duodenal diverticulum filled with a blood clot, which covered about 10 cm of the duodenal second portion. In addition, severe peridiverticular fibrosis and deformity were found to be causing duodenal second portion stricture. Through a longitudinal duodenotomy, a diverticular opening was identified at the 3rd portion of the duodenum that was more than 10 cm away from the ampulla of Vater. Neither dissection nor resection of the diverticulum was possible due to dense adhesion. Instead, suture ligation of the diverticular opening, with hematoma evacuation and gastrojejunostomy, was performed (Figure 4).

After the operation, she suffered from delayed gastric emptying, which caused symptoms such as postprandial bilious vomiting. However, the symptoms improved dramatically after several weeks of conservative treatment and, open discharge, she retained the ability to tolerate any meal without bleeding.

## DISCUSSION

We have presented a patient with duodenal obstruction after successful selective TAE for a duodenal diverticular hemorrhage. There are few reports on duodenal obstruction caused by duodenal stenosis from retroperitoneal or duodenal fibrotic encasement, while a few other reports describe the obstruction caused by intraluminal diverticular stone, bezoar, and ingested foreign bodies around diverticulum<sup>[1-3]</sup>. To the best of our knowledge, this is the first case report of duodenal

diverticular hemorrhage complicated by duodenal obstruction after TAE.

Only 5% to 10% of patients with duodenal diverticula suffer from clinical symptoms, of which 1% or less require treatment for various complications such as perforation, hemorrhage, and biliary/pancreatic/gastrointestinal obstruction<sup>[1,4]</sup>. In our patient, the patient suffered from both hemorrhage and obstruction. After a CT scan and from laboratory findings, we found that the symptoms of obstruction were caused by the diverticular hemorrhage and a surrounding retroperitoneal hematoma, which compressed the duodenal lumen. Angiography showed that the origin of the hematoma was the arterial pseudoaneurysmal rupture on the anterior wall of the duodenal diverticulum.

Use of arteriography to diagnose duodenal diverticular bleeding was first reported by Miller *et al*<sup>[5]</sup> in 1970. Since then, angiography and endoscopy have become the most useful modalities for diagnosing and managing gastrointestinal bleeding, suggesting TAE as the treatment of choice of duodenal diverticular hemorrhage<sup>[6,7]</sup>. However, due to the complex vascular anatomy of duodenum, TAE must be done with extreme caution. Pancreatitis, ischemic damage, and rebleeding have been reported as possible complications after superselective TAE. In our patient, the patient didn't suffer from any of these complications after the superselective TAE, and bleeding control was successful with no change in hemoglobin level during the follow-up period after the intervention. However, two weeks after admission, symptoms of obstruction were abruptly aggravated. CT, an upper gastrointestinal barium study, and gastroscopy suggested stricture of the second portion of the duodenum caused by duodenal wall thickening with deformity and dysmotility, which resulted from peridiverticular fibrosis, not by ischemic damage from the embolization. Therefore, great care should be taken after the TAE, not only to detect pancreatitis and rebleeding, but also for the symptoms of obstruction, so that the patient can be managed appropriately and timely.

According to previous reports, duodenal obstruction due to fibrotic encasement of the duodenum in most cases is self-limited by the end of the first month of treatment of nasogastric suction and total parenteral nutrition<sup>[8]</sup>. However, the patient suffered from repeated central line sepsis, therefore we concluded that the conservative treatment had failed and we decided to operate.

Various operations are considered for diverticulum based on the symptoms and operative field findings<sup>[1,9,10]</sup>. Surgical management of duodenal obstruction in duodenal diverticulum includes diverticulectomy through duodenotomy or duodenal resection with primary end-to-end anastomosis. However, inflamed duodenal diverticulum might cause adhesions, making it difficult to dissect, especially when the diverticulum is located in the second portion of the duodenum. The diversion of the upper gastrointestinal tract might be better than complete dissection and resection of the diverticulum, possibly preventing CBD or AoV injury. Therefore, a diversion procedure is more suggestive when there is severe inflammation or friability of the tissue. In

our patient, the diverticular opening was in the 3rd portion of the duodenum; however, due to severe dense adhesions and fibrosis of the duodenum, only suture ligation of diverticular opening and gastrojejunostomy were performed.

In conclusion, selective TAE should be considered as the first line treatment for duodenal diverticular hemorrhage. Complications of peridiverticular and retroperitoneal fibrosis around the resolving hematoma could happen after successful TAE; therefore the resolving hematoma should be thoroughly observed. In addition, conservative treatment should be considered before surgery to relieve the duodenal obstruction resulting from duodenal fibrotic encasement after a duodenal diverticular hemorrhage. If surgery is necessary, gastrointestinal diversion could be done instead of complete resection in cases with severe inflammation or tissue friability.

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# Percutaneous paraumbilical embolization as an unconventional and successful treatment for bleeding jejunal varices

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small bowel. Portal vein thrombosis was present. As he had severe coagulopathy and extensive paraumbilical varices, surgery was of high risk. He was not suitable for transjugular intrahepatic porto-systemic shunt as he had portal vein thrombosis. Percutaneous paraumbilical embolization *via* caput medusa was performed on day 9 of hospitalization. Following the embolization, the hematochezia stopped. However, he defaulted subsequent follow-up.

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**Key words:** Embolization; Jejunal varices; Obscure bleed; Capsule endoscopy; Angiography

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## Abstract

A 48-year-old Indian male with alcoholic liver cirrhosis was admitted after being found unresponsive. He was hypotensive and had hematochezia. Esophagogastroduodenoscopy (EGD) showed small esophageal varices and a clean-based duodenal ulcer. He continued to have hematochezia and anemia despite blood transfusions. Colonoscopy was normal. Repeat EGD did not reveal any source of recent bleed. Twelve days after admission, his hematochezia ceased. He refused further investigation and was discharged two days later. He presented one week after discharge with hematochezia. EGD showed non-bleeding Grade 1 esophageal varices and a clean-based duodenal ulcer. Colonoscopy was normal. Abdominal computed tomography (CT) showed liver cirrhosis with mild ascites, paraumbilical varices, and splenomegaly. He had multiple episodes of hematochezia, requiring repeated blood transfusions. Capsule endoscopy identified the bleeding site in the jejunum. Concurrently, CT angiography showed paraumbilical varices inseparable from a loop of small bowel, which had herniated through an umbilical hernia. The lumen of this loop of small bowel opacified in the delayed phase, which suggested variceal bleeding into the

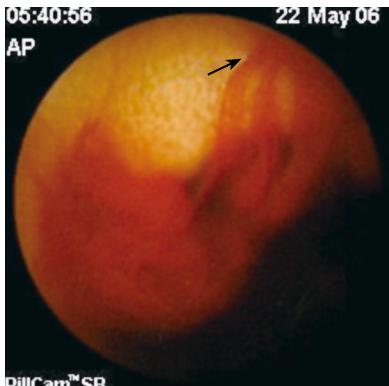
## INTRODUCTION

Bleeding jejunal varices can be difficult to diagnose and manage. We present a case of obscure overt gastrointestinal hemorrhage secondary to jejunal varices, detailing the extensive investigations and unconventional management.

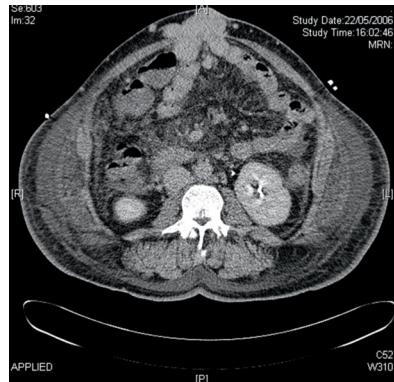
## CASE REPORT

A 48-year-old Indian male with alcoholic liver cirrhosis had been treated for ascites and encephalopathy for the prior 6 years. Variceal endoscopic ligation was performed 5 years previously for bleeding esophageal varices. In the same year, he underwent a laparotomy for repair of an incarcerated umbilical hernia. He defaulted follow-up thereafter.

Regarding the current episode, he was admitted after being found unresponsive. He was hypotensive



**Figure 1** Capsule endoscopy identified the bleeding site (arrow) in the jejunum.



**Figure 2** CT angiography showed variceal bleeding into the small bowel.



**Figure 3** Percutaneous paraumbilical embolization *via* caput medusae was performed.

and digital rectal examination showed hematochezia. His hemoglobin was 7.4 g/dL on admission. Esophago gastroduodenoscopy (EGD) showed small esophageal varices, and a 2 cm clean-based ulcer in the duodenal bulb. He continued to have hematochezia and anemia despite blood transfusions. Colonoscopy was normal up to the cecum. Repeat EGD again did not reveal any source of recent bleed. Twelve days after admission, his hematochezia ceased, and his hemoglobin was 8.4 g/dL. He refused further investigation and was discharged two days later.

He subsequently presented one week after discharge, complaining of abdominal pain and hematochezia for two days. His blood pressure was 120/80 mmHg, and heart rate was 110 beats/min. There was no encephalopathy. He had mild ascites. Digital rectal examination showed hematochezia. Hemoglobin was 4 g/dL, platelets  $63 \times 10^9/L$ , prothrombin time was 22 s and albumin 15 g/L. Diagnosis was Child's C alcoholic liver cirrhosis with possible variceal bleed. He was treated with IV esomeprazole, IV somatostatin and IV ceftriaxone and was transfused with fresh frozen plasma and packed cells. Emergency EGD showed non-bleeding Grade 1 esophageal varices and a clean-based duodenal ulcer. Colonoscopy was normal. Abdominal computed tomography (CT) showed liver cirrhosis with mild ascites, paraumbilical varices, and splenomegaly. He continued to have multiple episodes of hematochezia, requiring repeated blood transfusions.

Capsule endoscopy (Figure 1) identified the bleeding site in the jejunum. Concurrently, CT angiography was also carried out, having been negative on two prior episodes of hematochezia. However, the third

CT angiography (Figure 2), performed during active hematochezia, showed paraumbilical varices inseparable from a loop of small bowel which had herniated through an umbilical hernia. The lumen of this loop of small bowel opacified in the delayed phase, which suggested variceal bleeding into the small bowel. Portal vein thrombosis was present.

A decision was made for percutaneous paraumbilical embolization *via* caput medusae to be carried out on day 9 of hospitalization. The caput medusae near the umbilicus was punctured. When there was good return of blood, the guidewire was introduced and it was noted to pass freely into a vein. The guidewire was manipulated into the superior mesenteric and the splenic vein. Injection of contrast demonstrated the anatomy of the portovenous system clearly. The catheter tip was then positioned peripherally so that injection of contrast opacified only the veins near the ventral hernia leading into the caput. Three steel coils were placed into this vein. At the end of the study, injection of contrast under pressure did not demonstrate any retrograde flow, indicating that it was occluded (Figure 3).

Following the embolization, the hematochezia stopped. The patient remained well and somatostatin was stopped on day 12 of hospitalization. His hemoglobin rose to 9.0 g/dL and he was discharged. However, he defaulted subsequent follow-up.

## DISCUSSION

Obscure gastrointestinal bleeding (OGIB) is categorized into obscure overt and obscure occult bleeding based on the presence or absence of clinically evident bleeding<sup>[1]</sup>. Gastroscopy and colonoscopy should be repeated in OGIB, as a bleeding source can be identified on repeat endoscopies in some patients<sup>[2]</sup>. When repeat upper and lower endoscopies are negative, the small intestine should be investigated. Diagnostic accuracy for such investigations is limited mainly by the difficulty of performing adequate studies in this uncommon condition. In a meta-analysis, capsule endoscopy was shown to be superior to push enteroscopy and small bowel barium radiography for diagnosing clinically significant small bowel pathology in patients with OGIB, with incremental yields of 30% and 36%, respectively<sup>[3]</sup>. In a prospective study comparing capsule endoscopy with double-balloon enteroscopy in 35 patients with OGIB,

small-bowel abnormalities were detected using capsule endoscopy in 80% of cases, compared with 60% using double-balloon enteroscopy. The authors suggested that although the detection rate of capsule endoscopy was superior, the procedures were complementary, and that an initial diagnostic capsule endoscopy might be followed by therapeutic double-balloon enteroscopy<sup>[4]</sup>. In a prospective study of patients with lower gastrointestinal hemorrhage, sensitivity, specificity, and positive and negative predictive values of CT angiography for detection of colonic angiodysplasia were 70%, 100%, 100% and 57%, respectively, using colonoscopy and conventional angiography as reference standards<sup>[5]</sup>. A prospective study reported the diagnostic yield for OGIB of capsule endoscopy and CT enteroclysis to be 50% and 12.5%, respectively<sup>[3,6]</sup>. In another prospective study, diagnostic yield for OGIB of capsule endoscopy and MR enteroclysis was 36% and 0%, respectively<sup>[3,7]</sup>.

Capsule endoscopy, being relatively non-invasive, was a suitable investigation for this patient. CT angiography relies on an actively bleeding lesion to identify the bleeding site with the added advantage that CT scanning can visualize intra-mural and extra-intestinal disease. CT or MR enteroclysis use intravenous contrast and either neutral or positive ionic oral contrast delivered through a nasoenteric tube. The volume control distends the small bowel and the multi-sensor CT or MR with fine cuts provides sufficient detail to visualize intra-mural and extra-intestinal disease. Having more than one investigative procedure that use different methodologies increases the chances of success. There was overt and often profound gastrointestinal bleeding, making CT angiography a good candidate.

Once diagnosis is secured, then selection of a therapeutic approach is needed. This may indeed be challenging, balancing between risk and benefit in the context of the patient's general state of health. In the situation of this patient, variceal bleeding of the small bowel seemed most likely. A triad of portal hypertension, hematochezia without hematemesis, and previous abdominal surgery characterizes small intestinal varices<sup>[8]</sup>. Prior abdominal surgery predisposes the development of ectopic varices in adhesions. Possible physiological explanation could be the network of fine communication between the posterior abdominal wall and the parietal surface of the viscera, arising in the embryo due to the juxtaposition of the developing systemic and visceral venous plexus<sup>[9]</sup>. The patient had previous hernia repair, therefore satisfying the above-mentioned triad characteristic of small intestinal varices.

Options available for the treatment of jejunal varices include surgery, transjugular intrahepatic porto-systemic shunt (TIPS), enteroscopy and percutaneous embolization. Surgical treatment options for small intestinal variceal bleed include resection of the affected area of bowel, direct ligation of the bleeding varix, and re-anastomosis or portosystemic shunt insertion, which are associated with significant risk in a patient with advanced cirrhosis. TIPS with or without trans-catheter embolization of the varices is an alternative to laparotomy in some cases<sup>[10]</sup>.

Uncontrolled bleeding from ectopic varices located in rectum, colon, ileum, jejunum, duodenum, and stoma have been treated with TIPS successfully in 90% of patients<sup>[10]</sup>, and this is the option recommended for prevention of rebleeding from gastric and ectopic varices (including intestinal, stomal, and anorectal varices) by the American Association for the Study of Liver Diseases<sup>[11]</sup>.

As the patient had severe coagulopathy and extensive paraumbilical varices, surgery was deemed to be of high risk. He was not an optimal candidate for TIPS as he had portal vein thrombosis. Double-balloon enteroscopy was a suitable alternative, but this would have been a prolonged procedure, with increased technical difficulty posed by the expected large amount of blood in the small bowel. As such, percutaneous embolization was attempted first. Embolization using steel coils, gel foam, thrombin, collagen or autologous blood clot aims to occlude the feeding vein to the ectopic varices. Steel coils are available in a variety of sizes, allowing occlusion of large veins without difficulty, leading to a permanent focal occlusion, and are therefore the preferred embolic materials. Percutaneous embolization has been reportedly performed via the transjugular or transhepatic route. However, as he had portal vein thrombosis, these approaches were not straightforward. Unconventional approaches had to be considered. He had extensive caput medusae, and this provided us with an alternative and more direct access to the portal circulation.

The percutaneous paraumbilical embolization via caput medusae used to treat our patient has not been previously described, and could be a possible therapeutic option for cirrhotics with portal vein thrombosis and bleeding small intestinal varices not suitable for surgery. There is a case report of a patient with jejunal varices treated successfully with embolization<sup>[12]</sup>. A 79-year-old woman developed melena 2 years post-jejunal resection for a jejunal varix. Abdominal angiography revealed recurrence of the jejunal varix around the hepaticojjunostomy. She underwent a surgical procedure with intra-operative portography, and embolization of the jejunal varix. Our patient, in addition to having previous abdominal surgery, also had severe coagulopathy, which made any surgical approach a high risk procedure.

In summary, for this cirrhotic patient with portal vein thrombosis and previous abdominal surgery, ectopic jejunal varices were found to be the source of OGIB by the combination of CT angiography and capsule endoscopy. However, portal vein thrombosis precluded the recommended therapeutic choice, TIPS, and we used percutaneous embolization with coils via the paraumbilical approach as an unconventional and successful solution.

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## Mesenteric panniculitis: Various presentations and treatment regimens

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### Abstract

Mesenteric panniculitis is a rare, benign and chronic fibrosing inflammatory disease that affects the adipose tissue of the mesentery of the small intestine and colon. The specific etiology of the disease is unknown. The diagnosis is suggested by computed tomography and is usually confirmed by surgical biopsies. Treatment is empirical and based on a few selected drugs. Surgical resection is sometimes attempted for definitive therapy, although the surgical approach is often limited. We report two cases of mesenteric panniculitis with two different presentations and subsequently varying treatment regimens. Adequate response was obtained in both patients. We present details of these cases as well as a literature review to compare various presentations, etiologies and potential treatment modalities.

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**Key words:** Mesentery; Panniculitis; Colon; Computed tomography; Fibrosis; Inflammation

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### INTRODUCTION

Mesenteric panniculitis is an acute benign fibrosing

and inflammatory condition that involves the adipose tissue of the mesentery. It was first described by Jura in 1924 as "retractile mesenteritis" and further labeled as "mesenteric panniculitis" by Odgen later in the 1960s. Currently, it has several names: sclerosing mesenteritis, mesenteric lipodystrophy, mesenteric sclerosis, retractile mesenteritis, mesenteric Weber-Christian disease, liposclerotic mesenteritis, lipomatosis and lipogranuloma of the mesentery<sup>[1]</sup>. It can be categorized according to three pathological changes: chronic nonspecific inflammation, fat necrosis and fibrosis<sup>[2]</sup>. This varied terminology has caused considerable confusion, but the condition can now be evaluated as a single disease with two pathological subgroups. If inflammation and fat necrosis predominate over fibrosis, the condition is known as mesenteric panniculitis, and when fibrosis and retraction predominate, the result is retractile mesenteritis. The overall presence of some degree of fibrosis makes the pathological term sclerosing mesenteritis more accurate in most cases<sup>[3]</sup>.

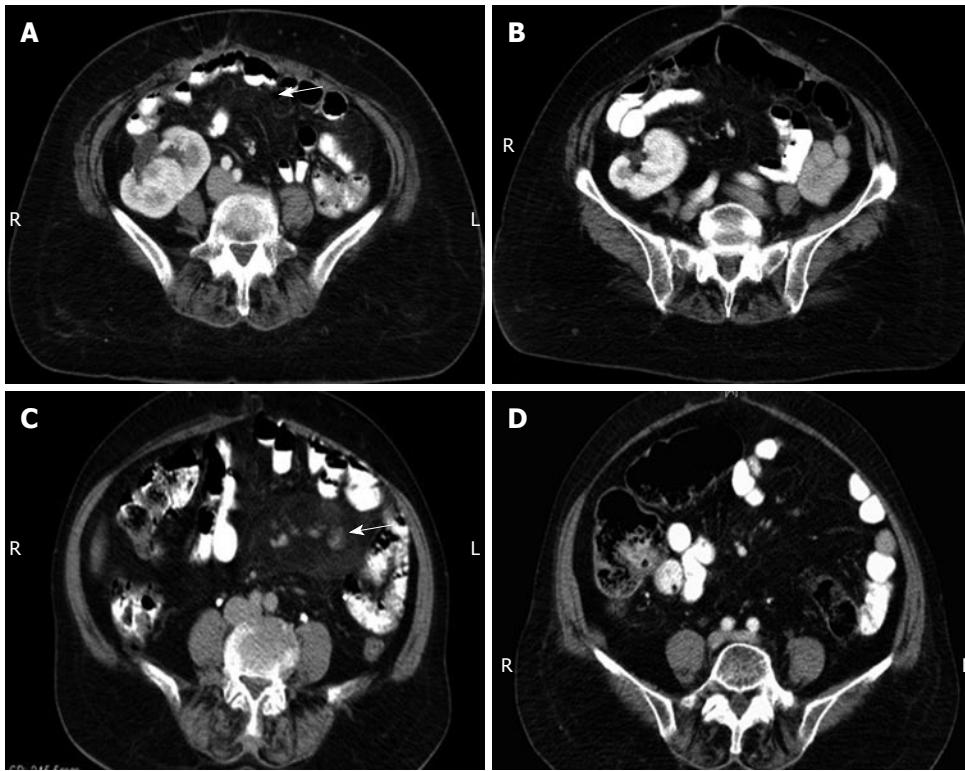
### CASE REPORT

#### Case 1

A 68-year-old female patient was admitted to our hospital with a 1-wk history of recurrent, right-sided abdominal pain, moderate in intensity, which lasted for many hours, and was associated with nausea but not vomiting. She had no change in bowel habits and was passing flatus and stools.

Her past medical history included hypertension for 10 years, dyslipidemia and diffuse diverticulosis with recurrent episodes of diverticulitis that necessitated left partial colectomy with primary anastomosis 4 years ago. As a result of disease progression, she underwent open total colectomy with an ileorectal anastomosis. She presented at 6 wk after surgery. Her medication history included valsartan, propranolol, fenofibrate, laxatives, antispasmodics and fibers. She had no known allergies, no significant family history, and a review of her systems was unremarkable.

Upon physical examination, the patient appeared well, in no acute distress and had stable vital signs. The remainder of the examination was unremarkable, except for moderate tenderness upon superficial and deep palpation of the abdomen (right quadrant, and a feeling of an ill-defined mass. Her laboratory profile was normal.



**Figure 1 High resolution computed tomography scan.** A: Smooth thickening evident between small bowel loops suggestive of a large inflammatory mass (arrow); B: Almost total disappearance of the inflammatory mass; C: A mass in jejunal compartment suggestive of mesenteric panniculitis. arrow shows a 9 mm lymph node; D: Complete disappearance of the previously described mass.

Computed tomography (CT) of the abdomen was performed using reconstructed slice thickness of 5 mm after oral and intravenous (*iv*) contrast administration, which showed a focal increase in density of the mesenteric fat with stranding in the supra-umbilical region, which was most probably inflammatory in origin and suggestive of mesenteric panniculitis (Figure 1A). This finding was surprising, especially in the light of a previous laparotomy a few weeks before, which revealed a clean abdomen.

The patient was started on prednisone 40 mg daily and was followed-up closely. Her symptoms gradually decreased in intensity and pain disappeared totally within 8 wk. Follow up CT 3 mo later showed a decrease in the mesenteric mass by 80%-90% (Figure 1B). However, she could not tolerate the steroids much longer because of peripheral neuropathy and hyperglycemia, therefore, she was switched to colchicine 100 mg daily orally.

Her status was reassessed 6 mo later, and CT showed persistence of the positive response and absence of the mass. Currently, she has been off treatment for 6 mo without recurrence of any symptoms.

### Case 2

A 74-year-old female patient presented to our care because of a chronic history of abdominal discomfort. Her symptoms were episodic and included discomfort that lasted a few minutes, which was associated with vomiting and followed by syncope of a few seconds duration. She had no abdominal pain, no change in bowel habits or hematochezia and no weight loss.

Her past medical history was significant for hypertension, herniated vertebral disc, peptic ulcer disease and diverticulosis. At the time of presentation, the patient was taking perindopril, levothyroxine and risedronate. She had no known allergies to any medication or substance, and no significant family history. A review of her systems was notable for nausea and occasional vomiting.

Upon physical examination, she appeared well, in no acute distress and had stable vital signs. Laboratory data revealed a normal complete blood count, blood chemistry and coagulation profile. Upper gastrointestinal endoscopy was performed and showed mild non-erosive gastritis with a positive urease test for *Helicobacter pylori*.

Spiral CT of the abdomen and pelvis, using a reconstructed slice thickness of 5 mm after oral and iv contrast administration showed a hazy, veil-like hyperattenuation of the mesenteric root and leaves, primarily seen in the jejunum. The radiological picture was highly suggestive of mesenteric panniculitis (Figure 1C). Interspersed subcentimetric mesenteric lymph nodes were also seen. The largest one was located in a lower jejunal mesenteric leaflet of the left lower quadrant, and reached 9 mm in the short axis. The rest of the examination was normal.

Treatment was started with 40 mg prednisone once daily and she was discharged home. She had an excellent response that was demonstrated in her follow-up CT scan 2 mo later. The previously described increased density of the peri-pancreatic and mesenteric fat caused by panniculitis was no longer present. The

mesenteric fat showed normal density with no evidence of inflammation or retroperitoneal or mesenteric adenopathy.

At this time, she had no abdominal complaints. She was still taking the same dose of steroids. Three months later, she was readmitted with acute herpes zoster infection. Treatment had to be aborted and prednisone was slowly tapered until discontinuation, and no other treatment was initiated. She was followed-up closely with no recurrence of symptoms and persistent radiological remission, as shown by CT repeated at 2 mo after steroid discontinuation (Figure 1D).

## DISCUSSION

Mesenteric panniculitis is a rare inflammatory condition that is characterized by chronic and nonspecific inflammation of the adipose tissue of the intestinal mesentery. So far, 130 cases have been reported in the literature under several names: retractile mesenteritis, sclerosing mesenteritis, liposclerotic mesenteritis, isolated lipodystrophy of the mesentery, mesenteric lipomatosis, and lipogranuloma of the mesentery, and mesenteric manifestations of Weber-Christian disease<sup>[4,5]</sup>. Most studies have indicated that the disease is more common in men, with a male/female ratio of 2-3:1, and several reports have indicated it to be more common in Caucasian men. Incidence increases with age, and pediatric cases are exceptional, probably because children have less mesenteric fat when compared to adults<sup>[6]</sup>.

The pathogenic mechanism of mesenteric panniculitis seems to be a nonspecific response to a wide variety of stimuli. Although various causal factors have been identified, the precise etiology remains unknown. Emory *et al*<sup>[2]</sup> have reported a series in which 84% of patients had a history of abdominal trauma or surgery. Furthermore, the disease is related to other factors, such as mesenteric thrombosis, mesenteric arteriopathy, drugs, thermal or chemical injuries, vasculitis, avitaminosis, autoimmune disease, retained suture material, pancreatitis, bile or urine leakage, hypersensitivity reactions, and even bacterial infection<sup>[6,7]</sup>. Other factors, such as gallstones, coronary disease, cirrhosis, abdominal aortic aneurysm, peptic ulcer, or chylous ascitis, have also been linked to this disease<sup>[8]</sup>. More recent studies have shown a strong relationship between tobacco consumption and panniculitis<sup>[7]</sup>.

Retractile mesenteritis has been associated with a number of malignant diseases such as lymphoma, lung cancer, melanoma, colon cancer, renal cell cancer, myeloma, gastric carcinoma, chronic lymphocytic leukemia, Hodgkin's disease, large cell lymphoma (giant-cell carcinoma), carcinoid tumor, and thoracic mesothelioma<sup>[2,6,7,9-11]</sup>.

In over 90% of cases, mesenteric panniculitis involves the small-bowel mesentery, although it may sometimes involve the sigmoid mesentery<sup>[10]</sup>. On rare occasions, it may involve the mesocolon, peripancreatic region, omentum, retroperitoneum or pelvis<sup>[12]</sup>.

The mean clinical progression is usually 6 mo,

**Table 1 Differential diagnosis of mesenteric panniculitis**

Lymphoma
Lymphosarcoma
Carcinoid tumors
Desmoid tumors
Infectious diseases (tuberculosis and histoplasmosis)
Amyloidosis
Peritoneal mesothelioma
Desmoplastic carcinoma metastases
Whipple's disease
Chronic inflammation due to foreign body
Reaction to adjacent cancer or chronic abscess
Retroperitoneal sarcoma

ranging from 2 wk to 16 years. The disease is often asymptomatic. When present, clinical symptoms vary greatly, and may include anorexia, abdominal pain, abdominal fullness, nausea, pyrexia, and weight loss<sup>[11]</sup>. On occasions, the disease may also present with merely a single or multiple palpable masses. Exceptionally, rectal bleeding, jaundice, gastric outlet obstruction, and even acute abdomen have been reported<sup>[2,9,12]</sup>. Such a wide variety of manifestations means that a large number of illnesses must be considered for differential diagnosis, therefore, careful assessment by the treating physician is strongly advised (Table 1).

Histologically, the disease progresses in three stages<sup>[6]</sup>. The first stage is mesenteric lipodystrophy, in which a layer of foamy macrophages replaces mesenteric fat. Acute inflammatory signs are minimal or non-existent; the disease tends to be clinically asymptomatic and prognosis is good. In the second stage, termed mesenteric panniculitis, histology reveals an infiltrate made up of plasma cells and a few polymorphonuclear leukocytes, foreign-body giant cells, and foamy macrophages. Most common symptoms include fever, abdominal pain, and malaise. The final stage is retractile mesenteritis, which shows collagen deposition, fibrosis, and inflammation. Collagen deposition leads to scarring and retraction of the mesentery, which in turn, leads to the formation of abdominal masses and obstructive symptoms. The exact diagnosis is often difficult and is usually made by finding one of three major pathological features: fibrosis, chronic inflammation, or fatty infiltration of the mesentery. To some extent, all three components are present in most cases<sup>[13]</sup>.

Blood tests tend to be within the normal range. Neutrophilia, increased erythrocyte sedimentation rate or anemia have been reported occasionally in the retractile mesenteritis stage<sup>[6]</sup>.

Some reports even go as far as stating that few or none of the patients with mesenteric panniculitis can be diagnosed correctly before operation<sup>[4,14]</sup>. However, with the advent of imaging technology like high-resolution CT or magnetic resonance imaging, distinguishing mesenteric panniculitis from other mesenteric diseases with similar imaging features such as carcinomatosis, carcinoid tumor, lymphoma, desmoid tumor, and mesenteric edema seems possible and feasible<sup>[15,16]</sup>. The imaging appearance of mesenteric

panniculitis varies depending on the predominant tissue component (fat necrosis, inflammation, or fibrosis)<sup>[17]</sup>. It is visualized usually as a heterogeneous mass with a large fat component and interposed linear bands with soft tissue density in cases of mesenteric panniculitis, or as a homogeneous mass of soft tissue density in cases of retractile mesenteritis. Colonoscopy is usually unrevealing, since mesenteric panniculitis is extrinsic to the bowel. Paracentesis that reveals inflammatory cell populations without mitotic figures can also aid diagnosis.

Mesenteric panniculitis resolves spontaneously in most cases, however, palpable masses may often be found between 2 and 11 years after diagnosis, especially in patients with associated comorbidity<sup>[6]</sup>. In such cases, several types of treatment have been proposed but no consensus has been established. In general, treatment has been reserved for symptomatic cases. Incidental masses may be observed and left untreated. Therapy is individualized on a case by case basis. Treatment may be attempted with a variety of drugs including steroids, thalidomide, cyclophosphamide, progesterone, colchicine, azathioprine, tamoxifen, antibiotics and emetine, or radiotherapy, with different degrees of success<sup>[18-20]</sup>. Surgery may be attempted if medical therapy fails or in the presence of life-threatening complications such as bowel obstruction or perforation<sup>[5]</sup>.

Our two cases showed different presentations: one was chronic and compatible with most published data, and the other was post-surgical, which makes it a very rare occurrence. Two different treatment regimens were used successfully in both cases. This should encourage us to review our approach to those cases that are always considered to be surgical, and leave room for medical treatment, which may be more effective than previously noted.

In conclusion, mesenteric panniculitis is a rare clinical entity that occurs independently or in association with other disorders. Diagnosis of this nonspecific, benign inflammatory disease is a challenge to gastroenterologists, radiologists, surgeons and pathologists. CT features of the disease, usually highly suggestive, have recently been delineated clearly. Open biopsy seems rarely necessary. There is no standardized treatment, and it may consist of anti-inflammatory or immunosuppressive agents. We recommend resection only when the advanced inflammatory changes become irreversible or in cases of bowel obstruction. Overall prognosis is usually good and recurrence seems to be rare.

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## Cavernous hemangioma arising from the gastro-splenic ligament: A case report

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### Abstract

We present a rare case of a 45-year-old woman who presented with epigastric pain associated with early satiety and weight loss. Imaging revealed a large intra-abdominal mass in the epigastrium. Despite intensive investigations, including ultrasound scanning, computed tomography, upper gastrointestinal endoscopy, and percutaneous biopsy, a diagnosis could not be obtained. A histological diagnosis of cavernous hemangioma arising from the gastro-splenic ligament was confirmed after laparoscopic excision and histological examination of the intra-abdominal epigastric mass.

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**Key words:** Hemangioma; Laparoscopy; Abdominal neoplasms; Vascular diseases; Gastrointestinal neoplasms

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### INTRODUCTION

Cavernous hemangiomas are congenital hamartomatous lesions that originate from the mesodermal tissues, which are composed of large dilated blood vessels and contain large blood-filled spaces that are caused by dilation and thickening of the walls of the capillary loops. They have been reported in different organs, including the liver, colon, retroperitoneum, spleen, adrenal glands, soft tissues, bone, central nervous system, and mediastinum<sup>[1]</sup>. However, it is extremely rare for these tumors to originate from the mesentery<sup>[2]</sup> or omentum<sup>[3]</sup>. In this case report, we present the clinical presentation, diagnosis and treatment of a cavernous haemangioma that arose from the gastro-splenic ligament.

### CASE REPORT

A 45-year-old woman presented with sudden-onset epigastric pain of 2 h duration after eating a meal. She described her pain as a dull ache that radiated to the back, which was associated with early satiety, retching, vomiting and recent weight loss of approximately 6.35 kg. Her past medical history included laparoscopic cholecystectomy and uterine fibroid embolization. She was taking regular omeprazole for dyspepsia. Upon examination, she had neither jaundice nor anemia, and her vital signs were within normal limits. She had no supraclavicular lymphadenopathy. The abdominal examination revealed epigastric tenderness with no guarding or rigidity, and fullness in the left upper quadrant. Her initial laboratory blood results including liver function tests, amylase and full blood count were within normal limits. An abdominal ultrasound scan revealed a large 10.5 cm × 7.5 cm × 6.5 cm solid mass in the epigastrium anterior and to the left of the tail of the pancreas, and a mildly enlarged spleen, normal liver, pancreas and kidneys, and a fibroid uterus with an intrauterine device *in situ*. The ultrasonic appearance of the epigastric mass was not typical of a gastrointestinal stromal tumor (GIST) or lymphoma. Subsequent computed tomography (CT) of the chest and abdomen showed a well-circumscribed mass of mixed attenuation in the left upper abdomen, which was probably related to the stomach, and measured 10 cm in maximum diameter (Figure 1). In order to rule out gastric cancer, she underwent upper gastrointestinal endoscopy that



**Figure 1** Computed tomography appearance of a well-circumscribed mass of mixed attenuation in the left upper abdomen, that was probably related to the stomach, and measured 10 cm in maximum diameter.

showed mild reflux oesophagitis and a normal stomach. The diagnosis remained unclear despite an ultrasound-guided biopsy of the epigastric mass, which showed ectatic lymphovascular vessels with a fibrous stroma, with no evidence of malignancy upon histology.

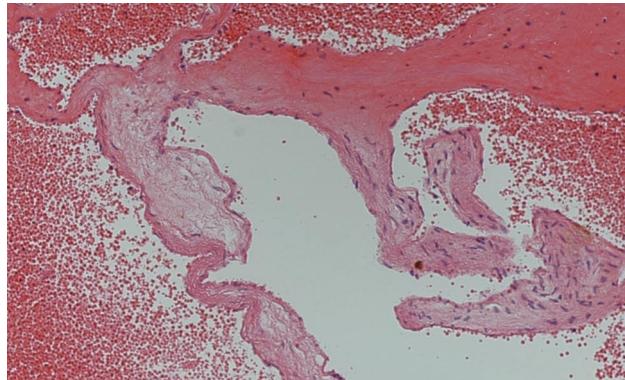
Laparoscopic excision of the intra-abdominal epigastric mass was performed. The intraoperative findings showed that the encapsulated mass, which was related to the omentum and gastro-splenic ligament, was adherent to but not directly invading the anterior wall of the stomach. The mass was dissected off the anterior wall of the stomach, and was mobilized from the omentum and gastro-splenic ligament with laparoscopic LigaSure Advance™ device (Covidien Ltd). The mass with an intact capsule was delivered through a Pfannenstiel incision in the suprapubic region. The microscopic specimen showed a hemorrhagic lesion that consisted of numerous thick-walled blood vessels filled with blood, surrounded by fibrosis and a few adjacent, benign-looking simple ductules, which were characteristic of cavernous hemangioma (Figure 2).

The patient was discharged 2 d postoperatively with no complications.

## DISCUSSION

Cavernous hemangiomas of the omentum and mesentery are extremely rare. These tumors frequently are asymptomatic and discovered incidentally at imaging, surgery or autopsy, but can have serious consequences. The clinical presentation of such a lesion varies depending on its location. Our patient presented with symptoms of abdominal pain, vomiting, early satiety and weight loss. In other case reports, these lesions presented as intermittent abdominal pain and dyspepsia<sup>[3]</sup>, palpable abdominal mass<sup>[2,4]</sup>, or acute abdomen caused by ruptured omental cavernous hemangioma<sup>[5]</sup>, ruptured mesoappendix cavernous hemangioma<sup>[6]</sup>, or an infected greater omental cavernous hemangioma<sup>[7]</sup>.

The diagnosis of such lesions can be challenging, and ultrasonography and CT have been used. Ultrasound scanning reveals a solid mass with a heterogeneous multinodular appearance, whereas CT reveals a



**Figure 2** Numerous thick-walled blood vessels lined by endothelial cells and containing red blood cells. Appearance was characteristic of cavernous hemangioma (HE,  $\times 100$ ).

homogenous or a heterogeneous mass<sup>[6]</sup>, or a mixed picture<sup>[4]</sup>.

Magnetic resonance imaging of cavernous hemangioma typically shows a uniform high signal intensity on T2-weighted images<sup>[3,4]</sup>, but occasionally shows heterogeneous signal intensity on T2-weighted images, as a result of fibrosis, hemorrhage or calcification<sup>[8]</sup>, or low signal intensity in T2-weighted images because the tumor is mainly occupied with old blood<sup>[2]</sup>. In hindsight, this resectable lesion should not be biopsied percutaneously, as it may spread the tumor through other anatomical planes, as in the case of malignant GIST. In addition, a small needle biopsy may produce a poor cellular yield, and the quality of the sample may not be adequate to make an accurate diagnosis. In this case of cavernous hemangioma, a biopsy may potentially cause a rupture or bleeding of the tumor.

In patients with gastrointestinal bleeding of obscure origin who are transfusion-dependent, wireless capsule endoscopy or double-balloon enteroscopy may allow examination of the entire small bowel and successful identification of hemangioma within the bowel lumen. Willert *et al*<sup>[9]</sup> have reported successful endoscopic treatment of multiple cavernous hemangioma during double-balloon enteroscopy of the small bowel. However, in our case report of cavernous hemangioma located at the gastro-splenic ligament, capsule endoscopy and double-balloon enteroscopy were unlikely to have been beneficial, as the lesion was located extraluminally. On the other hand, endoscopic ultrasound may help to characterize the extent of vascularity of the hemangioma, and allow selection of cases for endoscopic mucosal resection of cavernous hemangioma in the wall of the esophagus<sup>[10]</sup> or stomach<sup>[11]</sup>.

The treatment for cavernous hemangioma is surgical excision, and recurrence after complete resection has never been reported. Laparoscopic excision of this vascular lesion is feasible, and has the advantages of less pain and quicker recovery following surgery.

In conclusion, cavernous hemangioma of the omentum or mesentery is a rare tumor that can be very difficult to diagnose preoperatively, despite advanced

imaging techniques. As a result, it should be included in the differential diagnosis of any mass that arises in the mesentery or omentum. Surgical excision and histological examination may offer the only means of diagnosis.

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LETTERS TO THE EDITOR

## "Anti-HBc alone" in human immunodeficiency virus-positive and immuno-suppressed lymphoma patients

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### TO THE EDITOR

Patients with negative hepatitis B surface antigen (HBsAg) and positive antibody to hepatitis B core antigen (anti-HBc) but no antibody to hepatitis B surface antigen (anti-HBs) have traditionally been described as having occult hepatitis B virus (HBV) infection<sup>[1]</sup>. The recent article by Pérez-Rodríguez *et al*<sup>[2]</sup> in the issue of the *World J Gastroenterol* (March 14, 2009) described these patients as "anti-HBc alone", none of them was positive for serum HBV DNA. Of note, 10 of the 30 patients tested were taking lamivudine or tenofovir when the tests were performed. Furthermore, "anti-HBc alone" was associated with factors such as young age and HCV infection. They concluded that HBV DNA determination should not be performed in every "anti-HBc alone" patient, but only in those with unexplained clinical or analytical signs of liver injury.

Patients with prior exposure to HBV may subsequently clear their serum HBsAg, and become anti-HBc positive. Anti-HBs may be positive or negative, as anti-HBs may fall over time. Some authors considered patients with anti-HBc but no anti-HBs as having occult HBV infection<sup>[1,3-6]</sup>. Consequently, in a portion of these patients, HBV DNA may be detected. Biologically, it would be difficult to explain why this should not be the case in HIV patients as reported by Pérez-Rodríguez and colleagues<sup>[2]</sup>. It is uncertain what the authors meant when they considered that patients with defective immune response (anti-HBc) would have undetectable HBV DNA. On the contrary, if one considers that these HIV patients may have defective immune response (i.e. immunodeficiency), one might expect a higher HBV DNA level. We performed a similar study in lymphoma patients, who were HBsAg negative, anti-HBc positive, and tested for anti-HBs and HBV DNA at diagnosis ( $n = 89$ ). Among the 27 "anti-HBc alone" patients, who were anti-HBs negative as defined by the authors, 2 (7.4%) had a detectable HBV DNA level,

### Abstract

Hepatitis B virus (HBV) infection is endemic in various parts of the world. A proportion of patients have resolved prior exposure to HBV, as evidenced by the clearance of circulating hepatitis B surface antigen and the appearance of antibody to hepatitis B core antigen (anti-HBc), which could produce protective antibody to hepatitis B surface antigen (anti-HBs). With time, anti-HBs in some patients may become negative. Such patients are described as having occult HBV infection or "anti-HBc alone". In the context of immunodeficient patients, such as HIV patients or lymphoma patients undergoing immunosuppressive immunotherapy, the lack of protective anti-HBs may increase the risk of hepatitis B reactivation. Serum HBV DNA testing may be necessary in "anti-HBc alone" patients, to detect patients at a high risk of developing HBV infection allowing appropriate prophylactic management.

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**Key words:** Hepatitis B virus; Human immunodeficiency virus; Antibody to hepatitis B core antigen; Hepatitis B virus DNA; Viral hepatitis

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as compared to the negative results by Pérez-Rodríguez *et al*<sup>[2]</sup>. Although our results were observed in lymphoma patients, it is difficult to explain why this should be different in HIV patients. Interestingly, even among the 63 lymphoma patients with positive anti-HBs, 2 (3.2%) had detectable HBV DNA.

The negative HBV DNA results reported by Pérez-Rodríguez *et al*<sup>[2]</sup> could be confounded by the fact that 10 patients were on anti-viral agents, suppressing HBV DNA levels. It is also uncertain whether administration of anti-viral agents could have an impact on HBV DNA load, thus it will be important to re-examine the HBV profile of patients with HIV at diagnosis, prior to the usage of anti-viral agents, particularly lamivudine (which suppresses HBV) to confirm the results reported by the authors.

Furthermore, HBV DNA tests were not conducted for all "anti-HBc alone" patients due to non-medical reasons as reported by Pérez-Rodríguez *et al*<sup>[2]</sup>. Due to the relatively small number of patients tested ( $n = 30$ ), more patients need to be followed up.

It has also been reported that "anti-HBc alone" is associated with young age, which is consistent with the earlier findings<sup>[7]</sup>. However, this paper did not provide a biological explanation for the higher prevalence of the defective immune pattern observed in younger patients. Notably, the median age of our 27 patients was 69.3 (range 17.6-83.6) years whereas the mean age of the 59 lymphoma patients with negative HBsAg, positive anti-HBc and anti-HBs was 63.9 (range 24.2-86.5) years. Contrary to the results reported by Pérez-Rodríguez *et al*<sup>[2]</sup>, we observed a higher median age in lymphoma patients with the "anti-HBc alone" pattern.

In conclusion, it is important to verify the author's observations in a proportion of newly diagnosed HIV

patients, prior to the usage of anti-viral agents, particularly lamivudine (which could suppress HBV DNA levels) to confirm the results reported by the authors. Testing of HBV DNA in "anti-HBc alone" patients may be necessary.

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## Meetings

### Events Calendar 2009

January 12-15, 2009  
Hyatt Regency San Francisco, San Francisco, CA  
Mouse Models of Cancer

January 21-24, 2009  
Westin San Diego Hotel, San Diego, CA  
Advances in Prostate Cancer Research

February 3-6, 2009  
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)  
Second AACR Conference  
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009  
Hyatt Regency Boston, Boston, MA  
Translation of the Cancer Genome

February 8-11, 2009  
Westin New Orleans Canal Place, New Orleans, LA  
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009  
Hong Kong Convention and Exhibition Centre, Hong Kong, China  
19th Conference of the APASL  
<http://www.apasl2009hongkong.org/en/home.aspx>

February 27-28, 2009  
Orlando, Florida  
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009  
Vienna, Austria  
EASL/ AASLD Monothematic: Nuclear Receptors and Liver Disease  
[www.easl.ch/vienna2009](http://www.easl.ch/vienna2009)

March 13-14, 2009  
Phoenix, Arizona  
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009  
Marriott Wardman Park Hotel  
Washington, DC  
13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009  
Glasgow, Scotland  
British Society of Gastroenterology (BSC) Annual Meeting  
Email: bsg@mailbox.ulcc.ac.uk

April 8-9, 2009  
Silver Spring, Maryland  
2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009  
Colorado Convention Center, Denver, CO  
AACR 100th Annual Meeting 2009

April 22-26, 2009  
Copenhagen, Denmark  
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)  
<http://www.easl.ch/>

May 17-20, 2009  
Denver, Colorado, USA  
Digestive Disease Week 2009

May 29-June 2, 2009  
Orange County Convention Center Orlando, Florida  
45th ASCO Annual Meeting  
[www.asco.org/annualmeeting](http://www.asco.org/annualmeeting)

May 30, 2009  
Chicago, Illinois  
Endpoints Workshop: NASH

May 30-June 4, 2009  
McCormick Place, Chicago, IL  
DDW 2009  
<http://www.ddw.org>

June 17-19, 2009  
North Bethesda, MD  
Accelerating Anticancer Agent Development

June 20-26, 2009  
Flims, Switzerland  
Methods in Clinical Cancer Research (Europe)

June 24-27 2009  
Barcelona, Spain  
ESMO Conference: 11th World Congress on Gastrointestinal Cancer  
[www.worldgicancer.com](http://www.worldgicancer.com)

June 25-28, 2009  
Beijing International Convention Center (BICC), Beijing, China  
World Conference on Interventional Oncology  
<http://www.chinamed.com.cn/wcio2009/>

July 5-12, 2009  
Snowmass, CO, United States  
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009  
Aspen, CO, United States  
Molecular Biology in Clinical Oncology

August 1-7, 2009  
Vail Marriott Mountain Resort, Vail, CO, United States  
Methods in Clinical Cancer Research

August 14-16, 2009  
Bell Harbor Conference Center, Seattle, Washington, United States  
Practical Solutions for Successful Management  
<http://www.asge.org/index.aspx?id=5040>

September 23-26, 2009  
Beijing International Convention Center (BICC), Beijing, China  
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists(IASGO)  
<http://iasgo2009.org/en/index.shtml>

September 27-30, 2009  
Taipei, China  
Asian Pacific Digestive Week  
<http://www.apdwcongress.org/2009/index.shtml>

October 7-11, 2009  
Boston Park Plaza Hotel and Towers, Boston, MA, United States  
Frontiers in Basic Cancer Research

October 13-16, 2009  
Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States  
Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009  
Versailles, France  
Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009  
Boston, MA, United States  
The Liver Meeting

November 15-19, 2009  
John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States  
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009  
London, UK  
Gastro 2009 UEGW/World Congress of Gastroenterology  
[www.gastro2009.org](http://www.gastro2009.org)



### Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.



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- 1 Jung EM, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

- 2 Lin GZ, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

- 3 Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of

balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

*Organization as author*

- 4 **Diabetes Prevention Program Research Group.** Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

*Both personal authors and an organization as author*

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

*No author given*

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

*Volume with supplement*

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

*Issue with no volume*

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

*No volume or issue*

- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

**Books**

*Personal author(s)*

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

*Chapter in a book (list all authors)*

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

*Conference proceedings*

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

*Conference paper*

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*Patent (list all authors)*

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**Statistical data**

Write as mean  $\pm$  SD or mean  $\pm$  SE.

**Statistical expression**

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as  $\chi^2$  (in Greek), related coefficient as *r* (in italics), degree of freedom as *v* (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

**Units**

Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h, blood glucose concentration, *c* (glucose)  $6.4 \pm 2.1$  mmol/L; blood CEA mass concentration, *p* (CEA) =  $8.6 \pm 24.5$   $\mu$ g/L; CO<sub>2</sub> volume fraction, 50 mL/L CO<sub>2</sub>, not 5% CO<sub>2</sub>; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23243641.

The format for how to accurately write common units and quantums can be found at: <http://www.wjgnet.com/wjg/help/14.doc>.

**Abbreviations**

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**Italics**

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *argI*, *c myc*, *c fos*, etc.

Restriction enzymes: *EcoRI*, *HindIII*, *BamHI*, *KboI*, *KpnI*, etc.

Biology: *H pylori*, *E coli*, etc.

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## Baishideng's century goal: Editing and publishing high-quality articles

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### Abstract

Baishideng's goal over the next few years is to edit and publish high-quality articles through the open-access model, to maximize the benefits to members of the editorial board, authors and readers, as well as achieving social and economic benefits.

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**Key words:** Access to information; Baishideng; Maximization of the personal benefit; Editorial policies; Open access; Periodicals; Publishing

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### INTRODUCTION

Beijing Baishideng BioMed Scientific Co., Ltd was established on November 20, 2007. Its predecessor was The WJG Press, and its main task is to edit and publish biomedical journals. The predecessor to the currently published *Shijie Huaren Xiaohua ZaZhi* (*World Chinese*

*Journal of Digestology*) was the *Xin Xiaohuabingxue ZaZhi* (*New Journal of Gastroenterology*), which was launched in Shanxi on January 15, 1993. The predecessor of the weekly publication *World Journal of Gastroenterology* was the *China National Journal of New Gastroenterology*, which was launched in Shanxi on October 1, 1995. We are also preparing to publish the *World Journal of Hepatology*, *World Journal of Gastrointestinal Oncology*, *World Journal of Diabetes*, *World Journal of Gastrointestinal Endoscopy*, *World Journal of Stem Cells*, *World Journal of Gastrointestinal Surgery*, *World Journal of Gastrointestinal Pathophysiology*, and *World Journal of Gastrointestinal Pharmacology and Therapeutics* which will be launched successively in Beijing after September 15, 2009. Our goal is to edit and publish high-quality articles through the open-access (OA) model<sup>[1]</sup>, to maximize the benefits to members of the editorial board, authors and readers.

### MAXIMIZATION OF THE PERSONAL BENEFITS OF EDITORIAL BOARD MEMBERS

Our editorial board comprises 1126 invited experts in 60 countries, which means that we have access to outstanding scientists around the world, because first-class publications require a first-class editorial team<sup>[2,3]</sup>. Member of the editorial board should: (1) have attained a high level of scholarship and respect in their respective fields; (2) have a rigorous academic attitude and integrity; (3) participate actively in academic conferences and activities; (4) carry out peer review of six articles for the journal every year; and (5) write a commentary article or organize a series of "Highlight" articles for the journal every 2 years. Maximization of the interests of members of the editorial board is mainly reflected in the following.

#### Members of the editorial board review articles

(1) Responsibility to their own fields. A member of the editorial board rigorously evaluates a manuscript within a specified time; (2) Responsibility to the author. After agreeing to peer review a manuscript, a member of the editorial board makes a scientific evaluation of its accuracy, clarity, importance and its contribution to the scientific community; and (3) Responsibility to the journal. When a member of the editorial board cannot complete the review within the time set by the journal, he/she should decline the review and explain the reason.

Once a member of the editorial board agrees to review a manuscript, he/she is under an obligation to send their comments to the editorial department within the specified time. If the member of the editorial board cannot complete the review on time, he/she should inform the editorial department and ask whether to return the unreviewed manuscript or request for an extension of time. When a member of the editorial board receives a manuscript involving an apparent conflict of interest with his/her own research work or point of view, the member of the editorial board should decline to review the manuscript and explain the specific conflict of interest. The most common conflict of interest is private or professional relationships between the member of the editorial board and the organization undertaking the research or the author(s) that may affect the impartiality of the review, or competition between the member of the editorial board and those undertaking the research. When a member of the editorial board cannot return a reviewed manuscript to the editor for some reason, he/she can recommend one or two peer reviewers familiar with the topic of the paper to help the editor process the manuscript accurately and quickly. As a qualified member of the editorial board, he/she must abide by certain rules, deal with problems fairly and impartially, and avoid abuse of his/her position<sup>[4]</sup>. We publish all relevant information, including the conclusion of the peer review and the name and organization of the reviewer, along with the article. This is to control the quality of the peer reviewers, editors and publishers, ensure that each article is published after peer review, and reflect peer reviewers' responsibility.

#### **Members of the editorial board publish commentary articles**

Almost all the 1126 members of the *WJG* editorial board are leaders in their own fields. To mobilize the enthusiasm of the editorial board and maximize their interest in the journal, we set ourselves the following tasks. (1) We publish a list of members of the editorial board at a fixed location in the printed and online editions of each issue of the journal; (2) We express thanks to members of the editorial board whose reviewed manuscripts have been accepted, publish relevant information such as the name and organization of the reviewer along with the author's article, and send a PDF copy of the article to them; (3) We also express thanks to members of the editorial board whose evaluated manuscripts have been rejected; (4) We send a summary list of articles published to members of the editorial board each year, and invite them to write commentary articles for the coming year; and (5) We offer 50 US dollars for the author of every "Highlight" or "Observation" article. However, the majority of the members of the editorial board do not take the payment for articles solicited. A Canadian member of the editorial board, Professor H Freeman, University of British Columbia, said at the outset of the "Observation" articles that: "We need the spirit of Bethune to run *WJG* successfully"<sup>[5]</sup>. Professor Freeman has published 20 commentary articles in the observation column of which he is the chief editor. Scientists contribute to the

international community and they do not publish articles for economic gain. *WJG* published a variety of 1203 articles in issues 1-48 in 2008, of which there were 265 commentary articles written by members of the editorial board, which accounted for 22.08% of the published output. According to Journal Citation Reports published from 2006-2007 for *WJG* included in the Science Citation Index Expanded, the top three categories that contributed published articles in terms of the average number of times cited were reviews (8.63), editorials (6.24) and highlights (5.83)<sup>[6]</sup>.

#### **Members of the editorial board are ambassadors for the journal**

From 2007 to 2009, the editorial board of *WJG* had 1126 members in 60 countries. Where there are members of the *WJG* editorial board, there are also authors and readers of *WJG*. We decide on candidate members of the editorial board according to their citations in the ISI Web of Knowledge. Whether a person can be finally accepted as a member of the editorial board depends mainly on his/her resumé and job title. Only experts holding a rank higher than associate professor can serve as members of the *WJG* editorial board. International members of the editorial board are ambassadors who help with the global dissemination of our journals. They are also liaison officers who recommend persons in the same field as their own to contribute to *WJG*.

### **MAXIMIZING THE BENEFIT TO AUTHORS OF PUBLISHING ARTICLES**

One of the biggest advantages of open access publishing is that people around the world can have immediate access to peer-reviewed articles *via* the internet. This provides a new platform for the international community to have equitable access to the latest scientific developments, and to play a positive role in advancing scientific knowledge.

#### **Qualifications of authors for publishing articles**

We require authors to declare that they all meet the eligibility criteria below, no author who meets these criteria has been left out, and contributors and sponsors have been duly thanked. The eligibility criteria for authors proposed by the international committee of medical journal editors are<sup>[7]</sup>: (1) authors must have made a significant contribution to the concept and design of the research and the acquisition, analysis and interpretation of data; (2) draft articles and critically revise the article for important intellectual content; and (3) approve the final version of the article to be published.

#### **Editing and publishing quality of published articles**

Whether the editorial department of the journals receives high-quality international articles hinges on many factors. The editing and publishing quality of every article published in the journal needs to be guaranteed. Our approach is as follows. (1) We give our journals an international appeal, e.g. containing the word "World"

in the title, to attract international authors to contribute articles; (2) We accept online contributions, so that authors can submit a manuscript from any place that has an internet connection. The system replies automatically or the editorial department returns acknowledgement of the manuscript within 24 h; (3) Members of the editorial board participate in online peer review, which generally takes 2-4 wk; (4) Reject manuscripts or send those that are accepted back to the authors for revision; (5) Edit and ReleaseProcess manuscripts in a scientific manner; (6) Authors revise their articles; (7) Formal acceptance or rejection of manuscripts; (8) In processing manuscripts, the language editor solves common errors, including grammar, syntax, spelling, punctuation and logic; (9) Return manuscripts to the author for final revision; (10) The editorial director organizes the production; (11) Electronic editing and production, including redrawing of charts, graphs and automatic checking by the software Heima; (12) The electronic editor in charge carries out editing and typesetting; the science editor in charge carries out proofreading, including Heima proofreading; (13) The author carries out proofreading; (14) The editorial director and editor-in-chief carry out proofreading; (15) Produce and proofread films; (16) Printing; (17) Create the online edition; (18) The editorial director checks the online edition; and (19) Release the online edition. Every aspect of every article published through the above process is original. For example, charts and graphs are re-drawn, references are re-formatted using software, and Word documents are converted into XML format automatically to avoid human errors. In the entire process from editorial personnel receiving the manuscript to online publishing, we put service and quality first, and aim to establish the reputation of the journal so as to attract international contributors.

### **Comments accompanying original articles**

The accompanying comments for each article are written in an easy-to-understand manner to enable professional scientists, the public and media to understand more broadly the relevance of the article, and to play a role in promoting science to a wider audience. Accompanying comments cover the following. (1) Background information: summarizes relevant background that will give readers a better understanding of the article in an easy-to-understand manner to enable professional readers, the public and the media to understand more broadly relevant knowledge of the article and have a better understanding of the meaning of the article; (2) Research and development: aims to help readers understand how the article relates to the key areas of research in the field; (3) Relevant reports: references to other relevant papers that may help with understanding the article, or as pointers to wider reading on the subject of the article; (4) Inventory of innovations: summarizes the innovative aspects of the article, so that readers can see how the new study differs from and is related to previous work; (5) Main points of application: summarizes the practical applications of the article, and how it may improve existing or develop future applications. Hopefully, this may lead to the article being

cited extensively; (6) Glossary: a simple, straightforward and clear explanation of the terms in the article that are important for its understanding, as many readers may not be familiar with these terms or have a full understanding of them; and (7) Peer review: the editorial department summarizes the opinions of the peer reviewers, to demonstrate the significance of the article.

### **The core interests of authors in publishing articles**

Members of the editorial board publish a wide range of commentary articles in the form of invited papers. The purpose of these is to comment on the status quo, progress and problems in fast-developing fields, in an attempt to play a role in guiding clinical or basic research. Other contributing authors submit original articles for publication. The purpose of these is to report the latest developments in scientific research, which are innovative, of a high standard, and have considerable scientific significance in their own field. The core interests and values main purpose of members of the editorial board and contributing authors in publishing articles is to disseminate their peer-reviewed research results around the world, and hopefully, to have them cited by others in the same field, thereby contributing to scientific progress. If journal articles written by members of the editorial board and contributing authors do not achieve the above-mentioned core interests and values, the science editor and peer reviewer's judgment of the quality of the article will be considered to be at fault, which is caused mainly by subjectivity and bias. In this case, subjectivity means that all manuscripts are evaluated by peer reviewers based on their own expertise. Academic research can be controversial, and within a given subject area, the opinions of peer reviewers may be not be exactly same and may even be diametrically opposed. Some peer reviewers are relatively tolerant, while others are relatively harsh. These are all important factors affecting peer reviewers' opinions. Therefore, the review of manuscripts cannot be completely free of subjective assumption. Bias refers to non-academic influencing factors that result from the peer reviewers' different preferences or prejudices, including the authors' nationality, mother tongue, sex and organization for which they work<sup>[4]</sup>. *WJG* published 2506 articles in 2006-2007, of which 1963 (78.33%) have been cited in another 1597 journals. The self-citation rates for articles in *WJG* in 2006 and 2007 were 22.16% and 11.34%, respectively<sup>[6]</sup>.

### **International profile of articles published**

It is very disappointing for an author to see no reaction after his/her article is published in a journal. A major reason for this is the particular journal's lack of international profile. High-quality journals are an important indicator of a country's achievement in terms of scientific and technological research, therefore, we aim to create high-quality academic journals of global significance to increase our international profile. Influential high-quality scientific journals are an important platform for academic exchange around the world and an effective vehicle for cultivating innovative talent. They

also provide authoritative leadership in their particular discipline and give a lead in developing new areas of research<sup>[8]</sup>. To achieve these ends, we have adopted an OA publishing model with the following objectives. (1) Release the online edition of *WJG* on time. Up to July 12, 2009, the number of visits was 33947050, the maximum number of times an article was viewed was 50454, and the maximum number of times an article was downloaded in PDF format was 7653; (2) Upload PubMed abstracts on time. From 1998 to 2009, PubMed included 7977 articles from *WJG*. The number of visits by international readers in October 2008 was 76358; (3) Upload PMC full texts on time. From May 1, 2009 to June 11, 2009, 689 articles were included. The number of dedicated IP addresses visiting articles was 31491; the number of times articles were downloaded was 22133; and the number of times XML articles were viewed was 35479; (4) Upload EurekAlert! press releases on time. A total of 198 *WJG* scientific press releases were published from 2007 to 2009. The number of times press releases were viewed was 441128; and the maximum number of times a single press release was viewed was 4126. Through the above methods, *WJG* has increased greatly its international profile and has attracted more contributions from international authors. *WJG* published 1203 articles in issues 1-48 in 2008, which originated from 62 countries, and international authors accounted for 70.25% of the articles published. Editing and publishing high-quality articles and distributing them on time to various media is one of the important methods of increasing the international profile of the authors' work. We award 500 RMB to the electronic editor in charge of each issue for publishing on time and have achieved significant results, thus ensuring on-time publication of each issue in various media.

## MAXIMIZING THE BENEFITS TO READERS BY OBTAINING KNOWLEDGE

At present, universities and scientific research institutions have to spend vast sums of money to obtain access to academic scientific papers, and many small institutions cannot afford the high fees for access licenses. Implementation of the open access model around the world can increase the access of researchers and the general public to information about achievements in scientific research. The research report compares three kinds of publishing models. The open access model has the greatest advantage because research institutions or those funding the research have paid the publishing

costs, therefore, papers can be obtained free of charge<sup>[9]</sup>. The competitive edge of scientific journals depends on the commitment and loyalty of readers. The more readers, the bigger the market share for any given journal. We must establish a reader-centered service so that they accept and come to value our journals<sup>[10]</sup>. We make the online edition of 7977 articles published by *WJG* available to readers around the world. The full text of the articles is available for free download and > 20000 visits are received from readers each day. Readers around the world currently can read and download the latest articles published by *WJG* free of charge, through PubMed and PMC of the United States National Library of Medicine.

## CONCLUSION

The only way for Baishideng to achieve a lasting reputation and continue to develop is to publish high-quality articles and make the full text freely available around the world. This will allow us to develop a commercial publishing model that maximizes the interests of the editorial board, authors and readers.

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# Ascitic fluid analysis for diagnosis and monitoring of spontaneous bacterial peritonitis

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## Abstract

Polymorphonuclear (PMN) cell count in the ascitic fluid is essential for the diagnosis and management of spontaneous bacterial peritonitis (SBP). To date, PMN cell count is routinely performed by traditional manual counting. However, this method is time-consuming, costly, and not always timely available. Therefore, considerable efforts have been made in recent years to develop an alternative test for a more rapid diagnosis and monitoring of SBP. The use of urinary reagent strips was proposed to achieve an "instant" bedside diagnosis of SBP. A series of reports evaluated the urine strip test for SBP diagnosis and reported promising results. However, a recent large multicenter study revealed a surprising lack of diagnostic efficacy of the urine screening test for SBP diagnosis. Another method, more recently proposed as an alternative to the manual PMN count, is the measurement of lactoferrin in ascitic fluid, but the data available on the diagnostic value of this test are limited to a single study. However, both urinary reagent strips and ascitic lactoferrin tests are qualitative methods and need, therefore, to be further confirmed by standard cytology of the ascitic fluid. To date, the only quantitative method proposed as a valid alternative to manual PMN counting is automated blood cell counters, commonly used in all laboratories for blood cell counting. Data available in the literature on the diagnostic performance of this method are limited but very promising, and this tool seems to have the potential to replace the manual counting method.

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**Key words:** Ascites; Ascitic fluid lactoferrin; Automated

## INTRODUCTION

Spontaneous bacterial peritonitis (SBP) is a frequent and severe complication of decompensated cirrhosis. Since its first description in the 1970s, when the mortality rate exceeded 80%, a significant and substantial improvement in the prognosis of SBP has been noted. In more recent prospective studies the mortality rate was reported to be around 20%<sup>[1-3]</sup>. The widespread use of diagnostic paracentesis, as well as the prompt initiation of empiric antibiotic therapy, based on the results of ascitic polymorphonuclear (PMN) cell count, has contributed to an improvement in the survival of these patients.

## DIAGNOSIS AND TREATMENT OF SBP

The percentage of SBP in hospitalized cirrhotic patients with ascites ranges between 10% and 30%<sup>[4-9]</sup>, whereas the prevalence of SBP among outpatients with cirrhosis is markedly lower (approximately 3.5%)<sup>[10]</sup>. Four practical guidelines and experts' consensus reports on the diagnosis and management of SBP are currently available<sup>[11-14]</sup>, all of which suggest that the diagnosis of SBP should be based on PMN cell count in the ascitic fluid. The detection of a PMN cell count greater than 250 cells/mm<sup>3</sup> is highly suspicious of SBP and provides an indication to initiate empirical antibiotic treatment. Only a few SBP patients, in fact, have typical symptoms suggestive of peritoneal infection such as fever, abdominal pain and a high blood leukocyte count. More often, SBP is suspected when the patient develops signs of hepatic

encephalopathy or a rapid impairment of renal function without any apparent precipitating factor. In addition, in a significant percentage of cases, SBP may be completely asymptomatic and the diagnosis is made only by diagnostic paracentesis. The presentation of SBP recently observed in a consecutive series of hospitalized patients<sup>[15]</sup> is reported in Table 1. Following hospitalization of any cirrhotic patient with newly diagnosed ascites, even in the absence of symptoms or if admitted for other reasons, a diagnostic paracentesis is therefore advised, aimed at investigating the presence of SBP. To rule out the possible presence of SBP, a paracentesis tap should also be repeated in all ascitic cirrhotic patients with gastrointestinal bleeding or hepatic encephalopathy, and whenever patients develop clinical symptoms and signs of infection, renal impairment or an unexplained worsening of their clinical condition<sup>[11]</sup>.

Following the diagnosis of SBP, ascitic fluid collection and blood cultures should be carried out before initiating antibiotic administration. The commonest organisms isolated in patients with SBP include *Escherichia coli*, Gram-positive cocci (mainly *Streptococcus* species) and *Enterococci*. These organisms account for approximately 70% of all cases of SBP<sup>[16,17]</sup>. Antibiotic therapy, however, should not be delayed to the moment when microbiological results are available; with conventional culture techniques, the ascitic fluid culture may be negative in up to 60% of patients with SBP<sup>[11]</sup>. Antibiotic therapy should instead be initiated empirically immediately after evidence of SBP at diagnostic paracentesis, without the need to wait for identification of the causative organism and *in vitro* susceptibility. Cefotaxime is the antibiotic most extensively investigated in patients with SBP<sup>[18-20]</sup>, since it covers 95% of the flora isolated from the ascitic fluid and achieves high ascitic fluid concentrations during therapy<sup>[12,21]</sup>. The dose of cefotaxime is 4.8 g/d given intravenously for a minimum of five days. Other studies<sup>[22-26]</sup> have evaluated the efficacy of cephalosporins (such as ceftriaxone and ceftazidime) administered once daily; although data are still limited, they appear to be as effective as cefotaxime. The association between amoxicillin and clavulanic acid also proved to be as effective as cefotaxime, with a similar security profile<sup>[27,28]</sup>. In patients with uncomplicated SBP (i.e. no gastrointestinal bleeding, hepatic encephalopathy or renal failure), oral treatment with ofloxacin or other quinolones for eight days can be administered<sup>[29]</sup>. An intravenous → oral step-down schedule with ciprofloxacin (switch therapy) allows completion of the antibiotic treatment at home, once the patient is discharged. This regimen was recently shown to be as efficacious but more cost-effective than intravenous cefotaxime<sup>[30]</sup>. With the regimen described above, the resolution of SBP is achieved in approximately 90% of cases and 30-d survival is at least 80%<sup>[11]</sup>.

The empirical antibiotic treatment chosen should be continued until symptom resolution or should be modified according to the results of the microbiological test. However, since SBP is often asymptomatic and the ascitic fluid culture outcome is often negative, once again the PMN cell count should be considered as a diagnostic

**Table 1 Signs and symptoms of SBP at the time of hospital admission in a recently published paper<sup>[15]</sup>**

Patients/No. of episodes of SBP (32/38)	n
Clinical manifestations at hospital admission	
Fever	12
Abdominal pain	6
Leukocytosis	11
Hepatic encephalopathy	8
Renal failure	14

tool to objectively demonstrate the efficacy of the ongoing antibiotic treatment and, finally, resolution of the infection. According to the International Ascites Club guidelines published in 2000<sup>[11]</sup>, a diagnostic paracentesis should be repeated after two days during antibiotic therapy to assess treatment response. A PMN cell count reduction greater than 25% of the initial value was in fact suggested as the main criterion for establishing the efficacy of the antibiotic and the need for switching the therapy. This recommendation is very important, since there have been suggestions that the type and etiology of bacterial infections in cirrhosis may have changed during recent years<sup>[31,32]</sup>. An increasing incidence of SBP caused by Gram-positive bacteria in cirrhotic patients with ascites has been observed by different authors<sup>[33,34]</sup>. In addition, an increased frequency of bacteria resistant to multiple antibiotics was shown<sup>[35]</sup>. Our recent study<sup>[15]</sup> reported that, in clinical practice, a switch from cefotaxime to another antibiotic was necessary in more than 40% of SBP cases, thus supporting the possibility that the microbial etiology of SBP, as well as sensitivity to antibiotic therapy, is changing. A method aimed at objectively establishing resolution of the infection would be extremely useful; once again, the PMN cell count in the ascitic fluid obtained at diagnostic paracentesis was revealed to be the most reliable tool.

## THE DETERMINATION OF PMN CELLS IN THE ASCITIC FLUID

Unfortunately, in clinical practice, ascitic PMN cell count is not always possible within a few hours, thus causing an unacceptable delay in the diagnosis and treatment of this potentially fatal infection. At present, PMN cell count is routinely performed by a traditional hematological method with a light microscope in a manual counting chamber. For this method, 10 mL of ascitic fluid are collected in tubes containing ethylenediaminetetraacetic acid (EDTA) and centrifuged at 1500 r/min for 10 min. Nine milliliters of the supernatant are discharged and 40 µL of the remaining ascitic fluid are diluted with 800 µL of Turk's fluid, gently shaken and used to fill the counting chamber. The cells are counted (40 × objective) in one of the nine large squares and the number of white blood cells per cubic millimeter calculated. Another 10 mL sample of ascitic fluid is used for the PMN percentage determination (100 × objective), after centrifugation and May Grünwald-Giemsa staining. This method is presently considered the

**Table 2** Results of the urine reagent strips for the diagnosis of SBP

<b>Authors</b>	<b>Samples/SBP (n)</b>	<b>Strips/cut-off grade</b>	<b>Sensitivity</b>	<b>Specificity</b>	<b>PPV</b>	<b>NPV</b>
Vanbervliet et al <sup>[36]</sup> 2002	72/9	Multistix $\geq 1$	100	100	100	100
Castellote et al <sup>[37]</sup> 2003	228/52	Aution $\geq 3$	89	99	98	97
		Aution $\geq 2$	96	89	74	99
Thevenot et al <sup>[38]</sup> 2004	100/9	Multistix $\geq 3$	89	100	100	99
		Combur $\geq 2$	89	100	100	99
Butani et al <sup>[39]</sup> 2004	136/12	Multistix $\geq 2$	83	99	91	98
Sapey et al <sup>[40]</sup> 2005	184/13	Center 1/Nephur $\geq 1$	86	100	100	99
		Center 1/Multistix $\geq 1$	100	100	100	100
		Center 2/Nephur $\geq 1$	100	92.5	75	100
		Center 2/Multistix $\geq 1$	83	96	83	96
Sapey et al <sup>[41]</sup> 2005	245/17	Nephur $\geq 3$	88.2	99.6	93.8	99.1
		Multistix $\geq 1$	64.7	99.6	91.7	97.4
Kim et al <sup>[42]</sup> 2005	75/18	Uriscan $\geq 2$	100	100	100	100
		Multistix $\geq 3$	50	100	100	87
Sarwar et al <sup>[43]</sup> 2005	214/38	Combur $\geq 3$	97.7	89.4	90	97.7
Wisniewski et al <sup>[44]</sup> 2005	47/6	Multistix $\geq 1$	83	83	97	42
Campillo et al <sup>[45]</sup> 2006	443/46	Multistix $\geq 1$	69.6	94.9	64	96
		Combur $\geq 1$	80.4	90.4	52	97.3
		Multistix $\geq 2$	45.7	98	75	93.3
Rerknimitr et al <sup>[46]</sup> 2006	200/42	Combur $\geq 1$	88	81	55	96
		Combur $\geq 2$	63	96	82	81
Braga et al <sup>[47]</sup> 2006	100/9	Combur $\geq 2$	100	98.9	92.3	100
Torun et al <sup>[48]</sup> 2007	63/15	Aution $\geq 2$	93	100	100	98
Gaya et al <sup>[49]</sup> 2007	173/14	Multistix $\geq 1$	100	91	50	100
Nobre et al <sup>[50]</sup> 2008	109/9	Combina $\geq 2$	78	88	37	98
		Combina $\geq 3$	67	98	75	97
Ribeiro et al <sup>[51]</sup> 2007	200/14	Multistix $\geq 1$	86	96	60	99
		Multistix $\geq 2$	71	99	91	98
Nousbaum et al <sup>[52]</sup> 2007	1041/117	Multistix $\geq 3$	45.3	99.2	77.9	96.9

“gold standard” for the evaluation of ascitic PMN count, but this procedure is laborious and time-consuming. This results in the laboratory often providing the PMN count too late in the day, or sometimes even on the next day, when the patient may have progressed from an early stage to a fatal stage of infection. Moreover, the manual method is not always timely available in all hospitals, especially in those small patient care units with limited laboratory facilities, and the method cannot frequently be performed on an emergency basis (at night or on week-ends). This manual system, therefore, often delays the initiation of antibiotic therapy and may expose patients to a higher risk of death.

On the basis of these considerations, considerable efforts have been made in recent years to develop an alternative test for a more rapid diagnosis of SBP. This represents an interesting and promising area of investigation, which could determine the further optimization of SBP management and further improvement in its prognosis. The use of urinary reagent strips—which identify leukocytes by detecting their esterase activity *via* a colorimetric reaction—was proposed to achieve an “instant” bedside diagnosis of SBP. Their employment was tested for the rapid diagnosis of bacterial meningitis, pleural or synovial infections, thus suggesting that they could also be used for the rapid diagnosis of SBP. The first study<sup>[36]</sup>, published in 2002, involved 72 cirrhotic patients, nine of whom had SBP, reported a sensitivity and a specificity of 100% for the urinary strips in the diagnosis of SBP. After this successful result, in the following years a number of

reports<sup>[37-51]</sup> evaluated the urine strip test for the diagnosis of SBP (Table 2). These studies reported promising and encouraging results, with the sensitivity of urinary strips for the diagnosis of SBP ranging between 85% and 100% and the specificity between 90 and 100%. These published studies were, however, limited by the small number of SBP episodes and were restricted to one or two centers. Therefore, the need to validate this method in a larger population of cirrhotic patients in a multicenter study arose. A prospective multicenter (70 centers) study with a total of 2123 paracenteses performed in 1041 patients was published in 2007 by Nousbaum et al<sup>[52]</sup>. Surprisingly, this paper did not confirm the good and promising results of the previous studies, but confirmed a very high specificity for reagent strips in the diagnosis of SBP, and revealed a poor sensitivity of this test (45%). This study, therefore, reported the lack of diagnostic efficacy of the urine screening test and the authors concluded that a routine cytological examination remains mandatory for the diagnosis of SBP. After initial great enthusiasm, the use of reagent strips for SBP diagnosis has been disappointing.

Another method, more recently proposed as an alternative to the manual PMN count, is the measurement of lactoferrin in ascitic fluid. Lactoferrin is released by PMN leukocytes on the activation of these cells and its presence in body fluids is proportional to the flux of neutrophils. It was therefore hypothesized that its use could also be useful in the detection of SBP. The data available on the diagnostic value of this test for SBP diagnosis are limited to a single study<sup>[53]</sup>. This study, in

**Table 3 Results of the automated cell counter for the diagnosis of SBP**

Authors	Samples/SBP (n)	Automated cell counters	Diagnosis of SBP			
			Sensitivity	Specificity	PPV	NPV
Angeloni <i>et al</i> <sup>[54]</sup> 2003	130/11	Technicon System H*1	94	100	100	99.1
		Coulter	100	92	87	100
Cereto <i>et al</i> <sup>[55]</sup> 2004	133/48	UF-100	98	98	96	99
		ADVIA	100	95	95	100
Riggio <i>et al</i> <sup>[56]</sup> 2008	112/16	Technicon System H*1	100	97.7	94.1	100

which ascitic fluid lactoferrin was measured in a total of 218 ascites samples, reported that the sensitivity and specificity of the assay for SBP diagnosis were 95% and 97%, respectively. However, the quantitative lactoferrin assay used in this study is not commercially available. Qualitative and rapid tests are already commercially available for bedside measurement of lactoferrin concentration in the stool (to distinguish inflammatory from non-inflammatory bowel conditions). The authors hypothesized that qualitative tests able to detect lactoferrin levels in excess of a predetermined level for bedside diagnosis can easily be developed and with limited costs. They concluded that, although further confirmatory studies are needed, ascitic fluid lactoferrin could serve as an initial screening test for the detection of SBP in cirrhotic patients.

It is important to underline that both urinary reagent strips and ascitic lactoferrin tests are qualitative methods and need, therefore, to be further confirmed by standard cytology of the ascitic fluid. These tests could merely be a screening modality providing only a presumptive diagnosis, but they do not have the potential to replace the manual counting method. Moreover, a qualitative test does not have applicability in the clinical management of SBP, since a reduction of more than 25% of the initial PMN cell count value is, according to the current guidelines, the criterion to establish the efficacy of the ongoing antibiotic treatment. To date, the only quantitative methods proposed as a valid alternative to manual PMN counting are automated blood cell counters, commonly and largely used in all laboratories for blood cell counting. They offer accurate and rapid differential counts of leukocytes, including PMN cells. The published data available on the diagnostic performance of this method are limited but very promising (Table 3). The first study<sup>[54]</sup>, which tested an automated cell counter for the diagnosis of SBP, was published in 2003 by our group. This study, in which 130 ascitic fluid samples obtained from 74 cirrhotic patients were analyzed, demonstrated that the use of an automated cell counter provides an accurate determination of ascitic fluid PMN and has a reliable diagnostic accuracy when compared with manual counting. In fact, the automated blood cell counter had a sensitivity of 94% and a specificity of 100%, while positive and negative predictive values were 100% and 99.1%, respectively (Table 3). These findings were also confirmed by Cereto *et al*<sup>[55]</sup> (Table 3). More recently, a further study<sup>[56]</sup>, in which we analyzed 112 samples of ascitic fluid, showed that the manual and automated meth-

ods had good agreement not only in the first diagnostic paracentesis, but also in those performed during the follow-up. These results suggested that the automated cell counter is a useful and reliable tool not only for the diagnosis of SBP, but also in its management for monitoring the therapeutic efficacy of antibiotic treatment, as well as for establishing resolution of the infection. The use of automated PMN counting may provide several advantages for the diagnosis and management of SBP: it is commonly available in all patient care units, it is routinely accessible in emergencies (i.e. at night or on weekends) and provides the results in a few minutes, thus allowing the clinician to quickly decide whether to start empiric antibiotics or to change the ongoing antibiotic therapy. Finally, it is important to remember that all this involves a very low cost (only €1.38 for a single ascitic cell determination). Another, although less important, advantage of the automated cell counting method over urinary reagent strips, the manual method and the ascitic lactoferrin test, is that it is possible to precisely assess the amount of PMN in bloody (for a traumatic tap or a condition inducing bleeding) ascitic fluid. With the other methods, the amount of PMN derived directly from the blood spilled over into the ascitic fluid cannot be differentiated from the amount of PMN due to the infection. A correction factor of 1 PMN per 250 red blood cells has been proposed<sup>[11]</sup>, because this is the maximum expected ratio of PMN to red blood cells normally present in the peripheral blood. With automated cell counters, a measure of the amount of red blood cells and PMN in both the peripheral blood and the ascitic fluid can be obtained simultaneously. The amount of ascitic PMN due to the infection can therefore be calculated by the real PMN-to-red blood cells ratio in the blood and by the real erythrocytes and PMN number in the ascitic fluid.

## CONCLUSION

Although further confirmatory data are needed, automated cell counters offer a reliable, easier and quicker PMN count, they may also considerably simplify the diagnostic approach and clinical management of SBP and should be routinely adopted. The automated method has the potential to be an optimal substitute for manual PMN counting and could definitely replace it.

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TOPIC HIGHLIGHT

Damian Casadesus, MD, PhD, Series Editor

## Surgical resection of rectal adenoma: A rapid review

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inexpensive, and associated with the lowest complication rate. When the size and/or location of the tumor limits standard endoscopic resection, a number of different transanal approaches are used to remove adenomatous polyps in the lower rectum, but adenomas in the middle or upper rectum are difficult to remove using standard transanal excision (TE) instruments. Different techniques have been developed for the removal of rectal adenomas that are not amenable to removal during colonoscopy.

### TE

TE of lower rectal adenoma, first reported by Parks<sup>[2]</sup>, is one of the most frequently adopted approaches for excision of rectal polyps of any dimension, situated as far as 6 cm from the anal verge, and has very few complications, and low recurrence and mortality. In a review of the literature, bleeding after TE was present in up to 10% of the patients, perforation in up to 6%, and anal stenosis in up to 5%, with a mortality rate of 2%<sup>[3-6]</sup>. Recurrence is variable, at 3%-50% for adenoma from adenoma, and up to 3% for carcinoma from adenoma. In a long series of 117 procedures, Sakamoto *et al*<sup>[7]</sup> treated 27% of patients for residual disease and 30% for recurrence, with a 10% rate of serious complications. In a recent study, Pigot *et al*<sup>[8]</sup> obtained better results: they resected apparently benign rectal adenomas from 207 consecutive patients with a 3.6% recurrence rate, eight postoperative complications, and one death. Specific recurrence-free probability was 99.5% at 1 year, 96% at 5 years, and 95% at 10 years.

In the middle and upper third of the rectum, benign lesions are difficult to reach transanally, and standard radical surgical options, such as posterior trans-sphincteric resection, and low anterior resection can be offered to the patient; however, they have been associated with high morbidity, such as anastomotic leakage, sexual dysfunction, and fecal and urinary incontinence.

### Abstract

Transanal excision (TE), endoscopic transanal resection (ETAR) and transanal endoscopic microsurgery (TEM) can be used to remove adenomatous polyps. However, their use is limited by the size or location of the tumor. TE is limited to the lower rectum, TEM offers better access to lesions in the middle and upper rectum, and ETAR is used less frequently than it deserves for resection of rectal lesions.

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**Key words:** Adenoma; Colorectal surgery; Endoscopy; Gastrointestinal; Microsurgery; Rectal neoplasms

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### INTRODUCTION

The risk of carcinoma developing in a colorectal polyp of 1 cm or larger is 2.5% and 8% at 5 and 10 years, respectively<sup>[1]</sup>. This potential for malignancy of adenomatous colorectal polyps is an indication for their excision and the first-line treatment is endoscopic removal during the diagnostic procedure, which is safe, relatively

### POSTERIOR APPROACH

The posterior approach (represented by a combination of coccyx removal and partial sphincter division) has been progressively abandoned. It was indicated for extensive lesions in the anterior rectal wall at a distance of 8-10 cm from the anal verge. Variable rates of complications and very high rates of recurrence have been reported. In a

**Table 1** Resection of apparent adenoma polyps by ETAR

Ref.	Patients	ETAR	Recurrence	No. of complication patients	Failure	Follow-up (mo)	Related mortality
Tuech <i>et al</i> <sup>[11]</sup>	28	39	2	6	-	60 (2.5-10.5)	0
Beattie <i>et al</i> <sup>[12]</sup>	11	30	0	-	-	-	0
Wetherall <i>et al</i> <sup>[13]</sup>	23	38	2	-	1	4.5 (2-42)	0
Dickinson <i>et al</i> <sup>[14]</sup>	38	62	1	10	-	-	1
Sutton <i>et al</i> <sup>[15]</sup>	60	102	4	-	2	-	0
Bujanda <i>et al</i> <sup>[16]</sup>	13	13	1	2	-	15 (3-24)	-
Tsai <i>et al</i> <sup>[17]</sup>	131	180	27	7	-	60	0
Total	304	464	37	25	3	-	1

**Table 2** Adenoma resected by TEM

Ref.	No. of lesions	Residual adenoma in surgical margin (%)	Local recurrence (%)	Follow-up (mo)
Steele <i>et al</i> <sup>[20]</sup>	77	9	5.1	7.4
Farmer <i>et al</i> <sup>[21]</sup>	36	25	5.6	33
Nakagoe <i>et al</i> <sup>[22]</sup>	9	0	0	d.n.o
Cocilovo <i>et al</i> <sup>[23]</sup>	56	1.7	3.5	d.n.o
Neary <i>et al</i> <sup>[24]</sup>	21	0	4.7	d.n.o
Katti <i>et al</i> <sup>[25]</sup>	58	7	10	34
Vorobiev <i>et al</i> <sup>[26]</sup>	113	-	8.3	29.5
Bretagnol <i>et al</i> <sup>[27]</sup>	148	14.9	7.6	33
Endreseth <i>et al</i> <sup>[28]</sup>	64	20	13	24
Zacharakis <i>et al</i> <sup>[29]</sup>	48	4.2	6.3	37
Schäfer <i>et al</i> <sup>[30]</sup>	33	18	12	36.4
Røkke <i>et al</i> <sup>[31]</sup>	56	10	0	12
Guerrieri <i>et al</i> <sup>[32]</sup>	530	-	4.3	44
McCloud <i>et al</i> <sup>[33]</sup>	75	37.3	16	31
Whitehouse <i>et al</i> <sup>[34]</sup>	146	5.5	4.8	39
Platell <i>et al</i> <sup>[35]</sup>	62	-	2.4	24
Lloyd <i>et al</i> <sup>[36]</sup>	68	9	5.9	28.7
Ganai <i>et al</i> <sup>[37]</sup>	82	10	15	44

d.n.o: Data not obtained.

review of 360 cases treated for benign and malignant lesions, fecal fistula was the most frequent complication in 5%-70% of patients and fecal incontinence in 5%-25%<sup>[3,9]</sup>. Adenoma recurrence rate was up to 33% and a stoma was necessary in 20%-70% of patients, as a temporary stoma to avoid fecal fistula or as a therapeutic stoma after its appearance<sup>[3,9]</sup>.

## ENDOSCOPIC TRANSANAL RESECTION (ETAR)

ETAR was first described in 1977 by Lindenschmidt *et al*<sup>[10]</sup> and has been used in the resection of rectal adenoma. A review of 304 cases (464 procedures) from seven studies, most of them in the United Kingdom, with probable double recording of patients (Table 1), suggests that ETAR is valuable in the resection of rectal adenoma with low morbidity and mortality. This technique has shown low recurrence, however, it is impossible to know if the resection is complete and the margins are free of tumor after various procedures in the same patient from whom the adenoma is resected piecemeal. ETAR does not require surgical assistance, anesthesia, extreme positioning, new technology or special training. Nevertheless, this technique provides limited resection of mesorectal fat and lymph nodes,

limited histopathological information regarding extent of resection, and poor local disease control.

## TRANSANAL ENDOSCOPIC MICROSURGERY (TEM)

TEM has been used successfully in the management of rectal adenoma and in selected cases of rectal carcinoma, since it was introduced by Professor Buess in 1984<sup>[18]</sup>. It has been suggested that TEM enables local excision of adenomas up to 24 cm from the anal verge, and offers a minimally invasive alternative to TE, radical surgery and recurrence from previously used endoscopic resection, with superior endoscopic magnification and illumination, accuracy and complete resection, and secure suture closure.

TEM has produced satisfactory results, with better recurrence rates and low morbidity and mortality. In one of the largest studies of adenoma resection using TEM, the authors reported a 3.4% early postoperative complication rate and 1.2% and 7% recurrence rates after 1 and 5 years, respectively, in a series of 286 cases<sup>[19]</sup>. In a review of 1682 adenoma resection procedures from 18 studies (Table 2), an average of 11% of the patients were found to have residual adenoma in the surgical margin, 6.3% had recurrence, and the complication rate was up to 11% during a follow-up period of 12 mo in one series, and > 24 mo in the rest of the studies.

The histologically positive resection margin is highly significant in terms of local recurrence rates after adenoma resection. In a review of 18 studies with a minimum follow-up of 12 mo (Table 2), I have found that residual adenoma in the surgical margin varied from 0% to 37.3%. However, the recurrence rate was 0%-15%, predominantly in cases with positive or uncertain resection margins. In three large series, it has been found that adenoma recurrence rate is high in the incompletely resected group (37.7%, 46% and 25%) compared with that in patients with free histological margins after adenoma resection (4.3%, 3% and 3.7%, respectively)<sup>[28,33,34]</sup>. In another two studies, adenoma extending to the surgical margin of locally excised polyps was found in 25%<sup>[21]</sup> and 30.7% of cases<sup>[38]</sup>. The latter was a large series of 238 patients (226 patients treated by TEM and 12 by TE). It is remarkable that, with such high positive-residual-margin rates, the recurrence rates in these studies were only 5.6% after 33 mo follow-up<sup>[21]</sup> and 3.6% after 67.5 mo follow-up<sup>[38]</sup>, which are lower

than the rates reported in studies with lower positive-residual-margin rates. Røkke *et al*<sup>[31]</sup> did not observe adenoma recurrence after 12 mo follow-up in a series of patients in whom the residual adenoma in surgical margin was 10%.

Even for recurrent adenoma, TEM has now become an important alternative treatment. Five series have reported the use of TEM in the treatment of recurrent adenoma or residual disease without further recurrence<sup>[25,26,32,33,35]</sup>.

In five studies that have compared adenoma resection with TEM and another procedure, the lower or similar recurrence, residual tumor and early complication rates favor TEM resection<sup>[22,38-40]</sup>. Late complication rates were higher after TEM because of the increased incidence of transient incontinence in the postoperative period<sup>[39,40]</sup>. Recently, Moore *et al*<sup>[41]</sup> have compared rectal adenoma excision with TEM and TE, and concluded that TEM is more effective than the other technique, because they found a significantly higher rate of clear margin with less fragmented specimens after TEM, with low complication and recurrence rates.

## CONCLUSION

The transanal approach is a feasible, frequently used technique with few complications and low recurrence and mortality, but its use is limited to the lower rectum. The posterior approach with coccygeal resection, with or without sphincter section, can be mentioned only anecdotally in colorectal surgery. Probably very few surgeons are using it because of its high morbidity and recurrence and the use of colostomy. ETAR is a forgotten minimally invasive technique. It is a simple and safe method and technical expertise has already been acquired on urological conditions such as bladder and prostate tumors; however, it is used less frequently than it deserves for resection of rectal lesions. This is probably because few colorectal surgeons have much enthusiasm for the multiple sessions and piecemeal resection using a resectoscope. TEM offers better access to lesions in the middle and upper rectum, with superior endoscopic magnification and better illumination, superior visualization and pneumorectum, with accurate and complete resection allowing better histological analysis, and secure suture closure. The benefits of TEM are: few complications; low recurrence and mortality; less postoperative pain and use of analgesia; shorter time to the patient being able to walk, sit, eat and defecate; shorter hospital stay; and avoidance of colostomy. These benefits can compensate for the high price of the instruments.

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## Secondary hepatic resection as a therapeutic goal in advanced colorectal cancer

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### INTRODUCTION

Globally, one half of the nearly 1 million patients diagnosed with colorectal cancer annually will develop liver metastases during the course of the disease<sup>[1,2]</sup>. Autopsy findings suggest that approximately 50% of patients who die of colorectal cancer have liver metastases, which are the only site of metastatic disease in approximately 20%-30% of patients<sup>[3]</sup> and the cause of death in most of these patients<sup>[4]</sup>. In other types of cancer, liver metastases are a sign of distant dissemination, and surgery is not a curative option<sup>[5]</sup>. In colorectal cancer, however, portal vein drainage from the gastrointestinal tract to the liver favors metastasis to the liver without systemic dissemination<sup>[5]</sup>. Surgery therefore provides a potentially curative treatment option for patients with resectable liver disease<sup>[6]</sup>, in contrast with palliative chemotherapy for those with unresectable disease.

One of the most important recent advances in the management of advanced colorectal cancer is the concept of downstaging initially unresectable disease using chemotherapy so that more patients become eligible for potentially curative surgery. Current treatment guidelines<sup>[4]</sup> and substantial research highlight the importance of increasing the rates of secondary resection in initially unresectable disease. The addition of active targeted therapies, such as cetuximab and bevacizumab, to chemotherapy may further increase secondary resection rates. This article summarizes the current data on secondary resection of initially unresectable liver metastases of colorectal cancer using currently available systemic therapy regimens.

### HOW RESECTABILITY MAY BE ACHIEVED

Reported 5-year survival rates following hepatic resection generally range from 25% to 40% (Figure 1)<sup>[7-9]</sup>, though

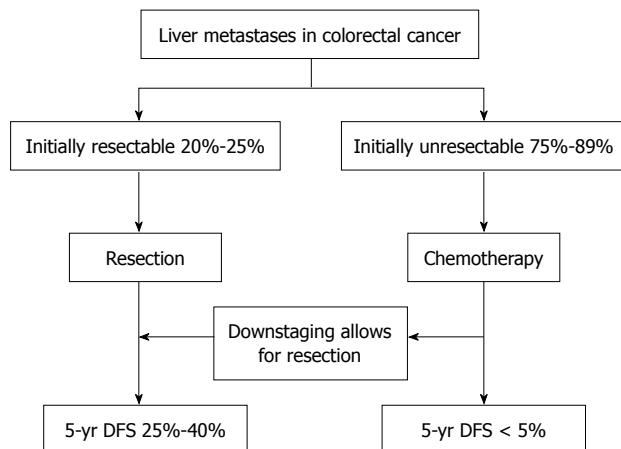
### Abstract

Surgery is the only curative option for patients with liver metastases of colorectal cancer, but few patients present with resectable hepatic lesions. Chemotherapy is increasingly used to downstage initially unresectable disease and allow for potentially curative surgery. Standard chemotherapy regimens convert 10%-20% of cases to resectable disease in unselected populations and 30%-40% of those with disease confined to the liver. One strategy to further increase the number of candidates eligible for surgery is the addition of active targeted agents such as cetuximab and bevacizumab to standard chemotherapy. Data from a phase III trial indicate that cetuximab increases the number of patients eligible for secondary hepatic resection, as well as the rate of complete resection when combined with first-line treatment with the FOLFIRI regimen. The safety profiles of preoperative cetuximab or bevacizumab have not been thoroughly assessed, but preliminary evidence indicates that these agents do not increase surgical mortality or exacerbate chemotherapy-related hepatotoxicity, such as steatosis (5-fluorouracil), steatohepatitis (irinotecan), and sinusoidal obstruction (oxaliplatin). Secondary resection is a valid treatment goal for certain patients with initially unresectable liver metastases and an important end point for future clinical trials.

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**Key words:** Colorectal cancer; Liver metastases; Liver resection; Cetuximab; Bevacizumab; Hepatotoxicity

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**Figure 1** Breakdown of the incidence of liver metastases of colorectal cancer and expected outcomes with current treatment.

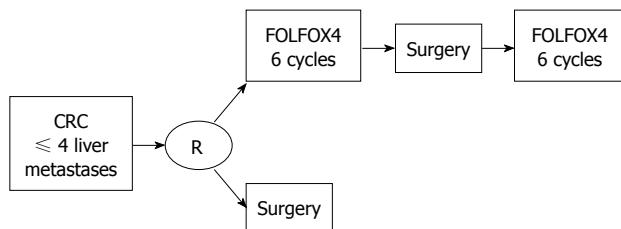
rates exceeding 50% have been observed in some studies<sup>[1,10,11]</sup>, and these outcomes may be improved by the use of preoperative or postoperative chemotherapy or both<sup>[12,13]</sup>. In a recent phase III trial, hepatic resection was compared with resection plus perioperative chemotherapy (6 cycles of FOLFOX4 before and 6 cycles after surgery) (Figure 2). Perioperative chemotherapy increased 3-year survival rates significantly in eligible patients (36.2% vs 28.1%) and resected patients (42.4% vs 33.2%), compared with surgery alone<sup>[2]</sup>.

Unfortunately, 80%-90% of patients with liver metastases are considered to have unresectable disease at the time of diagnosis<sup>[4,7]</sup>. For them, modern chemotherapy and biologic agents afford median survival times hovering around 15 to 20 mo (Figure 1)<sup>[14-20]</sup>.

Curative surgery remains an option if initially unresectable disease can be downstaged to allow for potentially definitive, ie, secondary, resection. Reported resectability rates after chemotherapy vary widely, depending on the patient population and definition of resectability. In unselected patients with unresectable liver metastases treated with chemotherapy, resection rates of 1% to 26% have been reported<sup>[21]</sup>. Higher resection rates (approximately 24%-54%) have been seen in patients with disease confined to the liver<sup>[21]</sup>.

Outcomes following secondary resection are comparable to those observed after primary resection, making resectability a high-priority therapeutic goal. In a study of 872 patients with colorectal liver metastases, 701 (80%) were deemed to have unresectable disease. Of these patients, 95 (13%) ultimately underwent secondary resection after downstaging with chemotherapy. The 5-year survival rate in patients undergoing secondary resection was the same as that in patients undergoing primary resection at the same institution for initially resectable disease (34%)<sup>[7]</sup>.

As investigators attempt to refine the use of resectability and collect information about resection as a clinical endpoint, a rough positive indicator of potential improvement in resectability rates is tumor shrinkage activity. It is therefore unsurprising that conversion to resectability correlates with response to



**Figure 2** Schema of the EORTC 40983 of perioperative chemotherapy<sup>[2]</sup>.

chemotherapy<sup>[21]</sup>. A retrospective review of 305 patients treated with preoperative irinotecan- or oxaliplatin-based chemotherapy followed by resection of liver metastases showed that pathologic response to chemotherapy is an independent predictor of survival<sup>[22]</sup>. Importantly, these findings support an aggressive approach in which optimal management aims to put curative options within reach by tailoring systemic therapy to achieve the highest response rate possible, particularly in patients with borderline resectable disease.

## SECONDARY RESECTION FOLLOWING STANDARD CHEMOTHERAPY

### Oxaliplatin- and irinotecan-based regimens

Several combinations of 5-fluorouracil and leucovorin with either oxaliplatin or irinotecan have established efficacy and are accepted as standard treatment for advanced colorectal cancer<sup>[4,14,15,17,22,23]</sup>. These regimens can also lead to secondary resection in some patients with initially unresectable liver metastases. In unselected populations with advanced colorectal cancer, oxaliplatin-based therapy produces secondary resection rates of 15%-22% and complete resection rates (R0) of 9%-13% (Table 1)<sup>[14,23-25]</sup>. Among patients treated with irinotecan-based regimens, approximately 9% become eligible for surgery and 7% will achieve complete resection (Table 2)<sup>[14,23,26-28]</sup>.

Comparative data from several studies suggest that oxaliplatin-based regimens may be more effective than irinotecan-based regimens in converting unresectable disease to resectable disease<sup>[14,29,30]</sup>, although this finding has not always been consistent<sup>[23]</sup>. Importantly in these studies, median survival times for resected patients reach 42-47 mo.

Higher resection rates have been reported in studies of selected patients, such as those with liver metastases only. In a phase II trial evaluating FOLFOX4 in patients with unresectable metastases confined to the liver, response rate was 60% and surgery was possible in 40% of patients<sup>[25]</sup>. Of the 17 patients who underwent surgery, 14 were free of residual disease (R0). The median survival was 26 mo. Additional studies have evaluated irinotecan-based regimens in selected patient populations; resection rates were generally 30%-40%<sup>[26-28]</sup>.

### FOLFOXIRI

More recently, several groups have evaluated the

**Table 1** Outcomes related to secondary hepatic resection in patients treated with oxaliplatin-based chemotherapy

Study	<i>n</i>	Regimen	Response rate (%)	Resection rate (%)	R0 rate (%)	MS (mo)
Unselected populations						
GERCOR <sup>[14]</sup>	111	FOLFOX6	54.0	22.0	13.0	NYR
Tournigand <i>et al</i> <sup>[24]</sup>	311	FOLFOX4	58.5	17.7	11.3	38.9
Tournigand <i>et al</i> <sup>[24]</sup>	309	FOLFOX7 <sup>1</sup>	59.2	15.2	9.4	43.0
Colucci <i>et al</i> <sup>[23]</sup>	182	FOLFOX4	34.0	4.4	NR	NR
Liver metastases only						
Alberts <i>et al</i> <sup>[25]</sup>	44	FOLFOX4	60.0	40.0	33.3	NR

<sup>1</sup>FOLFOX7 × 6 cycles, followed by treatment without oxaliplatin × 12 cycles, followed by FOLFOX7 until PD. MS: Median survival in resected patients; FOLFOX: Oxaliplatin plus infusional 5-fluorouracil/leucovorin (5-FU/LV); NYR: Not yet reached; NR: Not reported.

**Table 2** Outcomes related to secondary hepatic resection in patients treated with irinotecan-based chemotherapy

Study	<i>n</i>	Regimen	Response rate (%)	Resection rate (%)	R0 rate (%)	MS (mo)
Unselected populations						
Tournigand <i>et al</i> <sup>[14]</sup>	109	FOLFIRI	61.0	9.0	7.0	47
Colucci <i>et al</i> <sup>[23]</sup>	178	FOLFIRI	36.0	5.1	NR	NR
Liver metastases only						
Pozzo <i>et al</i> <sup>[26]</sup>	40	5-FU/LV, irinotecan	47.5	40.0	32.5	NYR
Zelek <i>et al</i> <sup>[27]</sup>	31	5-FU/LV, irinotecan, and HAI of pirarubicin	48.0	35.0	29.0	NYR
Ho <i>et al</i> <sup>[28]</sup>	40	5-FU/LV, irinotecan	55.0	10.0	NR	NR

HAI: Hepatic arterial infusion.

combination of 5-fluorouracil, oxaliplatin, and irinotecan (FOLFOXIRI)<sup>[16,31,32]</sup>, with the rationale of maximizing concurrent exposure to multiple active agents<sup>[14,33]</sup>. In a preliminary study in 39 patients with unresectable liver metastases, the response rate was 64% and secondary surgery was possible in 23 patients (59%)<sup>[32]</sup>. Of these patients, 84% achieved R0 resection.

Two randomized trials have compared FOLFOXIRI with FOLFIRI as first-line therapy for patients with initially unresectable advanced colorectal cancer<sup>[16,31]</sup>. Although the first study, of 283 patients, found no significant difference between the two treatment groups in response or survival<sup>[31]</sup>, the second study, involving 244 patients, found that FOLFOXIRI significantly increased response rates (60% vs 34%) and median survival (22.6 mo vs 16.7 mo)<sup>[16]</sup>. Both studies, however, showed that FOLFOXIRI increased secondary resection rates compared with FOLFIRI. Significantly more patients treated with FOLFOXIRI in the first study were eligible for secondary resection (14 vs 6)<sup>[31]</sup>. This included 14 patients with liver metastases (11 vs 3), of whom 11 achieved R0 resection (9 vs 2). In the second study, the rate of R0 secondary resection was significantly greater with FOLFOXIRI (15% vs 6%) overall and in those with liver metastases only (36% vs 12%)<sup>[16]</sup>.

Both randomized trials reported increased toxicity with FOLFOXIRI compared with FOLFIRI, including higher incidences of alopecia, diarrhea, neutropenia, and neurotoxicity, a factor to weigh against the benefits achieved<sup>[16,31]</sup>. Further investigation is needed to identify new strategies for increasing rates of secondary resection without increasing toxicity associated with standard chemotherapy agents.

## IMPROVING ON STANDARD CHEMOTHERAPY: ADDITION OF BIOLOGICS

Two monoclonal antibodies are approved for use in advanced colorectal cancer that have been shown to improve outcomes when combined with chemotherapy. Combining these agents with standard chemotherapy may represent a safe and effective strategy for increasing the proportion of patients eligible for potentially curative surgery.

### Cetuximab

Cetuximab blocks the activity of the epidermal growth factor receptor (EGFR) and is approved for use in the United States in combination with irinotecan in patients with irinotecan-refractory disease, or as monotherapy for patients who have failed both irinotecan- and oxaliplatin-based chemotherapy. Adam *et al*<sup>[34]</sup> assessed the ability of cetuximab-based therapy to downstage patients with unresectable disease and liver metastases refractory to prior chemotherapy to become eligible for surgery with curative intent. A total of 151 patients were treated either completely at the Paul Brousse Hospital (PBH, *n* = 133) or referred to PBH for hepatectomy after receiving cetuximab-based therapy elsewhere (*n* = 18). Of the 151 patients, 27 underwent surgery, of whom 20 had received cetuximab plus irinotecan; four, cetuximab plus oxaliplatin; and one, all three agents. Two-thirds of patients who underwent surgery had had at least two prior chemotherapy regimens. Of the 133 patients treated completely at PBH, 9 (7%) underwent surgery, which is encouraging, considering these were heavily pretreated and refractory patients.

**Table 3 Randomized trials comparing first-line chemotherapy with or without cetuximab or bevacizumab that report secondary resection rates**

<b>Study</b>	<b>n</b>	<b>Regimen</b>	<b>Response rate (%)</b>	<b>Resection rate (%)</b>	<b>R0 rate (%)</b>	<b>R0 rate in liver-only disease (%)</b>	<b>PFS (mo)</b>
Cetuximab							
CRYSTAL <sup>[37]</sup>	559	FOLFIRI	38.7	2.5	1.5	4.5	8.0
	559	FOLFIRI + cetuximab	46.9	6.0	4.3	9.8	8.9
Bevacizumab							
Hurwitz <i>et al</i> <sup>[38]</sup>	411	IFL	34.8	< 2%	NR	NR	6.2
	402	IFL + bevacizumab	44.8	< 2%	NR	NR	10.6
NO16966 <sup>[39,40]</sup>	701	CT <sup>1</sup>	49.0	4.9	NR	11.5	8.0
	699	CT <sup>1</sup> + bevacizumab	47.0	6.3	NR	12.3	9.4

<sup>1</sup>FOLFOX4 or XELOX. PFS: Progression-free survival in the study arm; FOLFIRI: Irinotecan plus infusional 5-fluorouracil/leucovorin (5-FU/LV); IFL: Irinotecan, fluorouracil, and leucovorin; CT: Chemotherapy.

In the first-line setting, an initial phase I / II trial of 21 patients with unresectable, EGFR-expressing disease who received cetuximab plus 5-fluorouracil, leucovorin, and irinotecan found a 67% response rate with median survival of 33 mo<sup>[19]</sup>. Five patients (24%) were eligible for secondary resection. A phase II study evaluated first-line cetuximab plus FOLFOX4 in 43 patients with unresectable, EGFR-expressing disease<sup>[35]</sup>. Confirmed responses were seen in 72% of patients and the disease control rate was 95%. Ten patients (23%) underwent potentially curative surgery and nine (21%) had no evidence of residual disease after surgery. Overall, the median survival was 30 mo.

A randomized phase II trial comparing FOLFOX4 with or without cetuximab (the OPUS trial) found that cetuximab significantly increases response rates in patients with good performance status when added to standard first-line chemotherapy (49.0% vs 36.8%)<sup>[36]</sup>. Resection rates were higher with cetuximab (6.5% vs 3.6%), as were R0 resection rates (4.7% vs 2.4%)<sup>[36]</sup>. Cetuximab was associated with an increase in skin reactions, infusion-related reactions, and hypomagnesemia, but no exacerbation of oxaliplatin-related toxicities.

The CRYSTAL study (*n* = 1198) investigating the addition of cetuximab to FOLFIRI was the first phase III trial to demonstrate that adding a therapeutic monoclonal antibody to standard chemotherapy may improve secondary resection rates (Table 3)<sup>[37-40]</sup>. Cetuximab significantly increased the overall secondary resection rate (6% vs 2.5%), and boosted the rate of complete resection (R0) 3-fold [4.3% vs 1.5%; odds ratio 3.0 (95% CI 1.4-6.5)]. As expected, higher rates of R0 resection were seen in the subgroup of patients with disease confined to the liver (approximately 20% of patients in each arm), for whom the addition of cetuximab also appeared to increase R0 resection rates compared with FOLFIRI alone (9.8% vs 4.5%) and was associated with a significant increase in progression-free survival (median, 11.4 mo vs 9.2 mo)<sup>[37]</sup>.

The second key finding of CRYSTAL revealed that the benefit from cetuximab was concentrated in the subgroup of patients with wild-type K-RAS on their tumors (64.4% of the initial evaluable population); this population experienced a reduction in the risk of

progression of 32% (HR = 0.68, *P* = 0.017), and 16% higher response rate (59.3% vs 43.2%, *P* = 0.0025)<sup>[42]</sup> compared with those with mutant K-RAS. The effect on patients with disease confined to the liver was also dramatic, with the response rate reaching 77% in the population with K-RAS wild type<sup>[41]</sup>.

These promising observations have propelled more focused investigation on the role of cetuximab as a conversion or downstaging agent. The CELIM study was a randomized phase II study (*n* = 111) in which patients with unresectable liver metastases received cetuximab with either FOLFOX or FOLFIRI. Of 106 patients evaluable for efficacy, a response rate of 74% was reported in both arms combined, 79% in those with K-RAS wild-type tumors. Confirmed response (by second CT scan according to RECIST criteria or by resection) rate was 62% in both arms combined, 70% in those with K-RAS wild-type tumors. The rate of resection was an encouraging 46%, and 34% of patients achieved R0 resections. Nineteen of 75 patients (25%) with unresectable disease at enrollment had resectable disease after 16 wk of therapy (*P* = 0.021)<sup>[42]</sup>.

### **Bevacizumab**

Bevacizumab binds to vascular endothelial growth factor (VEGF) and is approved for use in combination with 5-fluorouracil-based chemotherapy as first- or second-line treatment for metastatic colorectal cancer. This agent has been shown to improve outcomes when added to first-line chemotherapy for advanced colorectal cancer, increasing response rates by approximately 10% compared with chemotherapy alone and producing median survival rates of approximately 17-24 mo<sup>[20,38,43]</sup>. Nonrandomized trials evaluating the combination of bevacizumab and oxaliplatin-based chemotherapy as neoadjuvant therapy in patients with resectable disease have also produced promising results<sup>[13,44]</sup>.

Data from phase III trials suggest that bevacizumab does not increase secondary resection rates when added to standard chemotherapy (Table 3), both for irinotecan-based and oxaliplatin-based therapy, although the resected patients in bevacizumab are low to extract any firm conclusion<sup>[38,39]</sup>.

A non-randomized, uncontrolled phase IV study

(first BEAT) prospectively collected data on resection rates following bevacizumab plus various chemotherapy regimens as first-line treatment for advanced colorectal cancer. Of the 1914 evaluable patients, 215 (11.2%) underwent surgery after receiving systemic therapy and 170 (8.8%) achieved R0 resection. Preliminary data suggest a 2-year survival rate of 44% in the entire patient population and 82% in those who achieved R0 resection<sup>[40]</sup>.

Based on the findings described above, it appears that bevacizumab does not compromise the feasibility of secondary resection of metastatic disease; it is unclear, however, whether adding bevacizumab has the potential to improve upon the resectability rates achieved with chemotherapy alone<sup>[40]</sup>.

## THE ROLE OF LOCAL THERAPIES IN THE MANAGEMENT OF LIVER METASTASES

Local treatments may be combined with surgery with the potential to improve upon the therapeutic benchmarks achieved with surgery alone. Abdalla *et al*<sup>[1]</sup> reported on the use of radiofrequency ablation (RFA), alone or in combination with surgery in 158 patients in whom R0 resection was not possible. Outcomes were far superior for the 190 patients who underwent complete resection, with roughly double survival rates at 4 years (65% vs 22%-36% with RFA alone or RFA plus surgery). RFA seemed to offer a modest advantage over palliative chemotherapy only.

Stereotactic body radiation (SBRT) is a local treatment approach that may enhance outcomes in patients with liver metastases<sup>[45]</sup>, but it is too early to determine its relative efficacy compared with other modalities. A recent retrospective comparison of outcomes after surgery, RFA, or SBRT in patients whose liver disease recurred after initial partial resection, revealed equivalent outcomes across the three approaches<sup>[46]</sup>; well-controlled prospective comparisons are lagging.

Finally, selective internal radiation therapy (SIRT), using Yttrium-90 microspheres<sup>[47]</sup>, is also a feasible technology awaiting validation in this disease setting.

## TREATMENT DECISIONS, TOXICITY, FEASIBILITY AND OTHER CONCERNS

Considering the importance of achieving resectability as a therapeutic goal, its accurate assessment is key to delineate management strategies, and the availability of increasingly sophisticated diagnostic tests may help optimize evaluation approaches. Positron-emission tomography combined with computed tomography (PET-CT) with [<sup>18</sup>F]-fluoro-2'-deoxy-D-glucose (FDG) has an important role in staging patients with colorectal cancer, and in ruling out extrahepatic metastases in advanced disease, although prior chemotherapy may lower its sensitivity<sup>[48]</sup>. MRI is used for further evaluation of the liver in patients with metastatic colorectal cancer. A 2008 study of 20 consecutive patients with colorectal

cancer compared whole-body MRI *vs* PET-CT for staging of lymph nodes and distant metastases. Results suggested a preference for PET-CT in diagnosing lymph nodes, but a trend toward superiority for whole-body MRI in detecting metastases in liver, brain, and bone<sup>[49]</sup>. Finally, intraoperative ultrasound (IOUS) is a standard method to determine resection margin and identify previously undetected tumors<sup>[50-52]</sup>. Contrast-enhanced IOUS offers greater sensitivity, increasing identification of new metastatic lesions by approximately 20%<sup>[50,53,54]</sup>, and allows more time to find new metastases intraoperatively<sup>[51]</sup>. Zalinski *et al*<sup>[55]</sup> developed a new marking technique to help identify resection margins intraoperatively following preoperative chemotherapy: coils are placed behind the deep margin of lesions using a guide needle and CT or ultrasound guidance prior to systemic chemotherapy. The coils are then easily detected during surgery.

The relevance of resectability in disease prognosis recently became clearer. Patients with resectable metastases either hepatic or extrahepatic (but not both) had a 5-year overall survival rate of 54%, compared with 13% for patients with both resectable hepatic and extrahepatic disease and 0% for unresectable metastases, regardless of location<sup>[56]</sup>. The authors proposed the inclusion of resectability as a criterion to stratify stage IV colorectal cancer.

The degree of optimization of downstaging strategies in routine clinical practice, however, remains unclear. In a recent review of managed care records of nearly 500 patients with colorectal cancer who underwent hepatic resection, only 20% had received preoperative chemotherapy<sup>[57]</sup>. It is not known how many patients initially diagnosed with unresectable liver metastases are re-evaluated by a liver surgeon after first-line therapy to determine whether the patient's resectability status has changed. Several authors have emphasized the importance of a multidisciplinary approach to the management of colorectal liver metastases and close cooperation among radiologists, medical oncologists, and surgeons with expertise in liver resection<sup>[4,7,58]</sup>.

### Chemotherapy-related hepatotoxicity

Due to recent advances in surgical techniques and postoperative care, the safety of hepatic resection for colorectal liver metastases has improved considerably<sup>[1]</sup>. Despite the trend toward increasing extensiveness of resection, surgical mortality rates remain less than 5%<sup>[8,59]</sup>. In addition, use of portal vein embolism (PVE), a well-established and well-tolerated procedure, has been shown to reduce the risk of postoperative liver failure, with a low rate of complications in resection of colorectal liver metastases<sup>[60,61]</sup>.

However, systemic therapy may be associated with liver injury and affect surgical outcomes. Chemotherapy is known to cause pathologic changes in normal liver tissue, modifying gross appearance and impairing parenchymal hemostasis and regenerative capacity<sup>[2,12,62]</sup>. The impact of these effects on clinical outcomes in patients undergoing hepatic resection is unclear. In the EORTC 40983 trial

**Table 4** Liver injury by chemotherapy regimen<sup>[12]</sup>

Regimen	Sinusoidal dilation <sup>1</sup> (n = 22)			Steatosis > 30% (n = 36)			Steatohepatitis <sup>2</sup> (n = 34)		
	Yes (%)	No (%)	P value <sup>3</sup>	Yes (%)	No (%)	P value <sup>3</sup>	Yes (%)	No (%)	P value <sup>3</sup>
No CT	2	98		9	91		4	96	
FU	0	100	NS	17	83	NS	5	95	NS
IRI	4	96	NS	11	89	NS	20	80	0.001
OX	19	81	0.00001	4	96	NS	6	94	NS
Other	0	100	NS	8	92	NS	0	100	NS

<sup>1</sup>Rubbia: Brandt grade 2 or 3; <sup>2</sup>Kleiner score ≥ 4; <sup>3</sup>Presence of liver injury characteristic; comparison of each chemotherapy group vs no chemotherapy. FU: Fluorouracil; IRI: Irinotecan; OX: Oxaliplatin; NS: Not significant.

(Figure 2), six cycles of preoperative chemotherapy did not increase the rate of surgical mortality compared with surgery alone. Chemotherapy was associated with an increase in reversible postoperative morbidity that was generally within the expected range for hepatic resection (25% vs 16%)<sup>[2]</sup>. Karoui *et al*<sup>[63]</sup> retrospectively compared outcomes following hepatic resection in 45 patients who received preoperative chemotherapy and 22 who received no preoperative chemotherapy. Treatment with chemotherapy was associated with an increase in postoperative morbidity. Interestingly, morbidity correlated with the number of cycles of preoperative therapy given (≥ 6 cycles vs < 6 cycles), but not the type of chemotherapy used.

Others have reported that different chemotherapy regimens cause different types of liver injury, with varying effects on clinical outcomes (Table 4)<sup>[12]</sup>. Treatment with 5-fluorouracil-based treatment has been associated with an increase in steatosis, which is associated with increased postoperative morbidity<sup>[58,64]</sup>. In addition, longer duration of 5-fluorouracil-based therapy (≥ 9 cycles vs 1–8 cycles) was associated with significantly increased incidence of sinusoidal injury (42% vs 26%; P = 0.017) and postoperative liver insufficiency (11% vs 4%; P < 0.035), and was an independent predictor of postoperative liver insufficiency (P = 0.031; odds ratio, 3.90)<sup>[65]</sup>. Irinotecan has been associated with steatohepatitis, which in turn was correlated with increased mortality following hepatic resection, particularly due to liver failure<sup>[12]</sup>. Oxaliplatin therapy does not induce steatosis or steatohepatitis, but increases the risk of developing vascular lesions and sinusoidal obstruction syndrome; a clear link between these effects and postoperative morbidity or mortality rates has not been established<sup>[12,66]</sup>.

Given the varying effects of different chemotherapy agents on liver tissue, both the efficacy and safety of available regimens should be considered when selecting an appropriate regimen for use in an individual patient. It also is advisable limiting the number of cycles of preoperative chemotherapy in order to avoid extensive liver injury, and to perform surgery as soon as possible after achieving resectability.

#### Cetuximab and hepatic outcomes

The most common adverse event associated with cetuximab treatment is skin reactions. Less common but potentially serious adverse events include infusions

reactions, cardiopulmonary arrest, and hypomagnesemia.

There is little data specifically on the effects of cetuximab on the liver in this setting. In Adam *et al*<sup>[34]</sup>, nine patients who became eligible for surgery after cetuximab-based therapy had evidence of liver injury, but this was not attributable to cetuximab. Overall, one patient died postoperatively and approximately 50% of patients had surgical complications.

The addition of cetuximab does not appear to increase the incidence of adverse events typically associated with oxaliplatin-based therapy, such as neutropenia, diarrhea, fatigue, or neurotoxicity<sup>[35,36]</sup>. In the CRYSTAL trial, the addition of cetuximab to FOLFIRI in the first-line setting did not increase all-cause mortality rates, and there were no deaths attributed to cetuximab. Patients who received cetuximab and FOLFIRI had higher incidences of diarrhea and cetuximab-related skin reactions<sup>[37]</sup>.

#### Bevacizumab and hepatic outcomes

Treatment with bevacizumab is associated with increased incidence of hypertension and relatively low rates of certain potentially serious events, such as bleeding, gastrointestinal perforation, and arterial thromboembolism<sup>[20,38,40]</sup>. Recent results have found that bevacizumab reduces the incidence of oxaliplatin-related sinusoidal injury, although the exact mechanism for this effect is unknown<sup>[44,65]</sup>. It has been suggested that the antiangiogenic effects of bevacizumab can interfere with hepatic regeneration and wound healing, which is relevant to its use in the perioperative setting<sup>[13,67]</sup>. To avoid surgical morbidity, it is currently recommended to stop bevacizumab therapy at least 6 wk before surgery and resume treatment 28 d or more after surgery, provided that all incisions have healed completely<sup>[4,13,67]</sup>. Reddy *et al*<sup>[68]</sup> reported that complications are more prevalent in patients who undergo surgery within 8 wk of stopping bevacizumab, compared with those who discontinue bevacizumab more than 8 wk before surgery (62.5% vs 30.4%). In a study of preoperative chemotherapy plus bevacizumab, Gruenberger *et al*<sup>[13]</sup> reported that when bevacizumab was discontinued about 5 wk before surgery, the resulting rates of complications were similar to that achieved in a historical control group of patients who received chemotherapy alone. Whether the need to discontinue bevacizumab for several weeks perioperatively hinders its ability to allow for complete resection is unknown.

**Table 5 Secondary resection in patients with initially unresectable liver metastases**

Improve patient selection through early and continued consultation in a multidisciplinary team approach, including close cooperation among a radiologist, medical oncologist, and surgeon with experience in liver resection  
 Conduct surgical evaluation at baseline and, if disease is initially unresectable, reevaluation at intervals during therapy to determine if conversion to resectability has been achieved  
 Set appropriate goals of therapy (best response, conversion to resectable disease, or palliation)  
 Determine length of therapy, with consideration for the risk of potential toxicities  
 Consider the safety profile of individual agents and the risks of overtreatment, including hepatotoxicity  
 If the treatment goal is conversion to resection, treat to resectability and not to best response

## CONCLUSION

The use of systemic therapy to downstage unresectable liver metastases to achieve resectability offers a curative option with long-term outcomes similar to those achieved with primary resection. Therefore, secondary resection is a valid treatment goal for certain patients with initially unresectable liver metastases and an important end point for future clinical trials (Table 5).

Refinement of available first-line treatment options may increase secondary resection rates. Because response rates correlate with secondary resection rates, aggressive approaches that increase the likelihood of preoperative response seem warranted. The blurring of first-line therapy and neoadjuvant therapy underscores the need for clear definitions of treatment approach, ie, curative versus palliative, and the goals of therapy, e.g. best response, conversion to resectable disease, and palliation. Multidisciplinary involvement in the management of the patient is essential to implement a continued surgical evaluation approach as patients with borderline resectable/unresectable disease undergo systemic treatment.

The benefits of aggressive chemotherapy to downstage liver metastases should be weighed against the potential increase in adverse events, particularly those related to liver injury and surgical outcomes. Different chemotherapy agents have different effects on the liver. To avoid excessive liver injury and postoperative complications, the safety profile of individual agents should be considered when selecting a preoperative regimen; the duration of preoperative chemotherapy should be kept to a minimum; and surgery should be performed as soon as resectability has been achieved.

The addition of targeted therapies to current first-line chemotherapy platforms may improve secondary resection rates. The two leading candidates for this approach are cetuximab and bevacizumab. Data from a randomized trial indicate that cetuximab increases secondary resection rates when added to irinotecan-based therapy. The addition of cetuximab to preoperative chemotherapy does not appear to increase hepatotoxicity or mortality compared with chemotherapy alone. When discontinued at least 6 wk before surgery and reinitiated at least 28 d after surgery, bevacizumab also appears to be a safe treatment option in this setting, and there is some evidence to suggest that bevacizumab may reduce the risk of sinusoidal injury. Current studies evaluating cetuximab, bevacizumab, and both in combination are

underway and will help to further refine their role in the preoperative setting.

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# Overexpression of $\beta 3/\gamma 2$ chains of laminin-5 and MMP7 in biliary cancer

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The invasive front dominant type was associated with histologic type and advanced stage. MMP7 positivity was correlated with LN $\gamma 2$  or LN $\beta 3$  expression but not with clinicopathological characteristics. Active MMP7 detected by casein zymography was correlated with depth of invasion and advanced stage. Downregulation of MMP7 expression by siRNA resulted in a significant decrease in biliary tract cancer cell invasion *in vitro*.

**CONCLUSION:** Our results suggest that LN $\gamma 2$  and LN $\beta 3$ , in conjunction with MMP7, play a key role in the progression of biliary tract cancer.

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**Key words:** Biliary tract cancer; Laminin-5; Laminin  $\gamma 2$ ; Laminin  $\beta 3$ ; MMP7

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## Abstract

**AIM:** To clarify the clinicopathological significance of laminin-5  $\gamma 2$  (LN $\gamma 2$ ) and  $\beta 3$  (LN $\beta 3$ ) chains and MMP7 expression in biliary tract cancer.

**METHODS:** We analyzed the association between immunohistochemically detected LN $\gamma 2$ , LN $\beta 3$ , and MMP7 expression in biliary tract cancer and clinicopathological characteristics. Activity of MMP7 was analyzed by casein zymography. An *in vitro* invasion assay after treatment with MMP7-specific siRNA was performed.

**RESULTS:** LN $\gamma 2$  expression was predominantly observed in carcinoma cells at the invasive front. LN $\gamma 2$  expression was seen in 57% of patients with biliary tract cancer, and was associated with depth of invasion, histologic type, and advanced stage. The expression pattern of LN $\beta 3$  was classified into two types: invasive front dominant type (38%) and diffuse type (28%).

## INTRODUCTION

Despite recent advances in diagnosis and treatment, the prognosis of patients with biliary tract cancer is still poor. Surgical resection is possible in only a small proportion of patients<sup>[1,2]</sup>. Consequently, elucidating the biological characteristics of these carcinomas has become necessary to improve the prognosis of patients and to devise better treatment strategies.

Laminins are components of the extracellular matrix (ECM) that contribute to the architecture of the basal lamina surrounding the epithelial cells and mediate cell adhesion, growth, migration, proliferation, and differentiation. Laminins are heterotrimeric glycoproteins composed of three different polypeptide

chains ( $\alpha$ ,  $\beta$  and  $\gamma$ ) arranged in a cruciform structure. A separate gene encodes each polypeptide chain and different combinations of these chains lead to the 15 different laminin isoforms<sup>[3-5]</sup>. Laminin-5/laminin-332 (LN5), consists of  $\alpha_3$ ,  $\beta_3$ , and  $\gamma_2$  chains, which are encoded by three distinct genes (*LAMA3*, *LAMB3*, and *LAMC2*, respectively)<sup>[6]</sup>. LN5 has been shown to promote the adhesion, migration, and scattering of a variety of cultured cells, mainly through integrin  $\alpha_3\beta_1$ , more strongly than other ECM proteins<sup>[7]</sup>. Moreover, in hepatocellular carcinoma (HCC), LN5 reportedly plays an important role in epithelial-mesenchymal transition through down-regulation of E-cadherin and translocation of  $\beta$ -catenin into the nuclei<sup>[8]</sup>.

Expression of the three subunits of LN5 is regulated differentially in cancer cell lines and in normal and malignant tissues including HCC<sup>[9-11]</sup>. Indeed, LN $\gamma_2$  has been shown to be secreted as a single subunit in gastric cancer<sup>[12]</sup>. Several lines of evidence suggest that the tumor-derived LN $\gamma_2$  contributes to invasion of tumor cells. LN $\gamma_2$  expression has been immunohistochemically detected in various types of carcinomas, such as HCC, colorectum, stomach, and esophagus<sup>[9,12-16]</sup>. It is notable that LN $\gamma_2$  has been predominantly detected at the invasive front, where tumor cells with the most aggressive phenotype are localized<sup>[17]</sup>.

Degradation of ECM components is mostly controlled by proteolytic enzymes called matrix metalloproteinases (MMPs)<sup>[18]</sup>. Specific cleavage of LN5 ( $\gamma_2$  subunit at residue 587) by MMP2 has been shown to induce migration of breast epithelial cells<sup>[19]</sup>. This altered form of LN5 was found in tumors and in tissues undergoing remodeling, but not in quiescent tissues. LN5 is also converted into a migration-promoting substrate by MT1-MMP<sup>[20]</sup>. MMP7, also known as matrilysin, is a “minimal domain MMP” that exhibits broad proteolytic activity against components of the ECM and non-ECM<sup>[18]</sup>. MMP7 is often overexpressed at the invasive front in various types of human cancer and is associated with cancer progression<sup>[21,22]</sup>. Both LN $\gamma_2$  and MMP7 are targets of the Wnt/ $\beta$ -catenin pathway<sup>[23,24]</sup>.

Although there are only a few reports regarding LN $\beta_3$  expression in human cancer, coexpression of LN $\gamma_2$  and LN $\beta_3$  has been reported in HCC, squamous cell carcinoma of the tongue and colorectal carcinoma and basal cell carcinoma of the skin<sup>[9,25,26]</sup>. It has recently been reported that human LN5 is a ligand for MMP7 and that a specific cleavage occurs in its  $\beta_3$  chain<sup>[27]</sup>. These results are interesting because MMP7 is overexpressed in HCC and colorectal carcinoma<sup>[18,28]</sup>. However, expression of LN5 and MMP7 in biliary tract cancer has not been clearly addressed.

To clarify the possible involvement of LN5 and MMP7 in the progression of biliary tract cancer, we immunohistochemically analyzed these expressions in 61 primary biliary tract cancer. Activity of MMP7 was analyzed by casein zymography. An *in vitro* invasion assay of biliary tract cancer cell lines after treatment with MMP7-specific siRNA was performed.

## MATERIALS AND METHODS

### **Cell lines and tissue samples**

Human bile duct cancer cell lines (TFK-1, HuH-28, and MEC) were obtained from Cell Resource Center for Biomedical Research, Tohoku University. Bile duct cancer cell line TKKK and gallbladder carcinoma cell lines TGBC1TKB, TGBC2TKB, and TGBC14TKB were purchased from Riken Cell Bank (Tsukuba, Japan). Cells were cultured in RPMI1640 or DMEM supplemented with 10% fetal bovine serum. Formalin-fixed, paraffin-embedded sections of 61 biliary tract carcinomas (30 extrahepatic bile duct carcinomas, 18 gallbladder carcinomas, 13 carcinomas of the ampulla of Vater) were used for immunohistochemically. Sections containing the most invasive part of each tumor were used. Fresh specimens of extrahepatic bile duct carcinoma ( $n = 10$ ), gallbladder carcinoma ( $n = 7$ ), and carcinoma of the ampulla of Vater ( $n = 3$ ) were obtained from patients who had undergone surgical treatment. Specimens were immediately frozen in liquid nitrogen at the time of surgery and stored at -80°C. Each tissue specimen was used for casein zymography. Histopathological features of the specimens were classified according to the pathological tumor-node-metastasis (TNM) classification system of the International Union Against Cancer. Informed consent was obtained from each subject and the institutional review committee approved the experiments.

### **Semi-quantitative reverse transcriptase-PCR (RT-PCR) and real-time RT-PCR**

Semi-quantitative RT-PCR was carried out as described previously<sup>[29]</sup>. Total RNA was extracted from cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. cDNA was synthesized from 1  $\mu$ g of total RNA using SuperScript III reverse transcriptase (Invitrogen) with random hexamers. PCR was performed using primers specific for the *LAMA3*, *LAMB2* and *LAMC2* gene and the *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene. *GAPDH* served as an internal control of the reaction. Standard curves for semiquantitative RT-PCR were drawn as described previously<sup>[30]</sup>. All reactions were carried out at least in duplicate and controlled without reverse transcriptase. PCR products were electrophoresed in 2% agarose gels. Real-time RT-PCR was performed using the TaqMan real-time PCR system as described previously<sup>[31]</sup>.

### **Immunohistochemistry**

Immunohistochemistry was carried out as described previously<sup>[32]</sup>. The antibodies used were as follows: anti-LN $\alpha_3$  rabbit polyclonal antibody (1/100 dilution, Santa Cruz, CA, USA), anti-laminin5 ( $\gamma_2$  chain) mouse monoclonal antibody (1/50 dilution, Chemicon, Temecula, CA, USA), anti-LN $\beta_3$  rabbit polyclonal antibody (1/100 dilution, Santa Cruz), and anti-MMP7 mouse monoclonal antibody (1/50 dilution, Daiichi Fine Chemical, Takaoka, Japan). Normal mouse or rabbit

immunoglobulins were substituted for each primary antibody as negative controls. Cytoplasmic expression was defined as positive when immunoreactivity was observed in more than 10% of carcinoma cells. We defined the cells at the deepest invading part of the tumor as the invasive front.

### Casein zymography

Casein zymography was performed as previously described with some modifications<sup>[28]</sup>. Tissue extracts were electrophoresed on 8% polyacrylamide gel containing 1 mg/mL casein. After electrophoresis, gels were washed in 2.5% Triton-X 100 and incubated for 48 h at 37°C in 50 mmol/L Tris-HCl (pH 7.4), 10 mmol/L CaCl<sub>2</sub>, 1 mmol/L ZnCl<sub>2</sub>, and 0.02% NaNO<sub>3</sub>, followed by staining with 0.1% Coomassie brilliant blue.

### siRNA transfection

siRNA transfection was performed as described previously<sup>[33]</sup>. Levels of MMP7 inhibition were analyzed by RT-PCR and Western blotting. siRNA-transfected cells were used for the *in vitro* invasion assay.

### In vitro invasion assay

Assays were performed by the modified Boyden Chamber method as described previously<sup>[34]</sup>. Assays were also performed with 250 ng/mL of TIMP1, an MMP inhibitor. The results were presented as means  $\pm$  SD for each sample.

### Statistical analysis

Expression was assessed for associations with clinicopathological parameters using the chi-square two-tailed test or Fisher's exact test. A *P* value  $< 0.05$  was considered statistically significant. A *P* value between 0.05 and 0.10 was considered as a trend toward an association.

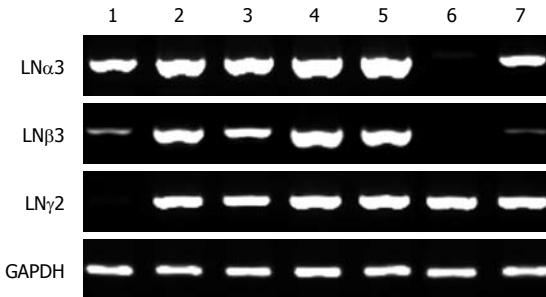
## RESULTS

### Expression of LN $\alpha$ 3, LN $\beta$ 3 and LN $\gamma$ 2, and MMP7 in cell lines

Expression levels of LN $\alpha$ 3, LN $\beta$ 3 and LN $\gamma$ 2, and MMP7 in cancer cell lines were analyzed using semi-quantitative RT-PCR (Figure 1). LN $\alpha$ 3 mRNA was detected in all seven cell lines but at very low levels in TGBC-14TKB. LN $\beta$ 3 mRNA was detected in six cell lines but at very low levels in MEC and TKKK. LN $\gamma$ 2 mRNA was detected in all seven cell lines but at very low levels in MEC. TFK-1, HuH-28, TGBC-1TKB, and TGBC-2TKB expressed considerable amounts of all 3 chains of LN5. MMP7 mRNA was detected in all seven cell lines. There were no significant correlations between these expression patterns and characteristics of the cell lines. Similar data were observed by real-time RT-PCR (data not shown).

### Overexpression of LN $\gamma$ 2, LN $\beta$ 3, and MMP7 in biliary tract cancer tissues

Expression of LN $\alpha$ 3 was detected in normal basement

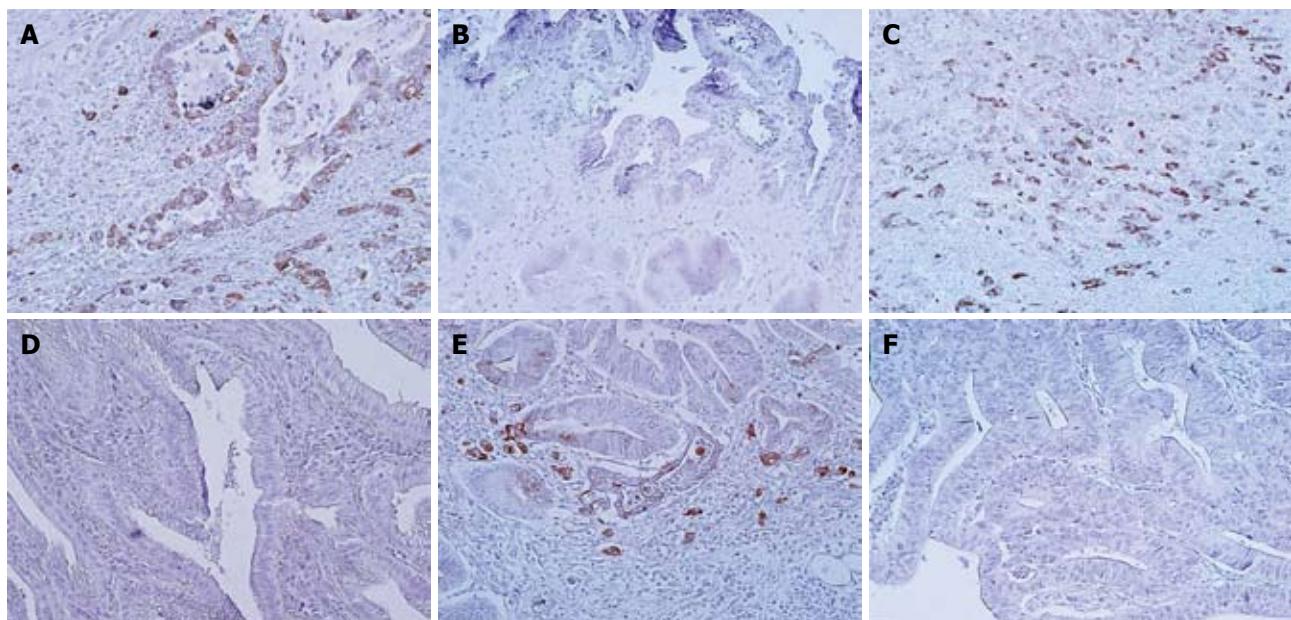


**Figure 1** RT-PCR analysis of the LN $\alpha$ 3, LN $\beta$ 3, LN $\gamma$ 2 genes in biliary tract cancer cell lines. 1: MEC; 2: TFK-1; 3: HuH28; 4: TGBC1TKB; 5: TGBC2TKB; 6: TGBC14TKB; 7: TKKK.

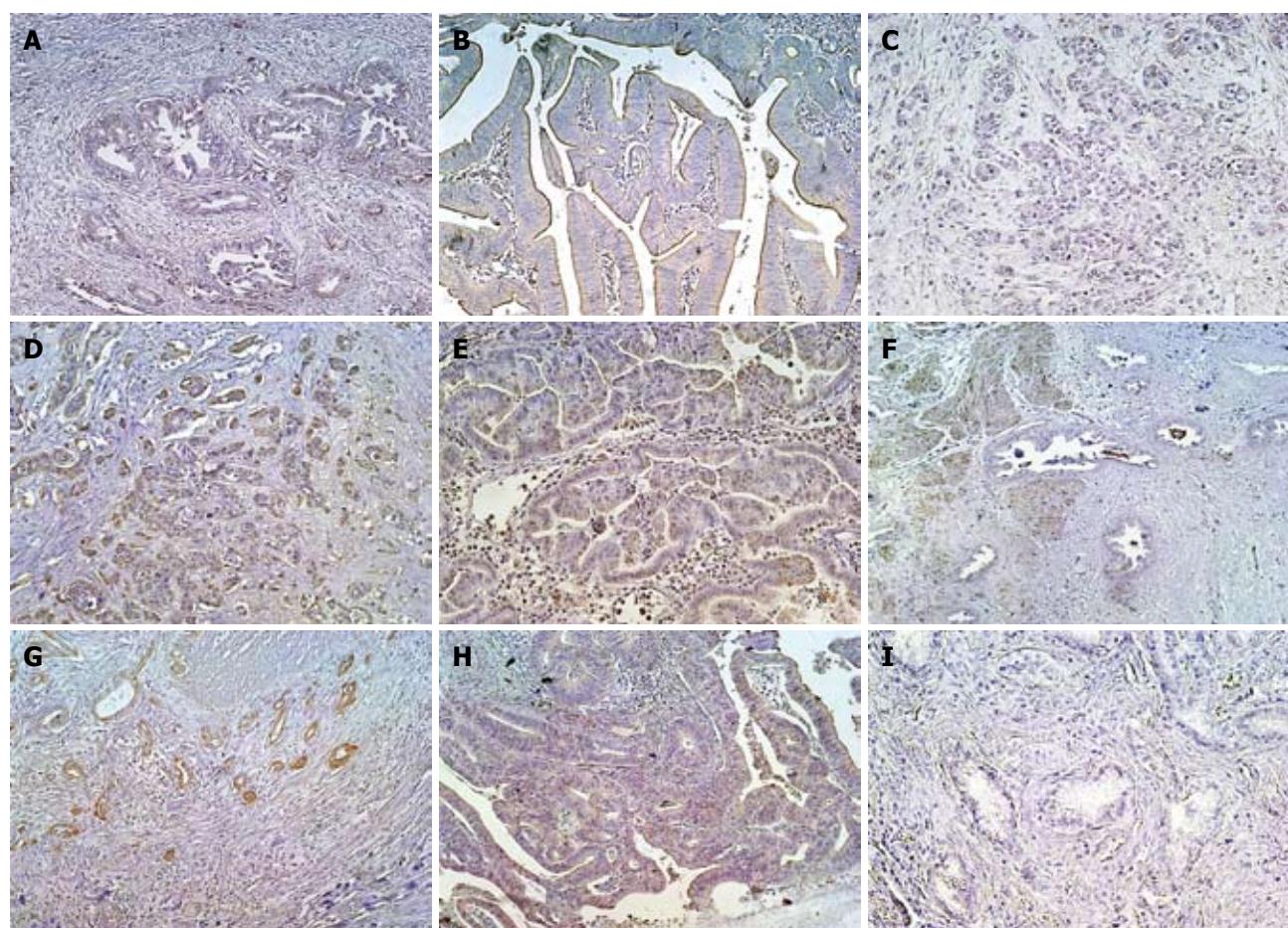
membranes but not in carcinoma cells (data not shown). Figure 2 shows representative results of immunohistochemistry for LN $\gamma$ 2 in biliary tract cancer. In carcinoma tissues, the cytoplasm of carcinoma cells was stained for LN $\gamma$ 2 at levels much stronger than those in normal basement membranes. The cytoplasmic immunoreactivity was often more intense at the invasive front. Cancer cells budding or dissociating from the tumor nests showed intense cytoplasmic staining. Sections with immunostaining signals in over 10% of carcinoma cells, which were observed in 35 (57%) of 61 cases, were judged to be positive for LN $\gamma$ 2. LN $\gamma$ 2 positivity was 67% in extrahepatic bile duct cancer, 50% in gallbladder cancer, and 46% in carcinoma of the ampulla of Vater. Figure 3 shows representative results of immunohistochemistry for LN $\beta$ 3 in biliary tract cancer. In carcinoma tissues, the cytoplasm of carcinoma cells was stained for LN $\beta$ 3 at levels much stronger than those in normal basement membranes. The expression pattern of LN $\beta$ 3 was classified into 2 patterns: invasive front dominant pattern and diffuse pattern. The invasive front dominant pattern and diffuse pattern were observed in 23 (38%) and 17 (28%) of 61 cases, respectively. Positivity for invasive front dominant pattern and diffuse pattern was 47% and 17% in extrahepatic bile duct cancer, 28% and 39% in gallbladder cancer, and 31% and 38% in carcinoma of the ampulla of Vater. Figure 4 shows representative results of immunohistochemistry for MMP7 in biliary tract cancer tissues. MMP7 immunoreactivity was intense at the invasive front in several cases. In general, MMP7 immunoreactivity was diffuse rather than invasive front dominant like LN $\gamma$ 2. Sections with immunostaining signals in over 10% of carcinoma cells, which were observed in 42 (69%) of 61 cases, were judged to be positive for MMP7. MMP7 positivity was 80% in extrahepatic bile duct cancer, 50% in gallbladder cancer, and 69% in carcinoma of the ampulla of Vater.

### Association of LN $\gamma$ 2, LN $\beta$ 3, and MMP7 expression with clinicopathological characteristics

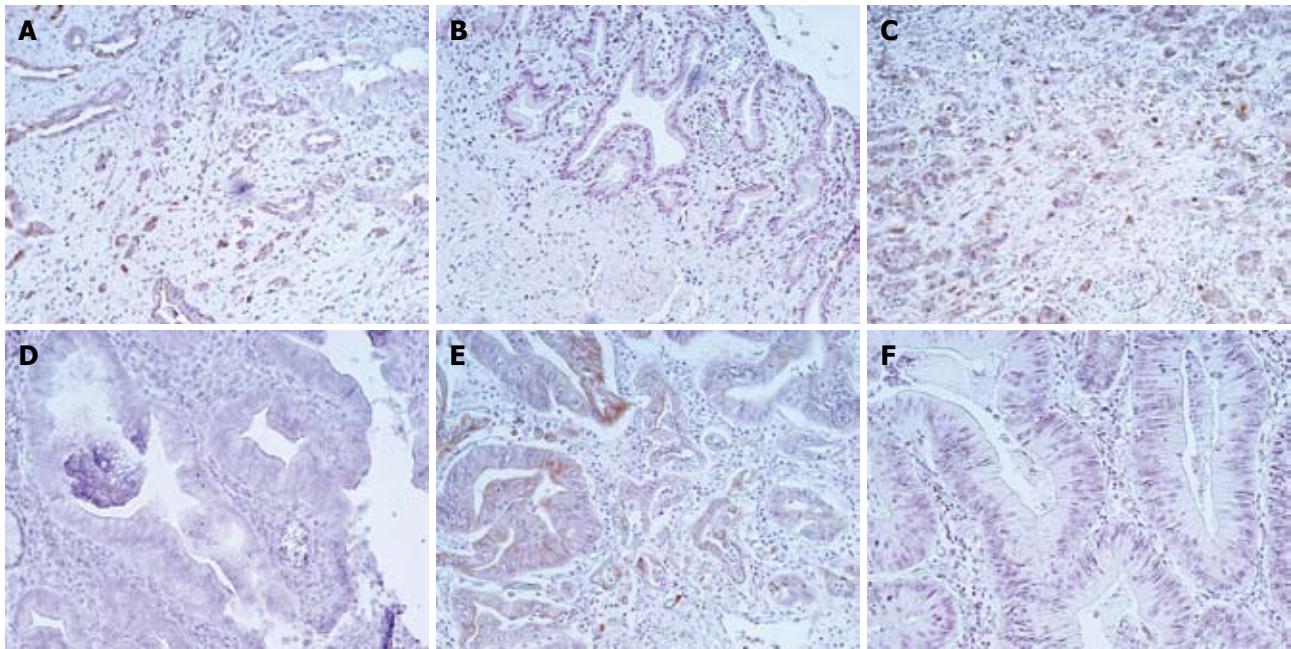
The relationship between LN $\gamma$ 2 positivity and clinicopathological characteristics is summarized in Table 1. LN $\gamma$ 2 positivity was significantly correlated with histologic type (less differentiated type), depth of invasion



**Figure 2 LN $\gamma$ 2 expression in biliary tract cancer tissues.** A, B: Extrahepatic bile duct cancer tissues; A: Moderately differentiated tubular adenocarcinoma positive for staining. Note that LN $\gamma$ 2 is strongly positive in tumor cells at the invasive front; B: Papillary adenocarcinoma negative for staining; C, D: Gallbladder cancer tissues; C: Moderately differentiated tubular adenocarcinoma positive for staining; D: Well differentiated tubular adenocarcinoma negative for staining; E, F: Carcinoma tissues of the ampulla of Vater; E: Moderately differentiated tubular adenocarcinoma positive for staining. Note that LN $\gamma$ 2 is strongly positive in tumor cells at the invasive front; F: Well differentiated tubular adenocarcinoma negative for staining.



**Figure 3 LN $\beta$ 3 expression in biliary tract cancer tissues.** A-C: Extrahepatic bile duct cancer tissues; A: Well differentiated tubular adenocarcinoma positive for invasive front dominant staining; B: Papillary adenocarcinoma positive for diffuse staining; C: Moderately differentiated tubular adenocarcinoma negative for staining; D-F: Gallbladder cancer tissues; D: Moderately differentiated tubular adenocarcinoma positive for invasive front dominant staining; E: Papillary adenocarcinoma positive for diffuse staining; G-I: Carcinoma tissues of the ampulla of Vater; G: Well differentiated tubular adenocarcinoma positive for invasive front dominant staining; H: Well differentiated tubular adenocarcinoma positive for diffuse staining; I: Well differentiated tubular adenocarcinoma negative for staining.



**Figure 4 MMP7 expression in biliary tract cancer tissues.** A, B: Extrahepatic bile duct cancer tissues; A: Moderately differentiated tubular adenocarcinoma positive for staining. Note that MMP7 is strongly positive in tumor cells at the invasive front; B: Papillary adenocarcinoma negative for staining; C, D: Gallbladder cancer tissues; C: Moderately differentiated tubular adenocarcinoma positive for staining; D: Well differentiated tubular adenocarcinoma negative for staining; E, F: Carcinoma tissues of the ampulla of Vater; E: Moderately differentiated tubular adenocarcinoma positive for staining. Note that MMP7 is strongly positive in tumor cells at the invasive front; F: Well differentiated tubular adenocarcinoma negative for staining.

(invasion into serosa), and advanced stage. The relationship between LN $\beta$ 3 positivity and clinicopathological characteristics is summarized in Table 2. LN $\beta$ 3 invasive front dominant pattern was significantly correlated with histologic type (less differentiated type) and advanced stage. There was a tendency that lymph node metastasis was more frequently observed in cases with LN $\beta$ 3 invasive front dominant pattern than in other cases ( $P = 0.063$ ). The relationship between MMP7 positivity and clinicopathological characteristics is summarized in Table 3. MMP7 positivity was not significantly correlated with clinicopathological characteristics. The expression of LN $\gamma$ 2, LN $\beta$ 3 invasive front dominant pattern, and MMP7 were correlated with each other (Tables 4–6).

#### MMP7 activity detected by zymography

Using casein zymography, the levels of secreted matrilysin were analyzed (Figure 5). Nontumorous tissues secreted neither latent (28 kDa) nor activated (19 kDa) MMP7 activity. Latent and activated forms of MMP7 were detected in 16 (80%) and 12 (60%) of 20 carcinoma tissues, respectively. The activity was eliminated by the addition of the metalloproteinase inhibitor EDTA (data not shown). The activated form but not the latent form was correlated with depth of invasion and advanced stage.

#### Suppression of cancer cell invasiveness by MMP7

##### siRNA treatment

*In vitro* invasion assays after treatment with specific siRNA for the MMP7 gene were carried out to assess the direct role of the expression of MMP7 in cancer

**Table 1 Correlation between laminin  $\gamma$ 2 staining and clinicopathologic factors n (%)**

	Case (n = 61)	Laminin $\gamma$ 2		<i>P</i> value
		Positive (n = 35)	Negative (n = 26)	
Age (yr)				
≤ 65	25 (41)	15 (60)	10 (40)	
> 65	36 (59)	20 (56)	16 (44)	0.47
Gender				
Male	41 (67)	25 (61)	16 (39)	
Female	20 (33)	10 (50)	10 (50)	0.29
Location				
Bile duct	30 (49)	20 (67)	10 (33)	
Gall bladder	18 (30)	9 (50)	9 (50)	
Ampulla of Vater	13 (21)	6 (46)	7 (54)	0.34
Maximum diameter (mm)				
< 30	41 (67)	25 (61)	16 (39)	
≥ 30	20 (33)	10 (50)	10 (50)	0.29
Histologic type				
Tub1 & pap	35 (57)	16 (46)	19 (54)	
Others	26 (43)	19 (73)	7 (27)	0.03
Depth				
Serosa negative	37 (61)	17 (46)	20 (54)	
Serosa positive	24 (39)	18 (75)	6 (25)	0.02
Lymph node metastasis				
Absent	29 (48)	16 (55)	13 (45)	
Present	27 (44)	17 (63)	10 (37)	0.37
Stage				
< IV	43 (70)	21 (49)	22 (51)	
≥ IV	17 (28)	13 (76)	4 (24)	0.046

cell invasiveness. Transfection efficiency determined by fluorescein isothiocyanate-labeled oligonucleotide uptake was  $84\% \pm 6\%$  in TFK-1 cells and  $86\% \pm 5\%$  in TGBC-

**Table 2 Correlation between parameters and the pattern of laminin  $\beta 3$  expression  $n$  (%)**

	Total ( $n = 61$ )	Laminin $\beta 3$			$P$ value
		Invasive ( $n = 23$ )	Diffuse ( $n = 17$ )	Negative ( $n = 21$ )	
Age (yr)					
$\leq 65$	25 (41)	9 (36)	8 (32)	8 (32)	
$> 65$	36 (59)	14 (39)	9 (25)	13 (36)	0.83
Gender					
Male	41 (67)	17 (41)	8 (20)	16 (39)	
Female	20 (33)	6 (30)	9 (45)	5 (25)	0.11
Location					
Bile duct	30 (49)	14 (47)	5 (17)	11 (37)	
Gall bladder	18 (30)	5 (28)	7 (39)	6 (33)	
Ampulla of Vater	13 (21)	4 (31)	5 (38)	4 (31)	0.40
Maximum diameter (mm)					
$< 30$	40 (66)	18 (45)	10 (25)	12 (30)	
$\geq 30$	21 (34)	5 (24)	7 (33)	9 (43)	0.27
Histologic type					
Tub1 & pap	35 (57)	9 (26)	14 (40)	12 (34)	
Others	26 (43)	14 (54)	3 (12)	9 (35)	0.02
Depth					
Serosa negative	37 (61)	11 (30)	13 (35)	13 (35)	
Serosa positive	24 (39)	12 (50)	4 (17)	8 (33)	0.18
Lymph node metastasis					
Absent	29 (48)	8 (28)	11 (38)	10 (34)	
Present	27 (44)	14 (52)	5 (19)	8 (30)	0.13
Stage					
$< IV$	43 (70)	10 (23)	15 (35)	18 (42)	
$\geq IV$	17 (28)	12 (71)	2 (12)	3 (18)	0.003

**Table 3 Correlation between MMP7 staining and clinicopathologic factors  $n$  (%)**

	Case ( $n = 61$ )	MMP7		$P$ value
		Positive ( $n = 42$ )	Negative ( $n = 19$ )	
Age (yr)				
$\leq 65$	25 (42)	17 (68)	8 (32)	
$> 65$	36 (58)	25 (69)	11 (31)	0.66
Gender				
Male	41 (67)	30 (73)	11 (27)	
Female	20 (33)	12 (60)	8 (40)	0.23
Location				
Bile duct	30 (49)	24 (80)	6 (20)	
Gall bladder	18 (30)	9 (50)	9 (50)	
Ampulla of Vater	13 (21)	9 (69)	4 (31)	0.09
Maximum diameter (mm)				
$< 30$	41 (67)	29 (71)	12 (29)	
$\geq 30$	20 (33)	13 (65)	7 (35)	0.43
Histologic type				
Tub1 & pap	35 (57)	23 (66)	12 (34)	
Others	26 (43)	19 (73)	7 (27)	0.37
Depth				
Serosa negative	37 (61)	25 (68)	12 (32)	
Serosa positive	24 (39)	17 (71)	7 (29)	0.58
Lymph node metastasis				
Absent	29 (48)	21 (72)	8 (28)	
Present	27 (44)	18 (67)	9 (33)	0.43
Stage				
$< IV$	43 (70)	31 (72)	12 (28)	
$\geq IV$	17 (28)	10 (59)	7 (41)	0.24

2TKB cells (data not shown). Transfection with siRNA resulted in over 80% inhibition of mRNA and protein expression (data not shown). Transfection with *MMP7*-

**Table 4 Expression of laminin  $\gamma 2$  and MMP7 in biliary tract carcinoma  $n$  (%)**

MMP7 expression	Laminin $\gamma 2$		$P$ value
	Positive ( $n = 42$ )	Negative ( $n = 19$ )	
Positive ( $n = 34$ )	28 (46)	6 (10)	
Negative ( $n = 27$ )	14 (23)	13 (21)	0.01

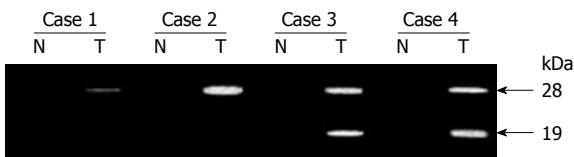
**Table 5 Expression of laminin  $\beta 3$  and MMP7 in biliary tract carcinoma  $n$  (%)**

MMP7 expression	Laminin $\beta 3$			$P$ value
	Invasive ( $n = 23$ )	Diffuse ( $n = 17$ )	Negative ( $n = 21$ )	
Positive ( $n = 34$ )	17 (28)	6 (10)	14 (23)	
Negative ( $n = 27$ )	6 (10)	11 (18)	7 (11)	0.037

**Table 6 Expression of laminin  $\beta 3$  and laminin  $\gamma 2$  in biliary tract carcinoma  $n$  (%)**

Laminin $\gamma 2$ expression	Laminin $\beta 3$			$P$ value
	Invasive ( $n = 23$ )	Diffuse ( $n = 17$ )	Negative ( $n = 21$ )	
Positive ( $n = 42$ )	18 (30)	8 (13)	9 (15)	
Negative ( $n = 19$ )	5 (8)	9 (15)	12 (20)	0.036

specific siRNA decreased invasiveness of TFK-1 cells compared with control siRNA-transfected counterparts ( $P < 0.01$ , Figure 6). This difference was significantly diminished by the addition of TIMP1. Similar results were observed in *MMP7*-specific siRNA-transfected TGBC-2TKB cells (data not shown).



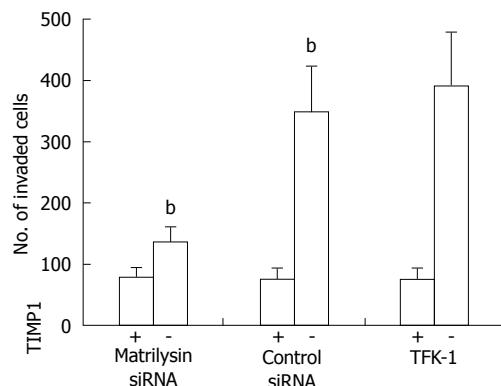
**Figure 5** Casein zymography of surgical specimen pairs of biliary tract carcinoma and adjacent nontumor tissue. N and T: Matched samples from nontumor and tumor tissue, respectively.

## DISCUSSION

In the current study, LN $\gamma$ 2 positivity in carcinoma cells at the invasive front was immunohistochemically observed in 57% of patients with biliary tract cancer, and was associated with histologic type (less differentiated type), depth of invasion, and advanced stage. LN $\beta$ 3 invasive front dominant pattern was immunohistochemically observed in 38% of patients with biliary tract cancer, and was associated with histologic type (less differentiated type) and advanced stage. These results suggest that LN $\gamma$ 2 and LN $\beta$ 3 expression in carcinoma cells at the invasive front contributes to the more aggressive phenotype of carcinoma cells, resulting in the progression of biliary tract cancer.

Preferential expression of LN $\gamma$ 2 and LN $\beta$ 3 in carcinoma cells at the invasive front and its correlation with tumor progression suggest that these molecules play a role in the acquisition of a migrating and invading epithelial cell phenotype that is a prerequisite for malignancy<sup>[13]</sup>. Also in metastatic HCC, LN5 was mainly distributed along the tumoral advancing edge<sup>[9]</sup>. The mechanism underlying the preferential distribution of LN $\gamma$ 2 and LN $\beta$ 3 at the invasive front in cancer is not known. It is known that activation of cancer-related genes in carcinoma cells affects their associated stromal cells. Certain stromal cell populations lying close to carcinoma cells may be induced to assist the invasion process by signals sent out by the cancer cells, stimulating the synthesis of gene products that facilitate cancer cell invasion and migration<sup>[35]</sup>. Interactions of carcinoma cells with stromal cells or with the surrounding extracellular matrix at the invasive front may result in an accumulation of LN $\gamma$ 2 and LN $\beta$ 3 at the invasive front, where they may play a direct role in tumor invasion processes<sup>[19]</sup>.

Although there are only a few reports regarding LN $\beta$ 3 expression in human cancer, coexpression of LN $\gamma$ 2 and LN $\beta$ 3 has been reported in HCC, squamous cell carcinoma of the tongue, colorectal carcinoma and basal cell carcinoma of the skin<sup>[9,25,26]</sup>. Sordat *et al*<sup>[14]</sup> reported that the heterodimer of the LN $\gamma$ 2 and LN $\beta$ 3 chains is accumulated in the cytoplasm of dissociating (or budding) tumor cells from neoplastic tubules of colon carcinomas. Since LN $\gamma$ 2 and LN $\beta$ 3 were not always coexpressed in biliary tract cancer, further analysis is necessary to elucidate the mechanism of overexpression and localization of LN $\gamma$ 2 and LN $\beta$ 3 in biliary tract cancer. LN5 reportedly plays an important role in epithelial-mesenchymal transition through downregulation of E-cadherin and translocation of  $\beta$ -catenin



**Figure 6** *In vitro* invasion assay with or without TIMP1 (250 ng/mL) in TFK-1 and siRNA transflectants. Each column indicates the means of three experiments; <sup>b</sup>*P* < 0.01.

into the nuclei<sup>[8]</sup>. It will be interesting to address this issue in biliary tract cancer in the near future.

In contrast to preferential expression of MMP7 in carcinoma cells at the invasive front in various carcinomas, the expression pattern of MMP7 was diffuse in biliary tract cancer. The mechanism underlying the differential distribution of MMP7 in carcinomas needs to be further analyzed. MMP7 positivity was not significantly correlated with clinicopathological characteristics. However, the expression of LN $\gamma$ 2, LN $\beta$ 3 invasive front dominant pattern, and MMP7 were correlated with each other. It has been suggested that the controlled up-regulation of gene products is one of the characteristics of invading cancer cells and that these gene products have functions crucial for the invasive phenotype of cancer cells<sup>[13]</sup>. It is notable that limited proteolysis of LN $\beta$ 3 by MMP7 increases the cell motility activity of LN5 in colon carcinoma cells<sup>[27]</sup>.

The activated form but not the latent form of MMP7 was correlated with depth of invasion and advanced stage, suggesting that active MMP7 plays an important role in the progression of biliary tract cancer. The implication of up-regulation of MMP7 expression in tumor progression was further substantiated by the *in vitro* invasion analysis. We revealed that down-regulation of MMP7 expression by siRNA resulted in a significant decrease in biliary tract cancer cell invasion *in vitro*, suggesting that up-regulation of MMP7 contributes to the more invasive phenotype of biliary tract cancer cells. Taken together, our results suggest that LN $\gamma$ 2 and LN $\beta$ 3, in conjunction with MMP7, play a key role in the progression of biliary tract cancer.

## COMMENTS

### Background

Biliary tract cancers are relatively rare human malignancies involving the gallbladder and/or the bile ducts, but the prognosis is poor. Understanding the molecular biological features of biliary tract cancer progression is necessary for improving the prognosis. The potential role of laminin-5 (LN5) and MMP7 in human cancer is receiving increasing attention.

### Research frontiers

Altered expression patterns of LN5, especially the LN $\gamma$ 2 chain, and MMP7 have been correlated with tumor behavior, such as invasiveness, vascularization, metastatic potential, and patients' poor prognosis. However, expression of LN5

and MMP7 in biliary tract cancer has not been clearly addressed. In this study, the authors demonstrate that LN $\gamma$ 2, LN $\beta$ 3, and active MMP7 play a key role in the progression of biliary tract cancer.

### Innovations and breakthroughs

This is the first study to report that invasive front dominant expression of LN $\gamma$ 2 and LN $\beta$ 3, and active MMP7 play a key role in the progression of biliary tract cancer. Furthermore, our *in vitro* studies suggest that MMP7 plays an important role in biliary tract cancer cell invasion.

### Applications

Detection of LN $\gamma$ 2, LN $\beta$ 3, and active MMP7 could be molecular markers for tumor aggressiveness in biliary tract cancer. Understanding how LN $\gamma$ 2, LN $\beta$ 3, and active MMP7 are induced and how their expression is blocked may represent a future strategy for therapeutic intervention in the treatment of patients with biliary tract cancer.

### Terminology

**Laminin:** A heterotrimeric glycoprotein composed of three different polypeptide chains ( $\alpha$ ,  $\beta$  and  $\gamma$ ), is a component of the extracellular matrix (ECM) that contributes to the architecture of the basal lamina surrounding the epithelial cells and mediates cell adhesion, growth, migration, proliferation, and differentiation. **LN-5/LN-332:** LN5, consists of  $\alpha$ 3,  $\beta$ 3 and  $\gamma$ 2 chains, and is involved in cell adhesion, migration, and scattering. Altered expression of LN-5 plays an important role in cancer. **MMP7:** Degradation of ECM components is mostly controlled by proteolytic enzymes called MMP. MMP7, also known as matrylsin, is a minimal domain MMP that exhibits broad proteolytic activity against components of the ECM and non-ECM.

### Peer review

This paper reports the expression of LN-5 chains and MMP-7 in biliary cancer. The authors showed that LN $\gamma$ 2 and LN $\beta$ 3, in conjunction with MMP7, play a key role in the progression of biliary cancer. The study sounds interesting and confirms the role of LN-5 and MMP7 in human cancer.

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ORIGINAL ARTICLES

# Peroxisome proliferator-activated receptor- $\gamma$ is essential in the pathogenesis of gastric carcinoma

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p27 expression was enhanced following 15d-PGJ<sub>2</sub> treatment in a dose-dependent manner in MGC803 cells. Interestingly, we also found that small interfering RNA for PPAR- $\gamma$  inhibited growth and induced apoptosis in MGC803 cells. The inhibition of PPAR- $\gamma$  function may be a potentially important and novel modality for treatment and prevention of gastric carcinoma.

**CONCLUSION:** A PPAR- $\gamma$  agonist inhibited growth of human gastric carcinoma MGC803 cells by inducing apoptosis and G<sub>1</sub>/G<sub>0</sub> cell cycle arrest with the involvement of survivin, Skp2 and p27 and not via PPAR- $\gamma$ .

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**Key words:** Peroxisome proliferator-activated receptor- $\gamma$ ; Gastric cancer; Apoptosis; Cell cycle

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## Abstract

**AIM:** To investigate whether peroxisome proliferator-activated receptor  $\gamma$  (PPAR- $\gamma$ ) is expressed in human gastric carcinoma and whether PPAR- $\gamma$  is a potential target for gastric carcinoma therapy.

**METHODS:** PPAR- $\gamma$  protein in gastric carcinoma was examined by immunohistochemistry. In the gastric carcinoma cell line MGC803, PPAR- $\gamma$ , survivin, Skp2 and p27 protein and mRNA were examined by Western blotting and real-time reverse transcription-polymerase chain reaction, respectively; proliferation was examined by MTT; apoptosis was examined by chromatin staining with Hoechst 33342 and fluorescence activated cell sorting (FACS); and cell cycle was examined by FACS; the knockdown of PPAR- $\gamma$  was done by RNA interference.

**RESULTS:** A high level of expression of PPAR- $\gamma$  was observed in human gastric carcinoma and in a human gastric carcinoma cell line MGC803. The PPAR- $\gamma$  agonist 15-deoxy- $\Delta$ 12,14-prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>) inhibited growth, and induced apoptosis and G<sub>1</sub>/G<sub>0</sub> cell cycle arrest in MGC803 cells in a concentration-dependent and time-dependent manner. The effect of 15d-PGJ<sub>2</sub> on MGC803 cells was not reversed by the selective and irreversible antagonist GW9662 for PPAR- $\gamma$ . Furthermore, survivin and Skp2 expression were decreased, whereas

## INTRODUCTION

Gastric carcinoma is the second most common cancer in the world and kills more than 600 000 people annually<sup>[1]</sup>. At present, the management of gastric carcinoma mainly includes surgery and chemotherapy, but the curative effect of the existing chemotherapeutic drugs is not effective and they have numerous side effects. Many studies have been performed to search for therapeutic targets and drugs capable of preventing and treating gastric carcinoma and other malignancies.

Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors, initially described as molecular targets for compounds, which induce peroxisomal proliferation<sup>[2]</sup>. Up to now, 3 different isotypes of PPARs have been identified in various species, being the products of distinct genes and generally designated as PPAR- $\alpha$ , PPAR- $\beta/\delta$ , and PPAR- $\gamma$ <sup>[2,3]</sup>. PPAR- $\gamma$  has been extensively studied. Similarly to other

members of the nuclear receptor gene family, PPAR- $\gamma$  is an agonist-activated transcription factor<sup>[3,4]</sup>. PPAR- $\gamma$  heterodimerizes with retinoid X receptor to bind to the PPAR response element, leading to the transcription of downstream genes<sup>[5]</sup>. Several specific agonists have been found, such as the thiazolidinediones (including pioglitazone, rosiglitazone, and troglitazone), 15-deoxy- $\Delta$ 12,14-prostaglandin-J<sub>2</sub> (15d-PGJ<sub>2</sub>), and certain polyunsaturated fatty acids. GW9662 has been shown to be a selective and irreversible antagonist of PPAR- $\gamma$ , and irreversibly binds within the agonist binding domain through covalent modification of a cysteine residue<sup>[6]</sup>.

PPAR- $\gamma$  is predominately expressed in adipose tissue and plays a central role in adipocyte differentiation and insulin sensitivity<sup>[7]</sup>. Recent studies have shown that, in addition to its classic role, PPAR- $\gamma$  is implicated as a putative therapeutic target for cancer in a variety of tumors as several observations suggest that stimulation of PPAR- $\gamma$  function may inhibit carcinogenesis and tumor cell growth<sup>[8,9]</sup>. However, the exact role of PPAR- $\gamma$  on carcinogenesis and tumor cell growth is still unclear<sup>[10-14]</sup>. Recent investigations by Morita *et al*<sup>[15]</sup> and Konturek *et al*<sup>[16]</sup> have shown that PPAR- $\gamma$  is implicated in *Helicobacter pylori*-related gastric carcinogenesis, and that PPAR- $\gamma$  agonists may have potential in a cancer therapeutic role.

We here demonstrate that PPAR- $\gamma$  may be involved in gastric carcinogenesis, and that the inhibition of PPAR- $\gamma$  may be of benefit in the treatment of gastric carcinoma.

## MATERIALS AND METHODS

### Chemicals

PPAR- $\gamma$  agonist, 15d-PGJ<sub>2</sub> and antagonist GW9662 were purchased from Cayman chemical (Ann Arbor, M1). Other drugs were reagent grade.

### Tissue samples

One hundred and thirty eight samples of surgically-resected primary gastric carcinoma tissues, 138 samples of paired adjacent mucosa (2-5 cm from the margin of the gastric carcinoma) and 138 samples of paired normal mucosa (histologically proven) at the surgical margin (at least 5 cm from the margin of the gastric carcinoma) tissues were obtained from the First Affiliated Hospital of Inner Mongolia Medical College in China from October 2004 to January 2005. All gastric carcinoma patients underwent total or subtotal gastrectomy, and no patient received any treatment for cancer before surgery. The patient series included 111 males and 27 females with a mean age of 58.5 years and a median age of 60 years (range, 36-78). After surgery, the gastric specimens were fixed in 10% neutral buffered formalin and embedded in paraffin for immunohistochemistry. The clinical stage of all gastric carcinoma specimens were determined according to new TNM criteria published by the Union International Centre Cancer in 1997. All pathological diagnoses were performed by 2 independent pathologists.

### Immunohistochemical staining of PPAR- $\gamma$

Sections of paraffin-embedded tissues (4  $\mu$ m) were

mounted on glass slides. The intracellular expression of PPAR- $\gamma$  was detected using a PPAR- $\gamma$  specific mAb (sc-7372, E-8, Santa Cruz Biotechnology, CA) with standard non-biotin horseradish peroxidase (HRP) 2-step immunostaining (Zymed) according to the manufacturer's instructions. The immunoreactive products were visualized using 3,3'-diaminobenzidine (DAB)/H<sub>2</sub>O<sub>2</sub>.

### Cell culture and treatment with PPAR- $\gamma$ agonists and antagonists

We used the human gastric carcinoma cell line MGC803 from Beijing Tumor Institute. It was maintained in RPMI1640 containing 10% fetal bovine serum (FBS) at 37°C under 5% CO<sub>2</sub>. A PPAR- $\gamma$  agonist or antagonist were added at the time of replating.

### Cell survival assay

Gastric carcinoma cells were treated with a PPAR- $\gamma$  agonist or antagonist dissolved in DMSO for 8, 18 and 24 h in culture medium in 96-well plates. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma] solution was then added to the well with gentle pipetting. After 4 h, the supernatant was removed after centrifugation at 1000 r/min for 5 min at room temperature, and 180  $\mu$ L DMSO was added to each well to solubilize the crystal products by shaking for 10 min. Absorbance at 570 nm was measured with an ELISA reader. The negative control had medium without serum and cells, and was used as the zero point of absorbance. Cell vitality of the control group was 100%. The inhibitory rate (IR) of cells was calculated according to the equation as follows: IR (%) = [1 - ( $A_{570}$  nm absorbance in 15d-PGJ<sub>2</sub> treated group/ $A_{570}$  nm absorbance in control group)]  $\times$  100%. Each assay was performed in triplicate.

### Apoptosis analysis

Apoptosis was analyzed by 2 different methods. First, chromatin staining with Hoechst 33342 was carried out. Briefly, MGC803 cells treated with 15d-PGJ<sub>2</sub> were cultured on Laboratory-Tek Chamber Slides for 24 h. The cells were then fixed with methanol for 10 min and rinsed. Chromatin staining was done with Hoechst 33342 (Sigma) to detect nuclear condensation. Secondly, cell apoptosis was measured by fluorescence activated cell sorting (FACS) using the Annexin-V FITC Apoptosis Detection Kit according to the manufacturer's protocol. The apoptotic rate was calculated as the percentage of annexin V-positive and propidium iodide (PI)-negative cells divided by the total number of cells in the gated region. The data was pooled from 3 independent experiments.

### Cell cycle examination

The effect of 15d-PGJ<sub>2</sub> on the cell cycle was examined with PI staining by FACS using Epics-XL (Beckman Coulter).

### Western blotting analysis

Cell total protein was extracted from MGC803 cells using

CytoBuster™ Protein Extraction Reagent (Novagen). The concentration of the protein was determined with Coomassie brilliant blue G-250. Cell total protein (50 µg) was separated by 12% SDS-PAGE. After electrophoresis, the proteins were transferred to a polyvinylidene fluoride membrane, blocked overnight in Tris buffered saline with 10% skim milk at 4°C. PPAR- $\gamma$  (Santa Cruz), Skp2 (Zymed), p27 (Santa Cruz) and  $\beta$ -actin (Santa Cruz) were detected using primary mouse mAb, and survivin (Santa Cruz) was detected using primary rabbit polyclonal Ab at a dilution of 1:2000, followed by HRP-conjugated anti-mouse IgG or anti-rabbit IgG at a dilution of 1:1500. Immunocomplexes were visualized using DAB.  $\beta$ -actin was used as the internal control.

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

Total RNA was extracted from MGC803 cells using TRIzol reagent (Gibco, USA). The resultant RNA samples were quantified using a spectrophotometer at a wavelength of 260 nm. The integrity of the isolated RNA samples was analyzed electrophoretically on agarose gel, followed by staining in ethidium bromide. An aliquot of 2 µg of total RNA from each sample was reversely transcribed and amplified using RT-PCR Enzyme Mix Kit (Sino-American Biotechnology Co.). For detection of human PPAR- $\gamma$  mRNA, a combination of a sense primer (5'-TCTCTCCGTAATGGAAGACC-3') and an antisense primer (5'-GCATTATGAGACATCCCCAC-3') were used. The reverse transcription was performed for 1 h at 37°C and amplification was done by initial denaturation at 95°C for 2 min, 40 cycles of amplification (95°C for 40 s, 55°C for 50 s, and 72°C for 50 s) and a final extension step of 7 min at 72°C was carried out in a 30 µL mixture containing 2 µg of total RNA, 25 pmol/L each of the sense and antisense primers, 2.0 mmol/L MgCl<sub>2</sub>, 0.1 mmol/L dNTP, 1.5 µL 20 × buffer without Mg<sup>2+</sup>, 2 µL RT-PCR Enzyme Mix and double-distilled H<sub>2</sub>O.  $\beta$ -actin sense primer, 5'-ATCTGGCACACACCTTC TACAATGAGCTGCG-3', and antisense primer 5'-CGTCATACTCCTGCTGCTGATCCACATCTGC -3' were used. The reverse transcription was done for 1 h at 37°C and amplification was performed by initial denaturation at 94°C for 5 min, 35 cycles of amplification (95°C for 15 s, 60°C for 1 min, and 72°C for 30 s) and a final extension step of 7 min at 72°C was carried out in a 30 µL mixture containing the same components as above. The length of the  $\beta$ -actin PCR product was 838 bp and was the internal control. The PCR reaction products were separated electrophoretically in a 2% agarose gel and stained with ethidium bromide for 40 min at 80 V, and observed and photographed. All primers were synthesized by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd.

#### **Construction of small interfering RNA (siRNA) expression vectors**

To further elucidate the effect of PPAR- $\gamma$  in gastric carcinoma, we applied the siRNA targeting approach. We used shRNA™ vector (provided by Wuhan Genesil

Biotechnology Co., Ltd.) in which shRNA expression is driven by the U6 RNA promoter to produce small and hair RNA transcripts. We generated 3 different shRNA™ vectors and an empty vector used as a control. The oligonucleotides 5'-GATCCGAACAGAT CCAGTGGTTGCTTCAAGA-CGGCAACCACT G G A T C T G - T T C T T T T T G T C G A C A - 3', 3'-GCTTGTCTAGGTACCAACGAAGTTCTGC CGTTGGTGACCTAGACAAGAAAAACAG CTGTTCGA-5' were used to generate anti-heparanase shRNA™ pSi1, and oligonucleotides 5'-GATCCGTAAA-GTGGAGCCTGCATCTTC AAGACGGATGCAGGCTCCACTTGATTTT TGTCGACA-3', 3'-GCAGTTCACCTCGGACGTAG AAGTTCTGCCTACGTCCGAGGTGAAACTAAAA AACAGCTGTTCGA-5' were used to generate anti-heparanase shRNA™ pSi2. Oligonucleotides 5'-GATCCG-ACAAACCTGCTACAAGCCCTCAAGAC GGG-GCTTGTAGCAGGTTGTCTTTTGT CGACA-3', 3'-GCTGTTGACGATGTTCG GGAAGTTCTGCCCGAACATCGTCCAACAGAA AAAACAGCTGTTCGA-5' were used to generate anti-heparanase shRNA™ pSi3.

#### **Transfection**

For the transfection, pSi1, pSi2 and pSi3 (1.0 µg) were added to RPMI1640 medium (without FBS) containing cation liposome vector (2.0 µL) and allowed to incubate for 20 min at room temperature to produce the transfection mixture. The transfection mixture was then added to the cells ( $1 \times 10^5$  per dish) in the serum-free medium. Six hours after the start of transfection, the medium was changed to RPMI1640 medium containing 10% FBS. RNA interference effect was examined by RT-PCR, Western blotting and MTT assay. Cell apoptosis determination was carried out at the final step.

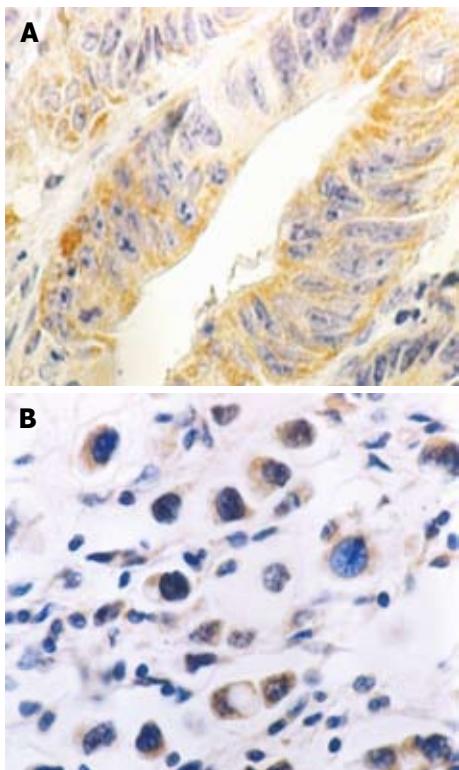
#### **Statistical analysis**

The results were expressed as mean  $\pm$  SD. Statistical analysis was performed using the Student's *t*-test,  $\chi^2$  test or one-way ANOVA and subsequent Fisher's LSD test.  $P < 0.05$  was considered statistically significant. Statistical analysis was performed using SPSS11.0 software.

## **RESULTS**

#### **Enhanced PPAR- $\gamma$ expression in gastric carcinoma tissue and MGC803 cells**

The positive rate of PPAR- $\gamma$  protein expression was significantly higher in gastric carcinoma (75.0%) than in paired mucosa adjacent to gastric carcinoma (27.5%) and in normal gastric mucosa at the surgical margin of gastric carcinoma (13.0%) ( $P < 0.001$ ). Mucosa adjacent to gastric carcinoma had significantly higher expression than normal gastric mucosa at the surgical margin of gastric carcinoma ( $P < 0.05$  and  $P < 0.001$ ) (Figure 1). The rate of PPAR- $\gamma$  protein expression was positively associated with histologic type and negatively associated with invasive depth, vessel invasion, lymph node metastasis, distant metastasis and clinical stage



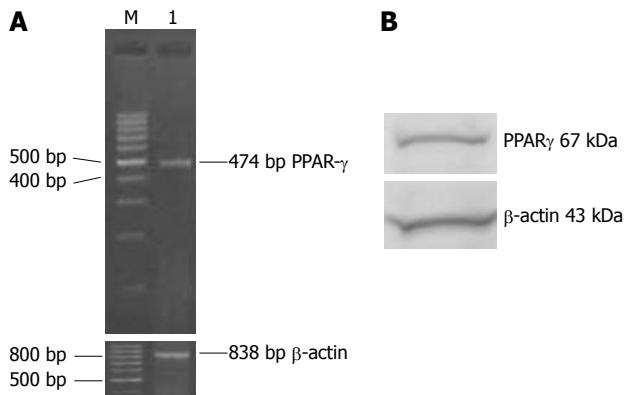
**Figure 1** Peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) protein in the cytoplasm of gastric carcinoma cells by immunohistochemistry (3,3'-diaminobenzidine and hematoxylin staining  $\times 400$ ). A: Well differentiated gastric carcinoma; B: Poorly differentiated gastric carcinoma.

**Table 1** Correlation of PPAR $\gamma$  protein expression in gastric carcinoma with clinicopathological features

Clinicopathological features	<i>n</i>	PPAR $\gamma$ protein		$\chi^2$	<i>P</i>
		+	-		
Histologic subtype	138			24.663	0.000
Intestinal type	63	60	3		
Diffuse type	75	44	31		
Depth of invasion	138			1.183	0.277
Serosal	24	16	8		
Out of serosa	114	88	26		
Vessel invasion	138			0.065	0.799
Absent	120	90	30		
Present	18	14	4		
Lymph node metastasis	138			0.309	0.578
Absent	36	28	8		
Present	102	76	28		
Distant metastasis	138			0.535	0.464
Absent	126	96	30		
Present	12	8	4		
Clinical stage <sup>1</sup>	126			0.017	0.897
I - II	39	30	9		
III-IV	87	61	26		

<sup>1</sup>Because clinical staging could not be performed because of the lack of accurate records about lymph nodes, data of 12 cases were not included in the statistical analysis.

( $P < 0.05$ ) of gastric carcinoma. The rate of PPAR- $\gamma$  protein expression was significantly higher in intestinal-type gastric carcinoma (95.2%) than in diffuse-type gastric carcinoma (58.7%) ( $P < 0.001$ ) (Table 1). Both PPAR- $\gamma$  mRNA and protein showed positivity in the human gastric carcinoma cell line MGC803 (Figure 2).



**Figure 2** PPAR- $\gamma$  mRNA and protein expression in gastric carcinoma cell line MGC803. A: PPAR $\gamma$  mRNA expression examined in MGC803 cells by reverse transcriptase-polymerase chain reaction. M: 100 bp DNA marker and 500 bp was the brightest; 1: MGC803 cells. B: PPAR- $\gamma$  protein expression examined by Western blotting in MGC803 cells.

#### Effect of 15d-PGJ<sub>2</sub>, GW9662 and 15d-PGJ<sub>2</sub> + GW9662 on growth of MGC803 cells

Treatment with a concentration of 0.01, 0.1 and 1  $\mu\text{mol/L}$  15d-PGJ<sub>2</sub> did not affect MGC803 cell growth. The inhibition of proliferation of MGC803 cells by 5, 10, 20, 30, and 40  $\mu\text{mol/L}$  15d-PGJ<sub>2</sub> was significantly higher than that of 0  $\mu\text{mol/L}$  ( $P < 0.001$ ) (Table 2). Correspondingly, inhibition was also enhanced with increasing 15d-PGJ<sub>2</sub> concentrations. Inhibition was  $5.98\% \pm 1.41\%$ ,  $13.64\% \pm 0.69\%$ , and  $34.64\% \pm 0.99\%$  after 8, 18, and 24 h, respectively, following 30  $\mu\text{mol/L}$  15d-PGJ<sub>2</sub> addition. It was significantly higher at 18 h than 8 h ( $P < 0.001$ ), and it was significantly higher at 24 h than 18 h ( $P < 0.001$ ); it was enhanced over a prolonged time. Thus 15d-PGJ<sub>2</sub> inhibited proliferation of MGC803 cells in a concentration-dependent and time-dependent manner (Figure 3A). The proliferation inhibition rate of MGC803 cells by GW9662 treatment for 24 h, showed no difference between 0.01, 0.1 and 1  $\mu\text{mol/L}$  and 0  $\mu\text{mol/L}$  concentrations (all  $P > 0.05$ ). Inhibition of proliferation was significantly higher at 2.5 and 5  $\mu\text{mol/L}$  compared to 0  $\mu\text{mol/L}$  (all  $P > 0.05$ ). The proliferation inhibition rate of MGC803 cells by 15d-PGJ<sub>2</sub> and GW9662 combination treatment for 24 h showed no difference between the 0.01, 0.1 and 1  $\mu\text{mol/L}$  GW9662 and 5, 10, 20, 30 and 40  $\mu\text{mol/L}$  15d-PGJ<sub>2</sub> combination groups and the 5, 10, 20, 30 and 40  $\mu\text{mol/L}$  15d-PGJ<sub>2</sub> group (Table 3).

#### Effect of 15d-PGJ<sub>2</sub> on apoptosis of MGC803 cells

15d-PGJ<sub>2</sub> is capable of inducing MGC803 cell apoptosis as seen by morphological observation (Figure 4) and flow cytometry (Figure 5). The apoptosis rate of MGC803 cells of 5, 10, 20, 30, and 40  $\mu\text{mol/L}$  15d-PGJ<sub>2</sub> was significantly higher than that of 0  $\mu\text{mol/L}$  ( $P < 0.001$ ) (Table 1); it was enhanced with increasing concentrations of 15d-PGJ<sub>2</sub>. The apoptosis rate was  $6.58\% \pm 0.62\%$ ,  $15.99\% \pm 1.73\%$ , and  $38.23\% \pm 1.36\%$  at 8, 18 and 24 h, respectively, following 30  $\mu\text{mol/L}$  15d-PGJ<sub>2</sub> addition, and increased with time. Thus, 15d-PGJ<sub>2</sub> induced

**Table 2** Effect of various concentrations of 15-deoxy- $\Delta$ 12,14-prostaglandin-J2 (15d-PGJ<sub>2</sub>) on proliferation, apoptosis and cell cycle of gastric carcinoma MGC803 cells after 24 h [(mean  $\pm$  SD) %]

15d-PGJ <sub>2</sub> concentration ( $\mu$ mol/L)	Proliferative inhibition rate	Apoptosis rate	Cell cycle distribution		
			G <sub>1</sub> /G <sub>0</sub>	S	G <sub>2</sub> /M
0	0.22 $\pm$ 0.11	0.37 $\pm$ 1.03	31.33 $\pm$ 2.31	60.33 $\pm$ 1.15	8.33 $\pm$ 1.15
0.01	0.21 $\pm$ 0.10				
0.1	0.21 $\pm$ 0.12				
1	0.23 $\pm$ 0.12				
5	7.47 $\pm$ 0.49 <sup>d</sup>	7.89 $\pm$ 1.07 <sup>d</sup>			
10	11.49 $\pm$ 1.00 <sup>d</sup>	11.41 $\pm$ 1.47 <sup>d</sup>			
20	14.36 $\pm$ 1.53 <sup>d</sup>	14.27 $\pm$ 1.62 <sup>d</sup>			
30	38.23 $\pm$ 1.36 <sup>d</sup>	34.64 $\pm$ 0.99 <sup>d</sup>	61.33 $\pm$ 1.53 <sup>d</sup>	36.00 $\pm$ 1.00 <sup>d</sup>	2.67 $\pm$ 0.58 <sup>b</sup>
40	57.00 $\pm$ 1.24 <sup>d</sup>	55.35 $\pm$ 1.88 <sup>d</sup>			

<sup>a</sup>P < 0.01, <sup>b</sup>P < 0.001 compared with 0  $\mu$ mol/L group.

**Table 3** Effect of GW9662 in various concentrations on the proliferation of MGC803 cells and on 15d-PGJ<sub>2</sub> inhibition of proliferation of MGC803 cells after 24 h [(mean  $\pm$  SD) %]

15d-PGJ <sub>2</sub> concentration ( $\mu$ mol/L)	Proliferative inhibition rate of GW9662 ( $\mu$ mol/L)					
	0	0.01	0.1	1	2.5	5
	0.19 $\pm$ 0.11	0.20 $\pm$ 0.12	0.20 $\pm$ 0.11	0.19 $\pm$ 0.12	3.20 $\pm$ 0.13 <sup>b</sup>	5.29 $\pm$ 0.14 <sup>b</sup>
5	7.47 $\pm$ 0.49	7.49 $\pm$ 0.52	7.50 $\pm$ 0.50	7.52 $\pm$ 0.49		
10	11.49 $\pm$ 1.00	11.50 $\pm$ 1.00	11.52 $\pm$ 1.03	11.53 $\pm$ 1.04		
20	14.36 $\pm$ 1.53	14.42 $\pm$ 1.62	14.44 $\pm$ 1.53	14.36 $\pm$ 1.53		
30	38.23 $\pm$ 1.36	38.33 $\pm$ 1.42	38.34 $\pm$ 1.36	38.43 $\pm$ 1.36		
40	57.00 $\pm$ 1.24	57.06 $\pm$ 1.34	57.10 $\pm$ 1.24	57.12 $\pm$ 1.34		

<sup>a</sup>P < 0.001 compared with 0  $\mu$ mol/L group of GW9662.

MGC803 cell apoptosis in a concentration-dependent and time-dependent manner (Figure 3B).

#### Effect of 15d-PGJ<sub>2</sub> on cell cycle of MGC803 cells

15d-PGJ<sub>2</sub> at 30  $\mu$ mol/L significantly increased the proportion of MGC803 cells in the G<sub>0</sub>/G<sub>1</sub> phase ( $P < 0.001$ ) and significantly decreased the proportion in the S and G<sub>2</sub>/M phases ( $P < 0.001$  and  $P < 0.01$ , respectively) (Figure 6).

#### Effect of 15d-PGJ<sub>2</sub> on survivin, Skp2 and p27 expression in MGC803 cells

Survivin and Skp2 protein were decreased and p27 protein was increased as the concentration of 15d-PGJ<sub>2</sub> increased as shown by Western blotting (Figure 7).

#### Effect of knockdown of PPAR- $\gamma$ by RNAi

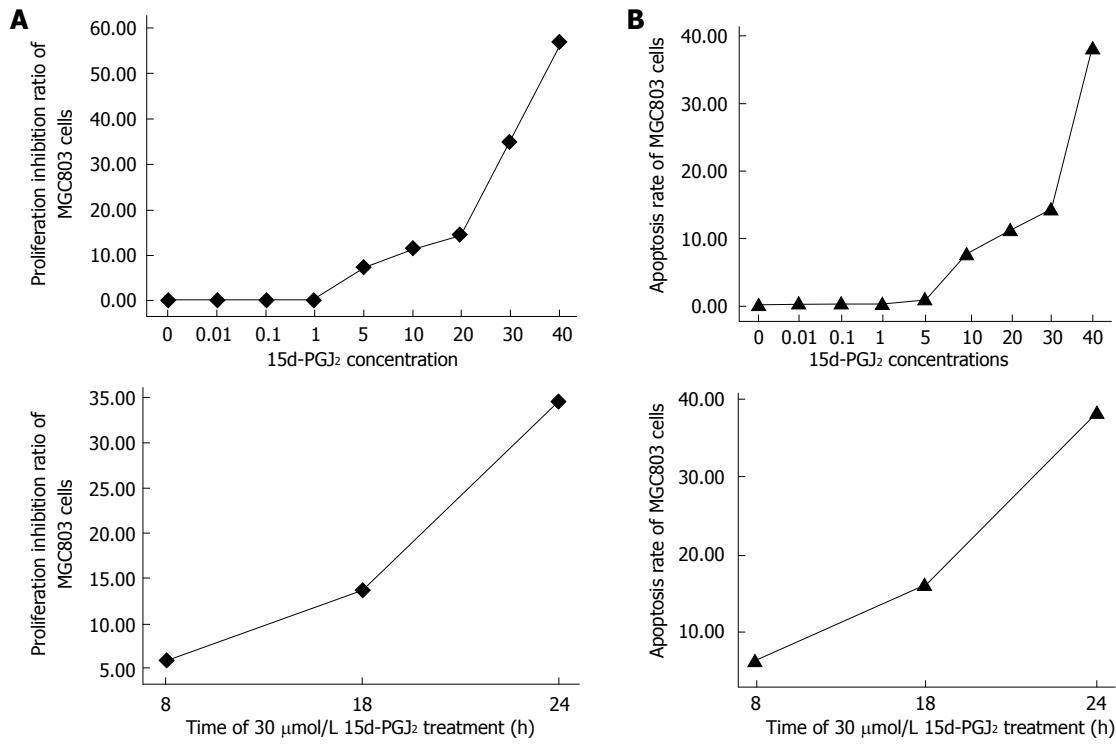
To examine whether the inhibitory effect of PPAR- $\gamma$  agonist 15d-PGJ<sub>2</sub> on the growth of MGC803 cells was via a PPAR- $\gamma$  specific pathway and influenced PPAR- $\gamma$  expression in the tumor cells, we used the RNAi approach for PPAR- $\gamma$ . Efficiency of transfection approximate 80% was evaluated by fluorescence microscopy 48 h after transfection of a vector containing the gene encoding green fluorescent protein (Figure 8). MGC803 cells were transiently transfected with pSi1, pSi2 and pSi3 or empty vectors by cation liposome vector, and the cells were tested for PPAR- $\gamma$  expression 48 h later. Of the cells transfected with pSi1, pSi2 and pSi3, pSi1 was most effective in silencing PPAR- $\gamma$  mRNA and

protein expression in MGC803 cells, as determined by semiquantitative RT-PCR (Figure 9A) and Western blotting (Figure 9B). PPAR- $\gamma$  specific RNAi effectively decreased PPAR- $\gamma$  mRNA and protein level in the MGC803 cells and showed inhibition of cell growth (Figure 10A) and induction of cell apoptosis (Figure 10B).

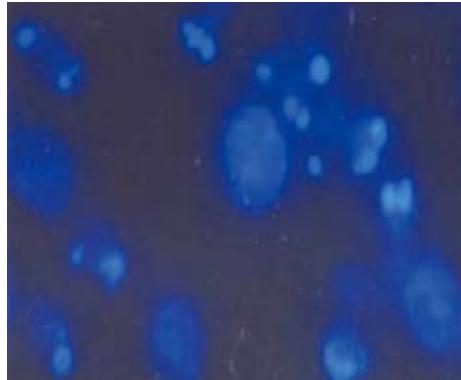
## DISCUSSION

Recently, the potential of PPAR- $\gamma$  as a target for the prevention and treatment of cancer has been widely studied<sup>[17]</sup>. However, the potential therapeutic role of PPAR- $\gamma$  agonists has been questioned, based on contradictory results. In experiments using animal models of colon cancer, PPAR- $\gamma$  agonists increased the development of colon tumors<sup>[10]</sup>. This contradictory result was supplemented by a recent report using transgenic mice that expressed a constitutive active form of PPAR- $\gamma$  in mammary glands, and showed that PPAR- $\gamma$  signaling accelerated tumor development in mammary glands<sup>[18]</sup>. The actual role of PPAR- $\gamma$  in cancer has been complicated by recent findings that PPAR- $\gamma$  agonists affect cancer cells independent of PPAR- $\gamma$ <sup>[19-22]</sup>, and silencing of PPAR- $\gamma$  and PPAR- $\gamma$  antagonists have been shown to inhibit cancer cell growth<sup>[13,14]</sup>. To date, the role of PPAR- $\gamma$  in gastric carcinogenesis remains unclear.

In the present study, PPAR- $\gamma$  was found to be expressed at higher levels in gastric carcinoma than in paired mucosa adjacent to gastric carcinoma, and in both tissues the PPAR- $\gamma$  levels were higher than in their



**Figure 3** 15d-PGJ<sub>2</sub> inhibited proliferation (A) and induced apoptosis (B) of MGC803 cells in a concentration-dependent and time-dependent manner.



**Figure 4** Morphological changes of MGC803 cells 24 h after treatment with 30  $\mu\text{mol/L}$  15d-PGJ<sub>2</sub>. Cell shapes were observed by fluorescence microscopy (Hoechst 33342 staining,  $\times 400$ ). Nuclear chromatin condensation, marginalization of nuclear chromatin and half moon formation, nuclear fragments with bright chromatin and apoptotic bodies were easily identified in some cells.

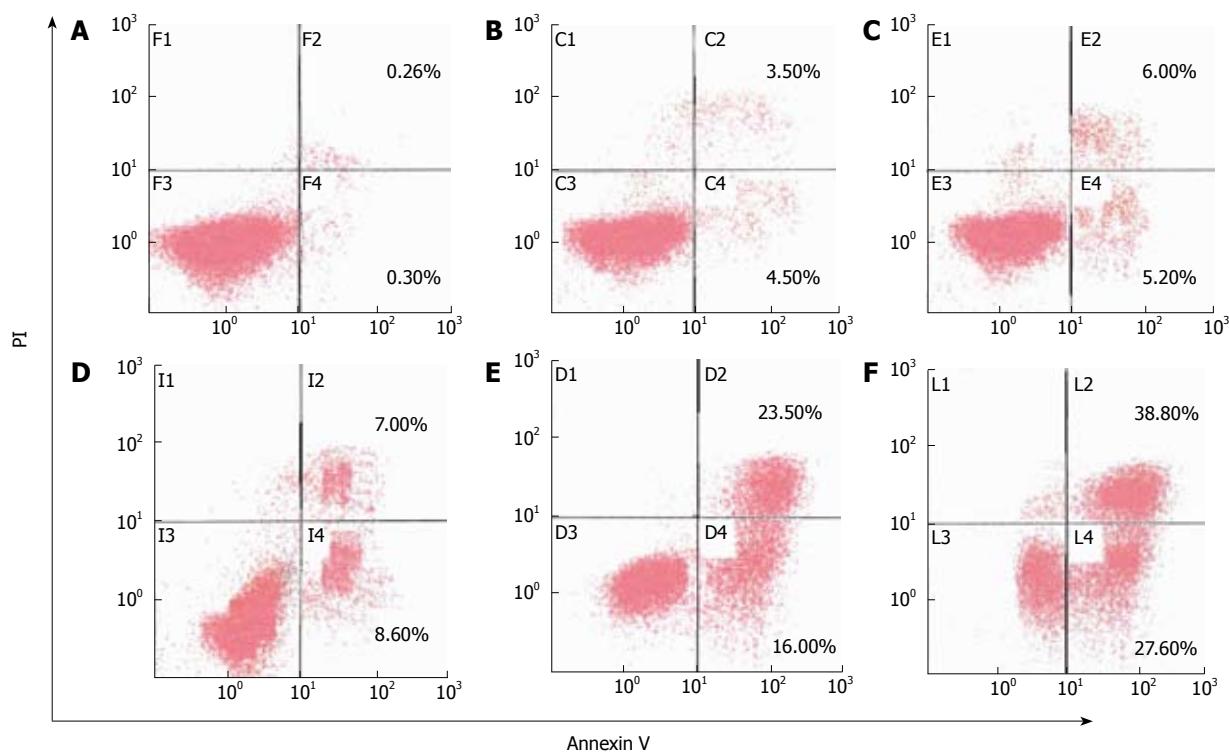
paired normal gastric mucosa at the surgical margin of the gastric carcinoma. Similar effects were also observed in gastric cancer in another study<sup>[16]</sup>. In addition, we used the siRNA approach to confirm the effect of PPAR- $\gamma$  on gastric carcinoma. The result showed that silencing PPAR- $\gamma$  clearly inhibited the growth and induced apoptosis of MGC803 cells. Similar effects were also observed in other cancers such as hepatocellular carcinoma<sup>[13]</sup>. These results indicated that PPAR- $\gamma$  may be involved in gastric carcinogenesis.

Here, we clearly showed that the PPAR- $\gamma$  agonist 15d-PGJ<sub>2</sub> inhibited growth of cultured gastric cancer MGC803 cells, and our study also demonstrated that the PPAR- $\gamma$  antagonist GW9662 did not interfere with this effect and that 2.5  $\mu\text{mol/L}$  GW9662 inhibited growth of

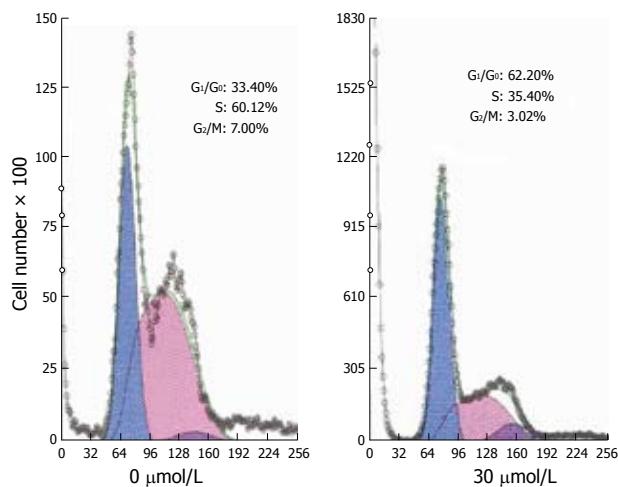
MGC803 cells. Furthermore, PPAR- $\gamma$  siRNA markedly inhibited the growth of MGC803 cells in our study. These results indicated that 15d-PGJ<sub>2</sub> inhibited growth of cultured gastric carcinoma MGC803 cells by a PPAR- $\gamma$ -independent pathway. These results also suggested that PPAR agonists may be useful in the chemoprevention or chemotherapy of gastric malignancies. Additionally, some recent studies reported that the biological effect of PPAR- $\gamma$  agonists is independent of PPAR- $\gamma$ <sup>[19,23-28]</sup>, but other research concluded that 15d-PGJ<sub>2</sub> inhibited growth of cancer cells and gastric cancer cells by activating the PPAR- $\gamma$  pathway<sup>[29-32]</sup>. What is the reason for the discrepancies between our data and the data found in other reports? This may be attributed to the fact that some investigators did not study the effect of PPAR antagonists on tumor cell growth and on the effect of PPAR agonists, as they only used PPAR agonists.

The inhibition of growth of cultured gastric cancer MGC803 cells by PPAR- $\gamma$  agonists is mainly a result of apoptosis, as evidenced by the data for nuclear condensation by chromatin staining with Hoechst 33342 and annexin V-FITC staining through FACS analysis. Mechanisms may exist by which apoptosis is induced by PPAR agonists. Furthermore, the inhibition of cell growth and induction of apoptosis by PPAR- $\gamma$  agonists were time- and concentration-dependent.

Survivin is an inhibitor of apoptosis, and is expressed during embryonal development but lacks expression in terminally differentiated adult tissues. Interestingly, it becomes re-expressed in transformed cell lines and in a variety of human tumors, and deserves growing attention as “an ideal target” for cancer therapy. Survivin is involved in the control of apoptosis and directly inhibits caspase 3 and 7 activities. Survivin is mostly



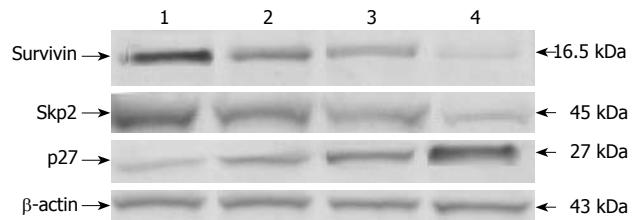
**Figure 5 Apoptosis rate of MGC803 cells detected by flow cytometry 24 h after treatment with 15d-PGJ<sub>2</sub> at various concentrations. A: 0 μmol/L; B: 5 μmol/L; C: 10 μmol/L; D: 20 μmol/L; E: 30 μmol/L; F: 40 μmol/L.**



**Figure 6 Cell cycle distribution of MGC803 cells by flow cytometry 24 h after treatment with 15d-PGJ<sub>2</sub> at various concentrations.** 15d-PGJ<sub>2</sub> at 30 μmol/L could significantly increase the proportion of cells in the G<sub>0</sub>/G<sub>1</sub> phase ( $P < 0.001$ ) and decrease the proportion in the S and G<sub>2</sub>/M phases in MGC803 cells ( $P < 0.001$  and  $P < 0.01$ ).

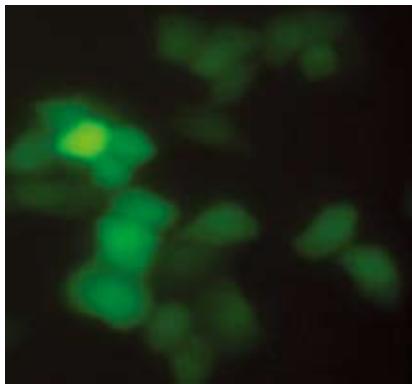
expressed in cell cycle G<sub>2</sub>/M and lacks expression in G<sub>1</sub>/G<sub>0</sub><sup>[33]</sup>. During tumorigenesis, survivin expression is negatively correlated with apoptosis but positively correlated with proliferation and angiogenesis<sup>[34]</sup>. Our study showed that the levels of survivin protein were decreased with the increase in 15d-PGJ<sub>2</sub> concentration, and were inversely associated with the apoptosis induced by 15d-PGJ<sub>2</sub>. These data suggest that 15d-PGJ<sub>2</sub> may inhibit survivin expression to induce apoptosis in gastric carcinoma MGC803 cells.

Our study showed, by flow cytometry, that 15d-PGJ<sub>2</sub>

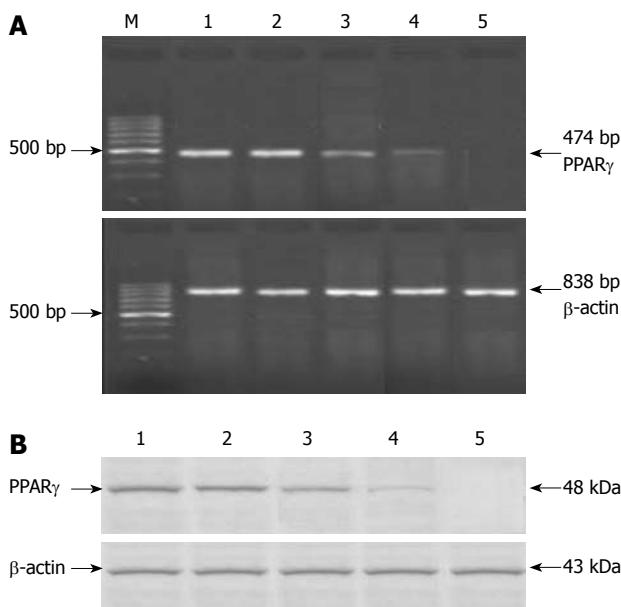


**Figure 7 Survivin, Skp2 and p27 protein expression by Western blotting in MGC803 cells 24 h after treatment with 15d-PGJ<sub>2</sub> at various concentrations.** Lane 1: 0 μmol/L; Lane 2: 5 μmol/L; Lane 3: 20 μmol/L; Lane 4: 30 μmol/L. Survivin and Skp2 protein decreased and p27 protein increased with increasing concentrations of 15d-PGJ<sub>2</sub>.

induced MGC803 cell arrest in the G<sub>1</sub>/G<sub>0</sub> cell cycle phase and reduced the percentage of cells in the S and G<sub>2</sub>/M cell cycle phases. This may be another reason for inhibition of the growth of gastric carcinoma MGC803 cells by 15d-PGJ<sub>2</sub>. Additionally, this may be in part the reason that 15d-PGJ<sub>2</sub> reduced the level of survivin, although we cannot exclude the possibility that 15d-PGJ<sub>2</sub> may have a direct effect on survivin expression. In eukaryotes, progression of the cell cycle is controlled by interactions between cell cycle control proteins (cyclins) and their catalytically active cyclin-dependent kinases (CDKs). The activity of each cyclin-CDK complex is in turn regulated by several different mechanisms, the most important being negative regulation by CDK inhibitors<sup>[35]</sup>. p27 is an inhibitor of cyclin E-CDK2 and cyclin A-CDK2, which drive cells from the G<sub>1</sub> phase to the S phase of the cell division cycle<sup>[36,37]</sup>, and p27 is expressed at its highest level in the G<sub>1</sub>/G<sub>0</sub> phase. The amount of p27 is mainly regulated by posttranslational

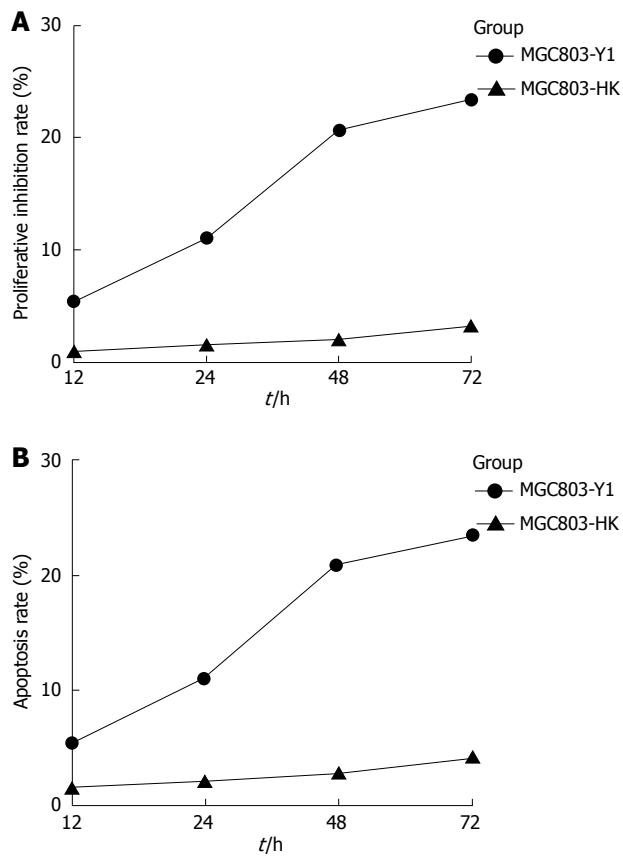


**Figure 8** Human gastric carcinoma MGC803 cells observed by fluorescence microscopy 48 h after pSi1 transfection.



**Figure 9** PPAR $\gamma$  after pSi transfection. A: PPAR $\gamma$  mRNA expression in various cells detected by RT-PCR 48 h after transfection; B: PPAR $\gamma$  protein expression in various cells 48 h after transfection as detected by Western blotting. Lane M: 100 bp DNA marker, 500 bp was the brightest; Lane 1: MGC803-N (normal MGC803 cells); Lane 2: MGC803-HK (MGC803 cells transfected by empty vector); Lane 3: MGC803-pSi3 (MGC803 cells transfected by pSi3 vector); Lane 4: MGC803-pSi2 (MGC803 cells transfected by pSi2 vector); Lane 5: MGC803-pSi1 (MGC803 cells transfected by pSi1 vector). The effect of pSi1 was best in silencing PPAR $\gamma$  mRNA and protein expression in MGC803 cells.

ubiquitin-proteasome-mediated proteolysis<sup>[38]</sup>. The cell cycle-dependent degradation of p27 is dependent on phosphorylation at Thr<sup>187</sup> in the late G<sub>1</sub> phase by CDK2, and Thr<sup>187</sup> phosphorylation is a necessary prerequisite for the sequential addition of ubiquitin molecules by a ubiquitin ligase complex, SCF<sup>Skp2</sup>, composed of Skp1, Cull, Rbx1, and the F-box protein Skp2<sup>[37]</sup>. Polyubiquitination of p27 then targets p27 for degradation in the proteasome, thus removing the p27 cell cycle “brake”, allowing cells to transition from G<sub>1</sub> to the S phase<sup>[39]</sup>. Some investigations have shown that Skp2 is a specific substrate-recognition subunit of SCF<sup>Skp2</sup>, the expression of Skp2 is required for the ubiquitination and subsequent degradation of p27 *in vitro* and *in vivo*<sup>[40-42]</sup>, and Skp2 knockout cells exhibit p27 at high levels<sup>[43]</sup>.



**Figure 10** Proliferative inhibition and apoptosis rate of MGC803-Y1 and of MGC803-HK cells after transfection. The proliferative inhibition (A) and apoptosis (B) rate of MGC803-Y1 cells was higher than that of MGC803-HK cells at 12-72 h after transfection.

Skp2 levels are cell cycle regulated, and Skp2 accumulates during the S phase. Inappropriate expression of Skp2 in G<sub>0</sub> cells can promote S phase entry concomitant with loss of p27. Recently, the targeted disruption of Skp2 resulted in an accumulation of p27, and cell cycle arrest in the G<sub>1</sub> cycle. The level of Skp2 protein was decreased, whereas the level of p27 protein was increased with increasing concentration of 15d-PGJ<sub>2</sub> treatment when examined by Western blotting. These data suggested that 15d-PGJ<sub>2</sub> induced MGC803 cell arrest in G<sub>1</sub>/G<sub>0</sub> by Down-regulating Skp2 expression and upregulating p27 expression. Furthermore, these results suggested that 15d-PGJ<sub>2</sub> may interfere with ubiquitin-proteasome-mediated proteolysis in gastric carcinoma cells.

In conclusion, our study suggests that PPAR- $\gamma$  may be involved in gastric carcinogenesis, and that 15d-PGJ<sub>2</sub> may inhibit the growth of human gastric carcinoma MGC803 cells by inducing apoptosis and G<sub>1</sub>/G<sub>0</sub> arrest, with the involvement of survivin, Skp2 and p27, but *via* a PPAR- $\gamma$ -independent pathway.

## COMMENTS

### Background

Many efforts have been made to search for therapeutic targets and drugs capable of preventing and treating gastric carcinoma and other malignancies. Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors, initially described as molecular targets for compounds, which induce peroxisomal proliferation.

## Research frontiers

Three different isotypes of PPARs have been identified in various species, being the products of distinct genes and generally designated as PPAR- $\alpha$ , PPAR- $\beta/\delta$ , and PPAR- $\gamma$ . PPAR- $\gamma$  is one that has been extensively studied. As with other members of the nuclear receptor gene family, PPAR- $\gamma$  is an agonist-activated transcription factor. PPAR- $\gamma$  heterodimerizes with retinoid X receptor to bind to the PPAR response element, leading to the transcription of downstream genes.

## Innovations and breakthroughs

Several specific agonists have been found, such as the thiazolidinediones (including pioglitazone, rosiglitazone, and troglitazone), 15-deoxy- $\Delta$ 12,14-prostaglandin-J<sub>2</sub> (15d-PGJ<sub>2</sub>), and certain polyunsaturated fatty acids. GW9662 has been shown to be a selective and irreversible antagonist of PPAR- $\gamma$ , and irreversibly binds within the agonist binding domain through covalent modification of a cysteine residue.

## Applications

By understanding how PPAR- $\gamma$  affects the growth of cells, this study may represent a future strategy for therapeutic intervention in the treatment of patients with gastric carcinoma.

## Terminology

PPAR- $\gamma$  receptors are nuclear hormone receptors, which induce peroxisomal proliferation. 15d-PGJ<sub>2</sub> is a specific agonist. Peroxisomal proliferation is thought to be crucial in gastric carcinoma.

## Peer review

PPAR- $\gamma$  receptors are nuclear hormone receptors. The authors examined whether PPAR- $\gamma$  may affect gastric carcinogenesis, and 15d-PGJ<sub>2</sub> may inhibit the growth of MGC803 cells by inducing apoptosis and G<sub>1</sub>/G<sub>0</sub> arrest, but through a PPAR- $\gamma$ -independent pathway. The result may be useful in treatment of gastric carcinoma.

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BRIEF ARTICLES

## Endotoxin receptor **CD14** gene variants and histological features in chronic HCV infection

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### Abstract

**AIM:** To analyze the correlation between CD14 rs2569190/C-159T single nucleotide polymorphism (SNP) and disease progression in chronic hepatitis C.

**METHODS:** Liver biopsy specimens from a total of 137 and 349 patients with chronic hepatitis C were separately evaluated with respect to necroinflammatory activity (grading) and architectural changes (staging). In one group, further histological lesions characteristic for hepatitis C, hepatitis C virus subtypes, and biochemical parameters of liver disease were also investigated. Samples of genomic DNA were genotyped for the respective SNP by 5'-nuclease assays using fluorescent dye-labeled allele-specific probes.

**RESULTS:** Genotype distribution did not deviate from the Hardy-Weinberg equilibrium. In the first group, patients homozygous for the variant allele T were found to be younger than C allele carriers ( $39.6 \pm 12.5$  vs  $45.7 \pm 11.5$ ,  $P = 0.008$ ). Among the histological lesions studied, portal lymphoid aggregates were more frequently observed among TT homozygotes than among C carriers ( $21/37$  vs  $32/100$ ,  $P = 0.008$ ). The presence of portal lymphoid aggregates was closely correlated with hepatic inflammation ( $P = 0.003$ ) and with bile duct damage ( $P < 0.001$ ). The degree of fibrosis, in contrast, was not found to be related to the **CD14** gene C-159T polymorphism.

**CONCLUSION:** The data suggest a possible relationship between CD14 C-159T polymorphism and the formation of portal lymphoid aggregates, but not liver fibrosis progression in chronic hepatitis C.

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**Key words:** CD14; Endotoxins; Hepatitis C virus; Inflammation; Lipopolysaccharides; Liver fibrosis; Portal system; Single nucleotide polymorphism

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### INTRODUCTION

Hepatitis C virus (HCV), which currently infects about 3% of the world's population (an estimated 210 million people), is a major cause of chronic viral liver disease<sup>[1]</sup>. Chronic hepatitis C is characterized by mostly mild hepatic inflammatory activity which does, however, hold a significant risk of proceeding to liver cirrhosis and hepatocellular carcinoma<sup>[2]</sup>. Further characteristic histological alterations may include steatosis, bile duct lesions, and portal lymphoid aggregates<sup>[3-5]</sup>. While steatosis, for instance, has been shown to be associated with HCV subtype infection and is suggested to be modulated by HCV proteins<sup>[4,6]</sup>, progression of fibrosis in chronic hepatitis C has been attributed to age, gender, or alcohol consumption<sup>[7]</sup>, and to host genetic factors<sup>[8]</sup>. Different genetic backgrounds have also been found to be associated with the susceptibility to HCV subtype infection<sup>[9,10]</sup>.

As a result of its anatomic links to the gut, the liver is constantly exposed to gut-derived bacterial products, e.g. lipopolysaccharides (LPS), which are suggested to be important cofactors in toxin- or ethanol-induced liver disease by exacerbating ongoing injury<sup>[11]</sup>. Endotoxemia arises from increased translocation of endotoxins from the gut lumen because of altered intestinal permeability and decreased hepatic clearance capacity<sup>[12]</sup>.

Hepatocytes and Kupffer cells, the resident liver macrophages which play a major role in the clearance of systemic bacterial infection, express the membrane-associated form of the endotoxin receptor CD14 (mCD14) at low levels in comparison to peripheral blood monocytes (reviewed by Schwabe *et al.*<sup>[12]</sup>). Moreover, sinusoidal endothelial cells and activated hepatic stellate cells, the main fibrogenic cell type in the injured liver, also express mCD14<sup>[12,13]</sup>. mCD14 is anchored by glycoprophosphatidyl inositol, being part of a cell surface receptor complex which additionally contains the dimerized Toll-like receptor 4 and MD-2 (reviewed by Pålsson-McDermott & O'Neill<sup>[14]</sup>). Furthermore, it also localizes with TLR3, the double-stranded RNA (dsRNA) receptor, in intracellular compartments enhancing dsRNA sensing and TLR3 signalling<sup>[15,16]</sup>. In addition to the membranous form, a soluble form of CD14 lacking the glycoprophosphatidyl inositol anchor is thought to modulate LPS responses *via* local stimulatory (promoting) and systemic anti-inflammatory (competing) mechanisms<sup>[17]</sup>.

Recently, a single nucleotide polymorphism (SNP) within the *CD14* gene, rs2569190/C-159T, has been demonstrated to be associated with the risk of developing liver cirrhosis, but not steatosis or less advanced stages of fibrosis, in patients with alcohol-induced liver disease (ALD)<sup>[18-20]</sup>. These results were attributed to the finding that, *in vitro*, the T allele is more actively transcribed than the C allele<sup>[21]</sup>, leading consequently to the assumption that the TT carriers' hepatic cells may be prone to enhanced inflammatory reactions after endotoxin exposure. The relationship between rs2569190 TT genotype and liver disease progression, however, was not observed in a study by von Hahn and colleagues, in which the variant allele T was alternatively shown to be solely associated with cryptogenic chronic liver disease<sup>[22]</sup>.

The current study aimed to investigate, in two different cohorts, whether the variant position rs2569190/C-159T within the *CD14* gene is associated with hepatitis C liver disease manifestations, and allowed replicated analysis as is demanded for genetic association studies<sup>[23]</sup>.

## MATERIALS AND METHODS

### Ethics

The study was approved by the local ethical committee and conformed to the ethical guidelines of the 2000 Declaration of Helsinki. Patients gave their informed consent.

### Patients

A total of 137 mainly Caucasian chronic hepatitis C patients (mean age  $44.0 \pm 12.0$  years, median 42 years) who consulted the Liver Unit of the Department of Gastroenterology and Endocrinology at the University Medical Center Goettingen (UMG), Germany, between 1993 and 2006 were enrolled. The chronic nature of infection was proven by detection of HCV-specific antibodies and HCV RNA in the patients' sera using a highly sensitive nested reverse transcription polymerase chain reaction (RT-PCR) over a period of at least 6 mo as described<sup>[24]</sup>.

As part of routine clinical evaluation, liver biopsy procedures were performed and liver disease was confirmed in the course of a defined histological evaluation as described below. Biochemical liver disease parameters, i.e. serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and  $\gamma$ -glutamyl transferase ( $\gamma$ -GT) were recorded in parallel. Patients with concomitant non-hepatitis C viral infections and those with continued alcohol or other drug abuse were excluded.

Samples and data from another 349 chronic hepatitis C patients (mean age  $45.8 \pm 13.5$  years, median 45 years) were kindly provided by the German Network of Competence for Hepatitis (Hep-Net)<sup>[25]</sup>.

### Determination of HCV genotype

HCV genotyping was performed for the 137 patients (UMG group) using the Innolipa HCV II line probe assay (Innogenetics, Ghent, Belgium).

### Histological evaluation

Before the start of therapy, liver biopsies were taken from patients for histological evaluation. In brief, sections (5–10  $\mu\text{m}$ ) from formalin-fixed and paraffin-embedded liver biopsies were stained with hematoxylin-eosin, trichrome, and Prussian blue. According to Desmet and colleagues, necroinflammatory activity (grading, score 1 to 3), and structural alterations (staging, score 0 to 4) were scored separately<sup>[26]</sup>. Other lesions typical of hepatitis C such as degree of steatosis (score 0 to 3), the presence or absence of portal lymphoid aggregates, and the presence or absence of bile duct damage were studied additionally as previously described<sup>[14]</sup>. Hep-Net samples were independently scored by two experienced pathologists according to the German guidelines with regard to inflammation activity and fibrosis progression<sup>[25,26]</sup>.

### Isolation of genomic DNA

Genomic DNA (gDNA) was purified from peripheral blood mononuclear cells (PBMCs) using the QIAamp DNA Mini Kit following the blood and body fluid spin protocol (Qiagen, Hilden, Germany). The concentration and the purity of the DNA isolated from PBMCs were determined spectrophotometrically by reading the absorbance levels at 260 and 280 nm. The integrity of gDNA was ascertained through electrophoresis using a 0.6% agarose gel. Alternatively, when PBMCs were not available, gDNA was purified from a 2 mL sample of serum by means of the QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

### SNP genotyping by 5'nuclease assay

gDNA (10 ng derived from PBMCs or an aliquot corresponding to 12.5  $\mu\text{L}$  serum) was amplified in a total volume of 20  $\mu\text{L}$  by real-time PCR using the TaqMan<sup>®</sup> Universal Master Mix (Applied Biosystems, Darmstadt, Germany) and 36  $\mu\text{mol/L}$  of primers each (*CD14*: forward 5'-CTAGATGCCCTGCAGAATCCTT-3', reverse 5'-CCCTTCCTTGAAATATTGCA-3'). Allelic discrimination was achieved by adding 8  $\mu\text{mol/L}$  differentially fluorescence dye-labeled allele-specific minor

**Table 1** Epidemiological characteristics of chronic hepatitis C patients with regard to CD14 rs2569190 genotype (UMG group)

	n	CD14 rs2569190 genotype			MAF	P
		CC	CT	TT		
Total number (%)	137	30 (21.9)	70 (51.1)	37 (27)	0.865 <sup>1</sup>	0.526
Gender (Female/male)	60/77	11/19	36/34	13/24	0.214 <sup>2</sup>	0.517/0.532
Age (mean ± SD)		46.5 ± 13.1	45.3 ± 10.8	39.6 ± 12.5	0.008 <sup>a</sup>	0.795 <sup>3</sup>
HCV subtype						
1a	31	7 (22.6)	17 (54.8)	7 (22.6)		
1b	73	18 (24.7)	38 (52.0)	17 (23.3)		0.500
1a + 1b	6	1 (16.7)	3 (50.0)	2 (33.3)		
					0.073 <sup>b</sup>	0.087 <sup>3</sup>
2a	1	0	0	1 (100.0)		
2b	4	2 (50.0)	1 (25.0)	1 (25.0)		0.630
3a	22	2 (16.7)	11 (50.0)	9 (33.3)		

MAF: Minor allele frequency. <sup>1</sup>Exact test for the Hardy-Weinberg equilibrium; <sup>2</sup>C carriers vs TT ( $\chi^2$  test); <sup>3</sup> $\chi^2$  test was applied; <sup>a</sup>C carriers vs TT (independent samples t-test); <sup>b</sup>C carriers vs TT, HCV type 1 vs non-type 1 infections ( $\chi^2$  test).

groove binder probes (CD14: VIC 5'-CCTGTTACGG TCCCCCTG-3', FAM 5'-CTGTTACGGCCCCCT-3'). Reactions and analyses were carried out in the sequence detection system ABI prism 7000 (Applied Biosystems, Darmstadt, Germany) according to the supplier's instructions.

### Statistical analysis

Quantitative parameters were described by mean and standard deviation or median and inter-quartile range, and the Kolmogorov-Smirnov test was applied to investigate whether these distributions were Gaussian. The UMG group and Hep-Net group were compared regarding age using the parametric independent samples *t*-test.

For describing the ordinal and nominal scaled parameters such as gender, HCV subtype, hepatitis activity, fibrosis, steatosis, portal lymphatic aggregates, and bile duct damage, absolute frequencies and percentages were determined.  $\chi^2$  tests were applied to investigate the association of these parameters with the genotype or minor allele frequency (MAF).

To avoid bias, data were also stratified by age (< 44 years, and ≥ 44 years), and parameters which showed significant correlations to the genotype in the univariate analysis were also analyzed using multivariate logistic regression. The results of stratifying and logistic regression were noted in the text and/or the tables where necessary.

All tests were performed two-sided and the level of significance was set at 0.05. The test results were interpreted in an exploratory way because no alpha adjustment for multiple testing was carried out. Statistical analyses were performed with the assistance of Medistat GmbH, Kiel, Germany, using PASW 17 for Windows (SPSS Inc., Chicago, IL).

## RESULTS

### Epidemiological characteristics

A total of 137 and 349 patients with chronic hepatitis C (UMG and Hep-Net groups, respectively) were genotyped for the bi-allelic SNP within the *CD14* gene,

rs2569190/C-159T. The variant allele T in the first group was found to be about as frequent as the wild-type C allele leading to a CC:CT:TT genotype distribution of 30:70:37 and a T allele frequency of 0.526 (Table 1). Hep-Net patients followed a distribution of 109:170:70 leading to a lower MAF of 0.444 (Table 2). MAFs were close to that given for Caucasians in public databases. The genotype distribution in both groups followed the Hardy-Weinberg equilibrium (Tables 1 and 2, respectively).

No significant difference was found between UMG and Hep-Net patients' regarding gender and age ( $\chi^2$  test,  $P = 0.781$ , independent samples *t*-test,  $P = 0.177$ , respectively). Epidemiological analysis revealed no significant relationships between the patients' CD14 rs2569190 genotype and gender (Tables 1 and 2). However, when analyzing patients' age with regard to the studied SNP genotypes, the UMG patients homozygous for the variant allele T were found to be on average 6.1 years younger than C carrier patients (mean age,  $45.7 \pm 11.5$  years) at the time of liver biopsy taken before the start of therapy (Table 1). This observation, however, was absent in Hep-Net patients (Table 2).

Similar to the European population, most UMG patients were infected with HCV subtype 1b, followed by 1a and 3a. No significant difference was found between the distribution of HCV type 1 and non-type 1 infections among the three SNP genotypes (Table 1).

### Biochemical parameters

Before the start of therapy, AST, ALT, and  $\gamma$ -GT serum activities were recorded for UMG patients as indicators of liver injury in chronic hepatitis C. The median levels of AST and ALT showed an increase from the wild-type to the variant type (Table 3). The number of TT patients with markedly elevated serum ALT activities, i.e. greater than two-fold the upper normal limit, was found to be markedly higher than the number of TT patients with normal ALT activities yielding a slightly increased T allele frequency among patients with markedly elevated ALT ( $\chi^2$  test,  $P = 0.044$ ) (Table 3). However, after stratification by age, this was no longer significant (data not shown).

**Table 2** Epidemiological characteristics of chronic hepatitis C patients with regard to CD14 rs2569190 genotype (Hep-Net group)

	n	CD14 rs2569190 genotype			MAF	P
		CC	CT	TT		
Total number (%)	349	109 (31.2)	170 (48.7)	70 (20.1)	0.828 <sup>1</sup>	0.444
Gender (Female/male)	148/201	47/62	66/104	35/35	0.151 <sup>a</sup>	0.459/0.433
Age (mean ± SD)		47.2 ± 13.5	44.6 ± 13.6	46.5 ± 13.3	0.616 <sup>b</sup>	0.484 <sup>2</sup>

<sup>1</sup>Exact test for the Hardy-Weinberg equilibrium; <sup>2</sup>χ<sup>2</sup> test was applied; <sup>a</sup>C carriers vs TT (χ<sup>2</sup> test); <sup>b</sup>C carriers vs TT (independent samples t-test).

**Table 3** Biochemical serum parameters and the number of patients with elevated parameters in chronic hepatitis C with regard to CD14 rs2569190 genotype (UMG group)

	CD14 rs2569190 genotype			MAF	²P
	CC	CT	TT		
AST (median, IQR)	21.0, 15.8-50.5	28.5, 17.0-52.3	32.0, 16.5-76.5		
<sup>1</sup> Number of patients with elevated/normal AST	9/21	29/41	19/18	0.159 <sup>a</sup>	0.588/0.481
ALT (median, IQR)	36.0, 25.8-85.5	46.5, 26.8-87.3	50.0, 34.5-153.5		
<sup>1</sup> Number of patients with elevated/normal ALT	11/19	38/32	23/14	0.171 <sup>a</sup>	0.583/0.462
γ-GT (median, IQR)	35.5, 13.8-58.8	27.0, 14.0-56.3	32.0, 15.5-53.5		
<sup>1</sup> Number of patients with elevated/normal γ-GT	10/20	19/51	11/26	0.934 <sup>a</sup>	0.513/0.531

AST: Aspartate aminotransferase. Upper normal limit is 19 U/mL for males, and 15 U/mL for females; ALT: Alanine aminotransferase. Upper normal limit is 23 U/mL for males, and 19 U/mL for females; γ-GT: γ-glutamyltransferase. Upper normal limit is 28 U/mL for males, and 18 U/mL for females; IQR: Inter-quartile range. <sup>1</sup>Markedly elevated serum activities of transaminases (> two-fold the upper normal limit) were considered; <sup>2</sup>χ<sup>2</sup> test was applied; <sup>a</sup>C carriers vs TT. After stratification by age, all P-values were non-significant.

### Hepatitis C disease activity and progression

Liver biopsy specimens were taken before the start of an interferon-based therapy and evaluated histologically. Hep-Net patients had higher frequencies of advanced degrees of hepatitis activity and fibrosis progression (Tables 4 and 5) (χ<sup>2</sup> test, P < 0.001 for both parameters). Both UMG and Hep-Net patients showed no correlation between their CD14 C-159T genotype and hepatitis activity or fibrosis (Tables 4 and 5, respectively). Other lesions typical and more characteristic of hepatitis C, namely the degree of hepatic steatosis, the presence or absence of lymphoid aggregates and bile duct damage, were additionally studied in UMG patients. With regard to the degree of steatosis and bile duct damage, no significant association with CD14 C-159T genotype distribution could be found (Table 4). T allele homozygous patients, however, were found to have portal lymphoid aggregates more frequently than C carriers (21/37 vs 32/100, respectively, χ<sup>2</sup> test, P = 0.008) (Table 4).

To avoid spurious findings, a separate analysis was carried out to identify other factors which might underlie the formation of portal lymphoid aggregates. No relationship was found between the presence or absence of portal lymphoid aggregates and sex, age, HCV subtype, biochemical parameters, the stage of fibrosis, or the degree of steatosis (logistic regression analysis, data not shown). In accordance with previous studies, however, a significant relationship between the presence of portal lymphoid aggregates and hepatic inflammatory activity (χ<sup>2</sup> test, P = 0.003) and bile duct damage (χ<sup>2</sup> test, P < 0.001) was found<sup>[3,5,27]</sup>. Nevertheless, even in the subgroups which had portal lymphoid aggregates with other lesions, a shift towards the T allele was always observed: 15 TT and 13 CT among the 32 patients who

had both a high grade of hepatitis activity and portal lymphoid aggregates: (MAF = 0.672 compared to 0.481 for the remaining patients); 13 TT and 17 CT among the 35 patients who presented with both bile duct damage and portal lymphoid aggregates: (MAF = 0.614 compared to 0.495 for the others) (data not shown).

### DISCUSSION

Among chronic hepatitis C patients, no evidence was found for a relationship between the endotoxin receptor CD14 rs2569190/C-159T genotype and the progression of liver fibrosis. This finding was obtained by analyzing two different patient cohorts, one derived from the UMG (n = 137) (Table 4), the other from the Hep-Net, a Germany-wide collection of samples (n = 349) (Table 5). It is in line with previous findings on Caucasian patients<sup>[20,22]</sup>.

By considering the sum of the evidence, the situation of chronic liver disease resulting from HCV infection appears to be different from the situation of chronic liver disease resulting from alcohol consumption: whereas in ALD, progression of fibrosis was shown to be associated with TT genotypes<sup>[18-20]</sup>, in chronic hepatitis C it did not appear to be related to this genetic variation.

Of note, a recent report found the influence of an environmental factor on the association of rs2569190/C-159T and total serum IgE levels in Russian children<sup>[28]</sup>. Depending on the *Helicobacter pylori* (*H pylori*) infection status, seronegative or seropositive, the T allele was associated with a decreased or an increased IgE serum concentration, respectively<sup>[28]</sup>. The observation of the lack of an association of rs2569190/C-159T genotype with chronic hepatitis C disease progression, but an

**Table 4** Histological features in chronic hepatitis C-infected patients with regard to CD14 rs2569190 genotype (UMG group) n (%)

Histological features	CD14 rs2569190 genotype			MAF	^P
	CC	CT	TT		
Hepatitis activity					
Mild	19 (25.0)	40 (52.6)	17 (22.4)		0.487
Moderate	10 (19.2)	24 (46.2)	18 (34.6)	0.172 <sup>b</sup>	0.152
Severe	1 (11.1)	6 (66.7)	2 (22.2)		
Fibrosis					
Absent	7 (38.9)	6 (33.3)	5 (27.8)		0.517
Mild	14 (20.6)	35 (51.5)	19 (27.9)		
Moderate	5 (19.2)	15 (57.7)	6 (23.1)	0.758 <sup>b</sup>	0.727
Marked	3 (21.4)	7 (50.0)	4 (28.6)		0.539
Cirrhosis	1 (9.1)	7 (63.6)	3 (27.3)		
Steatosis					
Absent	14 (23.3)	28 (46.7)	18 (30.0)		0.528
Mild	10 (20.8)	26 (54.2)	12 (25.0)		
Moderate	5 (26.3)	10 (52.6)	4 (21.1)	0.695 <sup>b</sup>	0.887
Marked	1 (10.0)	6 (60.0)	3 (30.0)		0.517
Portal lymphoid aggregates					
Absent	22 (26.2)	46 (54.8)	16 (19.1)		0.464
Present	8 (15.1)	24 (45.3)	21 (39.6)	0.008 <sup>b</sup>	0.623
Bile duct damage					
Absent	21 (23.6)	46 (51.7)	22 (24.7)		0.506
Present	9 (18.8)	24 (50.0)	15 (31.3)	0.411 <sup>b</sup>	0.563

<sup>a</sup>χ<sup>2</sup> test was applied to compare mild vs moderate and severe hepatitis activity, absent, mild vs moderate and marked fibrosis and cirrhosis, and absent, mild vs moderate and marked steatosis; <sup>b</sup>C carriers vs TT.

**Table 5** Hepatitis activity (grading) and fibrosis (staging) in chronic hepatitis C-infected patients with regard to CD14 rs2569190 genotype (Hep-Net group) n (%)

	CD14 rs2569190 genotype			MAF	^P
	CC	CT	TT		
Hepatitis activity					
Mild	19 (27.1)	35 (50.0)	16 (22.9)		0.479
Moderate	73 (32.2)	113 (49.8)	41 (18.1)	0.513 <sup>b</sup>	0.359
Severe	17 (32.7)	22 (42.3)	13 (25.0)		
Fibrosis					
Absent	4 (57.1)	2 (28.6)	1 (14.3)		0.449
Mild	35 (28.9)	61 (50.4)	25 (20.7)		
Moderate	44 (32.8)	63 (47.0)	27 (20.2)	0.928 <sup>b</sup>	0.837
Marked	18 (27.7)	37 (56.9)	10 (15.4)		0.441
Cirrhosis	8 (36.4)	7 (31.8)	7 (31.8)		

<sup>a</sup>χ<sup>2</sup> test was applied to compare mild vs moderate and severe hepatitis activity, absent, mild vs moderate and marked fibrosis and cirrhosis; <sup>b</sup>C carriers vs TT.

association with ALD and the dependency of the association on *H pylori* infection status for an atopy-related parameter, suggests that LPS sensitivity depends on both genetic and environmental factors (gene-environment interaction).

The lack of an association between this CD14 SNP and liver disease progression in chronic hepatitis C patients, is also in accordance with a finding by Huang *et al*<sup>[29]</sup> who, by functional genomic scanning, identified seven SNPs within seven genes which carry the most relevant risk for developing cirrhosis in Caucasian hepatitis C patients, with CD14 not being among them.

In contrast, a significantly higher frequency of rs2569190 T homozygote hepatitis C patients was found among patients with portal lymphoid aggregates (Table 4). As with lymphocyte infiltrations, primary follicles and secondary follicles in the lymph nodes, portal lymphoid aggregates have been described to

occur in various patterns ranging from vague lymphoid aggregation to round defined follicles to well-formed follicles with clearly identifiable germinal centers<sup>[30]</sup>. Their presence has been attributed to the participation of the host's immune system in liver disease pathogenesis<sup>[3,5]</sup>. To our knowledge, even today, the etiology and relevance of this manifestation for disease development and/or progression remains unclear. The presence of portal lymphoid aggregates was the only histological manifestation found to be related to CD14 rs2569190/C-159T genotypes (Table 4). As reported in other studies, the presence of portal lymphoid aggregates was found to be closely correlated with bile duct damage and the degree of inflammatory activity<sup>[3,5,27]</sup>, but not with sex, age<sup>[3,4]</sup>, or HCV subtype<sup>[4]</sup>. The finding of a correlation between portal lymphoid aggregates and HCV subtype 1b infection in Chinese<sup>[3]</sup> but not European patients is obviously related to a different

HCV subtype distribution. The comparison between subtype 1b- and type 2-infected patients, as is possible among Chinese patients, cannot be carried out among European patients because of the low proportion of type 2 infections. Portal lymphoid aggregates have also usually been found in the early stages of liver disease and to have disappeared in cirrhosis<sup>[27,31]</sup>. Thus, our finding of a positive association of TT status and the presence of portal lymphoid aggregates, on the one hand, and the absence of an association with disease progression, on the other hand, are concordant with that observation.

The relationship between TT genotype and younger age in the UMG group (Table 1) was neither observed among the patients from the Hep-Net group (Table 2) nor among patients from another German study<sup>[20]</sup>. In contrast to what might have been expected, these three cohorts do differ in demographic and clinical features. For instance, as outlined, the Hep-Net group comprised a higher proportion of patients with greater degrees of hepatic inflammatory activity and also more advanced stages of fibrosis than the UMG group (Tables 4 and 5). The cohort studied by Meiler *et al*<sup>[20]</sup> was found to differ significantly in age from the Hep-Net group and both in age and gender distribution from the UMG group (data not shown). Von Hahn's cohort was similar to the UMG and Hep-Net groups with regard to gender distribution, however, further demographic analysis, i.e. association of genotypes and age, was not given<sup>[22]</sup>.

In conclusion, in contrast to what was reported for ALD, our analyses did not reveal an increased risk for chronic hepatitis C patients, homozygous for the CD14 C-159T T allele, to develop more pronounced fibrosis but suggested a relationship of this variation in the formation of portal lymphoid aggregates.

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## COMMENTS

### Background

Progressive hepatic fibrosis develops in patients with chronic liver diseases irrespective of etiology and with a marked inter-individual variability. Different host genetic backgrounds have been shown to act as co-factors in promoting ongoing liver disease progression exacerbated by gut-derived bacterial lipopolysaccharides (endotoxins).

### Research frontiers

The variant allele T of a well-known single nucleotide polymorphism (SNP) in

the endotoxin receptor CD14 gene has been reported to be associated with increased alcohol-related liver cirrhosis. In this study, the authors show that the T allele is correlated with the presence of portal lymphoid aggregates rather than being associated with fibrosis progression in chronic hepatitis C virus (HCV) infection.

### Innovations and breakthroughs

Recent reports have shown a lack of an association between the T allele and liver fibrosis progression in the context of chronic HCV infection. Genetic association studies, however, require to be replicated. Apart from performing an analysis in two different cohorts, this study is the first to expand the analysis to further histological lesions typical of HCV infection with regard to CD14 rs2569190/C-159T.

### Applications

Understanding the mechanisms underlying chronic hepatocellular injury in hepatitis C is important for therapy applications. This study argues for a possible relationship of CD14 rs2569190 T allele in the formation of portal lymphoid aggregates, the presence of which has been attributed to the host's immunological participation in liver disease pathogenesis. Moreover, it excludes a possible role of the variation in promoting fibrosis progression.

### Terminology

Liver fibrosis is the excessive accumulation of extracellular matrix proteins including collagen. Cirrhosis is the last stage of fibrosis. A SNP is a variation of one base in the DNA; the nucleotide observed is different from the norm at this position. It occurs with a frequency of > 1% in the normal population. Portal lymphoid aggregates are defined as a densely packed collection of small lymphocytes within the portal tract without or with the formation of a germinal center. Lipopolysaccharide is the main component of the outer cell wall of gram-negative bacteria.

### Peer review

This is a valuable population-based association study, which is useful for examining a well-known genetic variation with a role in a common multifactorial disease that may have a strong environmental component. In contrast to the situation in alcoholic liver cirrhosis, the SNP rs2569190/C-159T is not related to chronic HCV-induced fibrosis progression, but to another histological feature, namely, the presence of portal lymphoid aggregates.

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BRIEF ARTICLES

## Anti-microbial antibodies in celiac disease: Trick or treat?

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Saccharomyces cerevisiae (gASCA), anti-laminaribioside (ALCA), anti-chitobioside, anti-mannobioside, anti-OMP antibodies and major NOD2/CARD15 mutations. Thirty out of 82 CD patients enrolled at the time of diagnosis were re-evaluated for the same antibodies after longstanding gluten-free diet (GFD).

**RESULTS:** 65.9% of the CD patients were positive for at least one of the tested antibodies at the time of the diagnosis. Except anti-OMP and ALCA, anti-microbial antibodies were exclusively seen in untreated CD; however, the overall sensitivity was low. Any glycan positivity (LR+: 3.13; 95% CI: 2.08-4.73) was associated with an increased likelihood ratio for diagnosing CD. Significant correlation was found between the levels of anti-glycan and anti-endomysial or anti-transglutaminase antibodies. Anti-glycan positivity was lost after longstanding GFD. Anti-glycan antibody titers were associated with symptoms at presentation, but not the presence of NOD2/CARD15 mutations. Patients with severe malabsorption more frequently had multiple antibodies at diagnosis ( $P = 0.019$ ).

**CONCLUSION:** The presence of anti-glycan antibodies in CD seems to be secondary to the impaired small bowel mucosa which can lead to increased antigen presentation. Furthermore, anti-glycan positivity may be considered an additional marker of CD and dietary adherence.

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**Key words:** Celiac disease; Glycans; Anti-Saccharomyces cerevisiae antibodies; Anti-outer membrane protein antibody; NOD2/CARD15; Gluten-free diet; Presenting symptoms; Bacterial translocation; Crohn's disease

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### Abstract

**AIM:** To determine the prevalence of a new set of anti-glycan and anti-outer membrane protein (anti-OMP) antibodies in a Hungarian cohort of adult Celiac disease (CD) patients.

**METHODS:** 190 consecutive CD patients [M/F: 71/119, age: 39.9 (SD: 14.1) years], 100 healthy, and 48 gastrointestinal controls were tested for glycan anti-

## INTRODUCTION

Celiac disease (CD) is a genetically determined chronic inflammatory disorder with autoimmune components induced by exposure to gluten<sup>[1]</sup>. The clinical presentation of the disease is highly variable and little is known about the factors that determine the type of symptoms. The inflamed and damaged small bowel mucosa as well as the clinical symptoms shows recovery in most affected subjects after a complete removal of gluten from their diet<sup>[2]</sup>. The presence of different autoantibodies is a typical feature of CD; however, the exact mechanism behind their production and potential role in the disease pathogenesis has not yet been fully understood<sup>[3]</sup>. Tissue transglutaminase 2 (TG2) is the main autoantigen of anti-endomysial antibodies (EMA) and anti-TG2/EMA are commonly used for screening and diagnosing CD<sup>[4]</sup>. More recently antibodies directed against synthetic deamidated gliadin peptides have also been suggested as a reliable tool for diagnosing CD<sup>[5]</sup>. Most of the other antibodies do not appear to have either high sensitivity or specificity for CD, but they may be associated with specific clinical presentations or extra-intestinal manifestations<sup>[6]</sup>. As a sign of the cytoskeleton and intercellular tight junction involvement, a high prevalence of IgA anti-actin antibodies was also reported which strongly correlated with the degree of villous atrophy, appearing in more severe forms of the disease<sup>[7]</sup>. Furthermore, production of anti-actin antibodies was gluten dependent. After strict adherence to gluten-free diet (GFD), they become undetectable within several months indicating that their appearance follows mucosal injury<sup>[8,9]</sup>. Similarly, also the occurrence of anti-zonulin antibodies was associated with active CD and disappearing during remission<sup>[10]</sup>.

The presence of serological responses to various microbial antigens [e.g. phosphopeptidomannan cell-wall component of anti-Saccharomyces cerevisiae (ASCA), outer membrane porin C transport protein of the *Escherichia coli* (OmpC) or the *Pseudomonas fluorescens* associated protein I2] is characteristic of Crohn's disease and has been intensively studied for its clinical value in this patient group<sup>[11]</sup>. The occurrence and magnitude of the seroreactivity are associated with complicated small bowel disease and the need for surgical intervention<sup>[12,13]</sup>. More recently, antibody formations against different glycans, which are common structures in the glycocalyx of pathogenic yeast and bacteria<sup>[14]</sup>, have also been reported in this patient group. Our group demonstrated that with the use of anti-glycan (g)ASCA and a panel of novel anti-glycan antibodies [anti-mannobioside (AMCA), anti-laminaribioside (ALCA), and anti-chitobioside (ACCA)], gave the same conclusions. Additionally, we evaluated the performance of all four anti-glycan antibodies and the traditional combination of ASCA IgG and IgA. Both panels equally identified 59.4% of all Crohn's disease patients. Eighty percent of these identified cases were the same patients while the remaining 10%-10% were detected by only one of the panels<sup>[15]</sup>.

Despite the fact that the exact mechanism behind antibody formation or their possible relevance in the

pathogenesis still needs to be elucidated, current data suggest that these markers are not an epiphenomenon related to the inflamed, leaky bowel mucosa<sup>[16-18]</sup> but reflect a loss of tolerance toward bacterial and fungal flora<sup>[19]</sup>. Furthermore, the anti-microbial antibodies might represent genetic susceptibility because patients who have positive antibodies often carry mutations in the NOD2/CARD15 gene<sup>[11,20,21]</sup>.

Anti-microbial antibody formation has also been reported in CD. ASCAs remain the most widely investigated antibodies<sup>[22-26]</sup> in this patient group but increasing experimental data are available on newly discovered antibodies such as anti-I2 or anti-OmpW (*Bacteroides caccae* TonB-linked outer membrane protein)<sup>[27,28]</sup>. The frequency of ASCA varied from 27% to 59% in various studies, owing to the differences in the number and the age of the patients as well as the commercial assays used for antibody detection. The frequency of seropositivity and serum levels of these antibodies clearly decreased during GFD.

We hypothesized that newly discovered inflammatory bowel disease (IBD)-associated antibodies (including anti-glycan antibodies and anti-OMP) may also be of importance in CD and aimed to determine the prevalence of these antibodies in a Hungarian cohort of adult CD patients in relation to clinical presentation, GFD and NOD2/CARD15 mutations.

## MATERIALS AND METHODS

### **Patients**

One-hundred and ninety consecutive, unrelated Hungarian adult patients with biopsy-proven CD (male/female: 71/119, mean age: 39.9 years, SD: 14.1) and 66 of their first degree relatives (siblings, mean age: 37.7 years, SD: 13.9) were investigated. The diagnosis of CD was based on small bowel biopsy showing severe villous atrophy with crypt hyperplasia (Marsh type III lesions)<sup>[29]</sup> and elevated serum levels of antibodies against transglutaminase (TGA) and endomysium (EMA). IgA and IgG class EMA were investigated on human umbilical cord substrate using indirect immunofluorescence method as previously described<sup>[30]</sup>. TGA were measured by enzyme-linked immunosorbent assay (ELISA) using human recombinant antigen expressed in *Escherichia coli*<sup>[31,32]</sup>.

Of the 190 patients, 82 patients' sera were obtained at the time of diagnosis (Group CD1) and in 30 of these 82 patients further serum samples were re-evaluated for the same antibodies after adherence to long-standing GFD. The median follow-up period between these blood samplings was 28.5 mo [interquartile range (IQR): 18-52]. In the 108 remaining cases the diagnosis of CD had been established prior to this study and they adopted a strict GFD. These 108 patients were divided into two separate groups according to their current TGA and EMA status and dietary compliance at the time of the sampling. Thirty-three patients still had positive EMA and TGA results (Group CD2) and the median duration was here 3.5 mo (IQR: 1-11). The adequate compliance was indicated by reduced antibody titers as compared to those at diagnosis. The remaining 75 patients had

negative EMA and normal TGA titers (Group CD3), median follow up: 21 mo (IQR: 6-85).

Detailed clinical data concerning the clinical presentation of CD at diagnosis were classified as follows: (1) severe generalized malabsorption (presence of at least four of the following five symptoms: diarrhea, abdominal distension, weight loss, anemia, hypoproteinemia); (2) non-specific gastrointestinal symptoms that did not compromise the general condition (diarrhea, constipation, bloating, recurrent abdominal pain or vomiting, esophageal reflux); (3) iron deficiency anemia without major gastrointestinal complaints; (4) dermatitis herpetiformis; (5) silent disease (population screening); (6) other (autoimmune diseases, reduced bone mineral density, liver disease, brain disease). Patients were assigned to one of these major presentation types in a prospective manner, based on clinical and routine laboratory results at diagnosis.

The control group consisted of 100 healthy, ethnically similar, blood donor individuals (male/female: 47/53, mean age: 36.6 years, SD: 9.1) who had normal findings on a thorough medical examination, blood pressure measurements, and routine laboratory tests. A second non-celiac gastrointestinal disease control group consisted of 48 patients with irritable bowel syndrome/diverticulosis without inflammation (male/female: 21/26, mean age 40.4 years, SD: 16.1). In controls, CD was excluded by the negativity of EMA and TGA. None of the control subjects had a family history of CD. Further comparisons were made with the previously published Crohn's disease cohort we investigated for the same antibodies earlier<sup>[15]</sup>.

The study protocol was approved by the Regional and Institutional Committee of Science and Research Ethics of the University of Debrecen (DEOEC RKEB/IKEB: 2600-2007). Informed consent was obtained from all patients and controls.

#### **Anti-microbial antibody assays**

gASCA IgG, AMCA IgG, ALCA IgG, ACCA IgA (IBDX®, Glycominds Ltd., Lod, Israel), ASCA IgG, ASCA IgA and OMP IgA (QUANTA Lite™ OMP PLUS, INOVA Diagnostics, San Diego, CA) were measured in sera according to the manufacturers' protocols. The results were presented as arbitrary units, which were calculated based on sample and calibrator optical density. Cut-off levels used for the determination of positivity were according to the manufacturers' guidelines: 50, 100, 60 and 90 U/mL for gASCA IgG, AMCA IgG, ALCA IgG, ACCA IgA, respectively, and 25 U/mL for ASCA IgG, ASCA IgA and OMP IgA.

#### **Detection of NOD2/CARD15 mutations**

One-hundred and thirty-four CD patients and 100 healthy control subjects were eligible for NOD2/CARD15 mutation analysis. Major NOD2/CARD15 mutations (SNP8, 12 and 13) were determined as previously described<sup>[13]</sup> by denaturing high-performance liquid chromatography (dHPLC, Wave DNA Fragment Analysis System, Transgenomic Limited, UK). Sequence variation, observed in the dHPLC profile, was sequenced on both

strands to confirm the alteration. Sequencing reactions were performed with the ABI BigDye Terminator Cycle Sequencing Kit v1.1 (Applied Biosystems, Foster City, CA) and samples were sequenced on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

#### **Statistical analysis**

Variables were tested for normality with Shapiro Wilk's  $W$  test.  $t$ -test with separate variance estimates,  $\chi^2$ -test,  $\chi^2$ -test with Yates correction and likelihood ratio (LR) test were calculated to evaluate differences between various groups of CD patients and controls, as well as within subgroups of CD patients, as appropriate. Sensitivities, specificities, positive and negative likelihood ratios were calculated to determine the predictive power of gASCA, AMCA, ALCA, ACCA, OMP or the combination of these markers in distinguishing between CD and controls. Spearman's rank order correlation was calculated to test the association between anti-glycan/OMP and TGA levels. Two-sided  $t$ -test for independent samples with separate variance estimates and ANOVA with post hoc Scheffe test were used to analyze the association between anti-glycan antibody titers and clinical symptoms at diagnosis. A  $P$  value of  $< 0.05$  was considered as significant. For statistical analysis, SPSS15.0 (SPSS Inc, Chicago, IL) was used with the help of a statistician (Dr. Peter Vargha).

## **RESULTS**

#### **Presence of anti-glycan and anti-OMP antibodies at the time of diagnosis of celiac disease**

The frequency and the mean titers of gASCA IgG, AMCA IgG, and ACCA IgA were significantly higher at the time of diagnosis of CD than in healthy and non-celiac gastrointestinal control groups (Table 1, Figure 1). However, the frequency of ALCA IgG and anti-OMP IgA positivity and also the mean titers in the patients were similar to those in control groups. No difference was found between healthy subjects and GI controls based on the presence of these antibodies. For that reason, we only used the healthy subjects as a control group in the subsequent comparisons.

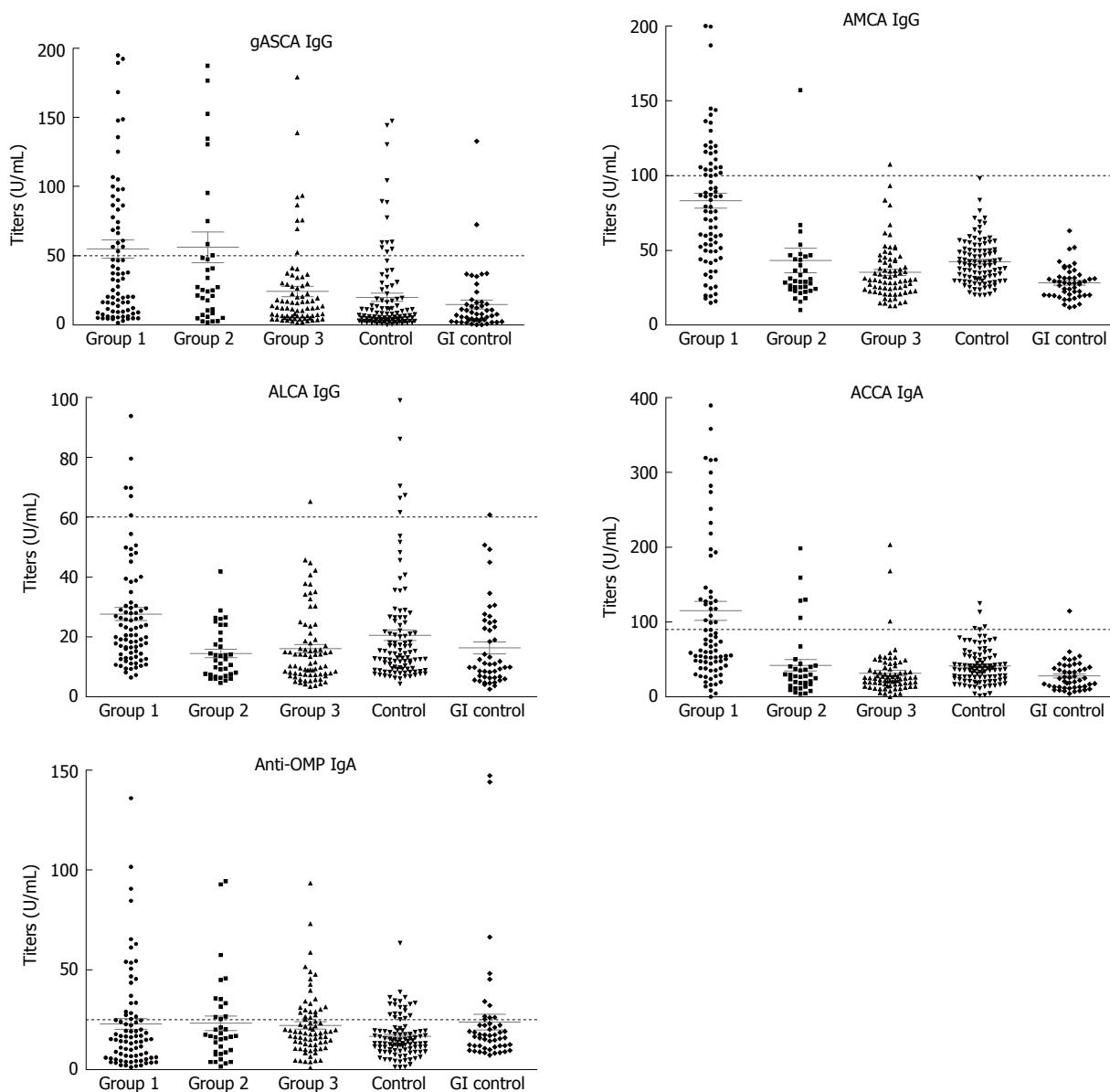
When calculating the sensitivity and specificity of the different markers based on the cut-off values suggested by the manufacturer, 65.9% of the CD patients were positive for at least one of the tested anti-microbial antibodies at the time of diagnosis. Except ALCA, all anti-glycan antibodies were specific for untreated CD. However, the overall sensitivity was low (gASCA: 39.0%, AMCA: 35.4%, ACCA: 37.8%). The above association was further tested by using the LR test. The sensitivity, specificity, positive and negative LR between CD at diagnosis and controls are presented in Table 2. Compared to healthy controls, gASCA, AMCA, and ACCA were associated with a moderate increase in the likelihood of CD, respectively. The positivity of any anti-glycan antibody significantly increased the likelihood for untreated CD (Table 2).

Detailed clinical data on the symptoms at the time

**Table 1 Frequency of anti-microbial antibodies in 190 patients with celiac disease and in control groups n (%)**

	Group 1	Group 2	Group 3	Control	GI control
n	82	33	75	100	48
TGA IgA (U/mL) (median; IQR)	54.3 (20.2-100.0)	32.2 (9.9-73.1)	2.7 (2.2-3.7)	-	-
gASCA IgG positive	32 (39.0) <sup>b,f,j</sup>	12 (36.3) <sup>d,i,l</sup>	10 (13.3)	14 (14)	2 (4.2)
AMCA IgG positive	29 (35.4) <sup>b,f,j,n</sup>	3 (9.1)	1 (1.3)	0 (0)	0 (0)
ALCA IgG positive	7 (8.5)	0 (0)	0 (0)	6 (6)	1 (2.1)
ACCA IgA positive	31 (37.8) <sup>b,f,j,n</sup>	4 (12.1)	3 (4.2)	6 (6)	1 (2.1)
Any glycan positive	54 (65.9) <sup>b,f,j,p</sup>	15 (45.4) <sup>d,f,l</sup>	15 (20)	21 (21)	4 (8.4)
Anti-OMP IgA positive	22 (26.8)	11 (33.3)	23 (30.7)	20 (20)	11 (22.9)

TGA: Antibodies against transglutaminase; Group 1: Celiac patients at the time of diagnosis; Group 2: Celiac patients after starting a gluten-free diet but still with celiac antibody positivity; Group 3: Celiac patients on a long-term strict gluten-free diet; Control: Healthy control; GI control: Non-celiac gastrointestinal disease control. Cut-off levels used for the determination of positivity were according to the manufacturers' guidelines: 50, 100, 60, 90 U/mL and 25 U/mL for gASCA IgG, AMCA IgG, ALCA IgG, ACCA IgA, and anti-OMP IgA, respectively. <sup>b</sup>P < 0.001, <sup>d</sup>P < 0.01, group 1 or Group 2 vs control; <sup>f</sup>P < 0.001, group 1 or Group 2 vs GI control; <sup>j</sup>P < 0.001, <sup>i</sup>P < 0.01, group 1 or Group 2 vs Group 3; <sup>n</sup>P < 0.001, <sup>p</sup>P < 0.03, group 1 vs Group 2. Using  $\chi^2$ -test with Yates correction.



**Figure 1 Anti-microbial antibody levels in 190 patients with celiac disease and in control groups.** Individual values are shown by black spots. Mean values with standard error bars are indicated in gray. Cut-off values for positivity are pointed out by dotted line and 50, 100, 60, 90 and 25 U/mL for gASCA IgG, AMCA IgG, ALCA IgG, ACCA IgA and OMP IgA, respectively.

**Table 2 Predictive power of serological markers for distinguishing between patients with celiac disease at the time of diagnosis and various control groups**

	Sensitivity (%)	Specificity (%)	95% CI	
			LR+	LR-
<b>Celiac disease vs healthy controls</b>				
gASCA	39	86	2.73 (1.57-4.76)	0.71 (0.59-0.86)
AMCA	35	100	-	-
ACCA	38	94	6.36 (2.76-14.4)	0.66 (0.56-0.79)
Any glycans	66	79	3.13 (2.08-4.73)	0.43 (0.31-0.59)
<b>Celiac disease vs non-celiac gastrointestinal controls</b>				
gASCA	39	96	9.36 (2.35-37.4)	0.64 (0.53-0.76)
AMCA	35	100	-	-
ACCA	38	98	18.1 (2.56-128.5)	0.64 (0.53-0.76)
Any glycans	66	92	7.90 (3.05-20.4)	0.37 (0.27-0.51)

**Table 4 Occurrence of multiple antibody responses to microbial antigens in untreated celiac disease patients in relation to the number of responses against microbial antigens n (%) (n = 78)**

	0	1	2 to 4	Total
Severe malabsorption	7 (22)	8 (25)	17 (53)	32 (40)
Non-specific gastrointestinal symptoms	13 (38)	12 (35)	9 (27)	34 (44)
Iron deficiency anemia	6 (67)	3 (33)	0	9 (12)
Others	2	0	1	3 (4)
Total	28 (36)	23 (29)	27 (35)	78 (100)

P = 0.019 by  $\chi^2$ -test. Clinical data were not available for 4 patients.

of diagnosis in Group CD1 was available in 78 patients out of 82. Of the 78 patients, 32 (41%) presented with severe malabsorption, 34 (43.6%) with non-specific or minor gastrointestinal symptoms, 9 (11.5%) with iron deficiency anaemia, and 3 (3.9%) with other symptoms. The titers of the anti-glycan antibodies varied according to the presenting symptoms (Table 3) by 2-sided *t*-test for independent samples with separate variance estimates. If the above association was tested by ANOVA and post hoc Scheffe-test only the association for gASCA ( $P = 0.027$ ) and AMCA ( $P = 0.03$ ) remained significant. Moreover, the clinical presentations of CD were distributed differently according to serological response (Figure 2, Table 4). Patients with severe malabsorption more frequently had multiple antibodies ( $P = 0.019$ ) while in those with non-specific gastrointestinal symptoms or iron deficiency anaemia no seroreactivity or reactivity against only one glycan components was more commonly seen (Table 4). Out of the CD patients with multiple antibodies positivity, 65.4% were diagnosed because of malabsorption, which was significantly higher than in CD patients with another serotype group (0 = 26.9%, or 1 = 34.8%,  $P = 0.019$ ) (Figure 2).

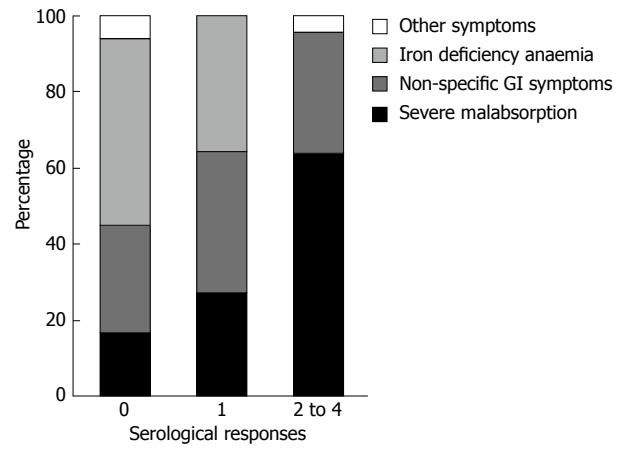
#### Correlation between anti-glycan and anti-OMP antibodies and TGA or EMA

A significant correlation was found between anti-glycan and TGA levels ( $P_{\text{gASCA}} < 0.001$ ,  $R = 0.39$ ;  $P_{\text{AMCA}} = 0.01$ ,  $R = 0.28$ ;  $P_{\text{ALCA}} = 0.006$ ,  $R = 0.23$ ;  $P_{\text{ACCA}} < 0.0001$ ,  $R =$

**Table 3 Association between the titer of anti-microbial antibodies and the leading clinical symptoms at the time of celiac disease presentation (in Group 1, n = 78)<sup>1</sup>**

	Malabsorption	Non-specific gastrointestinal	Anaemia
n	32	34	9
gASCA IgG	54.8 (16.4-99.4) <sup>b</sup>	21.3 (7.1-76.5)	12.2 (8.8-36.7)
AMCA IgG	90.3 (59.5-115.9) <sup>b</sup>	73.8 (52.3-104.4) <sup>a</sup>	44.1 (25.1-55.0)
ALCA IgG	20.6 (16.7-31.2) <sup>b</sup>	23.7 (16.5-39.1) <sup>a</sup>	12.8 (9.9-15.2)
ACCA IgA	103.8 (53.3-192.1) <sup>b</sup>	57.3 (37.4-100.5) <sup>a</sup>	26.8 (12.7-53.6)
OMP IgA	14.8 (5.2-33.2)	15.2 (8.2-24.2)	16.2 (2.9-34.1)

<sup>1</sup>Detailed clinical data were not available for 4 patients, data of 3 patients with other symptoms are not shown; <sup>a</sup> $P < 0.005$ , <sup>b</sup> $P < 0.01$ , between patients with non-specific gastrointestinal symptoms or malabsorption and anaemia by *t*-test for independent samples with separate variance estimates.

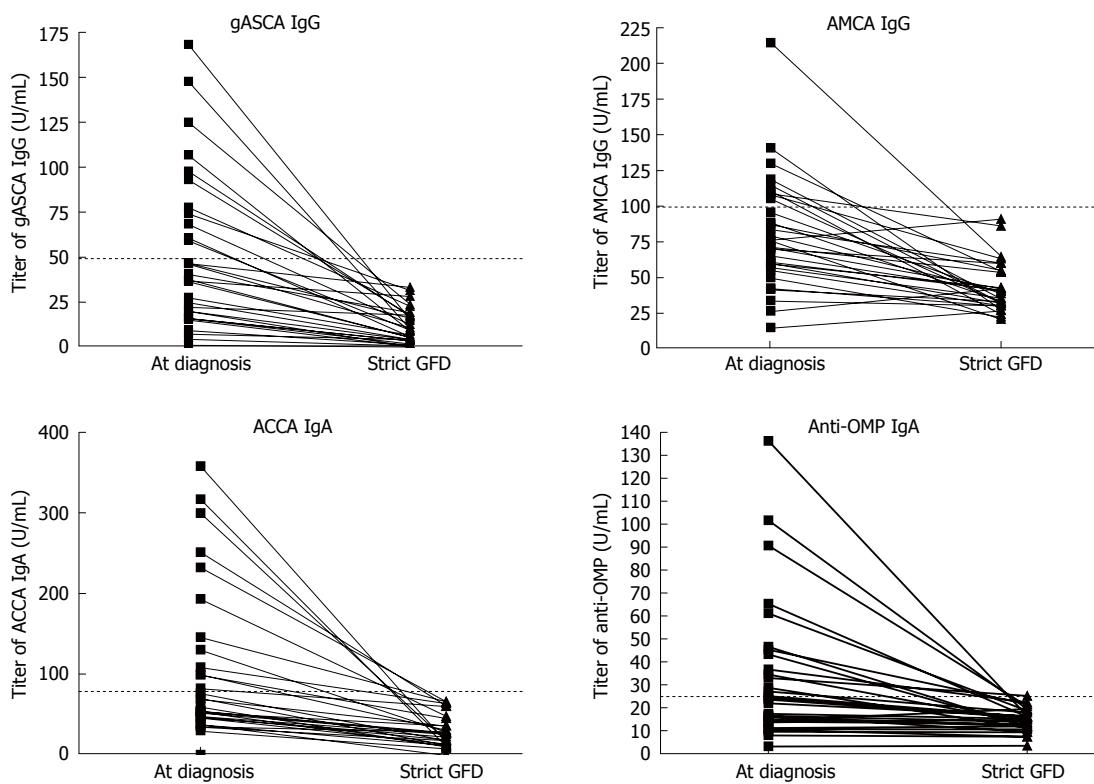
**Figure 2 Clinical presentation of celiac disease according to serological response.**

0.53;  $P_{\text{antiOMP}} = 0.001$ ,  $R = 0.25$  by Spearman's rank order correlation). Similarly, a positive association was found between EMA IgA and gASCA ( $P < 0.001$ ), AMCA ( $P < 0.001$ ), ACCA ( $P < 0.0001$ ), or any-glycan ( $P < 0.0001$ ) but not with anti-OMP positivity.

#### The effect of strict gluten-free diet on anti-glycan and anti-OMP antibody positivity

In the group of 30 patients who were evaluated both at diagnosis and following a long term GFD (subgroup of Group CD1), initial positivity for anti-glycan antibodies (gASCA in 12, AMCA in 9, and ACCA in 11 patients) observed at diagnosis was lost after GFD. The titer of each antibody decreased significantly after adherence to GFD ( $P < 0.001$  for each). Anti-OMP antibody positivity behaved similarly, with all but one of 14 patients positive at diagnosis becoming negative after GFD. The one patient who did not become negative during a 135 month-long period of GFD did in fact decrease during GFD from 33.2 to a borderline positive value of 25.4 units (a positive result is defined as  $\geq 25$  units).

The level of the different antibodies was also significantly lower after GFD ( $P < 0.001$  for each). Figure 3 shows individual anti-glycan and anti-OMP antibody titers at the time of diagnosis and their changes



**Figure 3** Individual anti-glycan and anti-OMP antibody titers at the time of the diagnosis and their variations after successful adherence of to the gluten-free diet (GFD). Mean follow up period of 49 [10-159] mo ( $n = 30$ ). Dotted lines show cut-off values for positivity.

after successful adherence of to the GFD.

The frequency of antibodies directed against glycans and the mean antibody titers were significantly lower in patients with successful adherence to GFD (Group CD3) than in untreated patients (Group CD1) (Table 2, Figure 1) and did not differ statistically from healthy controls.

#### Prevalence of NOD2/CARD15 mutations and their association with antibody titers and symptoms at presentation

The prevalence of NOD2/CARD15 mutation in CD (19/134, 14.2%) did not differ from that in the control group (16/100, 16%). Additionally, we did not observe any association between symptoms at presentation or anti-glycan antibody positivity and the presence of NOD2/CARD15 variants (data not shown).

#### DISCUSSION

This is the first report to investigate the complex associations between a panel of new serological markers, clinical presentation of the disease, and NOD2/CARD15 status in a relatively large cohort of CD patients. Furthermore, direct comparison between the anti-microbial responses in this CD group and our similarly tested previous Crohn's disease cohort<sup>[15]</sup> can be made to add new pieces to the puzzle of the anti-microbial antibody formation.

In this study, we demonstrated that the presence of anti-glycan antibodies (gASCA, ACCA, and AMCA) are associated with CD at the time of diagnosis. However

the prevalence of ALCA and anti-OMP did not differ from the results in the control group. The rate of gASCA positivity (39%) at the time of diagnosis of CD was comparable to the results in CD patients in previous studies<sup>[22-26]</sup>. Based on previous results a sample size of 42-66 celiac patients and controls would have been needed to confirm the above difference with an alpha error of 5% and a statistical power of 95%. In fact, in the present study, for celiac disease at diagnosis the alpha error was 3% and the statistical power 97%.

We could not concur with the findings of Candelli *et al*<sup>[26]</sup> and Barta *et al*<sup>[33]</sup>, which showed significant differences in the prevalence of ASCA IgG between CD and Crohn's diseases. In contrast, no significant difference was noted between the two groups (39% vs 50.5%,  $P = 0.091$ ). In the present study, except ALCA, the occurrence of other anti-glycan antibodies and their median titers in CD at diagnosis was also similar to those observed in Crohn's disease<sup>[13,34]</sup> (celiac disease gASCA: 33.1 U/mL, AMCA 79.3 U/mL, ALCA 21.5 U/mL, ACCA 68.4 U/mL vs Crohn's disease gASCA: 48.3 U/mL, AMCA 55.5 U/mL, ALCA 25.4 U/mL, ACCA 46.2 U/mL). In addition, the positivity rate for any anti-glycan antibody was also comparable in these patient groups (CD vs Crohn's disease: 65.9% vs 59.4%,  $P = \text{NS}$ ). In addition, sensitivity, specificity, positive and negative likelihood ratios in celiac disease are comparable to that observed in Crohn's disease. Consequently, in patients with gastrointestinal symptoms, the presence of gASCA, AMCA, or ACCA may not only suggest underlying Crohn's disease but may also be associated with untreated CD. At the same time, and based on our

results, ALCA and anti-OMP proved to be specific but relatively non-sensitive markers for Crohn's disease.

Current data advocate that in both CD and Crohn's disease patients have a primary defect in intestinal permeability that is also shared by a subgroup of relatives. In CD, it is also apparent that the exposure to gluten results in mucosal inflammation and the consequent tissue damage further abrogating the primary gut barrier defect, while gluten removal resolves the enhanced intestinal permeability<sup>[35,36]</sup>. These gliadin-induced mechanisms are proposed to be the cause of the anti-microbial antibodies formation in the disease and is strongly supported by the association found between anti-glycan markers and TGA or EMA in the present study and also that the antibody status is substantially altered following the introduction of GFD. gASCA and other positive anti-glycan antibodies were entirely lost in our cohort of CD patients, after strict adherence to long-term GFD. These results are concordant with previous findings<sup>[20,22]</sup>. In the study of Mallant-Hent *et al*<sup>[24]</sup>, ASCA IgG or IgA positivity disappeared in a substantial number but not the all of the 111 patients on a strict GFD (from 28.8% to 8.1%). A possible explanation for this difference could be that the mean follow up period after GFD was longer in our study [49 (10-159) mo vs 33 (range 3-113) mo]. These results suggest that as the period of strict GFD increases, so is the greater disappearance of antibody positivity, which will supposedly lead to entire mucosal healing in the small intestine. The higher prevalence of ASCA in adults compared to children further underlines the important role of long-lasting inflammation and consequently antigen exposure in the formation of anti-microbial antibodies.

In the present study, we also established that the kinetics of antibody disappearance is variably sensitive to the length of GFD. Of the anti-glycan antibodies, AMCA and ACCA declined most rapidly, right after the TGA titer started to diminish. In Group CD2, the prevalence of these antibodies had already changed as compared to Group CD1, from approximately 36% to 11%, while the frequency of gASCA and anti-OMP remained unchanged. Among those CD patients who adopted a strict GFD, the duration of GFD was the shortest in this group. In the group of patients with a successful response to GFD (Group CD3), the frequency of gASCA as well as AMCA and ACCA was also lower. At the same time, the overall frequency of anti-OMP did not change, either in group CD2 or in CD3 (Table 1). We showed however, that the level of anti-OMP clearly declined to normal in 13 of the 14 anti-OMP positive CD patients when specific patients in group CD1 were followed (Figure 2). The explanation of this supposedly inconsistency may be that the mean follow-up in both groups CD2 and CD3 was significantly shorter than in those in group CD1 participating in intra-individual longitudinal monitoring, suggesting that anti-OMP requires the longest time to disappear completely and this occurs long after the normalization of TGA and EMA. The differences in the evolution of anti-OMP and anti-glycan antibodies in IBD has also justified our findings in this patient group<sup>[15]</sup>.

We evaluated the possible relationship between sero-

logical response and the clinical presentation of the disease. Patients with multiple seroreactivity to glycans, more commonly presented with severe malabsorption as compared to those without any reactivity against any glycan at all (63% vs 22%,  $P = 0.019$ ), and accounted for 53% of all malabsorption cases. Among the patient groups, the TGA titer reached the highest value (115.9 U/mL vs others: 60.9 U/mL,  $P = 0.016$ ) in those presenting with malabsorption, further supporting enhanced intestinal permeability as a likely component involved in antibody formation. It is well known that the intestinal damage is most pronounced in the malabsorption cases and TGA is a good marker for tissue injury<sup>[37]</sup>. We must note however, that the number of subjects in different clinical presentation groups were limited, thus further studies with a larger cohort of CD patients are needed to confirm these findings.

Recent data suggest that the presence of anti-microbial antibodies might be linked to genetic susceptibility. In patients with Crohn's disease an association was found between antimicrobial formation and the carriage of mutations in innate immunity receptor genes (NOD2/CARD15 or toll-like receptor)<sup>[15,20]</sup>. However, in the absence of NOD2 variants in our Crohn's patients' cohort, the gASCA and the any-glycan positivity was also reasonably high (43.5% and 53.7%, respectively). Furthermore, among CD patients in the present study we found that these antibodies occur with the same frequency and magnitude as in patients with Crohn's disease, albeit the occurrence of NOD2/CARD15 mutation was significantly lower. These findings - alongside with the fact that there is no association between TLR4 variants and CD<sup>[38,39]</sup> - do not support the primary role of genetic predisposition in antibody formation. Nevertheless, the presence of NOD2/CARD15 was associated with an increased antibody formation in Crohn's disease and an apparent link was also reported between increased permeability and NOD2/CARD15 3020insC mutation<sup>[40]</sup>. We did not observe any association between anti-glycan antibody positivity and the presence of NOD2/CARD15 variants in CD. However, the limited number of subjects carrying NOD2/CARD15 mutations might not have allowed us to recognize significant differences in serological response in this patients group. An inheritable trait of anti-microbial antibody formation is unlikely in CD, since we did not find a higher prevalence of ASCA (9.1% vs 14%) and anti-OMP (12.1% vs 20%) as compared to the controls in the 66 unaffected, first-degree relatives (siblings) of this cohort.

On the basis of significant similarity in the qualitative and quantitative serological response in the two patients' groups, we hypothesize a similar mechanism for the formation of the anti-microbial antibody formation in both celiac disease and Crohn's disease. The presence of serological response might be the reflection of the sustained exposure to the constituent of the gut microflora due to the enhanced bacterial translocation. The known predisposing factors for bacterial translocation, such as bacterial overgrowth in the small bowel (secondary to intestinal dysmotility)<sup>[41-43]</sup>, the damage to the integrity of the gut

mucosa (secondary to alterations of the local intestinal microvasculature)<sup>[44,45]</sup>, which results in reduced oxygen delivery and an increased formation in oxygen radicals<sup>[46]</sup> as well as the upregulation of the proinflammatory cytokines, such as tumor necrosis factor  $\alpha$ , interleukin-17 or interferon gamma in active lesions<sup>[47]</sup>, and the defective mucosal immunological defense<sup>[21,48]</sup> are all typical features in both clinical conditions. The significance of the enhanced bacterial translocation out of the small bowel in the anti-microbial antibody formation is further supported by the fact that the presence of the serological response among patients with Crohn's disease is mainly characteristic for those with complicated (stricture or penetrating) small bowel involvement and is rarely observed in the isolated colonic disease or in patients with ulcerative colitis. At the same time, the recovered gut barrier function protects against the invasion of microbes or their components leading to the cessation of anti-microbial antibody formation. In CD, this process may be justified by the observation that the serological response is a temporary phenomenon. As a result of the discontinuation of gliadin exposure and the subsequent mucosal healing, the antibodies disappear completely. Confirming this aspect of our hypothesis is much more complicated in Crohn's disease. First of all, the pathogenetic processes are not only multifaceted but also less characterized as compared to CD. The complete elimination of the causative agents is not possible. Moreover, no such reliable serological markers are available reflecting the extent of gut inflammation as TGA and anti-actin IgA antibodies in CD. Finally, in terms of the complete loss of microbial seroreactivity, the long-lasting complete remission (without mucosal inflammation) is mandatory but rarely reached in patients with Crohn's disease as compared to CD patients adhering to a strict GFD. In this point of view, findings reporting a lack of solid correlation between disease activity and the presence or the magnitude of seroreactivity<sup>[17,49]</sup> in Crohn's disease can not be in opposition to our hypothesis any more. The advent of the new biological treatments might answer this unresolved question, since the complete mucosal healing in Crohn's disease can be achieved with this therapy in a greater proportion of cases than with classical drugs. At this moment, however, no data from prospective studies are available addressing the effect of the biological therapy on antibody stability. Our data also call for additional basic research to explore the exact mechanism of immune responses to commensal enteric bacteria as well as the possible clinical significance of the bacterial translocation in the pathogenesis or the complications of these diseases as it is well established in other clinical conditions such as liver cirrhosis, acute pancreatitis or sepsis<sup>[50]</sup>.

In conclusion, our results suggest that ASCA and other anti-glycan antibodies may be considered as additional markers for CD and adherence to a GFD. Furthermore, the presence and the magnitude of response to microbial components is associated with a more severe clinical course but not with mutations in NOD2/CARD15. This seroreactivity may be the consequence of the enhanced bacterial translocation through the impaired small bowel mucosa.

## COMMENTS

### Background

Anti-microbial antibody formation has been reported in celiac disease. Relatively high positivity rates were observed for the conventional antibodies, for example, anti-Saccharomyces cerevisiae (ASCA), anti-OmpW, and anti-I2, and they were known to decrease after a successful gluten free diet.

### Research frontiers

Newly discovered inflammatory bowel disease-associated antibodies (including anti-glycan antibodies and anti-OMP) may also be of importance in celiac disease, however, not studied thus far in the published literature. The presence of anti-microbial antibodies in relation to clinical presentation of the disease and NOD2/CARD15 mutations was also not investigated.

### Innovations and breakthroughs

Anti-glycan antibody positivity is a common feature of celiac disease at the time of diagnosis and is lost after long-term gluten-free diet. The positivity rate and titers at diagnosis are as high as observed in Crohn's disease. The presence of anti-glycan antibodies is associated with the presenting symptoms, especially with severe malabsorption but not with mutations in NOD2/CARD15. We did not find a higher prevalence of anti-microbial antibodies in the unaffected, first-degree relatives of this patient cohort.

### Applications

The data may add new pieces to the puzzle of the anti-microbial antibody formation and also assist to re-evaluate recently proposed mechanisms. Serological response to various microbial antigens might be considered a universal marker of the enhanced translocation of the gut microflora through the impaired small bowel mucosa both in celiac and Crohn's disease patients.

### Terminology

Serology markers: anti-endomysial antibodies, synthetic deamidated gliadin peptides, antibodies against microbial antigens such as cell wall component of *Saccharomyces cerevisiae*, outer membrane porin C transport protein of the *Escherichia coli* (OmpC) or the *Pseudomonas fluorescens* associated protein (I2). anti-glycan antibodies: glycan-ASCA (gASCA), anti-mannobioside (AMCA), anti-laminaribioside (ALCA), anti-chitobioside (ACCA).

### Peer review

Papp et al studied the prevalence of antimicrobial antibodies in celiac disease patients. The most relevant finding is that anti-glycan antibody titers were associated with symptoms at presentation and their positivity was lost after longstanding gluten free-diet as well as patients with multiple anti-glycan antibodies at diagnosis had more frequently severe malabsorption.

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## Different patterns of intestinal response to injury after arterial, venous or arteriovenous occlusion in rats

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### Abstract

**AIM:** To investigate the differences in injury patterns caused by arterial, venous or arteriovenous mesenteric occlusion.

**METHODS:** Male Wistar rats were separated equally into four groups. Occlusion was performed by clamping the superior mesenteric artery (A), the mesenteric vein (V) or both (AV) for 30 min, followed by 60 min of reperfusion. A control group received sham surgery only. Intestinal sections were examined for histological damage and serum tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), endothelin-1 (ET-1), P-selectin, antithrombin III (ATIII) and soluble intracellular adhesion molecule-1 (ICAM-1) concentrations were measured.

**RESULTS:** All groups showed significant mucosal injury compared to controls. Furthermore, mucosal injury was significantly more severe in the V and AV groups

compared to the A group ( $3.6 \pm 0.55$ ,  $3.4 \pm 0.55$  and  $2 \pm 0.71$ , respectively,  $P = 0.01$ ). ICAM-1 was similarly elevated in all groups, with no significant differences between the groups. P-selectin levels were significantly elevated in the V and AV groups but not the A group ( $1.4 \pm 0.5$  ng/mL,  $2.52 \pm 0.9$  ng/mL and  $0.02 \pm 0.01$  ng/mL, respectively,  $P = 0.01$ ) and ET-1 was significantly elevated in the A and V groups but not the AV group ( $0.32 \pm 0.04$  pg/mL,  $0.36 \pm 0.05$  pg/mL and  $0.29 \pm 0.03$  pg/mL, respectively,  $P = 0.01$ ) compared to sham controls. ATIII levels were markedly depleted in the V and AV groups, but not in the A group ( $29.1 \pm 5.2$  pg/mL,  $31.4 \pm 21.8$  pg/mL and  $55.8 \pm 35.6$  pg/mL, respectively,  $P = 0.01$ ), compared to controls. Serum TNF- $\alpha$  was significantly increased in all groups compared to sham controls ( $1.32 \pm 0.87$  ng/mL,  $1.79 \pm 0.20$  ng/mL and  $4.4 \pm 0.69$  ng/mL, for groups A, V and AV, respectively,  $P = 0.01$ ), with higher values in the AV group.

**CONCLUSION:** Different patterns of response to ischemia/reperfusion are associated with venous, arterial or arteriovenous occlusion. Venous and arteriovenous occlusion was associated with the most severe alterations.

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**Key words:** Arterial occlusion; Rat; Intestine; Ischemia/reperfusion; Venous occlusion

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### INTRODUCTION

Acute mesenteric ischemic disease can be either arterial or venous in etiology. Acute arterial ischemia results

from reduced blood flow caused by mesenteric artery thrombosis or embolism, leading to bowel necrosis and high mortality<sup>[1]</sup>. Mesenteric vein occlusion is usually due to thrombosis, is less common than arterial occlusion and carries a lesser risk of bowel necrosis and therefore reduced mortality<sup>[2,3]</sup>. Greater knowledge of the differences between these conditions would aid the search for therapeutic interventions, and also expand our understanding of ischemia/reperfusion (I/R) pathophysiology. This information would also be relevant for transplantation and aortic reconstruction procedures, which are invariably related to periods of I/R<sup>[4]</sup>.

The intestine is very susceptible to ischemic injury. Ischemia causes mucosal damage through the production of substances like oxygen-derived free radicals, pro-inflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and the complement system<sup>[5]</sup>. The pattern of injury has been found to be different after experimental arterial or venous occlusion of the mesenteric circulation followed by reperfusion. Generally, the histological injury grade is thought to be greater after venous occlusion, even though energy metabolism is altered to a lesser extent than in arterial occlusion<sup>[6]</sup>. However, another study found that venous congestion alone could not cause reperfusion injury, while arterial occlusion could do so easily<sup>[7]</sup>. This issue could be resolved if the differences between the injury response to these two situations could be found.

During I/R, endothelial functions are altered, resulting in microcirculatory dysfunction which modulates mucosal integrity<sup>[8]</sup>. Endothelial injury and increased vascular permeability also occur<sup>[9]</sup>. The vascular endothelium regulates vascular tone, leukocyte adhesion and the coagulation cascade, among other factors. Endothelial function serum marker levels such as intracellular adhesion molecule-1 (ICAM-1), endothelin-1 (ET-1) and coagulation factors have also been found to be altered after I/R. One study showed that ET-1 modulates intestinal I/R injury in rats<sup>[10]</sup>, while another showed that ICAM-1 is overexpressed, possibly contributing to injury by promoting leukocyte infiltration<sup>[11]</sup>. Another adhesion molecule, P-selectin, is also thought to modulate intestinal I/R injury, through complement deposition and leukocyte interaction<sup>[12]</sup>. Intestinal I/R also causes hemostatic alterations, namely coagulation induction, thrombin generation, and fibrin deposition, which correlate with the extent of injury<sup>[13]</sup>.

In this study we evaluate the possibility that arterial, venous and arteriovenous occlusion, followed by reperfusion, might cause different types of response to I/R injury in the intestine. These differences were investigated by measuring ET-1, antithrombin III (ATIII), soluble ICAM-1 and P-selectin serum concentrations, soluble markers of endothelial function, as well as by measuring TNF- $\alpha$ , a cytokine that plays a central role in I/R injury.

## MATERIALS AND METHODS

### **Animal procedures**

Animal procedures were performed in accordance with

the proper use and care of laboratory animals, approved by the ethics committee of our institution. Experiments were performed on 20 male Wistar rats weighing 200–250 g. Animals were maintained under standard conditions, such as stable room temperature (24 ± 3°C), a 12 h light/12 h dark cycle, and access to commercial rat pellets and water *ad libitum*.

### **Animal model**

Briefly, after pentobarbital sodium anesthesia (Anestesal, Pfizer Inc, Mexico) (35 mg/kg, i.p), a midline laparotomy was performed, and the small intestine was externalized and wrapped in humid gauze. Animals were placed under a heating lamp in order to preserve core body temperature at (37°C). The cecum was occluded using a 2-0 nylon suture to prevent collateral blood flow, and ischemia was induced by obstructing either the superior mesenteric artery, the mesenteric vein before its union with the portal system, or both, with microvascular clamps for 30 min (ischemia), and removed to allow 60 min reperfusion. Immediately after clamping the mesenteric vessels, the intestines were returned to the peritoneal cavity where they remained for the entire duration of the procedure. Occlusion was confirmed by loss of pulsation and characteristic red dark coloring of the intestine in the venous group, or pale pink coloring and loss of pulsation in the arterial group.

Rats were divided in four groups ( $n = 5$ ). The first group received only sham surgery (Sham), where laparotomy was performed but intestines were only manipulated but not made ischemic. Group A was subjected to arterial occlusion and reperfusion as described above. Group V was subjected to venous occlusion and reperfusion as described above. Group AV was subjected to both arterial and venous occlusion and reperfusion as described above.

### **Morphological examination**

Immediately after concluding the reperfusion period, rats were sacrificed by exsanguination from the aorta and tissue samples were obtained and fixed in 10% neutral buffered formalin overnight. Samples were then embedded in paraffin, and 4 µm-thick sections were stained with hematoxylin and eosin (H/E) and examined under a light microscope by a blinded pathologist. The Chiu score of mucosal injury was used to evaluate the degree of histological damage<sup>[14]</sup>. The scale consists of values from 0 to 5, where 0 is normal mucosa; 1, development of sub epithelial (Gruenhagen's) spaces; 2, extension of the sub-epithelial space with moderate epithelial lifting from the lamina propria; 3, extensive epithelial lifting with occasional denuded villi tips; 4, denuded villi with exposed lamina propria and dilated capillaries; and 5, disintegration of the lamina propria, hemorrhage, and ulceration.

### **Inflammatory cell infiltration**

Under light microscopy, H/E stained tissue samples were analyzed for leukocyte infiltration by a blinded pathologist. The predominant type was recorded in both mucosa and

muscular layers, and intensity of infiltration was quantified with a numerical scale with values from 0 to 3, where 0, no infiltration; 1, mild infiltration; 2, moderate infiltration and 3, severe infiltration. Two slides and five high power fields per slide were examined and averaged.

#### **Serum levels of ET-1, AT<sub>III</sub>, P-selectin, ICAM-1 and TNF- $\alpha$**

At the end of the reperfusion period 3 mL of blood was obtained from each rat and left to clot for serum acquisition. Serum levels of ET-1 were determined using a rat ET-1 EIA kit (Immuno-Biological Laboratories, Japan). Serum levels of AT<sub>III</sub> were determined using a rat AT<sub>III</sub> ELISA kit (GenWay Biotech, USA). Serum levels of soluble ICAM-1 were determined using a quantikine rat sICAM-1 ELISA kit (R & D Systems, USA). Serum levels of P-selectin were determined using a quantikine rat P-selectin ELISA kit (R & D Systems, USA). These were used as markers of endothelial function. Serum concentrations of TNF- $\alpha$  were determined using a rat TNF- $\alpha$  Elisa kit (PeproTech, Mexico).

#### **Statistical analysis**

SPSS 11.0 statistical software (SPSS Inc. Software, Chicago, Illinois, USA) was used to analyze data using one-way analysis of variance (ANOVA) and with the Tukey-Kramer post-hoc test so as to determine comparisons between the groups, and differences between the groups, respectively. The relationships between the variables studied were determined by the calculation of Pearson correlation coefficients. All values are expressed as mean  $\pm$  SD and  $P < 0.05$  was considered statistically significant.

## **RESULTS**

#### **Morphological examination**

The sham group showed normal intestinal mucosa (Chiu score  $0.2 \pm 0.45$ ). All occlusion groups had significantly higher injury scores compared to the sham group (Figure 1,  $P < 0.01$ ). Groups V and AV had the highest injury scores ( $3.6 \pm 0.55$  and  $3.4 \pm 0.55$ , respectively), showing severe epithelial lifting and severe capillary congestion (Figure 1), with no difference observed between these two groups. However, both the AV and V groups had significantly higher scores compared to group A ( $2 \pm 0.71$ ,  $P < 0.01$ ), which showed moderate lifting and very little congestion.

#### **Inflammatory cell infiltration**

Compared to the sham group ( $1.2 \pm 0.45$ ), groups V and AV ( $2.6 \pm 0.55$  and  $2.6 \pm 0.55$ ) both had a significantly higher leukocyte infiltration intensity (Figure 1,  $P < 0.05$ ). Group A ( $2 \pm 0.7$ ) was not statistically different from either the V or sham group. The infiltrate type was similar in all groups, and was composed mostly of neutrophils, with few macrophages or lymphocytes observed.

#### **Serum markers of endothelial function**

The sham group had soluble P-selectin serum concentrations of  $0.83 \pm 0.47$  ng/mL. In group A the levels remained

**Table 1 Pearson's correlation coefficient between the variables studied**

	<b>TNF-<math>\alpha</math></b>	<b>ET-1</b>	<b>P-selectin</b>	<b>ICAM-1</b>	<b>AT<sub>III</sub></b>
Chiu Score	0.715 <sup>a</sup>	0.714 <sup>b</sup>	0.552 <sup>a</sup>	0.608 <sup>b</sup>	-0.633 <sup>a</sup>
TNF- $\alpha$	x	0.26	0.646 <sup>b</sup>	0.337	-0.534 <sup>a</sup>
ET-1		x	-0.015	0.655 <sup>b</sup>	-0.409
P-selectin			x	0.044	-0.275
ICAM-1				x	-0.532 <sup>a</sup>

<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ . Data are presented as correlation coefficients ( $r$ ).

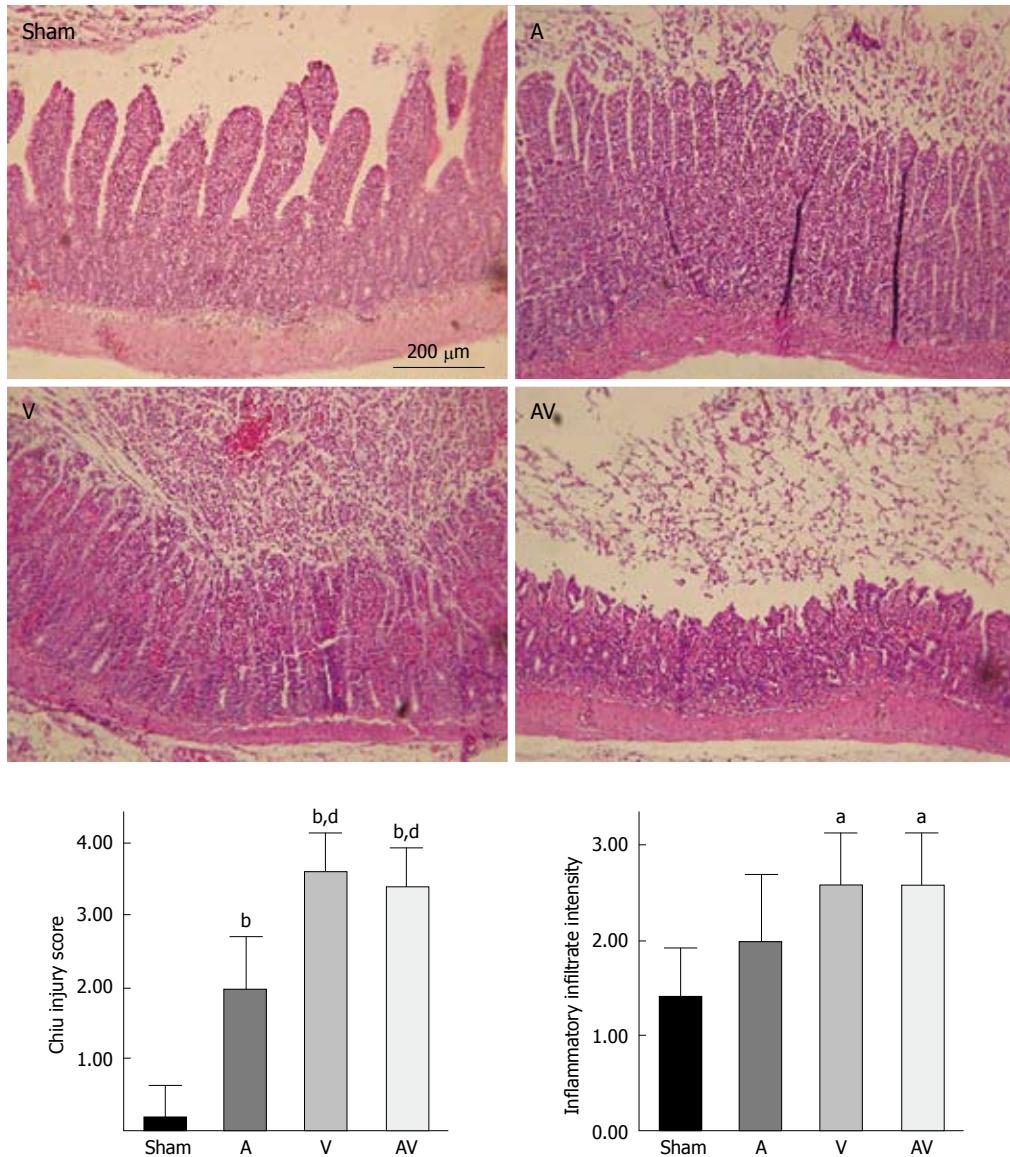
barely detectable, and were not statistically different to the sham group ( $0.02 \pm 0.01$  ng/mL). Groups V and AV had significantly higher values compared to the sham group ( $1.4 \pm 0.5$  ng/mL and  $2.52 \pm 0.9$  ng/mL, respectively,  $P < 0.01$ ), with the latter being significantly higher than the former ( $P < 0.05$ , Figure 2). AT<sub>III</sub> serum concentrations were significantly depleted in groups V and AV compared to the sham group ( $29.1 \pm 5.2$  pg/mL and  $31.4 \pm 21.8$  pg/mL vs  $85.3 \pm 17.4$  pg/mL, respectively,  $P < 0.01$ ), while in group A they remained within the sham group range ( $55.8 \pm 35.6$  pg/mL). However, there was no difference between the V or AV groups (Figure 2). No difference was found between ET-1 levels in the sham and AV groups ( $0.23 \pm 0.01$  pg/mL vs  $0.29 \pm 0.03$  pg/mL, respectively, Figure 2). Levels in both the A and V groups were significantly elevated compared to the sham group ( $0.32 \pm 0.04$  pg/mL and  $0.36 \pm 0.05$  pg/mL, respectively,  $P < 0.01$ ), with levels in the V group also being significantly higher than in that in the AV group, but not the A group. Finally, all groups had significantly elevated soluble ICAM-1 serum concentrations compared to the sham group (A:  $569.2 \pm 170.8$  pg/mL, V:  $529 \pm 191.5$  pg/mL and AV:  $483.9 \pm 41.6$  pg/mL vs  $194 \pm 38.9$  pg/mL, respectively,  $P < 0.01$ ), but no differences were found between each of these three groups (Figure 2). In summary, venous and arteriovenous occlusion elevated P-selectin, and depleted AT<sub>III</sub>, to a greater extent than arterial occlusion only. Venous and arterial occlusion, but not arteriovenous occlusion, elevated ET-1 levels compared to sham groups, with no differences between them. ICAM-1 levels were elevated in all groups compared to the sham group, with no differences between them.

#### **TNF- $\alpha$ serum concentrations**

The sham group had TNF- $\alpha$  serum concentrations of  $0.11 \pm 0.14$  ng/mL. In groups A, V and AV the levels were significantly increased compared to sham controls ( $1.32 \pm 0.87$  ng/mL,  $1.79 \pm 0.20$  ng/mL and  $4.4 \pm 0.69$  ng/mL, respectively,  $P < 0.01$ , Figure 2). TNF- $\alpha$  serum concentrations were significantly higher in the AV group compared to both the A and V group, with no differences observed between these two groups.

#### **Linear correlations between variables**

Linear correlations were observed between the systemic concentrations of endothelial function markers, TNF- $\alpha$  and injury scores. These correlations are shown in Table 1. All the measured variables, except for AT<sub>III</sub>, showed a significant positive correlation with the histological injury score. AT<sub>III</sub> showed a significant negative correlation with



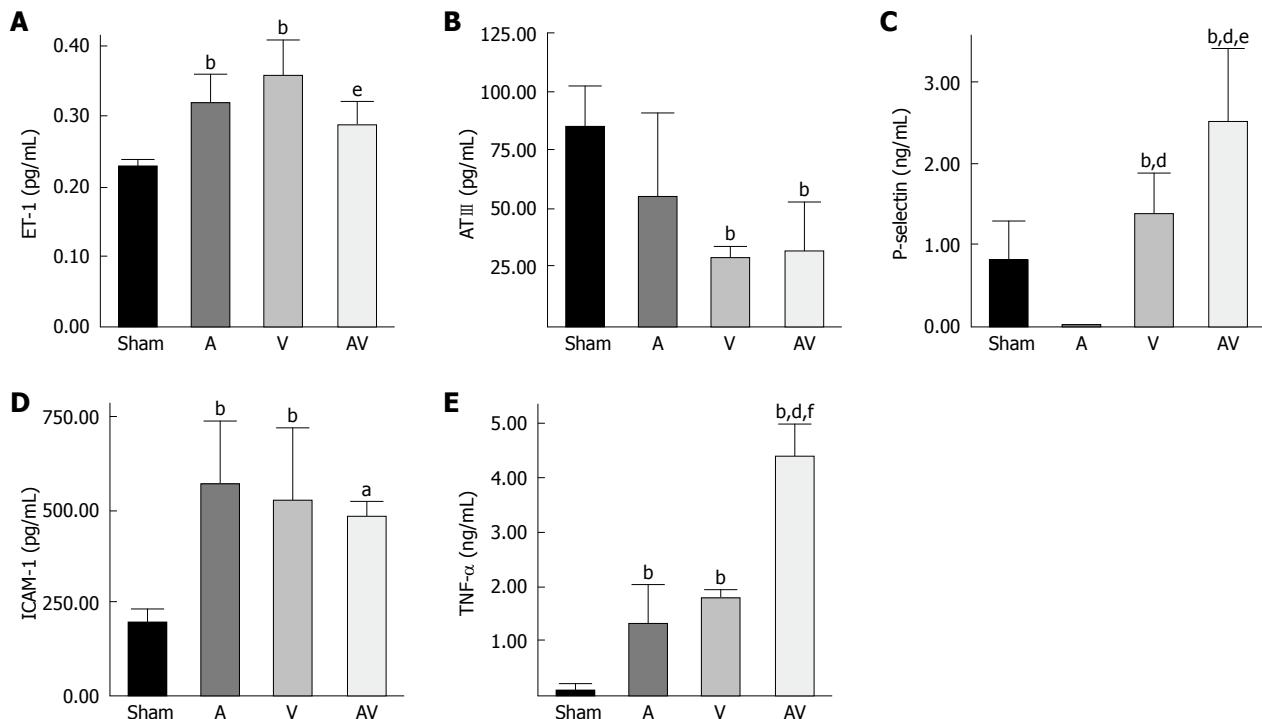
**Figure 1** Histological findings (H & E) and injury scores. A: Arterial occlusion and reperfusion group; V: Venous occlusion and reperfusion group; AV: Both arterial and venous occlusion and reperfusion group. <sup>a</sup>P < 0.05 vs sham; <sup>b</sup>P < 0.01 vs sham; <sup>c,d</sup>P < 0.01 vs A.

the injury score ( $r = 0.608$ ,  $P < 0.01$ ). TNF- $\alpha$  was the variable most strongly correlated with injury score ( $r = 0.715$ ,  $P < 0.01$ ).

## DISCUSSION

Ricci *et al*<sup>[15]</sup> found greater mortality in rats after intestinal arterial versus arteriovenous occlusion. However, Yano *et al*<sup>[16]</sup> found that the degree of intestinal damage due to venous occlusion was greater than the damage resulting from both arteriovenous and arterial occlusion, with no difference between the latter two procedures. Greater intestinal hemorrhage has also been observed after venous occlusion compared with arterial occlusion<sup>[6]</sup>. Other studies confirmed these findings, showing that venous occlusion could induce intestinal injury as early as within 5 min, while arterial occlusion could not even at longer time periods<sup>[17]</sup>. Some of the molecular bases of these differences have been investigated. Venous congestion has been shown to induce increased levels of inflammatory

cytokines, in a similar way to arterial occlusion<sup>[18]</sup>. However, in one I/R study, intestinal malonyldialdehyde levels (as an index of free radicals) were found to be higher in venous occlusion compared to arterial occlusion, despite no obvious difference in tissue injury<sup>[19]</sup>. In other organs, such as the kidney, renal vein occlusion has been found to induce a stronger inflammatory response (TNF- $\alpha$ , free radicals, but not ICAM-1), neutrophilic infiltration, and greater functional damage, compared to arterial occlusion<sup>[20]</sup>. In our study, I/R due to venous occlusion was shown to cause greater damage to the intestinal mucosa than arterial occlusion alone, in agreement with the previous studies. However, we found no difference between the type or the intensity of injury between venous and arteriovenous occlusion. Arterial occlusion caused limited but significant histological damage and was associated with little leukocyte infiltration. The pro-inflammatory cytokine TNF- $\alpha$  is one of the main orchestrators of the inflammatory response and a key mediator of injury during I/R<sup>[21]</sup>. In our study,



**Figure 2** Serum concentrations of endothelial function markers and TNF- $\alpha$ . A: ET-1 serum concentrations; B: ATIII serum concentrations; C: P-selectin serum concentrations; D: Serum levels of soluble ICAM-1; E: TNF- $\alpha$  serum concentrations.  $^aP < 0.05$  vs sham;  $^bP < 0.01$  vs sham;  $^dP < 0.01$  vs A;  $^eP < 0.05$  vs V;  $^fP < 0.01$  vs V.

TNF- $\alpha$  levels were significantly elevated after all three occlusion methods, with the highest levels observed in the arteriovenous occlusion group. TNF- $\alpha$  concentrations were strongly correlated to injury intensity.

Intestinal I/R affects the microvasculature, endothelial cells, and endothelial cell function<sup>[8]</sup>. ET is a vasoactive substance which induces intense vasoconstriction of blood vessels, and its activation is thought to play an important part in the development of these microvascular changes<sup>[22]</sup>. ET-1 participates in intestinal I/R injury by modulating leukotriene production<sup>[10]</sup>. Additionally, it has been demonstrated that ET-1 is involved mainly in post-reperfusion induced vasospasm and damage, and ET receptor antagonists are able to protect against mucosal injury<sup>[23]</sup>. In agreement with previous reports, we found that I/R due to arterial occlusion induces elevations in ET-1 levels, and further, we demonstrated that venous occlusion has a similar outcome, although there was a tendency for higher levels in the venous occlusion group. Surprisingly, ET-1 elevation was not present when both arterial and venous occlusion was performed simultaneously. Other factors capable of explaining this result might be at play. It is known that reductions in perfusion pressure during mesenteric ischemia cause diminished arteriolar resistance and vasodilation as a compensatory mechanism<sup>[24]</sup>. While venous occlusion alone causes severe congestion and increased intravascular pressure, arterial occlusion alone might only cause reduced inflow perfusion with normal outflow. Supporting this idea, Tsunada *et al*<sup>[18]</sup> reported a more pronounced reduction of blood flow and increased subsequent flow recovery after reperfusion with arterial occlusion, compared to venous occlusion. We speculate

that arteriovenous occlusion might balance inflow and outflow alterations, resulting in intravascular pressure and tone equilibrium, possibly explaining the absence of ET-1 activation which we observed. Further experiments are required to assess this possibility and the role of ET-1 in regulating post-ischemic vascular tone.

P-selectin is an adhesion molecule which modulates endothelial-leukocyte interactions. During intestinal I/R, P-selectin mRNA expression is upregulated in the microvascular endothelium<sup>[25]</sup> and P-selectin immunoreactivity is increased in the intestinal mucosa<sup>[26]</sup>. I/R studies have shown that P-selectin blockade or genetic deficiency result in diminished intestinal mucosa damage after I/R in rodent models, further supporting the idea that P-selectin participates in the pathophysiology of I/R injury<sup>[27,28]</sup>. In our study, I/R in the arteriovenous occlusion group caused higher elevations of soluble P-selectin serum concentrations than in the venous occlusion group. Moreover, arterial occlusion was unable to alter these values compared to the sham group. P-selectin levels were also found to be correlated to histological injury scores.

The coagulation cascade is promoted by I/R<sup>[13]</sup>. AT III is an endogenous anticoagulation molecule produced in lung, liver and endothelium which limits thrombin formation. Thrombin activation and the formation of thrombin-antithrombin complex is increased after intestinal I/R, contributing to cytokine production and inflammatory injury<sup>[29]</sup>. Furthermore, these alterations would theoretically result in endogenous ATIII depletion. One rodent study found that ATIII levels were indeed depleted after intestinal I/R, and that they were restored by protective therapy<sup>[30]</sup>. Additionally, ATIII

administration can protect the intestinal mucosa against I/R injury<sup>[31]</sup>. We found that I/R due to both venous and arteriovenous occlusion caused ATIII depletion whereas arterial occlusion alone did not. In our study, a negative correlation between ATIII levels and injury scores was observed, suggesting that depleted ATIII levels are indeed reflective of damage intensity. A possible explanation for our results is that the microvascular congestion and venous stasis caused by the venous occlusion component promotes the coagulation cascade to a greater extent than the arterial component during ischemia and reperfusion. Indeed, temporal and histological differences have been found to exist between thrombus generation in arterial and venous endothelium<sup>[32]</sup>. While stasis seems to be a more important factor in veins, impaired laminar flow and plaque formation seem to be essential for arterial thrombus generation<sup>[33]</sup>. Furthermore, studies have shown that decreased levels of ATIII after major surgery can predict higher complication and mortality rates<sup>[34]</sup>. Therefore, the differences in ATIII depletion between arterial or venous mesenteric ischemia might be of clinical relevance. The physiopathological consequences of these results warrant further investigation.

ICAM-1 is another adhesion molecule that mediates leukocyte reactivity and adhesion to endothelium. Intestinal I/R is also associated with an upregulation of ICAM-1 and subsequent leukocyte infiltration<sup>[35]</sup>. Systemic ICAM-1 activity is also upregulated, possibly contributing to multisystem failure and remote organ injury<sup>[11]</sup>. Serum levels of ICAM-1 have been shown to be markedly elevated after intestinal I/R, and low serum concentrations are associated with beneficial outcomes after the administration of protective agents<sup>[36]</sup>. The inhibition of ICAM-1 with monoclonal antibodies is able to reduce the intensity of the functional and morphological alterations in the intestine subjected to I/R<sup>[37,38]</sup>. In our study, we found that soluble ICAM-1 serum concentrations were elevated by intestinal I/R, in agreement with the previous reports. However, there was no difference between any of the occlusion methods employed, suggesting that soluble ICAM-1 is non-specifically elevated in arterial, venous or arteriovenous I/R.

In conclusion, we showed that a different pattern of response to I/R characterize different forms of mesenteric occlusion methods. Venous occlusion seems to cause more severe tissue damage and changes in P-selectin and ATIII depletion compared to arterial occlusion. This suggests that these molecules are sensitive to changes in the intensity of the histological injury and therefore might be partly responsible for some of the pathophysiological differences between venous and arterial occlusion. Arteriovenous occlusion showed greater tissue damage and TNF- $\alpha$  levels compared to arterial occlusion alone, but only differences in P-selectin and ET-1 were found between them. Finally, arterial occlusion was related to the least severe changes compared to controls, and ICAM-1 values were not different between the different I/R groups.

We discussed the possibility of these differences being dependent on local microvascular factors, but since

serum concentrations are indicative of the systemic state, the explanation could lie in differences in the systemic consequences of the occlusion methods employed. Studies on the local and remote tissue expression of these molecules would be helpful in clarifying these issues. However, our results confirm that there are differences in the inflammatory responses associated with venous, arterial or arteriovenous occlusion that could partly explain the molecular bases of the clinical differences between these conditions.

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## COMMENTS

### Background

Intestinal ischemia can occur when blood flow from the mesenteric vasculature is obstructed. Obstructions can be embolic, atherosclerotic or vasospastic in etiology. Mesenteric veins or arteries can be affected. Ischemia and reperfusion also takes place in transplant procedures and states of hypoperfusion (shock). The pathophysiological differences between venous, arterial or total ischemia of the intestine are not fully understood. In this study, the aim was to investigate these differences using a rat model.

### Research frontiers

Ischemia/reperfusion is an important topic of research. The molecular mediators responsible for ischemia/reperfusion injury could be modulated to improve transplantation outcomes.

### Innovations and breakthroughs

In this study, it is demonstrated that venous, arterial or arteriovenous occlusion followed by reperfusion, cause different patterns of injury and serum elevations of inflammatory markers.

### Applications

To understand the pathophysiological bases of ischemia/reperfusion injury could lead to novel treatments, specifically tailored for conditions of venous, arterial or total ischemia.

### Peer review

This is a very interesting study which examines changes in rat intestinal histology and inflammatory serum cytokine markers subsequent to arterial, venous or concomitant arterial and venous occlusion and reperfusion. The authors demonstrated the differences in injury patterns caused by arterial, venous or arteriovenous mesenteric occlusion. This study is well-investigated and has a novel finding.

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BRIEF ARTICLES

## Effects of Chinese herbs on salivary fluid secretion by isolated and perfused rat submandibular glands

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**Author contributions:** Murakami M provided the experimental system including vital reagents and analytical tools, was also involved in the whole experimental design, and editing the manuscript; Wei MX was involved in choosing the effective Chinese herbs and in research; Ding W performed research and analyzed the data; Zhang QD performed research on herb choice.

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overloading of CCh promoted fluid secretion in 15 of 20 CHs. This promotion was classified into four patterns, which were eventually related to the categories of CH: Overall sustained phase was continuously raised (*Yin*-nourishing, fluid production-promoting and heat-clearing agents); The sustained secretion rose to reach a maximum then decreased (*Qi*-enhancing agent); Sustained secretion rose to reach the highest maximum and was then sustained with a slight decline (swelling-reducing, phlegm-resolving and pus-expelling agents); Stimulation of salivary secretion without any added stimulants. Addition of CCh raised the fluid secretion to reach the highest maximum then sharply decreased to a lower sustained level (blood activating agent).

**CONCLUSION:** The present findings lead to the conclusion that various CHs have different promotional effects directly on the salivary gland.

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**Key words:** Chinese herbs; Salivary secretion; Submandibular glands; Xerostomia

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### Abstract

**AIM:** To determine whether Chinese herbs (CHs) relieve xerostomia (dry mouth) by increasing salivary secretion.

**METHODS:** The submandibular glands of Wistar rats were surgically isolated and perfused arterially with buffered salt solution. After control perfusion, recording started 5 min prior to the start of stimulation. After fluid secretion was induced by 0.2 μmol/L carbamylcholine (CCh) in the perfusate for 10 min, Chinese herb (CH) was added in the perfusion for 5 min. CCh was then overloaded at 0.2 μmol/L in the perfusion for 20 min. The volume of salivary fluid secretion was recorded by a computer-controlled balance system.

**RESULTS:** Saliva secretion formed an initial ephemeral peak at 30 s followed by a gradual increase to a sustained level. CH alone induced no or little saliva in all types of CH selected. During perfusion with CH,

### INTRODUCTION

Xerostomia (dry mouth) is caused by salivary hypo-function (reduction in salivary fluid secretion). Severe xerostomia induces speech difficulty, infection of the oral cavity and is associated with systemic disease<sup>[1]</sup>. Salivary hypofunction is associated with aging and with various diseases including diabetes, hypertension, dehydration, drugs, radiation therapy, autoimmune diseases, irradiation, sarcoidosis and many other renal, neurologic and skin diseases<sup>[2]</sup>. Xerostomia may be transient and/or reversible as in the case of medications but relatively permanent in

the case of autoimmune diseases, damage due to radiation therapy and aging. The present therapeutic procedures for xerostomia are limited and include the supplemental use of artificial saliva and the use of stimulants such as pilocarpine and cevimeline.

Therapeutic use of muscarinic agents is sometimes avoided due to underlying diseases. The use of artificial saliva may bother patients during talking. Clinical research on the gene transfer of aquaporin has recently taken place<sup>[3,4]</sup>, and is still in progress.

For thousands of years, Traditional Chinese Medicine (TCM) has been used in the treatment of xerostomia. TCM does not follow the western style of diagnosis such as whether the symptom is primary or secondary, but is based on the classical theory-Syndrome Differentiation and Treatment. However, both ancient and contemporary practitioners of TCM have no standard classification of xerostomia symptoms, because all herbalist doctors have their own understanding and clinical experiences of syndrome differentiation and herb-choice. Following a comparison of the types of classifications, we can summarize xerostomia symptoms and classify them as follows: Yin-deficiency and thin fluid-deficiency; Yin-deficiency and dryness-heat; lack of activation due to Qi-deficiency; internal block of blood stasis; accumulation of damp-heat; phlegm accumulating with blood stasis<sup>[5-7]</sup>.

In order to choose herbs, all herbalist doctors obey the principle of therapy "Supply the deficiency, Reduce the excess". Because all practitioners of Chinese Medicine have their own preferences for particular herbs, overall, they are likely to choose herbs for xerostomia as follows: *Qi*-enhancing agents, Body fluid-regenerating agents, *Yin*-nourishing agents, Heat-clearing agents, Dampness-eliminating agents, Blood-activating agents, and Phlegm-resolving agents<sup>[8-11]</sup>.

Several thousand types of Chinese herbs are now in clinical use. By combining our previous findings<sup>[12]</sup> with reports by other herbalist doctors, we chose the following twenty herbs: *Yuzhu* (YZ, *Rhizoma polygonati odorati*<sup>[13,14]</sup>), *Shihu* (SH, *Herba dendrobii*<sup>[15,16]</sup>), *Shashen* (SS, *Radix glehniae*<sup>[17,18]</sup>), *Maimendong* (MD, *Radix ophiopogonis*<sup>[10,18]</sup>), *Tianmendong* (TD, *Radix asparagi*<sup>[17,18]</sup>) as *Yin*-nourishing agents; *Gegen* (GG, *Radix puerariae*<sup>[5]</sup>), *Wumei* (WM, *Fructus mume*<sup>[10,19]</sup>) as Body fluid-regenerating agents; *Shengdihuang* (SD, *Radix rehmanniae*<sup>[19,20]</sup>), *Xuanshen* (XS, *Radix scrophulariae*<sup>[17,18]</sup>), *Chishao* (CS, *Radix paeoniae rubra*<sup>[5,21]</sup>) as Heat-clearing agents; *Huangqi* (HQ, *Radix astragali*<sup>[8,14]</sup>), *Tai Zi Shen* (Tzs, *Radix pseudostellaris iae*<sup>[8]</sup>), *Gancao* (GC, *Radix glycyrrhizae*<sup>[18,22]</sup>) as *Qi*-enhancing agents; *Danshen* (DS, *Radix salviae miltiorrhizae*<sup>[17,18]</sup>) as Blood-activating agents; *Zaoci* (ZJC, *Fructus gleditsiae abnormalis*<sup>[19]</sup>), *Tianhuafen* (THF, *Radix trichosanthis*<sup>[20,23]</sup>), *Ziyuan* (ZY, *Radix asteris*<sup>[24,25]</sup>) as Phlegm-resolving agents; *Taoren* (TR, *Semen persicae*<sup>[13,24]</sup>), *Chuanshanjia* (CSJ, *Squama manis*<sup>[23]</sup>) as Body mass-softening and resolving agents; *Dandiquionyu* (DDQY<sup>[26]</sup>) as a mixture.

However, the effects of these CHs have not been evaluated quantitatively and their mechanisms have not been widely investigated. In particular, it is unknown

whether the effects of these CHs act directly on the salivary glands or indirectly via neural or hormonal controls.

The present study examines whether these CHs have a direct effect on the salivary gland to increase salivary fluid secretion. Effective CHs can induce fluid secretion solely or accelerate fluid secretion by muscarinic agents such as carbachol. For this purpose, we used isolated and arterially perfused submandibular salivary glands from rats. The results provided us with quantitative data on how much CHs can accelerate salivary secretion.

## MATERIALS AND METHODS

### Materials

Granular extracts of the Chinese herbs (CHs): YZ, SH, SS, MD, TD, SD, XS, CS, GC, WM, HQ, Tzs, GG, DS, TR, ZJC, ZY, THF and CSJ were provided by Tian Jiang Pharmaceutical Co., Ltd., Jiang Yin, China. The preparations were in strict standard control according to the industry standards.

Just before each experiment, the extract equivalent to 10 g of each CH was dissolved in distilled water by ultrasonic wave concussion. After centrifugation at 5000 r/min for 10 min, the supernatant was collected and adjusted to 0.5 g/mL as the CH stock solution. Before use, the stock solution was diluted 100 times into the perfusate to obtain a final concentration of 5 g/L. Then, this CH-containing perfusate was filtered through a 0.22 µm pore size filter (Sterivex-GV, Millipore, MA, USA). The clinical dose of CH ranges from 10 to 50 g/person because the recipe is a mixture of several CHs (each CH dose is 5 g). For experimental convenience, we used an average dose of 25 g/animal for each experiment. Assuming that all CHs will move to the systemic circulation (5 L for 60 kg body weight), the concentration of CHs in the blood will be 5 g/L. We took this as the concentration of CH in the perfusion buffer.

DDQY has been proved effective in promoting saliva secretion. The ingredients of DDQY are as follows: *RenShen* 5 g, *ShengDi* 20 g, *FuLing* 10 g, *DanShen* 15 g, *MaiDong* 10 g. All the ingredients were added with 200 mL distilled water and were decocted twice for half an hour each time and the solution after each decoction was mixed and filtered. The final solution obtained was 96.7 mL. For convenience, we blended the solution with distilled water until the CH concentration of the stock solution was 0.5 g/mL. All the above ingredients were provided by JingQuan Group Chinese Traditional Medication Decoction Pieces Co., Ltd., AnHui, China.

Salts, glucose, carbamylcholine chloride (carbachol, CCh) were from Sigma, MO, USA. A fluorine-fiber tube (EXLONTM) was purchased from Iwase Co. Ltd. (Atsugi, Japan).

### Methods

Wistar rats were fed a standard pellet diet and water *ad libitum*. The rats were anesthetized with pentobarbitone sodium (50 mg/kg body weight, by intraperitoneal injection). The submandibular glands (120-180 mg) were surgically isolated as previously described<sup>[12]</sup> and the

attached sublingual gland was removed after ligation of the feeding arteries, draining vein and sublingual duct. The extralobular main duct from the submandibular gland was cannulated with a fluorine-fiber tube (0.3 mm × 0.5 mm O.D.) for sampling. The artery distal to the glandular branch was cannulated with a stainless steel catheter (26G) connected to the infusion line for perfusion. The vein from the gland was cut free. The gland was isolated and transferred to an organ bath (37°C), where the arterial catheter was connected to the perfusion apparatus. The drained venous effluent was continuously removed.

The glands were perfused arterially at a rate of 2 mL/min using a peristaltic pump (Cole-Palmer) to supply enough oxygen even without a specific oxygen carrier during the secretory period<sup>[12]</sup>. The perfusion fluid was a buffered salt solution of the following composition (mmol/L): Na<sup>+</sup>, 145; K<sup>+</sup>, 4.3; Ca<sup>2+</sup>, 1.0; Mg<sup>2+</sup>, 1.0; Cl<sup>-</sup>, 148.3; glucose, 5.0. This solution was buffered at pH 7.4 with 10 mmol/L HEPES. The solution was prepared from stock solutions for each experiment and placed in a reservoir where it was equilibrated with 100% oxygen at 37°C.

To measure fluid secretion rate, the ductal cannula was filled with perfusate buffer and the tip placed under the water surface in a cup on an electronic balance (minimum digit was 0.1 mg, Shimadzu AEG-220), avoiding any contact with the bottom of the cup. Then, when salivary secretion started, the cumulative secreted mass could be measured. Cumulative weight was automatically measured every 3 s and transferred to a computer. The rate of fluid secretion was calculated from time-differentiation of the cumulative volume of saliva assuming a saliva specific gravity of 1.0.

Following control perfusion for longer than 20 min, recording was started 5 min prior to the start of stimulation. Fluid secretion was induced by the addition of 0.2 μmol/L carbamylcholine (CCh) in the perfusate. Following control stimulation with CCh for 10 min, CCh was removed from the perfusion line by perfusion with control perfusate for 5 min. Then only the CH was added to the perfusion for 5 min. This was to observe the effect of only the CH. After CH perfusion for 5 min, CCh was overloaded at 0.2 μmol/L in the perfusion for 20 min. To observe the accelerative effect of the CH, the dose of CCh used was 0.2 μmol/L to induce a moderate fluid secretion (c.f. 1.0 μmol/L is a supramaximal dose). The digital noise was smoothed by taking the moving average of every 11 data sets.

### Statistical analysis

To reduce the variance among individual glands, the value of the flow rate was shown as mean ± SE. The statistical significance of the changes were assessed by double-tailed Student's *t*-test in a comparison between the control values (4.95–5 min) and the data sets obtained at 19.95–20 min, 24.95–25 min, 29.92–30 min and 34.95–35 min, along the time course. The induction of salivary secretion

by CH only was determined by a comparison between the data-sets at 9.95–10 min and 14.95–15 min. *P*-value < 0.1 were considered statistically significant.

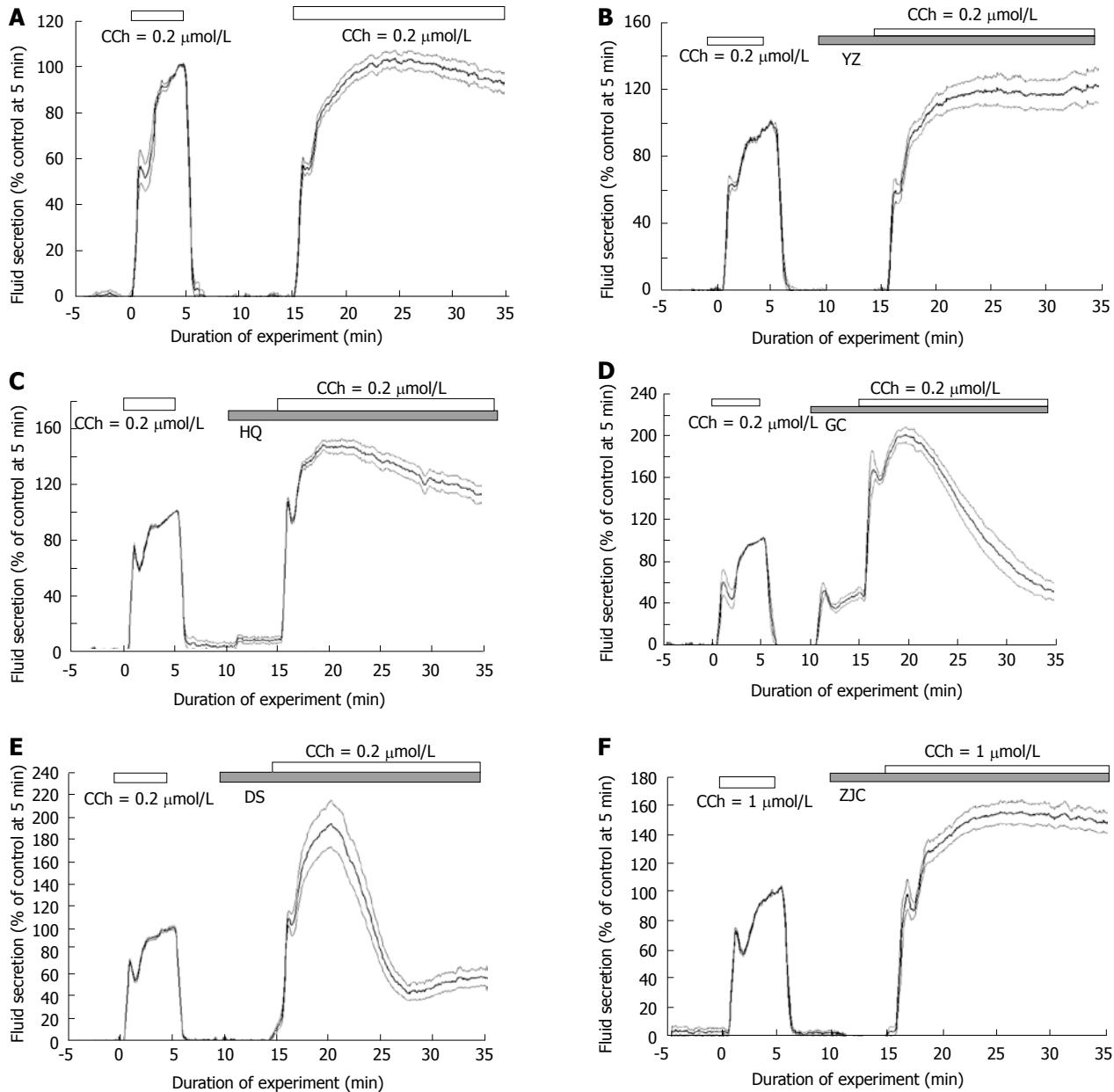
## RESULTS

### **Control stimulation with CCh**

The isolated perfused rat submandibular gland had almost no spontaneous fluid secretion. To standardize secretion in each gland, carbachol (CCh) stimulation was applied for 5 min. This initial phase included an initial transient increase and a pre-sustained stage of fluid secretion around 5 min from the start of CCh stimulation ( $71.4 \pm 8.5 \mu\text{L/g}$  per min). After washing with CCh for 5 min, CHs were applied for 5 min to check if the single application of the CH could induce fluid secretion. The period of 5 min allows the CHs and secretagogues to fully circulate in the gland. Because physiological neural reflex stimulation was applied to the gland under circulation of CH, we overloaded CCh on the CH circulated gland to determine the promotional effect of CH. Figure 1A shows the CCh-induced fluid secretion without addition of CHs. A single application of CCh showed a sustained plateau phase with a slight hump after the initial phase of secretion. For statistical examination of the promotional effect we compared how much the percentage of fluid secretion changed at 5, 10, 15 and 20 min from the start of the second CCh stimulation. The control stimulation of CCh showed no statistical difference to that of the 5-min value for the first stimulation (Figure 1A, *P* > 0.05).

### **Yin-nourishing agents**

YZ, SH, SS, MD and TD induced no secretion on single application (Figure 1B). Pre-loading of YZ did not increase the initial transient response to CCh (Figure 1B and Table 1). The initial transient peak ( $37.2 \pm 12.7 \mu\text{L/g}$  per min at 16.2 min, *n* = 6) and the maximal response ( $68.1 \pm 12.4 \mu\text{L/g}$  per min at 23.7 min) were similar to control values ( $36.9 \pm 7.0 \mu\text{L/g}$  per min at 1.1 min) and the value at 4.85 min ( $71.6 \pm 7.6 \mu\text{L/g}$  per min). Pre-loading of MD and TD increased the secretory response to CCh (Table 1). The initial transient peak ( $50.9 \pm 5.4 \mu\text{L/g}$  per min at 16.2 min, *n* = 6;  $41.4 \pm 6.0 \mu\text{L/g}$  per min at 17.55 min, *n* = 5) was higher than the control response to CCh ( $36.9 \pm 7.0 \mu\text{L/g}$  per min at 1.1 min;  $36.6 \pm 12.5 \mu\text{L/g}$  per min at 1.55 min), and the maximal response ( $82.2 \pm 6.1 \mu\text{L/g}$  per min at 23.05 min;  $73.2 \pm 3.6 \mu\text{L/g}$  per min at 27.2 min) was also higher than the control response to CCh ( $72.3 \pm 7.7 \mu\text{L/g}$  per min at 23.05 min;  $64.1 \pm 10.7 \mu\text{L/g}$  per min at 5.35 min). During CCh stimulation, fluid secretion was maintained at a higher level. On the other hand, pre-loading of SH did not change the responses to CCh. Pre-loading of SS decreased the responses to CCh (Table 1). The initial transient peak ( $25.3 \pm 5.8 \mu\text{L/g}$  per min at 16.4 min, *n* = 6) and the maximal response ( $43.8 \pm 5.3 \mu\text{L/g}$  per min at 27.35 min) were lower than the control value ( $45.4 \pm 6.1 \mu\text{L/g}$  per min at 1.1 min) and the value at 5.3 min ( $70.4 \pm 11.5 \mu\text{L/g}$  per min).



**Figure 1** Time course of fluid secretion during stimulation. A: 0.2  $\mu\text{mol/L}$  carbamylcholine (CCh); B: YZ + 0.2  $\mu\text{mol/L}$  CCh; C: HQ + 0.2  $\mu\text{mol/L}$  CCh; D: GC + 0.2  $\mu\text{mol/L}$  CCh; E: DS + 0.2  $\mu\text{mol/L}$  CCh; F: ZJC + 0.2  $\mu\text{mol/L}$  CCh. The values (%) were normalized by the value at 5 min from the first CCh stimulation (indicated by an open bar on the top of the graph). After washing the CCh for 5 min, Chinese herbs (CHs) were added in perfusion (indicated by a shaded bar). After another 5 min, the second CCh stimulation was added (an open bar, in Figure 1A without CH) to CHs perfusion. The second CCh stimulation was applied (open bar). The values for fluid secretion were statistically unchanged at 5, 10 and 15 min after the start of the second stimulation. The overall sustained phase was continuously raised. The average values (bold line,  $n=16$  from 8 glands in A;  $n=12$  from 8 glands in B;  $n=12$  from 6 glands in C;  $n=10$  from 5 glands in D;  $n=12$  from 6 glands in E;  $n=10$  from 5 glands in F) and the mean of standard error (mean  $\pm$  SE, thin line) are shown.  $n$ : The number of sampling points.

### Body fluid-regenerating agents

GG and WM did not induce secretion on a single application. Pre-loading of GG decreased the responses to CCh (Table 2). The initial transient peak ( $45.5 \pm 8.8 \mu\text{L/g}$  per min at 16.5 min,  $n=6$ ) was lower than the control initial transient peak ( $59.3 \pm 7.9 \mu\text{L/g}$  per min at 1.1 min). The maximal response ( $68.2 \pm 13.4 \mu\text{L/g}$  per min at 21 min) was also lower than the control response at 5 min ( $83.0 \pm 11.4 \mu\text{L/g}$  per min). After reaching a maximum, the flow rate was slowly decreased to  $52.9 \pm 16.0 \mu\text{L/g}$  per min at 35 min even during sustained stimulation. Thus, GG did not show a promotional effect on CCh-induced fluid secretion. Pre-loading of

WM increased the secretory response to CCh (Table 2). The initial transient peak ( $61.3 \pm 16.2 \mu\text{L/g}$  per min at 16.2 min,  $n=6$ ) was higher than the control response ( $53.9 \pm 16.1 \mu\text{L/g}$  per min at 1.1 min), and the maximal response ( $89.8 \pm 15.6 \mu\text{L/g}$  per min at 25.95 min) was also higher than the control response ( $72.3 \pm 7.7 \mu\text{L/g}$  per min at 5.4 min). During CCh stimulation, the fluid secretion was maintained at a higher level ( $83.2 \pm 10.2 \mu\text{L/g}$  per min at 35 min).

### Heat-clearing agents

SD, XS and CS did not induce secretion on a single application. Pre-loading of SD and XS decreased the

**Table 1** Fluid secretion induced by CCh with/without *Yin-nourishing* CHs in SMG

Time (min)	YZ (n = 12)		SS (n = 12)		MD (n = 12)		TD (n = 10)	
	FS (μL/g per min)	% of FS at 5 min	FS (μL/g per min)	% of FS at 5 min	FS (μL/g per min)	% of FS at 5 min	FS (μL/g per min)	% of FS at 5 min
(1) 4.95-5	63.8 ± 11.5	100.6 ± 0.5	69.4 ± 7.0	99.8 ± 0.4	68.3 ± 5.9	100.3 ± 0.3	64.7 ± 5.3	100.9 ± 0.4
(2) 19.95-20	65.3 ± 8.7	112.4 ± 6.3 <sup>b</sup>	42.4 ± 3.4 <sup>a</sup>	65.4 ± 6.4 <sup>a</sup>	77.7 ± 3.8	122.0 ± 11.1 <sup>a</sup>	62.0 ± 2.6	103.2 ± 11.6
(3) 24.95-25	67.0 ± 7.9	118.9 ± 8.1 <sup>a</sup>	45.2 ± 3.4 <sup>a</sup>	69.7 ± 6.7 <sup>a</sup>	80.8 ± 4.1 <sup>b</sup>	125.4 ± 9.3 <sup>a</sup>	75.0 ± 2.8 <sup>b</sup>	124.6 ± 13.9 <sup>b</sup>
(4) 29.95-30	65.8 ± 7.5	117.7 ± 8.8 <sup>b</sup>	40.6 ± 3.6 <sup>a</sup>	62.8 ± 7.5 <sup>a</sup>	80.7 ± 4.7 <sup>b</sup>	123.6 ± 6.9 <sup>a</sup>	77.0 ± 2.7 <sup>a</sup>	127.6 ± 13.5 <sup>b</sup>
(5) 34.95-35	67.9 ± 7.5	122.5 ± 10.2 <sup>a</sup>	32.3 ± 4.9 <sup>a</sup>	49.4 ± 8.3 <sup>a</sup>	78.7 ± 5.3	119.2 ± 6.0 <sup>a</sup>	75.4 ± 3.5 <sup>b</sup>	125.3 ± 14.5 <sup>b</sup>
(6) 9.95-10	-0.6 ± 0.4	-0.9 ± 1.1	0.8 ± 1.2	1.3 ± 1.7	0.1 ± 0.4	0.2 ± 0.6	-0.3 ± 0.5	-0.1 ± 1.1
(7) 14.95-15	-0.5 ± 1.4	-0.9 ± 3.4	-1.2 ± 0.5	-1.7 ± 0.5	-1.3 ± 0.81	-1.2 ± 1.2	-0.5 ± 0.3	-0.8 ± 0.4

Fluid secretion (FS) induced by (CCh) with/without Chinese herbs (CHs) in SMG. Fluid secretion was expressed in an absolute value (FS in  $\mu\text{L}/\text{min}$  per g wet weight of the gland) and % of the control value obtained at 5 min from the start of CCh perfusion (mean ± SE). n: The number of sampling points. Column (1) shows the average and SE from data set obtained at 4.95 and 5 min from the start of CCh application. Columns (2)-(5) show the values during addition of CH on CCh stimulation. Columns (6)-(7) show the values 5 min after CCh washing (6) and that under CH addition without CCh (7). t-tests between the control and the values at a fixed time during addition of CH on CCh stimulation are shown. The control and the values at a fixed time during addition of CH on CCh stimulation are shown <sup>b</sup>P < 0.1, <sup>a</sup>P < 0.05 vs FS at 4.95-5 min. YZ: *Yuzhu*; SS: *Shashen*; MD: *Maimendong*; TD: *Tianmendong*.

**Table 2** Fluid secretion induced by CCh with/without body fluid-regenerating/heat-clearing CHs in SMG

Time (min)	GG (n = 12)		WM (n = 12)		SD (n = 12)		CS (n = 12)	
	FS (μL/g per min)	% of FS at 5 min	FS (μL/g per min)	% of FS at 5 min	FS (μL/g per min)	% of FS at 5 min	FS (μL/g per min)	% of FS at 5 min
(1) 4.95-5	82.9 ± 7.2	99.9 ± 0.2	66.9 ± 9.7	107.0 ± 3.8	58.5 ± 5.7	97.7 ± 0.3	66.4 ± 6.9	100.6 ± 0.5
(2) 19.95-20	64.0 ± 8.1 <sup>b</sup>	78.4 ± 6.8 <sup>a</sup>	80.9 ± 10.1	116.2 ± 4.2	51.7 ± 6.0	86.9 ± 5.1 <sup>a</sup>	86.0 ± 5.6 <sup>a</sup>	140.1 ± 10.4 <sup>b</sup>
(3) 24.95-25	60.4 ± 8.5 <sup>a</sup>	76.5 ± 8.6 <sup>a</sup>	88.4 ± 9.9	130.0 ± 6.5 <sup>a</sup>	56.2 ± 4.9	95.4 ± 3.2	101.2 ± 4.7 <sup>a</sup>	168.0 ± 14.5 <sup>b</sup>
(4) 29.95-30	55.4 ± 8.2 <sup>a</sup>	70.4 ± 8.4 <sup>a</sup>	85.3 ± 7.3	129.3 ± 8.7 <sup>a</sup>	54.5 ± 3.4	94.2 ± 4.4	106.7 ± 5.5 <sup>a</sup>	179.4 ± 17.9 <sup>b</sup>
(5) 34.95-35	50.8 ± 8.2 <sup>a</sup>	63.7 ± 8.0 <sup>a</sup>	83.2 ± 6.6	127.5 ± 9.6 <sup>b</sup>	49.7 ± 3.2	85.8 ± 3.4 <sup>a</sup>	109.6 ± 6.3 <sup>a</sup>	184.6 ± 18.5 <sup>b</sup>
(6) 9.95-10	1.6 ± 0.7	2.2 ± 1.2	-0.9 ± 0.3	-1.1 ± 0.3	-0.4 ± 0.7	-1.1 ± 1.1	-0.9 ± 0.4	-1.3 ± 0.6
(7) 14.95-15	-1.7 ± 0.6 <sup>c</sup>	-2.4 ± 1.0 <sup>c</sup>	-0.7 ± 0.2	-1.1 ± 0.3	-1.7 ± 1.4	-3.5 ± 2.3	0.6 ± 1.6	1.8 ± 3.1

t-tests between the control and the values at a fixed time during addition of CH on CCh stimulation are shown <sup>b</sup>P < 0.1, <sup>a</sup>P < 0.05 vs FS at 4.95-5 min, <sup>c</sup>P < 0.05 vs FS at 9.95-10 min. GG: *Gegen*; WM: *Wumei*; SD: *Shengdihuang*; CS: *Chishao*.

responses to CCh (Table 2). The initial transient peak of SD ( $34.4 \pm 7.5 \mu\text{L}/\text{g}$  per min at 16.2 min, n = 5) and XS ( $42.9 \pm 7.9 \mu\text{L}/\text{g}$  per min at 16.2 min, n = 6) were similar to or lower than the control values ( $34.7 \pm 7.3 \mu\text{L}/\text{g}$  per min at 1.15 min;  $61.0 \pm 13.6 \mu\text{L}/\text{g}$  per min at 1.15 min), whereas the maximal response of both SD and XS ( $56.9 \pm 7.6 \mu\text{L}/\text{g}$  per min at 24.5 min;  $61.2 \pm 14.2 \mu\text{L}/\text{g}$  per min at 21.5 min) were lower than the control maximal value ( $72.3 \pm 7.7 \mu\text{L}/\text{g}$  per min at 5.1 min;  $81.0 \pm 15.2 \mu\text{L}/\text{g}$  per min at 5 min). The response gradually declined during stimulation. Thus, SD and XS did not show a promotional effect on CCh-induced fluid secretion. On the other hand, pre-loading of CS increased the secretory response to CCh (Table 2). The initial transient peak ( $57.9 \pm 7.4 \mu\text{L}/\text{g}$  per min at 17.2 min, n = 6) was higher than the control response ( $44.7 \pm 8.5 \mu\text{L}/\text{g}$  per min at 1.25 min), and the response increased  $86.0 \pm 8.7 \mu\text{L}/\text{g}$  per min at 20 min, which was also higher than the corresponding response to single stimulation with CCh ( $66.6 \pm 10.8 \mu\text{L}/\text{g}$  per min at 5 min). During CCh stimulation, fluid secretion continued to increase to the highest value,  $109.2 \pm 9.9 \mu\text{L}/\text{g}$  per min at 35 min.

### Qi-enhancing agents

HQ did not induce statistically significant fluid secretion (comparison between the values at 10 min and 12 min). Pre-loading of HQ enhanced the secretory response to

CCh (Figure 1C and Table 3). Both the initial transient peak ( $85.9 \pm 6.8 \mu\text{L}/\text{g}$  per min at 16 min, n = 6) and the maximal response ( $116.9 \pm 5.1 \mu\text{L}/\text{g}$  per min at 19.4 min) were higher than the control CCh stimulation ( $60.9 \pm 7.3 \mu\text{L}/\text{g}$  per min at 1 min and  $80.3 \pm 5.7 \mu\text{L}/\text{g}$  per min at 5.15 min, respectively). After reaching a maximum, fluid secretion declined slowly to a similar level to that of the control response (Figure 1C and Table 3). *Taizishen* (Tzs) did not induce secretion on a single application. Pre-loading of Tzs increased the secretory response to CCh (Table 3). Both the initial transient peak ( $71.1 \pm 16.2 \mu\text{L}/\text{g}$  per min at 17.25 min, n = 5) and the maximal response ( $104.9 \pm 13.7 \mu\text{L}/\text{g}$  per min at 24.05 min) were higher than the control CCh stimulation ( $43.5 \pm 6.3 \mu\text{L}/\text{g}$  per min at 1.2 min and  $72.2 \pm 7.8 \mu\text{L}/\text{g}$  per min at 5.05 min, respectively). After reaching a maximum, fluid secretion declined gradually. *Gancao* (GC) induced a significant secretion even on a single application (Figure 1D) with an initial transient peak ( $27.0 \pm 4.7 \mu\text{L}/\text{g}$  per min at 11.5 min, n = 5) and sustained secretion ( $25.6 \pm 4.6 \mu\text{L}/\text{g}$  per min at 15 min). Pre-loading of GC promoted the secretory response to CCh (Figure 1D and Table 3). Both the initial transient peak ( $85.1 \pm 13.2 \mu\text{L}/\text{g}$  per min at 16.65 min) and the maximal response ( $102.3 \pm 14.4 \mu\text{L}/\text{g}$  per min at 19.4 min) were higher than the control CCh stimulation ( $39.0 \pm 3.5 \mu\text{L}/\text{g}$  per min at 1.15 min and  $62.6 \pm 7.8 \mu\text{L}/\text{g}$  per min at 5.15 min, respectively). After fluid secretion increased to reach a value double the level of stimulation without GC,

**Table 3** Fluid secretion induced by CCh with/without *Qi-enhancing/Blood-activating* CHs in SMG

Time (min)	HQ (n = 12)		TZS (n = 10)		GC (n = 10)		DS (n = 12)	
	FS (μL/g per min)	% of FS at 5 min	FS (μL/g per min)	% of FS at 5 min	FS (μL/g per min)	% of FS at 5 min	FS (μL/g per min)	% of FS at 5 min
(1) 4.95-5	79.1 ± 3.5	99.6 ± 0.1	80.3 ± 7.0	101.1 ± 0.5	51.8 ± 4.3	101.7 ± 0.6	71.6 ± 8.0	101.9 ± 1.8
(2) 19.95-20	115.5 ± 3.6 <sup>a</sup>	146.8 ± 4.4 <sup>a</sup>	95.7 ± 8.8	122.7 ± 7.0 <sup>a</sup>	101.5 ± 8.9 <sup>a</sup>	204.4 ± 6.8 <sup>a</sup>	126.5 ± 11.5 <sup>a</sup>	194.8 ± 20.4 <sup>a</sup>
(3) 24.95-25	107.6 ± 4.2 <sup>a</sup>	137.2 ± 6.0 <sup>a</sup>	103.6 ± 8.3 <sup>b</sup>	135.3 ± 9.5 <sup>a</sup>	69.2 ± 6.2 <sup>a</sup>	144.0 ± 14.1 <sup>a</sup>	50.3 ± 10.4	75.0 ± 11.7 <sup>a</sup>
(4) 29.95-30	96.6 ± 3.5 <sup>a</sup>	123.6 ± 6.0 <sup>a</sup>	100.5 ± 7.9 <sup>b</sup>	132.0 ± 11.0 <sup>a</sup>	38.3 ± 4.7 <sup>a</sup>	82.3 ± 15.3	31.3 ± 3.7 <sup>a</sup>	50.7 ± 8.1 <sup>a</sup>
(5) 34.95-35	86.9 ± 3.8	112.0 ± 6.1 <sup>a</sup>	91.7 ± 7.1	121.6 ± 10.2 <sup>b</sup>	24.0 ± 3.5 <sup>a</sup>	50.0 ± 7.9 <sup>a</sup>	35.1 ± 3.0 <sup>a</sup>	56.9 ± 7.3 <sup>a</sup>
(6) 9.95-10	2.2 ± 2.5	2.2 ± 2.6	-0.2 ± 0.3	-0.6 ± 0.2	-0.2 ± 0.2	-6.3 ± 3.5	0.7 ± 0.5	1.5 ± 1.0
(7) 14.95-15	5.9 ± 2.0	7.4 ± 2.3	-0.4 ± 0.3	-0.1 ± 0.5	25.4 ± 2.7 <sup>c</sup>	50.5 ± 4.7 <sup>c</sup>	7.4 ± 1.8 <sup>c</sup>	12.0 ± 3.1 <sup>c</sup>

The control and the values at a fixed time during addition of CH on CCh stimulation are shown <sup>b</sup>P < 0.1, <sup>a</sup>P < 0.05 vs FS at 4.95-5 min, <sup>c</sup>P < 0.05 vs FS at 9.95-10 min. HQ: Huangqi; TZS: Taizishen; GC: Gancao; DS: Danshen.

**Table 4** Fluid secretion induced by CCh with/without *Phlegm-resolving/Body mass-softening/resolving* CHs in SMG

Time (min)	ZJC (n = 10)		ZY (n = 12)		TR (n = 10)		CSJ (n = 10)	
	FS (μL/g per min)	% of FS at 5 min	FS (μL/g per min)	% of FS at 5 min	FS (μL/g per min)	% of FS at 5 min	FS (μL/g per min)	% of FS at 5 min
(1) 4.95-5	67.0 ± 7.9	100.5 ± 0.7	63.5 ± 6.4	101.7 ± 0.3	53.8 ± 4.4	102.1 ± 0.8	46.2 ± 5.6	100.3 ± 0.4
(2) 19.95-20	85.9 ± 7.0 <sup>b</sup>	135.2 ± 6.3 <sup>a</sup>	70.8 ± 2.9	121.0 ± 8.5 <sup>a</sup>	57.6 ± 4.2	111.0 ± 5.9	49.8 ± 6.7	107.2 ± 2.7 <sup>a</sup>
(3) 24.95-25	98.3 ± 7.8 <sup>a</sup>	154.7 ± 7.1 <sup>a</sup>	83.8 ± 4.7 <sup>a</sup>	141.0 ± 7.6 <sup>a</sup>	66.0 ± 5.4 <sup>b</sup>	126.0 ± 8.5 <sup>a</sup>	55.4 ± 8.3	118.8 ± 8.0 <sup>a</sup>
(4) 29.95-30	97.1 ± 7.8 <sup>a</sup>	152.4 ± 6.4 <sup>a</sup>	85.6 ± 5.6 <sup>a</sup>	142.9 ± 7.1 <sup>a</sup>	63.5 ± 5.2	121.9 ± 8.1 <sup>a</sup>	54.1 ± 7.6	117.0 ± 8.9 <sup>b</sup>
(5) 34.95-35	94.1 ± 7.4 <sup>a</sup>	148.1 ± 6.9 <sup>a</sup>	80.5 ± 5.0 <sup>a</sup>	134.3 ± 6.5 <sup>a</sup>	59.9 ± 5.4	114.3 ± 7.2 <sup>b</sup>	50.8 ± 7.3	109.9 ± 9.2
(6) 9.95-10	1.6 ± 1.0	2.5 ± 1.4	1.4 ± 1.5	3.5 ± 3.1	-1.2 ± 0.7	-2.4 ± 1.5	0.5 ± 0.6	0.2 ± 0.9
(7) 14.95-15	1.0 ± 0.5	2.2 ± 1.3	-0.9 ± 0.3	-1.5 ± 0.6	-2.2 ± 1.0	-4.5 ± 2.0	2.3 ± 1.4	3.4 ± 1.8

The control and the values at a fixed time during addition of CH on CCh stimulation are shown <sup>b</sup>P < 0.1, <sup>a</sup>P < 0.05 vs FS at 4.95-5 min. ZJC: Zaojoci; ZY: Zi-yuan; TR: Taoren; CSJ: Chuanshanjia.

secretion then declined gradually to half the maximal value (24.0 ± 5.3 μL/g per min at 35 min).

### Blood-activating agents

DS induced significant secretion even on a single application (Figure 1E) with a long delay of 3-4 min (8.0 ± 2.9 μL/g per min at 15 min, n = 6) and then increased markedly to a maximum and thereafter declined (data are not shown, as the overload of CCh was started at 15 min in the present series of experiments). Pre-loading of DS promoted the secretory response to CCh (Figure 1E and Table 3). Both the initial transient peak (76.2 ± 11.5 μL/g per min at 16.05 min) and the maximal response (124.0 ± 17.5 μL/g per min at 19.3 min) were higher than the control CCh stimulation (52.1 ± 10.0 μL/g per min at 1.05 min, 72.3 ± 12.6 μL/g per min at 5.15 min) as shown in Figure 1E. Fluid secretion then increased to reach a value double the level of stimulation without DS, and declined gradually to half this value (36.6 ± 6.1 μL/g per min at 35 min).

### Phlegm-resolving agents

ZJC did not induce secretion on a single application (Figure 1F). Pre-loading of ZJC increased the secretory response to CCh (Figure 1F, Table 4). The initial transient peak (60.9 ± 7.1 μL/g per min at 16.8 min, n = 5) and the maximal response (99.1 ± 11.8 μL/g per min at 25.3 min) were higher than the control CCh stimulation (45.2 ± 7.0 μL/g per min at 1.2 min, 65.8 ± 12.5 μL/g per min at 5.25 min) as shown in Figure 1F. Fluid secretion tended to decline gradually (93.9 ± 11.8 μL/g per min at

35 min) during stimulation. THF and ZY did not induce secretion on a single application. Pre-loading of THF and ZY (Table 4) increased the secretory response to CCh. The initial transient peak (45.1 ± 9.8 μL/g per min at 16.25 min n = 6; 55.7 ± 11.0 μL/g per min at 16.55 min, n = 6) were higher than the control CCh stimulation (43.3 ± 13.2 μL/g per min at 1.15 min, 45.3 ± 9.3 μL/g per min at 1.2 min); the maximal response (77.0 ± 11.0 μL/g per min at 26.3 min; 86.9 ± 9.7 μL/g per min at 28.95 min) was also higher than the control CCh stimulation (65.4 ± 15.4 μL/g per min at 5.15 min, 63.9 ± 10.6 μL/g per min at 5.3 min). Fluid secretion tended to decline gradually (73.2 ± 10.0 μL/g per min at 35 min; 80.5 ± 8.0 μL/g per min at 35 min) during stimulation.

### Body mass-softening and resolving agents

TR and CSJ did not induce secretion on a single application. Pre-loading of TR increased the secretory response to CCh (Table 4). The initial transient peak (47.6 ± 8.6 μL/g per min at 16.4 min, n = 5) was higher than the control CCh stimulation (43.5 ± 6.3 μL/g per min at 1.1 min). However, the maximal response (66.1 ± 8.6 μL/g per min at 1.1 min) was similar to the control CCh stimulation (71.8 ± 7.6 μL/g per min at 5.25 min). Fluid secretion was sustained at the same level during stimulation. Pre-loading of CSJ did not increase the secretory response to CCh (Table 4). The initial transient peak (38.3 ± 8.6 μL/g per min at 16.65 min, n = 5) was similar to the control response (39.0 ± 3.5 μL/g per min at 16.65 min), and the maximal response (53.3 ± 12.7 μL/g per min at 25.75 min) was lower than the control response (71.8 ± 7.4 μL/g per min at 5.2 min). Because the

control values for fluid secretion in response to CCh were lower in the series using CSJ, the percentage change ( $118.8 \pm 8.0\%$  at 25 min) showed promotion by CSJ (Table 4).

### **Herb mixture**

DDQY did not induce secretion on a single application. Pre-loading of DDQY increased the secretory response to CCh. The initial transient peak ( $40.9 \pm 9.8 \mu\text{L/g}$  per min at 16.35 min,  $n = 5$ ) and the maximal response ( $78.0 \pm 7.9 \mu\text{L/g}$  per min at 27.45 min) were higher than the control CCh stimulation ( $25.9 \pm 4.7 \mu\text{L/g}$  per min at 1.45 min,  $51.3 \pm 8.7 \mu\text{L/g}$  per min at 5.25 min). Fluid secretion tended to decline gradually ( $74.2 \pm 4.7 \mu\text{L/g}$  per min at 35 min) during stimulation.

## **DISCUSSION**

### **Selection of CHs which possibly increase salivary fluid secretion**

Disorders of salivary secretion are the result of various causes and mechanisms in modern medicine, while in TCM, saliva is a body fluid and its deficiency is related to the deficiency of *Yin*-fluid, the vigor of internal heat and the dysfunctional distribution of *Yin*-fluid. TCM regards the body as a whole, and holds that the normal secretion of saliva needs the generational effect of *Qi* as well as its promotional effect. Once there is dysfunctional secretion of body fluid, the oral cavity cannot be moistened, and new pathologic features such as phlegm and excessive fluid will occur, leading to stagnation, which incurs another secondary injury which we call internal block (e.g. blood stasis),

In the treatment of secretion disorders, Body fluid-regenerating, *Yin*-nourishing, and Heat-clearing agents are commonly used and are reported in the literature<sup>[9,10]</sup>. *Qi*-enhancing agents are often prescribed simultaneously<sup>[8]</sup>. In chronic patients with a long disease course, Blood-activating agents are usually used, and sometimes even stasis-resolving<sup>[17,18]</sup>, hard mass-softening and block-dispelling agents<sup>[19,23]</sup>. In conclusion, the therapeutic concept of TCM is to identify suitable herbs which can resolve the pathologic status of patients so as to reach a new balance, which is called Syndrome Differentiation and Treatment.

### **Classification of responses**

It is interesting to find in our experiments that the promotional patterns of CH on secretion were classified into four patterns, which were eventually related to the categories of CH: overall sustained phase was continuously raised (*Yin*-nourishing, fluid production-promoting and heat-clearing agents). Although they belong to different CH types, they are closely correlated in the TCM theory, that is, *Yin*-nourishing and fluid production-promoting agents are similar in function, while heat-clearing agents can reserve body fluid which is reduced by internal heat; Sustained secretion rose to reach a maximum then decreased, this pattern was observed in HQ and TZS. These are *Qi*-enhancing agents; Sustained secretion rose to reach the highest

maximum then was sustained with a slight decline (swelling-reducing, phlegm-resolving and pus-expelling agents). These 3 types of herbs have one thing in common, in that they all aim to dispel pathologic features inside the body. It is possible that they may have a similar promotional pattern; Stimulation of salivary secretion without any added stimulants. The addition of CCh promoted fluid secretion to reach the highest maximum then inhibited secretion to a lower sustained level (blood activating agent DS and GC). GC, a *Qi*-enhancing herb, is usually used as a conciliatory agent, and is quite different from the common *Qi*-enhancing agents such as HQ.

The present findings at an organ level lead to the conclusion that various CHs have different promotional mechanisms and target sites. There are scientific essences in the TCM theory and its herb classifications, however further investigations are needed, including dose-dependency of the promotional pattern of each CH.

### **CHs which did not show potential in promoting salivary fluid secretion**

The isolated and arterially perfused salivary gland can induce fluid secretion either by neural stimulation and/or by arterial application of secretagogues. If there is no stimulation then no secretion occurs. In the present study, SH, XS, GG, SS, and DH did not show any potential in promoting fluid secretion in the salivary gland. This suggests no salivary promotional effects directly on the salivary glands by SH, XS, GG, SS, and DH. However, we have to examine the dose-response of these CHs, then we might expect to see that these CHs have indirect effects on the neural system and/or hormonal system, and indirectly affect salivary fluid secretion.

### **Possible mechanisms for the promotion of salivary fluid secretion by CHs**

Fluid secretion by salivary glands has been recognized as the transcellular movement of electrolyte/water at secretory endpiece cells (or acinar cells). Recently, the paracellular component of fluid secretion was also taken into account. The initial fluid secretion in response to CCh is estimated mainly due to transcellular fluid movement, whereas 60%-70% of fluid secretion is due to the paracellular component during the sustained stimulation period 1 min after the start of stimulation<sup>[27]</sup>. The initial transient peak of fluid secretion is thought to be due mainly to transcellular fluid secretion. Whereas, fluid secretion during the sustained phase depends both on transcellular and paracellular components.

One possible mechanism for the promotion through transcellular movement is activation of receptors to increase cytosolic  $\text{Ca}^{2+}$  which is mobilized from  $\text{Ca}^{2+}$  store/ $\text{Ca}^{2+}$  entry. In addition, the activation of transporters for  $\text{Cl}^-$  entry is possible.  $\text{Cl}^-$  channels allow  $\text{Cl}^-$  release across luminal membrane, and the activation of  $\text{Na}^+/\text{K}^+$  ATPase can increase  $\text{Na}^+$ -coupled  $\text{Cl}^-$  entry. The activation of unknown receptors can also play a role in these possible mechanisms.

In paracellular mechanisms, opening of junctional complexes is possible. In addition, increases in unknown

driving forces related to paracellular fluid transport needs further study.

Research to clarify the mechanisms of promotion is tightly linked to the search for control points of fluid secretion mechanisms. We could check cytosolic  $\text{Ca}^{2+}$  and  $\text{Na}^+$ -coupled  $\text{Cl}^-$ -entry in transcellular mechanisms by measuring the ouabain sensitive component of oxygen consumption. We could also determine the junctional complexes in paracellular mechanisms by measuring the secretion of fluorescent dye which cannot enter the cell. These possible mechanisms require further study.

## COMMENTS

### Background

Xerostomia (dry mouth) is caused by salivary hypofunction (reduction in salivary fluid secretion). Traditional Chinese Medicine (TCM) has been used in the treatment of xerostomia. The aim of the present study was to determine if Chinese herbs (CHs) had a direct effect on the salivary gland to increase salivary fluid secretion.

### Research frontiers

The present therapeutic procedures for xerostomia are limited and include the supplemental use of artificial saliva and the use of stimulants. TCM is an effective therapy with few side effects. However, there is little research on the effects of CHs on salivary secretion. Our study aims to identify the effects of CHs on salivary secretion.

### Innovations and breakthroughs

By combining our previous findings with reports by other herbalist doctors, we chose twenty herbs and used isolated and arterially perfused rat submandibular salivary glands to examine if CHs had direct effects on the salivary gland to increase salivary fluid secretion. The results provided us with quantitative data how much CHs could accelerate salivary secretion.

### Applications

The quantitative results of this study showed how much CHs could accelerate salivary secretion. This information could possibly provide a theoretical guide for choosing herbs to relieve xerostomia in TCM practices.

### Peer review

The manuscript by Murakami et al, describes the results of studies on the effect of several types of traditional Chinese herb remedies, commonly used to relieve the effects of dry mouth disease (xerostomia), on salivary fluid secretion in the perfused rat submandibular salivary gland. It has some merits as it addresses the problem of xerostomia, a condition affecting quite a large segment of the world population including millions of elderly women.

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BRIEF ARTICLES

## Soluble intercellular adhesion molecule-1, D-lactate and diamine oxidase in patients with inflammatory bowel disease

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### Abstract

**AIM:** To study the levels of serum soluble intercellular adhesion molecule-1 (sICAM-1), plasma D-lactate and diamine oxidase (DAO) in patients with inflammatory bowel disease (IBD), and the potential clinical significance.

**METHODS:** Sixty-nine patients with IBD and 30 healthy controls were included in this study. The concentration of sICAM-1 was detected with enzyme-linked immunosorbent assay, the level of D-lactate and DAO was measured by spectroscopic analysis, and the number of white blood cells (WBC) was determined by routine procedure.

**RESULTS:** The levels of sICAM-1, DAO, and WBC in IBD patients were significantly higher than those in the control group ( $P < 0.01$ ). sICAM-1 in IBD patients was found to be closely related to the levels of DAO and D-lactate ( $212.94 \pm 69.89$  vs  $6.35 \pm 2.35$ ,  $P = 0.000$ ), DAO  $212.94 \pm 69.89$  vs  $8.65 \pm 3.54$ ,  $P = 0.000$ ) and WBC ( $212.94 \pm 69.89$  vs  $7.40 \pm 2.61$ ,  $P = 0.000$ ), but no significant difference was observed between patients with ulcerative colitis and patients with Crohn's disease. The post-treatment levels of sICAM-1, D-lactate and WBC were significantly lower than before treatment

(sICAM-1  $206.57 \pm 79.21$  vs  $146.21 \pm 64.43$ ,  $P = 0.000$ ), (D-lactate  $1.46 \pm 0.94$  vs  $0.52 \pm 0.32$ ,  $P = 0.000$ ) and (WBC  $7.24 \pm 0.233$  vs  $5.21 \pm 3.21$ ,  $P = 0.000$ ).

**CONCLUSION:** sICAM-1, D-lactate and DAO are closely related to the specific conditions of IBD, and thus could be used as a major diagnostic index.

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**Key words:** Inflammatory bowel diseases; Intercellular adhesion molecule-1; D-lactate; Diamine oxidase

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### INTRODUCTION

The barrier function of the intestinal mucosa is of vital importance in inflammatory bowel diseases (IBD). Analysis of this function provides an important basis in the diagnosis of intestinal mucosal barrier dysfunction. Therefore, the importance of a timely, correct assessment of intestinal mucosal barrier function cannot be overestimated in judging the patient's disease state, estimating the prognosis and determining a comprehensive treatment program. However, direct observation of the intestinal barrier function involves many difficulties, so observation mostly needs to be made indirectly.

D-lactate, a metabolic end product of gastrointestinal bacteria, can be produced by many of these bacteria. As mammals do not have an enzyme system capable of its decomposition, D-lactate will enter the blood when the intestinal barrier function is damaged, and as they do

not have D-lactate dehydrogenase, a rise in the level of D-lactate can be detected when the permeability of the intestinal mucosa is increased. Therefore, examination of peripheral blood can reveal the degree of damage of the intestinal mucosa and the change in its permeability<sup>[1]</sup>. Diamine oxidase (DAO) is a highly active intracellular enzyme in the cytoplasm of the upper chorial cells of the intestinal mucosa. In cases where the intestinal mucosal epithelial cells and barrier function are damaged, the release of DAO is increased, and DAO enters the extracellular space, lymphatic vessels and bloodstream, thus increasing the level of DAO in the plasma. As the activity of DAO is stable, its concentration in the blood can reflect the damage and restoration of the intestinal cavity<sup>[2]</sup>.

Overactivation of leukocytes is an important pathological process of IBD. The stagnation and infiltrative exosmosis of leukocytes depend on the expression and function of the intracellular adhesion molecules (ICAMs) of leukocytes and endotheliocytes at the inflammatory location.

ICAMs are a type of glycoprotein synthesized by cells and assembled on the cell surface or secreted to the cell epimatrix, and can promote the adhesion between cells or between cells and the epimatrix. The role of ICAM-1, belonging to the immunoglobulin superfamily, in the development of IBD, has been receiving more and more attention in recent years. The ICAM-1 of normal tissues is usually expressed at low levels in vascular endothelial cells, and in mononuclear macrophagocytes in the intestinal mucosal lamina propria and lymph. In the intestinal tissues of patients with IBD, the expression and distribution of ICAM-1 is significantly increased and is closely related to the degree of inflammation of the tissues<sup>[3]</sup>. The adhesive molecules in vascular endothelial cells, leukocytes or other cells can be swallowed into the cells, or peel off into the blood circulation, becoming soluble intercellular adhesion molecule-1 (sICAM-1). The post-translation product of the mRNA of some cells, are possibly not expressed on the cellular surface, but are directly secreted into the blood and is thus another important source of sICAM-1<sup>[4,5]</sup>. The increase in sICAM-1 in the serum is a marker of the damage or activation of endotheliocytes. Therefore, the level of the sICAM-1 in the serum is a significant index in the detection of some diseases<sup>[6,7]</sup>.

In this research, sICAM-1, D-lactate and DAO of 69 patients with IBD were measured quantitatively with the objective of assessing their changes and clinical significance.

## MATERIALS AND METHODS

### Materials

**Subjects:** Test group including 69 patients, aged 18-60 years, with IBD hospitalized in our department [41 cases of ulcerative colitis (UC), 27 males and 13 females; 19 cases of Crohn's disease (CD), 11 males and eight females]. All patients were diagnosed by enteroscopy and were given the standard treatment set by the National

Symposium on IBD, 2000 for 15 d before reassessment. Thirty healthy blood donors are used as control group.

**Reagents and apparatus:** Human serum sICAM-1 enzyme-linked immunosorbent assay (ELISA) kit (purchased from Boehringer Mannheim, Germany); D-lactate standard solution and D-lactic acid dehydrogenase, O-dianisidine, cadaverine dihydrochloride, horseradish peroxidase and DAO standard solution (all purchased from Sigma). The following analytical reagents were prepared in the laboratory: methotrexate injection (purchased from Zhejiang Wanma Pharmaceutical Ltd.); sulfasalazine tablets (purchased from Shanghai Sanwei Pharmaceutical Ltd.); superoxide dismutase and myeloperoxidase kit (purchased from Nanjing Jiancheng Biological Institute).

**Major analysis apparatus:** 721 spectrophotometer (Shanghai Sophisticated Scientific Instruments Ltd.).

### Methods

A 3 mL sample of venous blood was collected from each subject of the test and control groups. After the sample was injected into dry test tubes and the serum was separated centrifugally, the serum was stored at -20°C for examination.

The level of sICAM-1 in the serum was detected with an ELISA, the plasma level of D-lactate was determined, after the plasma was deproteinized with perchloric acid, using enzyme-coupled UV-spectrophotometry<sup>[8]</sup>; the serum DAO was determined by spectrophotometry as by Luk *et al*<sup>[2]</sup>; the number of white blood cells (WBC) was detected routinely.

### Statistical analysis

All the data are presented as mean ± SD. Statistical methods used included the *t*-test, analysis of variance and linear correlation analysis. Statistical software SPSS (version 10.0) was employed for data analysis.

## RESULTS

### Comparison between the test group and the control group

As is shown in Table 1, the levels of sICAM-1 (206.57 ± 79.21 vs 107.25 ± 52.41), D-lactate (1.46 ± 0.94 vs 0.82 ± 0.47), DAO (9.91 ± 5.64 vs 2.04 ± 0.95), and WBC (7.24 ± 2.33 vs 4.82 ± 1.46) in IBD patients were significantly higher than those of the control group (*P* < 0.01).

### Comparison of WBC, sICAM-1, DAO and D-lactate between the 49 UC cases of UC and 19 CD cases

As is shown in Table 2, no distinct difference was demonstrated in the levels of all four substances in the UC group and CD group (*P* > 0.05). Analysis of the levels of WBC, sICAM-1, DAO and D-lactate of the test group demonstrated that sICAM-1 was related to the levels of DAO, with the correlation coefficient being 0.321 and *P* < 0.01, and also to the level of D-lactate, with the correlation coefficient being 0.412, and *P* < 0.01.

**Table 1 Comparison of the values of WBC, serum sICAM-1, DAO and plasma D-lactate between the test IBD group and control group (mean  $\pm$  SD)**

Group	n	WBC ( $\times 10^9/L$ )	sICAM-1 (ng/mL)	DAO (U/mL)	D-lactate ( $\mu\text{g/mL}$ )
Test	69	7.24 $\pm$ 2.33	206.57 $\pm$ 79.21 <sup>a</sup>	9.91 $\pm$ 5.64 <sup>a</sup>	1.46 $\pm$ 0.94 <sup>a</sup>
Control	30	4.82 $\pm$ 1.46	107.25 $\pm$ 52.41	2.04 $\pm$ 0.95	0.82 $\pm$ 0.47

<sup>a</sup>P < 0.01 vs control group. sICAM-1: Soluble intercellular adhesion molecule-1; DAO: Diamine oxidase; WBC: White blood cells; IBD: Inflammatory bowel disease.

**Table 2 Comparison of the values of the 4 substances in the ulcerative colitis (UC) group and Crohn's disease (CD) group (mean  $\pm$  SD)**

Group	n	WBC ( $\times 10^9/L$ )	sICAM-1 (ng/mL)	DAO (U/mL)	D-lactate ( $\mu\text{g/mL}$ )
UC	49	7.40 $\pm$ 2.61	212.94 $\pm$ 69.89 <sup>a</sup>	8.65 $\pm$ 3.54 <sup>a</sup>	6.35 $\pm$ 2.35 <sup>a</sup>
CD	20	7.29 $\pm$ 2.25	208.31 $\pm$ 51.05 <sup>b</sup>	8.58 $\pm$ 2.49 <sup>b</sup>	6.32 $\pm$ 2.23 <sup>b</sup>
Control	30	4.82 $\pm$ 1.46	107.25 $\pm$ 52.41	2.04 $\pm$ 1.35	0.82 $\pm$ 0.17

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

**Table 3 Comparison of WBC, sICAM-1, DAO and D-lactate of the IBD group before and after treatment (mean  $\pm$  SD)**

IBD	WBC ( $\times 10^9/L$ )	sICAM-1 (ng/mL)	DAO (U/mL)	D-lactate ( $\mu\text{g/mL}$ )
Pre-treatment	7.24 $\pm$ 2.33	206.57 $\pm$ 79.21	9.91 $\pm$ 5.64	1.46 $\pm$ 0.94
Post-treatment	5.21 $\pm$ 3.21	146.21 $\pm$ 64.43 <sup>a</sup>	6.42 $\pm$ 2.18 <sup>a</sup>	0.52 $\pm$ 0.32 <sup>a</sup>

<sup>a</sup>P < 0.01 vs pre-treatment.

### Comparison of WBC, sICAM-1, DAO and D-lactate of the test group before and after treatment

The levels of WBC, sICAM-1, DAO and D-lactate of the test group were examined after the subjects were treated and the IBD was shown to be improved substantially by clinical symptoms and enteroscopy. As is shown in Table 3, the post-treatment levels of sICAM-1 (206.57  $\pm$  79.21 vs 146.21  $\pm$  64.43), D-lactate (1.46  $\pm$  0.94 vs 0.52  $\pm$  0.32), DAO (9.91  $\pm$  5.64 vs 6.42  $\pm$  2.18) and WBC (7.24  $\pm$  2.33 vs 5.21  $\pm$  3.21) were significantly lower than before treatment (P < 0.01).

## DISCUSSION

In case of an acute attack of IBD, the structure and function of the intestinal mucosa of the patient can be seriously damaged, resulting in intestinal barrier dysfunction and thus release of intestinal bacteria, and may even induce multi-organ failure thus endangering life.

Presently, whether IBD is active is comprehensively judged primarily from clinical symptoms, erythrocyte sedimentation rate, C-reactive protein, endoscopic observations and pathology. This method can, in most cases, determine the activity of IBD quite accurately. However, for a small number of patients, those with the disease in the early stage and those with recurrence of the disease, the biochemical features are not conspicuous enough and endoscopic examination fails to display the acute activity of IBD, thus missing the opportunity of treatment because of the lack of a timely diagnosis; therefore, working out a set of monitoring indexes which are more

sensitive is of substantial importance to clinical work.

The permeability of the intestinal mucosa has been used for the assessment of the development and prognosis of some diseases, such as CD, UC, etc. The prospective research by Tibble *et al*<sup>[9]</sup> showed that 12 mo's duration of the alteration in intestinal mucosal permeability worsens the disease, with the sensitivity and specificity being 84% and 61%, respectively. In addition, this index can also be used in prediction of the recurrence of the disease. The multi-parameter regression analysis of 47 cases of intensive care patients<sup>[9,10]</sup> demonstrated that the intestinal mucosal permeability is the only index which can predict the development of multi-organ function failure syndrome in critical patients.

Injury is a process of inflammation, during which the adhesion of leukocytes and the damage to intestinal endothelial cells will increase the level of DAO and D-lactate. In this study, the serum DAO and D-lactate of the 69 patients with IBD before treatment were higher than those of the control group (P < 0.01), but no distinct difference was observed between the UC group and the CD group. The levels of DAO and D-lactate after treatment saw a marked decline. This offers proof to the fact that the levels of DAO and D-lactate, which reflect the changes in the disease condition, increase with activity of IBD but decline when the damage to the intestinal mucosa is restored after treatment.

Sans *et al*<sup>[11]</sup> found that sICAM-1 increased in the inflammatory intestinal mucosa of patients with IBD and was related to the concentration of sICAM-1 in the blood. The research by Mack *et al*<sup>[12]</sup> revealed that when

the expression of endotheliocyte ICAM-1 was lowered, the interaction between endotheliocytes and leukocytes weakened, thus alleviating the inflammatory activity. Taniguchi *et al*<sup>[13]</sup> relieved the symptoms of colitis induced by dextran sulphate sodium by adopting preventive treatment using anti-ICAM-1 monoclonal antibody.

In our research, it was found that the content of DAO and D-lactate of IBD patients were markedly higher than those of the control group, showing that damage to a certain degree existed in the intestinal mucosa of IBD patients in the acute stage ( $P < 0.01$ ), and the permeability increased. In addition, the post-treatment content of both DAO and D-lactate decreased, indicating that the permeability had somewhat improved.

It was also demonstrated in the research that the IBD patients' leukocyte number and sICAM-1 level before treatment were markedly higher than those of the control group ( $P < 0.01$ ) but markedly declined after treatment ( $P < 0.01$ ). This testified to the fact that the sICAM-1 level was obviously related to the condition of the disease. The research on anti-intracellular adhesion molecule monoclonal antibody in the treatment of IBD also pointed to the role of sICAM-1 in IBD.

To sum up, the tissue damage caused by the adhesion between leukocytes and endotheliocytes is one of the common pathologies in IBD produced by bacteria and cytokines. As high expression of sICAM-1 is the important molecular basis for the increase of the adhesion between leukocytes and endotheliocytes, blocking this adhesion has become an effective preventive and therapeutic method; therefore, sICAM-1, DAO and D-lactate in the serum can be utilized as important monitoring indexes in the treatment of IBD, and sICAM-1 may be a promising molecular indicator of the activity of IBD.

## COMMENTS

### Background

The barrier function of the intestinal mucosa is of vital importance in inflammatory bowel diseases (IBD). Detection of this function provides an important basis in the diagnosis of intestinal mucosal barrier dysfunction.

### Research frontiers

The importance of a timely correct assessment of the intestinal mucosal barrier function cannot be overestimated in judging the patient's disease state, estimating the prognosis and determining a comprehensive treatment program. However, direct observation of the intestinal barrier function involves many difficulties, so the observation mostly needs to be made indirectly.

### Innovations and breakthroughs

Presently, whether IBD is active is comprehensively judged primarily from clinical symptoms, erythrocyte sedimentation rate, C-reactive protein, endoscopic observations and pathology. This method can, in most cases, determine the activity of IBD quite accurately. However, for a small number of patients, those with the disease in the early stage and those with recurrence of the disease, the biochemical features are not conspicuous enough and endoscopic examination will fail to display the acute activity of IBD, thus missing the opportunity of treatment because of the lack of a timely diagnosis; therefore, determination of a set of monitoring indexes which are more sensitive is of substantial importance to clinical work.

### Applications

Serum soluble intercellular adhesion molecule-1 (sICAM-1), plasma D-lactate and diamine oxidase can be used as very important diagnostic indices, and sICAM-1 may be a useful molecular marker of the activity of IBD.

### Peer review

In this manuscript, Song *et al* report that serum levels of sICAM-1, D-lactate, and diamine oxidase are increased in IBD patients. Although the sample numbers tested seem to be relatively low, I feel that this manuscript contains potentially attractive data that would be of benefit to both clinical and basic scientific fields of IBD.

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BRIEF ARTICLES

## Barrier-focused intervention to increase colonoscopy attendance among nonadherent high-risk populations

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excluded from the study. The colonoscopy attendance rate was 23.04% (428/1858) during the first 12 mo without intervention, and 37.69% (539/1430) during the second 12 mo with intervention ( $P < 0.001$ ). Logistic regression analysis showed that the intervention was more effective among subjects with only objective barriers (OR: 34.590, 95% CI: 23.204-51.563) or subjects with some specific high-risk characteristics: first-degree relatives diagnosed with CRC (OR: 1.778, 95% CI: 1.010-3.131), personal history of intestinal polyps (OR: 3.815, 95% CI: 1.994-7.300) and positive result for immunochemical fecal occult blood testing (OR: 2.718, 95% CI: 1.479-4.996).

**CONCLUSION:** The barrier-focused telephone or on-site consultation intervention appears to be a feasible means to improve colonoscopy attendance among nonadherent high-risk subjects for CRC screening in China.

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**Key words:** Colorectal cancer; Screening; Intervention; Colonoscopy; Attendance

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### Abstract

**AIM:** To increase attendance for colonoscopy among nonadherent high-risk individuals for colorectal cancer (CRC) screening in China.

**METHODS:** During the first 12 mo without intervention, only 428 of the 2398 high-risk subjects attended a scheduled colonoscopy examination. The 1970 subjects who did not attend for CRC screening were enrolled in the present study. Prior barrier investigation was performed to ascertain the reasons for nonadherence. A barrier-focused intervention program was then established and implemented among eligible nonadherent subjects by telephone interviews and on-site consultations. The completion rates of colonoscopy during the first 12 mo without intervention and the second 12 mo with intervention were compared. Variations in the effect of the intervention on some high-risk factors and barrier characteristics were analyzed using logistic regression.

**RESULTS:** 540 subjects who were not eligible were

### INTRODUCTION

In 2002, colorectal cancer (CRC) was the fourth most common cancer diagnosed worldwide in men and the third in women, over 1 000 000 new cases were diagnosed, and more than 500 000 deaths resulted<sup>[1]</sup>. CRC screening can reduce mortality and incidence<sup>[2-5]</sup>. Data from 1991 to 2004 for the United States show that advances in prevention, early detection, and treatment of cancer have resulted in an approximately 14% decrease

in overall cancer mortality, with remarkable declines for lung, colorectal, breast and prostate cancer<sup>[6]</sup>. This evidence demonstrates that changes in lifestyle and/or cancer screening have been responsible for the decline in cancer mortality and incidence. The data from Chinese CRC screening practice have also shown decreased mortality and incidence rate in Jiashan and Haining<sup>[7,8]</sup>. A two-step screening method has been recommended for community-based CRC screening in China: immunochemical fecal occult blood testing (iFOBT) and investigation by questionnaire of high-risk factors, followed by colonoscopy examination.

Although attendance rate for colonoscopy examination is crucial to the screening effect, it has remained disappointing in our urban CRC screening. Low attendance for CRC screening has also been found in other urban areas in China<sup>[9]</sup>. Therefore, it is necessary to establish effective CRC screening intervention methods to increase attendance. The reasons for low attendance<sup>[10-16]</sup> can be categorized into subjective or objective barriers. In this study, we evaluated the effects of a barrier-focused intervention program on colonoscopy attendance among nonadherent high-risk subjects undergoing community-based CRC screening in an urban area.

## MATERIALS AND METHODS

### **CRC screening protocol in China**

The protocol for CRC mass screening in China is as follows<sup>[17]</sup>. Primary screening: subjects aged 40-74 years, who are positive for one or more of the following items are considered to be at high-risk for CRC: (1) immunochemical fecal occult blood testing (iFOBT); (2) first-degree relatives (FDRs) with CRC; (3) personal history of cancer or colorectal polyps; (4) two or more of the following items: (I) chronic diarrhea; (II) chronic constipation; (III) mucous and bloody stools; (IV) personal history of appendicitis or appendectomy; (V) personal history of chronic cholecystitis or cholecystectomy<sup>[18]</sup>; (VI) history of psychiatric trauma (e.g. divorce or death of relatives). Secondary screening: subjects who are considered to be at high risk for CRC should undergo colonoscopy examination.

### **Enrollment**

From the end of July 2006, we conducted population-based CRC screening according to Chinese screening guidelines in Xiacheng District, Hangzhou, China. All participants provided written informed consent. The 2398 subjects who were positive for the questionnaire and/or iFOBT were regarded as the high-risk population and were invited to follow-up colonoscopy. Only 428 (17.85%) high-risk subjects had attended a scheduled colonoscopy examination by the end of July 2007. The remained 1970 nonadherent high-risk subjects who did not attend colonoscopy examination were enrolled as the target population for our intervention. The cost of screening and intervention were supported by our research funding.

**Table 1 Main barriers to colonoscopy among high-risk subjects (*n* = 407)**

Barriers	<i>n</i> (%)
Subjective barriers	
"I am not at risk for CRC": no symptoms	185 (45.45)
Fear of finding cancer and subsequent surgery	10 (2.46)
Fear of some complications related to colonoscopy	9 (2.21)
Other personal health concerns <sup>1</sup>	9 (2.21)
Embarrassment	2 (0.49)
Total	215 (52.83)
Objective barriers	
Intolerance of pain or discomfort	112 (27.52)
Busy on working days	50 (12.29)
Intolerance of bowel preparation or diet control	14 (3.44)
Inconvenience and complexity of colonoscopy procedure	14 (3.44)
Transportation problems	2 (0.49)
Total	192 (47.17)

<sup>1</sup>Subjects preferred to focus on current health problems, such as diabetes, and fractures. CRC: Colorectal cancer.

### **Barrier investigation**

To obtain in-depth information about the reasons for nonadherence, we selected 500 subjects from the target population and conducted questionnaire surveys by telephone or on-site interviews about the barriers to adherence. The questionnaires contained some acknowledged barrier options. Barriers were classified as subjective or objective. Only the most influential barrier was recorded. Four hundred and seven valid questionnaires were obtained. Table 1 lists the main barriers among high-risk nonadherent subjects.

### **Population classification**

We planned to classify the target population into three groups for later evaluation of intervention effects. (1) Subjects with one or more subjective barriers (such as "I am not at risk for CRC"), with or without objective barriers. At least, the subjective barriers prevented them from attending colonoscopy examination. (2) Subjects with one or more objective barriers (such as intolerance of pain), without subjective barriers. They recognized the screening benefits but the objective barriers prevented them attending. (3) Subjects who simply forgot about or missed their colonoscopy examination. They were prepared to undergo colonoscopy.

### **Establishment and implementation of intervention**

Based on the results of barrier investigation, a barrier-focused intervention program was established. The program included general and special measures. The general measures were performed among all eligible subjects and the special measures were performed selectively on each subject according to his/her specific barriers (Table 2). For subjects with subjective barriers, an explanation and education were used. For subjects with only objective barriers, the aim was mainly to adopt definite goals to improve objective conditions. For subjects without barriers, only general measures were used.

The intervention program was started at the end

**Table 2** Barrier-focused intervention program

Barriers	Special measures	General measures
Subjective barriers	Explanation and education	
"Not at risk for CRC": no symptoms	Explain benefits and importance of screening, and give results of previous screening	Answering questions about the screening
Fear of finding cancer	Explain that early detection of CRC leads to good prognosis	Reminding of upcoming appointments
Fear of complications or perforation	Explain that complications in colonoscopy examination are rare	Arranging on-site consultations from the physician and epidemiologists if necessary
Embarrassment	Ensure that the patient's confidence will be well protected	Providing guidance
Objective barriers	Improving objective condition	
Intolerance of pain	Arranging painless colonoscopy <sup>1</sup>	
Lack of time on working days	Arranging examination at weekends	
Intolerance of bowel preparation	Simplifying bowel preparation	
Inconvenience and complexity of colonoscopy procedure	Providing guidance	

<sup>1</sup>Painless colonoscopy: Intravenous general anesthesia was performed during colonoscopy.**Table 3** Baseline characteristics of eligible subjects (*n* = 1430)

Characteristics	<i>n</i> (%)
Age (yr)	
40-59	687 (48.04)
60-74	743 (51.96)
Sex	
Male	511 (35.73)
Female	919 (64.27)
High-risk factors	
FDRs diagnosed with CRC	240 (16.78)
Personal history of intestinal polyps	105 (7.34)
Personal history of other cancers	136 (9.51)
Positive for questionnaire <sup>1</sup>	839 (58.67)
Positive for iFOBT	241 (16.85)
Characteristics of barriers	
With subjective barriers	646 (45.17)
With objective barriers	714 (49.93)
Without barriers	70 (4.90)

<sup>1</sup>Positive for questionnaire refers to having ≥ 2 of the following six items: (1) chronic diarrhea; (2) chronic constipation; (3) mucous or bloody stools; (4) personal history of appendicitis or appendectomy; (5) personal history of chronic cholecystitis or cholecystectomy; (6) history of psychiatric trauma. iFOBT: Immunochemical fecal occult blood testing.

of August 2007. The 1970 subjects received a series of telephone interviews or on-site interviews. The prevention care managers carried out the following: (1) explained the study purpose and obtained consent; (2) assessed eligibility by inquiring about patient disease history, and it was suggested that those with a disease history should be evaluated by specialists to make sure that they would tolerate the colonoscopy examination; (3) identification of the specific individual barriers to colonoscopy; (4) implementation of intervention measures predesigned to reduce these barriers; (5) arranged on-site consultations, during which the oncology physician and epidemiologists answered questions and tried to persuade subjects to participate in the colonoscopy examination; and (6) scheduled the colonoscopy appointments if the subjects consented.

The prevention care managers reminded the subjects by telephone 2 d ahead of the examination and provided guidance on the day of examination. Colonoscopy was

performed by gastroenterologists in the endoscopy units of local hospitals, and the results were retrieved via manual review of the medical records.

The intervention process was terminated in the following situations: (1) the subject attended a scheduled colonoscopy; (2) the subject definitely refused our invitation; and (3) the subjects were lost to follow-up after at least three calls. The intervention program was terminated at the end of August 2008.

### Statistical analysis

The  $\chi^2$  test was used to evaluate statistical differences between the colonoscopy completion rates. Logistic regression analysis was used to evaluate the variation in the effects of the intervention on different characteristics of barriers and among subgroups with different high-risk factors. All data were analyzed using SPSS version 16.0.

## RESULTS

### Baseline characteristics

Five hundred and forty subjects were excluded from the study because of death (*n* = 16), medical unfitness to undergo colonoscopy (*n* = 154), movement from the community (*n* = 260), or other reasons (*n* = 110). Table 3 provides the baseline characteristics.

### Intervention effect

Twenty-five subjects were lost to follow-up during the intervention, and we assumed that these subjects refused the colonoscopy invitation. The above 540 subjects were excluded from the study. The colonoscopy attendance rate was 23.04% (428/1858) during the first 12 mo without intervention, and 37.69% (539/1430) during the second 12 mo with intervention ( $P < 0.001$ ). Among the eligible 1430 subjects, attendance rate post-intervention was 5.11% (33/646) for subjects with subjective barriers, 62.32% (445/714) for those with objective barriers, and 87.14% (61/70) for those with no barriers.

As shown in Table 4, the intervention was more effective for subjects with only objective barriers (OR:

**Table 4** Variation in intervention effects (*n* = 1430)

Characteristics	Adjusted OR	95% CI	P value
Age (yr)			
40-59	1.000	0.643-1.133	0.273
60-74	0.853		
Sex			
Male	1.000		
Female	0.955	0.706-1.292	0.767
High-risk factors			
FDRs diagnosed with CRC	1.778	1.010-3.131	0.046
Personal history of intestinal polyps	3.815	1.994-7.300	0.000
Personal history of other cancers	1.444	0.745-2.799	0.277
Positive for questionnaire <sup>1</sup>	1.589	0.925-2.727	0.093
Positive for iFOBT	2.718	1.479-4.996	0.001
Characteristics of barriers			
With subjective barriers	1.000		
With objective barriers	34.590	23.204-51.563	0.000
Without barriers	132.421	59.709-293.681	0.000

<sup>1</sup>Positive for questionnaire refers to having  $\geq 2$  of the following six items: (1) chronic diarrhea; (2) chronic constipation; (3) mucous or bloody stools; (4) personal history of appendicitis or appendectomy; (5) personal history of chronic cholecystitis or cholecystectomy; (6) history of psychiatric trauma. FDRs: First-degree relatives.

34.590, 95% CI: 23.204-1.563) and those without barriers (OR: 132.421, 95% CI: 59.709-293.681), compared to those with subjective barriers. The intervention was also more effective for subjects with FDRs diagnosed with CRC (OR: 1.778, 95% CI: 1.010-3.131), personal history of intestinal polyps (OR: 3.815, 95% CI: 1.994-7.300) or positive results for iFOBT (OR: 2.718, 95% CI: 1.479-4.996).

## DISCUSSION

In previous studies on CRC screening intervention, higher attendance was achieved by some intervention practices such as health-care-provider-directed intervention<sup>[19]</sup>, telephone support<sup>[20,21]</sup>, psychoeducational intervention<sup>[22,23]</sup>, tailored guidance<sup>[24,25]</sup>, patient-physician communication<sup>[26]</sup>, motivational interviewing<sup>[27]</sup>, physician reminder<sup>[28,29]</sup>, community volunteers<sup>[30]</sup>, and mailed brochure<sup>[31]</sup>. However, one-sided intervention methods may not achieve a satisfactory effect among nonadherent high-risk subjects.

In this study, we first identified the main barriers to colonoscopy examination, and then established a multifaceted barriers-focused intervention program that targeted objective and subjective barriers. The results indicated that the intervention program effectively increased the completion rate of colonoscopy among nonadherent high-risk subjects. The attendance rate of colonoscopy screening significantly increased during the intervention compared with the first 12 mo without intervention (23.04% vs 37.69%, *P* < 0.001), which reduced more effectively the mortality and incidence in the screening area, because more positive lesions were detected<sup>[2,32]</sup>. Moreover, the targeted subjects during the intervention were nonadherent and did not respond to the examination invitations during the first 12 mo, which

made the intervention more difficult.

There were several barriers for any one subject, which made evaluation of each specific intervention measure complicated and inaccurate. We simplified the evaluation by classifying the target population into three behavioral groups according to their respective barriers. Colonoscopy completion rate in the population with objective barriers was higher than that in those with subjective barriers (62.32% vs 5.11%), which indicates that measures that target objective barriers are more effective. The colonoscopy completion rate in the population without barriers was as high as 87.14%, which indicates that only general intervention measures achieve satisfactory results among these individuals. Logistic regression also showed that the intervention was more effective for subjects with objective barriers and with no barriers compared to those with subjective barriers. Intervention measures that target subjective barriers should be improved to further increase the uptake rate.

Previous studies on barriers to CRC screening have noted that younger age is a predictor of receiving a physician recommendation for screening<sup>[33,34]</sup>. However, in our study, logistic regression showed that there was no significant difference in intervention effects between middle-aged (40-59 years) and aged (60-74 years) populations. This may have resulted from the differences between our study population and those of previous studies in terms of ethnicity, socioeconomic status and other sociodemographic characteristics.

Our findings suggested that several high-risk factors were associated positively with intervention effect. The intervention was more effective among subjects with a history of intestinal polyps. Most of these subjects had been advised by their physicians to take regular examinations, which may have contributed to their higher compliance with our intervention. Subjects with positive results for iFOBT showed better compliance. The perceived value of different high-risk factors may explain partially the variation in intervention effect. According to the medical knowledge of high-risk subjects, some items in the questionnaire (e.g. personal history of appendicitis or appendectomy, history of psychiatric trauma) seem to be less specific to CRC than iFOBT is. This may reduce the perceived importance of questionnaire investigation and cause the subjects to consider iFOBT more valuable in CRC screening. However, further investigation is needed to confirm this explanation. The poor compliance of subjects with positive questionnaire results highlighted the importance of tailoring education programs to address questionnaire investigation of CRC. Poor participation in CRC screening in FDRs of patients with CRC has been reported previously<sup>[35]</sup>. In our study, the subjects with FDRs diagnosed with CRC showed slightly better compliance. The possible reason was that the physicians who diagnosed and treated the index patients also explained the increased risk for the FDRs and advised CRC screening to increase their awareness and attendance.

Our study had several strengths: (1) we recruited

nonadherent high-risk subjects among whom intervention for colonoscopy examination has not been widely studied before; (2) the sample number was large, the consent rate was high, and few were lost to follow-up; and (3) the community setting of the study provided ready access to the subjects.

There were also some limitations to the study: (1) there was no comparison group, and one group pre- and post-test was studied; (2) it did not have saturation coverage of the communities, and it may not have included completion of all colonoscopy examinations, because the duration of the intervention was short; and (3) the population classification was based on self-reported barriers, which may have potential bias.

In summary, this barrier-focused intervention in nonadherent high-risk subjects may increase colonoscopy attendance in urban CRC screening. We found that our intervention in individuals with subjective barriers was not as effective or practical as in those with objective barriers. The next steps are to modify the intervention methods to better overcome subjective barriers. The ultimate goal is to apply the multifaceted barriers-focused intervention to other urban CRC screening regions in China.

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## COMMENTS

### Background

Colonoscopy examination is regarded as the gold standard for mass screening of colorectal cancer (CRC). Although attendance rate for colonoscopy examination among high-risk populations is crucial for screening success, it has remained disappointing for CRC screening. Therefore, it is necessary to establish effective screening intervention methods to increase colonoscopy compliance.

### Research frontiers

In previous studies on CRC screening intervention, higher attendance was achieved by some intervention practices such as health-care-provider-directed intervention, or telephone support. However, one-sided intervention methods may not achieve a satisfactory effect among nonadherent high-risk subjects. Many studies have reported that subjective or objective barriers prevent high-risk subjects from undergoing colonoscopy examination, which indicates that barrier-focused intervention might be effective. However, such barrier-focused intervention has not been reported previously.

### Innovations and breakthroughs

This is believed to be the first study to establish a barrier-focused intervention program and evaluate its effects on colonoscopy attendance in CRC screening. Moreover, in this study, nonadherent high-risk subjects were recruited, among whom, intervention for colonoscopy examination has not been widely studied before.

### Applications

The study suggests that barrier-focused intervention is an effective method and may be used to increase colonoscopy attendance in other CRC screening regions.

### Terminology

Barrier-focused intervention in CRC screening is an intervention method that

aims to increase colonoscopy attendance by removing the barriers that prevent the subjects from undergoing colonoscopy examination. Barrier-focused intervention must be tailored or individualized because every subject may have different barriers.

### Peer review

The authors performed barrier-focused intervention to increase colonoscopy attendance among nonadherent high-risk populations in China. The colonoscopy attendance rate was 23% during the first 12 mo without intervention and 38% during the second 12 mo with intervention. The authors concluded that the barrier focused telephone or on-site consultation intervention appears to be a feasible means to improve colonoscopy attendance among nonadherent high-risk populations for CRC screening. Improving colonoscopy attendance in CRC screening is an important issue, and the results of this study are interesting.

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BRIEF ARTICLES

## Prognostic impact of dissected lymph node count on patients with node-negative gastric cancer

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### Abstract

**AIM:** To investigate the long-term effect of the number of resected lymph nodes (LNs) on the prognosis of patients with node-negative gastric cancer.

**METHODS:** Clinical data of 211 patients with gastric cancer, without nodal involvement, were analyzed retrospectively after D2 radical operation. We analyzed the relationship between the number of resected LNs with the 5-year survival, the recurrence rate and the post-operative complication rate.

**RESULTS:** The 5-year survival of the entire cohort was 82.2%. The total number of dissected LNs was one of the independent prognostic factors. Among patients with comparable depth of invasion, the larger the number of resected LNs, the better the survival ( $P < 0.05$ ). A cut-point analysis provided the possibility to detect a significant survival difference among subgroups. Patients had a better long-term survival outcomes with LN counts  $\geq 15$  for pT1-2,  $\geq 20$  for pT3-4, and  $\geq 15$  for the entire cohort. The overall recurrence rate was 29.4% within 5 years after surgery. There was a statistically significant, negative correlation between the number of resected LNs and the recurrence rate ( $P < 0.01$ ). The post-operative complication rate was 10.9% and was not significantly correlated with the number of dissected LNs ( $P > 0.05$ ).

**CONCLUSION:** For node-negative gastric cancer, sufficient number of dissected LNs is recommended during D2 lymphadenectomy, to improve the long-term survival and reduce the recurrence. Suitable increments of the dissected LN count would not increase the post-operative complication rate.

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**Key words:** Stomach neoplasms; Lymph nodes; Gastrectomy; Lymphadenectomy; Prognosis

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Huang CM, Lin JX, Zheng CH, Li P, Xie JW, Lin BJ, Lu HS. Prognostic impact of dissected lymph node count on patients with node-negative gastric cancer. *World J Gastroenterol* 2009; 15(31): 3926-3930 Available from: URL: <http://www.wjgnet.com/1007-9327/15/3926.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.3926>

### INTRODUCTION

Gastric cancer is a common malignancy worldwide. Despite its declining incidence, mortality of gastric cancer remains high<sup>[1]</sup>. It is generally accepted that a higher survival rate benefits from a standardized pattern of lymph node dissection<sup>[2-4]</sup>. Many studies favor an extended lymphadenectomy at the time of potentially curative gastrectomy for node-positive gastric cancer, and the risk of long-term death tends to decrease when the number of resected lymph nodes increases to about 25<sup>[5-7]</sup>. However, there are few studies on how many nodes should be removed and examined when performing a radical gastrectomy for node-negative gastric cancer. Therefore, the aim of this retrospective study was to evaluate the long-term effect of the number of resected lymph nodes (LNs) on the prognosis of patients with node-negative gastric cancer.

### MATERIALS AND METHODS

#### Materials

Between January, 1995 and March, 2004, 211 patients diagnosed with primary gastric cancer were treated with curative resection at the Department of Oncology,

Affiliated Union Hospital of Fujian Medical University, Fuzhou, China. The surgical procedure was defined as curative when no grossly visible tumor tissue (metastasis or LN involvement) remained after resection and when the resection margins were histologically normal. There were 164 males and 47 females whose age ranged from 29 to 84 years ( $57.4 \pm 11.3$  years). All patients received a D2 dissection according to the Japanese Classification of Gastric Carcinoma<sup>[8]</sup>. Lymph nodes were meticulously dissected from the en bloc specimens, and the classification of the dissected LNs was determined by specialist surgeons who reviewed the excised specimens after surgery, based on the Japanese Classification of Gastric Carcinoma<sup>[8]</sup>. All resected lymph nodes were proven to be without nodal involvement. The tumors' size was  $4.2 \pm 2.1$  cm. Patients were divided according to the primary site of gastric cancer: 75 upper third (U) tumors; 40 middle third (M) tumors; and 96 lower third (L) tumors. Based on the Japanese Classification of Gastric Carcinoma<sup>[8]</sup>, there were 69 at stage pT1, 58 at stage pT2, 62 at pT3 and 22 at pT4. As far as the histological grades were concerned, there were 55 differentiated and 156 undifferentiated cases. Total gastrectomy, proximal subtotal gastrectomy and distal subtotal gastrectomy were carried out in 99, 15 and 97 cases, respectively. A total of 4649 LNs were taken for histological examination, and the median number of LN per patient was 22 (range, 6-48; mean  $22.7 \pm 7.0$ ). Routine follow-up consisted of physical examination, laboratory tests (including measurement of carcinoembryonic antigen, CA19-9 and CEA levels), chest radiography, and abdominopelvic ultrasonography or computed tomography (CT). At the early stage (pT1), patients were followed every 6 mo during the first 2 years, and then yearly beyond the third year; at more advanced stages (pT2 or greater), follow-up was every 3 mo during the first year, every 6 mo or yearly beyond the second year, for a total of 5 years. Endoscopy was performed every 6 mo or yearly. All surviving patients were followed for more than five years. The survival time was the time from diagnosis until the last contact, the date of death, or the date that the survival information was collected. The rate of follow-up visits was 92.9%, with 196 cases involved.

## Methods

Patients were stratified into five groups, based on the total number of LNs removed, as follows: < 15 LNs (18 cases), 15-19 LNs (49 cases), 20-24 LNs (63 cases), 25-29 LNs (44 cases) and  $\geq 30$  LNs (37 cases). The statistical analysis was performed with the Statistical Package for Social Science (SPSS), version 13.0 for Windows. Actuarial survival was determined via the Kaplan-Meier method, with univariate comparisons between groups through the log-rank test. Cox regression was used for multivariate analysis, with a backward elimination model for all covariates. Spearman rank correlation coefficient was used to analyze the relationship between the number of dissected LNs and recurrence rate. The correlation between the number of resected LNs and post-operative complication rate was analyzed with logistic regression model. Significance of differences was assumed at  $P < 0.05$ .

**Table 1 Univariate analysis of variables in patients with node-negative gastric cancer**

Characteristics	n	5-year survival rate (%)	$\chi^2$	P value
Gender			1.887	0.170
Male	164	81.7		
Female	47	84.0		
Age (yr)			0.619	0.431
< 60	109	84.2		
$\geq 60$	102	79.9		
Tumor size (cm)			4.538	0.033
$\leq 4$	94	88.9		
> 4	117	76.8		
Tumor location			1.681	0.432
Upper	75	80.1		
Middle	40	81.7		
Lower	96	84.0		
Depth of invasion			27.698	0.000
T1	69	92.4		
T2	58	83.9		
T3	62	76.4		
T4	22	57.8		
Pathology			0.212	0.645
Differentiated	55	88.6		
Undifferentiated	156	80.0		
Surgical type			0.219	0.640
Total gastrectomy	99	80.5		
Subtotal gastrectomy	112	83.6		
Number of resected LNs			45.219	0.000
0-14	18	43.2		
15-19	49	76.8		
20-24	63	84.5		
25-29	44	90.6		
$\geq 30$	37	94.5		

**Table 2 Multiple stepwise regression analysis with the Cox proportional hazards model**

Characteristics	$\beta$	SE	Wald	P	RR	95% CI
Tumor size (cm)	0.223	0.319	0.487	0.485	1.249	0.668-2.335
Depth of invasion			8.802	0.032		
T2 vs T1	0.789	0.483	2.666	0.103	2.200	0.854-5.670
T3 vs T1	0.945	0.464	4.138	0.042	2.572	1.035-6.391
T4 vs T1	1.466	0.500	8.605	0.003	4.334	1.627-11.545
Number of resected LNs	-0.641	0.141	20.628	0.000	0.527	0.399-0.695

$\beta$ : Coefficient of regression.

## RESULTS

### Univariate survival analysis

The five-year overall survival rate of the entire cohort was 82.2%. The clinicopathological variables tested in the univariate analysis are shown in Table 1. Factors influencing the 5-year survival rate were tumor size ( $P = 0.033$ ), depth of invasion ( $P < 0.001$ ) and number of resected LNs ( $P < 0.001$ ). The covariates age ( $P = 0.431$ ), gender ( $P = 0.170$ ), tumor location ( $P = 0.432$ ), pathological types ( $P = 0.645$ ) and type of gastrectomy ( $P = 0.640$ ) had no significant influence on the survival.

### Multivariate survival analysis

Multiple survival analysis was calculated by the Cox's proportional hazard regression model. The prognostic

**Table 3 Five-year overall survival by stage subgroups and total number of resected LNs n (%)**

Depth of invasion	n	OS					P
		0-14	15-19	20-24	25-29	≥ 30	
T1	69	4 (37.5)	19 (83.9)	20 (88.9)	17 (100.0)	9 (100.0)	0.003
T2	58	4 (25.0)	14 (70.1)	20 (89.4)	11 (90.9)	9 (100.0)	0.005
T3	62	7 (57.1)	12 (72.7)	19 (83.5)	10 (88.9)	14 (92.3)	0.022
T4	22	3 (33.3)	4 (75.0)	4 (50.0)	6 (66.7)	5 (80.0)	0.045
Total	211	18 (43.2)	49 (76.8)	63 (84.5)	44 (90.6)	37 (94.5)	0.000

OS: Overall 5-year survival rate.

**Table 4 Overall survival by total LN count and cut-point analysis per each stage subgroup**

Depth of invasion	≤ 14 vs ≥ 15		≤ 19 vs ≥ 20		≤ 24 vs ≥ 25		≤ 29 vs ≥ 30	
	χ <sup>2</sup>	P						
pT1-2	19.953	0.000	13.595	0.000	5.249	0.022	2.216	0.137
pT3-4	11.916	0.001	14.196	0.000	12.262	0.000	6.483	0.011
Total	32.824	0.000	26.679	0.000	16.109	0.000	7.572	0.006

**Table 5 Impact of number of resected LNs on recurrence rate n (%)**

Depth of invasion	No. of resected LNs	n	Recurrence rate <sup>2</sup>	r	P
pT1-2	0-14	8	5 (62.5)	-0.253	0.001 <sup>1</sup>
	15-19	33	11 (33.3)		
	20-24	40	8 (20.0)		
	25-29	28	5 (17.8)		
	≥ 30	18	1 (5.6)		
pT3-4	0-14	10	8 (80.0)	-0.405	0.000 <sup>1</sup>
	15-19	16	9 (56.2)		
	20-24	23	8 (34.8)		
	25-29	16	4 (25.0)		
	≥ 30	19	3 (15.8)		

<sup>1</sup>Significance of differences was assumed at P < 0.01; <sup>2</sup>Recurrence within 5 years after surgery.

factors considered at univariate analysis were analyzed first by stepwise regression, including tumor size, depth of invasion and number of resected LNs. As a result, there were two independent, statistically significant prognostic parameters: depth of invasion ( $P = 0.032$ ) and number of resected LNs ( $P < 0.001$ ). The risk ratios and their 95% confident interval were listed in Table 2.

#### Impact of total LN counts by univariate survival analysis

The five-year survival, based on T category, showed considerable variations with increasing counts of resected LNs. An obvious trend toward better survival was observed for higher numbers of resected LN (Table 3).

#### Cut-point survival analysis

A cut-point analysis was performed to determine the numeric LN value that determines the greatest actuarial survival difference between pT1-2 and pT3-4 subgroups. We selected the ability to detect differences between groups based on the magnitude of the log-rank test  $\chi^2$  statistic. Results for all relevant cut points and stage subgroups are listed in Table 4. The cut-point analysis yielded the greatest survival difference at the levels of 15 (pT1-2), 20 (pT3-4) and 15 (entire cohort).

#### Impact of the number of resected LNs on recurrence rate

Within 5 years after surgery, a recurrence was confirmed by clinical, radiological examination or reoperation in 62 patients, with an overall recurrence rate of 29.4%. The number of patients with peritoneal and lymph nodes recurrence were 28 and 13 respectively. The most common site of haematogenous recurrence was the liver, occurring in 12 patients. Other patterns of recurrence were bone recurrence in 5 patients and lung recurrence in 4 patients. The relationship between the number of resected LNs and recurrence rate was analyzed by Spearman rank correlation coefficient (Table 5). For either pT1-2 or pT3-4 subgroups, there was a statistically significant, negative correlation between the number of dissected LNs and the recurrence rate ( $P < 0.01$ ).

#### Correlation between number of resected LNs and post-operative complication rate

Twenty-three of the 211 patients (10.9%) experienced postoperative complications. These included pancreatitis (4 cases), anastomotic leakage (3), intra-abdominal abscess (4), postoperative ileus (1), stenosis (3), countercurrent esophagus (2), pancreatic leakage (2) and pneumonia (4). Logistic regression analysis revealed that the number of dissected LNs was not significantly correlated with the post-operative complication rate ( $P = 0.214$ ).

## DISCUSSION

It is commonly regarded that lymph node metastases is one of the most important prognostic factors in patients with gastric cancer after curative operation<sup>[9,10]</sup>. Bruno *et al*<sup>[11]</sup> pointed out that patients with node-negative gastric cancers have clinicopathological features similar to those with early gastric cancer and they showed also how those patients have a better prognosis than those with node-positive gastric carcinoma. The 5-year survival rate of patients with node-negative gastric carcinoma was 89.5%, based on the work by Wu *et al*<sup>[12]</sup>, whereas, based on our present data, this rate is

82.2%. The only potentially curative treatment for this disease is complete surgical resection, with an en bloc LN dissection. D2 lymph node dissection can increase the long-term survival of gastric cancer patients with lymph node metastases and it has become a standard surgical procedure for curative treatment in Korea and Japan<sup>[13]</sup>. D2 lymph node dissection requires not only a specific anatomical extension (i.e. it must include both perigastric and suprapancreatic nodes), but also a certain number of resected LNs (at least 15). The number of dissected LNs is closely related to the postoperative pathologic staging and the prognostic assessment. Bouvier *et al*<sup>[14]</sup> investigated 749 cases with gastric cancer, and the results showed that the risk of misclassification became significant, with an error rate of 47.1% in the pathology reports, when fewer than 10 lymph nodes were examined. They concluded that, in the latter cases, staging is not reliable. Karpeh *et al*<sup>[15]</sup> reviewed 1038 patients with gastric cancer who had undergone gastrectomy with curative intent. They found that removal of  $\geq 15$  LNs appears to provide a considerable survival advantage, in comparison with the removal of  $< 15$  LNs. In an attempt to improve staging accuracy, it was recommended that a minimum of 15 lymph nodes be examined. Based on our data, the median number of total LNs examined was 22 (mean  $22.7 \pm 7.0$ ) per patient. In addition, the number of LNs dissected emerges as one of the most important prognostic indicators. Our present study shows that, with comparable depth of invasion, patients with a larger number of dissected LNs have a better survival rate ( $P < 0.05$ ), consistent with the results of Smith *et al*<sup>[16]</sup>. The contribution of negative LN counts to the prognosis of patients is partly due to LN micrometastases. In patients without LN metastases identified by HE staining, about 20% had LN micrometastases<sup>[17]</sup>. Thus, it can be concluded that an extended lymphadenectomy contributes to decrease the number of residual tumor cells, leading to better prognosis. However, it is unclear how many LNs need to be removed in a D2 dissection in patients with node-negative gastric cancer. Giuliani *et al*<sup>[18]</sup> suggested that patients with node-negative gastric cancer should undergo adequate lymphadenectomy to permit examination of  $\geq 23$  LNs, which would allow accurate identification of prognostic variables. From the cut-point analysis, we suggest that, to be adequate, the resection, at the time of a D2 radical dissection, should involve 15 LNs for pT1-2 and 20 LNs for pT3-4, and this based on an accurate survival estimates in patients with node-negative gastric cancer.

Despite improved prognosis, as a result of early diagnosis, radical operations and the development of adjuvant therapy<sup>[9,19,20]</sup>, death from gastric cancer is still almost entirely due to recurrent disease. About 5% of patients with early gastric cancer and 50% of patients with advanced gastric cancer die with recurrent disease, as reported by Böhner *et al*<sup>[21]</sup>. In a large set of follow-up data from Japan, among 14 000 patients with recurrent gastric cancer, the rate of loco-regional recurrence following potentially curative resection was 25.6%<sup>[22]</sup>. This

may be due to the insufficient anatomical extension of the dissection at the time of the first surgery, including an insufficient gastrectomy or insufficient lymphadenectomy. The randomized Dutch gastric carcinoma study compared D1 and D2 lymphadenectomy and appeared to observe a significant reduction of the loco-regional recurrence rate after a more extended lymphadenectomy: following D1 lymphadenectomy, 36% of patients died with local recurrence, compared with 27% when a D2 lymphadenectomy was performed ( $P < 0.05$ )<sup>[23]</sup>. Our present study shows that the overall recurrence rate is 29.4% within 5 years after surgery. The Spearman rank correlation coefficient analysis revealed that there was a statistically significant, negative correlation between the number of dissected LNs and the recurrence rate ( $P < 0.01$ ). A lower recurrence rate was observed in cases with higher total LNs, indicating that a sufficient lymphadenectomy contributed to reduce the number of residual tumor cells. While correlating the number of dissected LNs with the post-operative complication rate, Zilberstein *et al*<sup>[24]</sup> did not find any difference between the average number of dissected LNs in patients with and without complications. These authors suggested that the higher morbidity and mortality rates were not due to the extended lymphadenectomy. Those rates may be more likely correlated with the level of experience in gastroesophageal surgery, with the presence of an appropriate learning curve for radical gastrectomy and with the experience in the management of postoperative complications. The post-operative complication rate in our study was 10.9% and the logistic regression analysis showed that the number of dissected LNs did not correlate with the post-operative complication rate ( $P > 0.05$ ). According to our results, it appears that a suitable increment of the number of dissected LN would not increase the post-operative complication rate if the surgery is performed by a trained surgical team.

## COMMENTS

### Background

Many studies have favored an extended lymphadenectomy at the time of a potentially curative gastrectomy for node-positive gastric cancer, and the risk of long-term death tends to decrease when the number of resected lymph nodes increases to about 25. However, few studies have assessed the relative contribution of the number of total resected lymph nodes to the outcome in patients with node-negative gastric cancers.

### Research frontiers

It is generally accepted that a higher survival rate benefits from a standardized pattern of D2 radical dissection, which requires the removal of Group 1 and Group 2 lymph nodes (LNs). The number of dissected LNs is strongly correlated with patients' survival.

### Innovations and breakthroughs

The authors retrospectively reviewed 211 patients with node-negative gastric cancer, who were treated with D2 radical resection at a hospital in Fujian between 1995 and 2004, to assess how the number of dissected LNs may affect their survival rate.

### Applications

The authors suggest that importance should be attached to removing a sufficient number of LNs in order to improve the long-term survival and reduce the recurrence rate of patients with node-negative gastric cancer. Moreover, suitable increments of the number of dissected LNs would not increase the post-operative complication rate.

**Peer review**

The authors are to be congratulated on this paper, recording the effect of the number of lymph nodes resected in a large number of patients (211) with node negative gastric cancer and a remarkable survival rate of 82.2% at 5 years. The number of lymph nodes resected influenced the prognosis of the patients with node negative gastric cancer. This finding has important information for gastrointestinal surgeons.

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# Tacrolimus dosage requirements in living donor liver transplant recipients with small-for-size grafts

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graphic characteristics, acute rejection rates, liver and renal function test results, or the number of potentially interacting medications administered between the two groups. The tacrolimus dosage requirements in group S were significantly lower than group N at 2 wk ( $2.8 \pm 0.4$  mg/d vs  $3.6 \pm 0.7$  mg/d,  $P = 0.006$ ), 3 wk ( $2.9 \pm 0.7$  mg/d vs  $3.9 \pm 0.8$  mg/d,  $P = 0.008$ ), 4 wk ( $2.9 \pm 0.8$  mg/d vs  $3.9 \pm 1.0$  mg/d,  $P = 0.023$ ) and 2 mo ( $2.8 \pm 0.7$  mg/d vs  $3.8 \pm 1.1$  mg/d,  $P = 0.033$ ). Tacrolimus 12-h trough concentrations were similar between the two groups at all times except for 2 wk post-transplantation, when the concentrations were significantly greater in group S recipients than in group N recipients ( $11.3 \pm 4.8$  ng/mL vs  $7.0 \pm 3.8$  ng/mL,  $P = 0.026$ ).

**CONCLUSION:** SFS grafts recipients have significantly decreased tacrolimus dosage requirements compared with non-SFS grafts recipients in AALDLT during the first 2 mo post-surgery.

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**Key words:** Blood concentration; Liver transplantation; Living donor; Small-for-size graft; Tacrolimus

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## INTRODUCTION

With the shortage of cadaveric donor organs, living donor liver transplantation (LDLT) is generally accepted for end-stage liver disease. In LDLT, the graft size is inevitably small and requires regeneration, especially in small-for-size (SFS) grafts. Various studies have indicated that liver regeneration and function return rapidly in both donor and recipient after transplantation<sup>[1-3]</sup>. However, several studies have shown that living donor liver transplant

**RESULTS:** There were no differences in the demo-

recipients required smaller doses of tacrolimus compared with deceased donor liver transplant patients<sup>[4,5]</sup>, which indicated that liver regeneration could affect the metabolism of tacrolimus in LDLT. The liver is a large metabolic pool which metabolizes many drugs including immunosuppressive drugs in humans. Tacrolimus, a calcineurin inhibitor, is predominantly metabolized in the liver by cytochrome P450 3A4 (CYP3A4)<sup>[6]</sup>. Kishino *et al*<sup>[7]</sup> reported that inter- and intra-individual variations in CYP3A4 activity were caused by differences in the actual ratio of graft volume (GV) to standard liver volume (SV) and donor age. Furthermore, Fukatsu *et al*<sup>[8]</sup> reported that graft hepatic weight was significantly correlated with clearance of tacrolimus. One possible explanation for the high variability of the optimal tacrolimus dose and its pharmacokinetics is the difference in graft size. In recent years, LDLT in adult patients with SFS grafts has become increasingly accepted<sup>[9]</sup>. However, questions related to this technique have arisen: What are the tacrolimus dosage requirements in SFS grafts which require adequate liver regeneration? Does liver regeneration of SFS grafts have any impact on tacrolimus metabolism? Are there any differences in tacrolimus dosage requirements between SFS grafts and non-SFS grafts in LDLT? The answers to these questions are unknown because there are few studies on tacrolimus dosage requirements in LDLT with SFS grafts in the existing literature. Hence, the purpose of this study was to determine the tacrolimus dosage requirements and blood concentrations in adult-to-adult right lobe living donor liver transplantation (AALDLT) recipients with SFS grafts.

## MATERIALS AND METHODS

### Inclusion and exclusion criteria

Patients were included in this analysis if they were 18 years or older, had their transplantation performed at West China Hospital, received tacrolimus as basic immunosuppression and were followed up for at least 6 mo post-surgery. Exclusion criteria: (1) Patients receiving dual liver grafts. (2) Patients who had undergone combined liver and kidney transplantation. (3) Patients who were followed up for less than 6 mo and who were lost to follow up. (4) Patients who underwent retransplantation. And (5) patients who received cyclosporine as basic immunosuppression.

### Recipients and donors

According to the inclusion and exclusion criteria, the study enrolled 54 AALDLT recipients and 54 donors who underwent right lobe hepatectomy at West China Hospital of Sichuan University during January 2007 and October 2008.

The 54 donors consisted of 34 males and 20 females. Donor age ranged from 19 to 53 years (mean, 30.9 ± 8.5 years). The relationships of the donors to the respective patients were: four fathers, three mothers, 11 offspring, 16 brothers, eight sisters, five wives, two husbands and five friends.

All the recipients underwent LDLT using right lobe graft without a middle hepatic vein. Of the 54 recipients, 45

(83.3%) were male and 9 (16.7%) were female. The average age of the recipients was 43 (27-64) years. Indications for transplantation were: chronic hepatitis B with liver cirrhosis (18 cases); fulminant hepatic failure (six cases); hepatocellular carcinoma (28 cases) and other causes (two cases). The pre-transplantation MELD scores of the recipients were 1-13 in 30 patients, 14-24 in 16 patients, and ≥ 25 in eight patients.

According to the GRBW, the 54 AALDLT recipients were divided into two groups: group S (GRBW < 0.8%), eight cases and group N (GRBW ≥ 0.8%), 46 cases. Written informed consent was obtained from both donors and recipients before surgery, and all the AALDLTs were approved by the Ethics Committee of West China Hospital.

### Immunosuppression protocols

Basic postoperative immunosuppression consisted of corticosteroids and tacrolimus (Prograf; Fujisawa, Osaka, Japan). Supplemental immunosuppression consisted of mycophenolate mofetil (for benign diseases) or azathioprine (for malignant diseases). Initial steroid tapers consisted of 1000 mg of intravenous (IV) methylprednisolone intraoperatively, followed by a 7-d taper (50 mg Q6h day 1, 40 mg Q6h day 2, 30 mg Q6h day 3, 20 mg Q6h day 4, 20 mg Q8h day 5, 10 mg Q6h day 6 and 10 mg Q8h day 7) to 20 mg of oral prednisone once daily. The corticosteroid was withdrawn from all patients within 3 mo after transplantation. Initial suspected or biopsy-proven rejections were treated with two 500-mg IV methylprednisolone boluses on consecutive days, followed by a 7-d taper to 20 mg of oral prednisone once daily.

Severe or steroid-resistant rejections were treated with a 7- to 14-d course of muromonab-CD3 (Orthoclone OKT3; Orthobiotech Products, Raritan, NJ, USA). Initial tacrolimus doses were 0.05-0.1 mg/kg per day divided into twice-daily dosing. Tacrolimus doses were adjusted to achieve a target 12-h trough concentration of 10-15 ng/mL for the first 3 mo post-transplantation, followed by 5-10 ng/mL thereafter. Tacrolimus 12-h trough concentrations were analyzed by the IMx assay (Abbott Laboratories, Chicago, IL, USA).

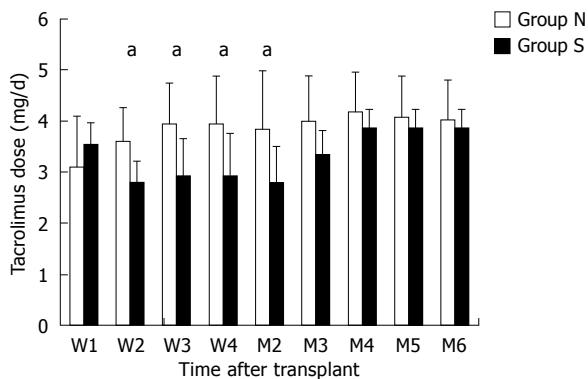
### Data collection

Tacrolimus daily doses and 12-h trough blood levels were recorded in both group S and group N at the following intervals after transplantation: weeks 1, 2, 3 and 4 and months 2, 3, 4, 5 and 6. At each interval, serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TB), albumin (Alb), and creatinine (Cr) levels were recorded. Tacrolimus 12-h trough concentrations, AST, ALT, TB, Alb and Cr were measured twice weekly in the first month post-surgery, and weekly from month 2 to 6. At each interval, values recorded for dose, level, and respective liver function and kidney function test results represent the mean value over the past days. Meanwhile, acute rejection rates and the number of potentially interacting medications were determined at each interval in the two groups. Body weight was also recorded for each patient at each interval.

**Table 1 Patient demographics**

	<b>Group S</b>	<b>Group N</b>	<b>P</b>
Recipients' age (yr)	41.9 ± 11.6	43.4 ± 7.3	NS (0.695)
Donors' age (yr)	28.6 ± 4.4	31.8 ± 9.5	NS (0.246)
Sex (Male/female)	6/2	39/7	NS (0.864)
MELD score			NS (0.911)
1-13	5	25	
14-24	2	14	
≥ 25	1	7	
Indications for LDLT			NS (1.000)
Cirrhosis	3	15	
HCC	4	24	
FHF	1	5	
Others	0	2	
Rejection	1/8	4/46	NS (0.567)
Body weight	66.6 ± 7.6	61.7 ± 9.8	NS (0.244)
Biliary anastomosis			NS (0.484)
Roux-en-Y	1 (12.5)	3 (7)	
Choledochocholeodochostomy	7 (87.5)	43 (93)	

NS: Not significant.

**Figure 1** Tacrolimus dosage requirements (week 1 to month 6). <sup>a</sup>P < 0.05.

### Statistical analysis

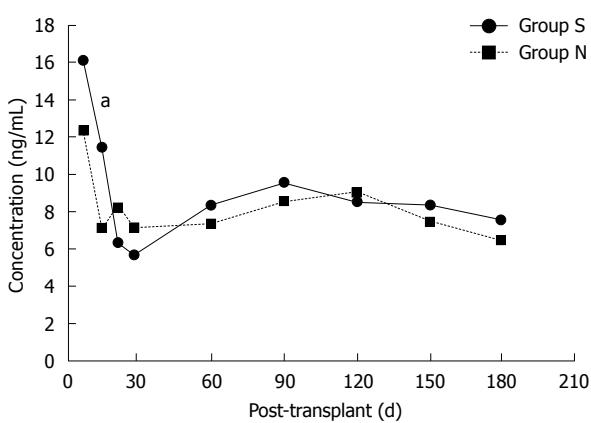
Quantitative descriptive data were expressed as mean ± standard deviation (SD) or median (minimum to maximum). Qualitative descriptive data were expressed as percentages. Fisher's Exact,  $\chi^2$ , Student's *t* and rank tests were used for statistical analysis. Tacrolimus doses and 12-h trough concentrations were compared weekly for the first month post-transplantation. For months 2-6 post-transplantation, tacrolimus doses and 12-h trough concentrations were compared monthly. ALT, AST, TB, Alb, and Cr were also compared between the two groups over the 6-mo period. All statistical analyses were performed using SPSS version 16.0 for Windows statistical software (SPSS Inc., Chicago, IL, USA). Differences with a *P* value < 0.05 were considered significant.

## RESULTS

The demographics of group S and group N cohorts are listed in Table 1. There were no differences between the two groups with regard to recipients' age, donors' age, sex, and MELD score. In addition, there were no differences between the two groups in indications for transplantation or number of treated rejection episodes. There were no

**Table 2 Medication interactions**

<b>Medication interaction factors</b>	<b>Group S</b>	<b>Group N</b>	<b>P</b>
Patients administered interacting drugs	2/8	7/46	NS
Total number of treatment courses	4	9	NS
Interacting drugs			
Fluconazole	1	3	NS
Felodipine	1	2	NS
Lansoprazole	2	4	NS
Average length of therapy (d)	7	9.5	NS
No. of treatment courses on postoperative day			NS
1-30	3	5	
31-60	1	3	
61-180	0	1	

**Figure 2** Tacrolimus 12-h trough level. <sup>a</sup>P < 0.05.

differences between the two groups with regard to the number of patients administered an interacting drug, total number of courses of an interacting drug, or duration of therapy (Table 2). Mean body weight for SFS grafts recipients undergoing AALDLT was slightly higher (66.6 kg) than for non-SFS grafts recipients undergoing AALDLT (61.7 kg, *P* = 0.244 not significant). The proportion of group S who underwent Roux-en-Y anastomosis was greater than that in group N, however, not statistically significant (12.5% vs 7%, *P* = 0.484).

Tacrolimus dosage requirements for each group over the first 6 mo post-transplantation are shown in Figure 1. There was no significant difference in doses during the first week. The tacrolimus dosage requirements in group S were significantly lower than those in group N at 2 wk ( $2.8 \pm 0.4$  mg/d vs  $3.6 \pm 0.7$  mg/d, *P* = 0.006), 3 wk ( $2.9 \pm 0.7$  mg/d vs  $3.9 \pm 0.8$  mg/d, *P* = 0.008), 4 wk ( $2.9 \pm 0.8$  mg/d vs  $3.9 \pm 1.0$  mg/d, *P* = 0.023) and 2 mo ( $2.8 \pm 0.7$  mg/d vs  $3.8 \pm 1.1$  mg/d, *P* = 0.033). At 3, 4, 5 and 6 mo post-transplantation, there was no statistically significant difference between the two groups with regard to tacrolimus dosage requirements.

Despite having lower dosage requirements at 2, 3 and 4 wk and 2 mo post-transplantation, tacrolimus 12-h blood concentrations were not significantly different between the two groups except for week 2 post-transplantation ( $11.3 \pm 4.8$  ng/mL vs  $7.0 \pm 3.8$  ng/mL, *P* < 0.05, Figure 2). In

**Table 3** Liver function and kidney function test values

	Month					
	1	2	3	4	5	6
Group N						
ALT (IU/L)	115.9	67.9	43.6	39	38	37.7
AST (IU/L)	52.7	47	34.9	39.3	36.1	38.9
TB ( $\mu\text{mol/L}$ )	23.1	18.5	17.2	14.9	17.6	17.9
Alb (g/L)	35.1	40.7	42.9	41.2	43	42.7
Cr ( $\mu\text{mol/L}$ )	55.5	55.1	59	55.7	54.1	52.3
Group S						
ALT (IU/L)	124.2	78.2	46.8	41.5	34.4	39.7
AST (IU/L)	62.6	53.4	42.5	39.9	38.8	39.3
TB ( $\mu\text{mol/L}$ )	33.2	22.5	19.6	18.1	16	15.6
Alb (g/L)	33.6	39.7	42.3	42.7	43.9	42.9
Cr ( $\mu\text{mol/L}$ )	56.9	58.3	61	61.7	64.5	63

addition, there were no significant differences between the two groups with regard to ALT, AST, TB, Alb, or Cr over the entire study period (Table 3).

## DISCUSSION

LDLT has emerged as an effective alternative strategy to overcome donor organ shortage. In recent years, LDLT in adult patients with SFS grafts has become increasingly accepted<sup>[9]</sup>. With SFS grafts, it is considered that reduced functional liver mass is a necessity for adequate liver regeneration. Nevertheless, the liver is a large metabolic pool which metabolizes many drugs including immunosuppressant drugs in humans. Tacrolimus (FK506) is a potent immunosuppressive agent that is widely used in organ transplantation<sup>[10]</sup>. Because FK506 has serious side-effects (such as nephrotoxicity, hypertension and neurotoxicity) and a narrow therapeutic window (5–20 ng/mL)<sup>[11]</sup>, the desired blood concentration should be achieved as soon as possible. However, tacrolimus dosage requirements and blood concentrations in LDLT with SFS grafts have not yet been reported.

Ours results indicated that tacrolimus dosage requirements were substantially reduced in AALDLT recipients with SFS grafts compared with non-SFS grafts recipients during the first 2 mo post-transplantation despite having similar 12-h trough concentrations. Beyond 2 mo post-transplantation, dosage requirements between the two groups were similar.

The reason for the relatively low tacrolimus dosage requirements in SFS grafts recipients during the first 2 mo post-transplantation is not clear. However, there are several possibilities. We analyzed pharmacokinetic variables between group S and group N (such as absorption, volume of distribution, and clearance) to explain the dosage requirement difference. Tacrolimus dosage requirements may be reduced by increased absorption, decreased volume of distribution, or decreased clearance. Neither increased absorption nor decreased volume of distribution in group S was a possible explanation. The only difference in group S and group N that could impact on absorption was the greater proportion of AALDLT recipients with a Roux-en-Y anastomosis. The proportion of group S who

underwent Roux-en-Y anastomosis was greater than that in group N (12.5% vs 7%), but this was not statistically significant ( $P = 0.484$ ). Moreover, a Roux-en-Y anastomosis is associated with decreased immunosuppressive absorption<sup>[12]</sup>. The volume of distribution of immunosuppressants was larger rather than smaller in group S because of their heavier body weight (SFS grafts recipients were 4.9 kg heavier than non-SFS grafts recipients).

Another explanation for the lower tacrolimus dosage requirements in group S might be reduced immunosuppressive clearance, which may be explained by two possible mechanisms. The first possible mechanism is that as the graft size in group S was smaller than in group N, it did not meet the demands of metabolism. SFS grafts have a relatively small liver mass because the GRBW is less than 0.8% and severe ischemic injuries caused by decreased hepatic arterial inflow or even hepatic artery thrombosis can occur<sup>[13-15]</sup>. Fukatsu *et al*<sup>[8]</sup> reported that graft hepatic weight was significantly correlated with clearance of tacrolimus in adult patients who had undergone LDLT, and Sugawara *et al*<sup>[16]</sup> reported that the optimal tacrolimus dose was best correlated with GV/SV ratio. Lee *et al*<sup>[17]</sup> reported that the clearance of tacrolimus was related to GRWR and the clearance of tacrolimus was decreased in patients with a small graft. Kishino *et al*<sup>[18]</sup> reported that the mean  $T_{1/2}$  of tacrolimus in patients with SFS grafts (GV/SV ratio: smaller than 50%) was significantly ( $P < 0.05$ ) longer than that in patients with GV/SV larger than 51%. These results suggest that graft size is important and could influence the clearance of tacrolimus. The reason why graft size can influence the clearance of tacrolimus is not very clear. CYP3A4 content and activity in liver grafts may partially explain the problem. Tacrolimus is mainly metabolized by CYP3A4 in the liver and intestine<sup>[6,19]</sup>. Powis *et al*<sup>[20]</sup> reported that CYP3A4 content and activity in human liver tissue decreased rapidly after surgical removal, and Kishino *et al*<sup>[7]</sup> reported that inter- and intra-individual variations in CYP3A4 activity were caused by differences in GV/SV ratio and donor age. In other words, the reduced CYP3A4 content and lower CYP3A4 activity in SFS liver grafts could partially explain the difference in tacrolimus dosage requirements between group S and group N.

Another explanation for reduced immunosuppressive clearance in SFS liver grafts may be related to several cytokines and growth factors. Several studies<sup>[21,22]</sup> have revealed that a SFS liver graft retained the capacity to regenerate faster by modulation of the expression pattern of HGF, IL-6 and TGF- $\beta$ 1 immediately after LDLT and both the regeneration rates and the levels of cytokines and growth factors were higher in SFS liver grafts than normal sized liver grafts. These markers have also shown an ability to decrease cytochrome P450 3A activity in the liver in both mice and humans<sup>[23-25]</sup>, which may decrease its ability to metabolize drugs. In other words, there were many more cytokines and growth factors (such as HGF, IL-6) in SFS liver grafts, which could decrease the clearance of tacrolimus.

In order to eliminate the impact of drug interactions, the potential medications which could affect tacrolimus level were determined in the two groups. It was noted in several studies that fluconazole, felodipine and lansoprazole could elevate tacrolimus blood level by inhibiting the activity of CYP3A4<sup>[26-28]</sup>. In the present study, the majority of patients in both groups were administered interacting drugs in the first month post-transplantation. However, there were no differences between the two groups with regard to the number of patients administered an interacting drug, total number of courses of an interacting drug, or duration of therapy. As a result, the impact of drug interactions was balanced in the two groups.

To make a rough determination of hepatic function and kidney function in group S and group N, we measured serum AST, ALT, TB, Alb, and Cr levels at each interval post-transplantation in both groups of patients. AST, ALT, and TB reflected the hepatic metabolic function while Alb was responsible for the synthetic function of the liver. AST, ALT, and TB levels were slightly greater at almost all follow-up intervals and Alb level was slightly lower in group S in the first 3 mo post-transplantation, although there was no statistical significance. This could not explain the difference in tacrolimus dosage requirements between group S and group N, however, more sensitive measures of hepatic function, i.e. galactose, caffeine, indocyanine green and monoethylglycinexylidide clearance test, should be performed to confirm the hypothesis.

There are several limitations to this study. Firstly, the sample size was small in group S. In addition, we did not study tacrolimus pharmacokinetics in this analysis which may have strongly affected the difference in the tacrolimus dosage requirements between the two groups.

In conclusion, AALDLT recipients with SFS grafts have significantly decreased tacrolimus dosage requirements compared with non-SFS grafts recipients during the first 2 mo post-surgery, in spite of having similar tacrolimus concentrations. We recommend that relatively low initial doses of tacrolimus should be administered to these patients.

## COMMENTS

### Background

Several studies have shown that living donor liver transplant recipients required smaller doses of tacrolimus compared with deceased donor liver transplant patients, which indicated that liver regeneration could affect the metabolism of tacrolimus in living donor liver transplantation (LDLT). However, there are few studies in the existing literature on tacrolimus dosage requirements in LDLT with small-for-size (SFS) grafts which require adequate liver regeneration.

### Research frontiers

In recent years, LDLT in adult patients with SFS grafts has become increasingly accepted. The administration of immunosuppressants for SFS grafts has not been explicitly reported due to limited studies. In this field, the research goal was to determine the optimal initial tacrolimus dose for LDLT recipients with SFS grafts.

### Innovations and breakthroughs

Several studies have shown that living donor liver transplant recipients required smaller doses of tacrolimus compared with deceased donor liver transplant patients. This is the first study to compare tacrolimus dosage requirements in

patients who received a graft with adequate hepatic mass and patients who received a SFS graft in LDLT.

### Applications

This study revealed that relatively low initial doses of tacrolimus should be administered to patients who receive a SFS graft in LDLT, which is a good guideline to follow for the rational administration of tacrolimus in future LDLT recipients with SFS grafts.

### Terminology

SFS graft: SFS liver graft, this is generally considered when a graft has a graft-recipient body weight ratio (GRBW) less than 0.8% in LDLT. GRBW: Graft-recipient body weight ratio.

### Peer review

The current paper is well-written and informative.

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CASE REPORT

## Celecoxib-induced cholestatic liver failure requiring orthotopic liver transplantation

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### INTRODUCTION

The primary effect of non-selective non-steroidal anti-inflammatory drugs (NSAIDs) is to inhibit cyclooxygenase (prostaglandin synthase), thereby impairing the ultimate transformation of arachidonic acid to prostaglandins, prostacyclin, and thromboxanes. The extent of enzyme inhibition varies among the different NSAIDs. At least two isoforms of cyclooxygenase enzymes have been described. While cyclooxygenase-1 (COX-1) is constitutively expressed in most normal tissues, cyclooxygenase-2 (COX-2) expression is predominantly induced during inflammation and tissue injury<sup>[1]</sup>. Most of the side effects associated with the use of non-selective NSAIDs are thought to be due to inhibition of COX-1. Therefore, selective COX-2 inhibitors have been developed in order to minimize some of the NSAID-associated adverse effects. Celecoxib is a widely used COX-2 inhibitor with high levels of patient acceptability and satisfaction, which may result from its combination of efficacy and relatively benign adverse effect profile. The daily recommended adult dose ranges between 100 and 800 mg/d for various clinical indications including osteoarthritis, ankylosing spondylitis, rheumatoid arthritis, chemoprevention of familial polyposis, primary dysmenorrhea, and acute pain<sup>[2]</sup>.

Recent reports described liver injuries in association with COX-2 inhibitors ranging from acute liver failure<sup>[3-6]</sup> to varying degrees of transient cholestatic<sup>[7-10]</sup> and/or hepatocellular injuries<sup>[11,12]</sup>. We report a case of celecoxib-associated acute cholestatic hepatitis progressing to liver failure requiring transplantation.

### Abstract

Selective cyclooxygenase-2 (COX-2) inhibitors are widely used due to their efficacy and good safety profile. However, recent case reports have described varying degrees of liver injuries associated with the use of COX-2 inhibitors. We report the case of a patient who developed acute cholestatic hepatitis progressing to hepatic failure requiring liver transplantation, following a 3-d course of celecoxib for treatment of generalized muscle aches and pains. The clinical presentation, the laboratory data, as well as the liver histopathology were supportive of the putative diagnosis of drug induced liver injury.

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**Key words:** Celecoxib; Cholestatic hepatitis; Liver failure; Liver transplantation

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El Hajj II, Malik SM, Alwakeel HR, Shaikh OS, Sasatomi E, Kandil HM. Celecoxib-induced cholestatic liver failure requiring orthotopic liver transplantation. *World J Gastroenterol*

### CASE REPORT

A 52-year-old Caucasian female was in her usual state of health until she developed generalized muscle aches and pains 1 d after performing yard work. Over the next 3 d, she took a total of eight 200 mg-tablet of the prescription drug Celecoxib. On the 3rd day, she developed fatigue, loss of appetite, intense pruritus and dark-brown (“coke”) colored urine. The patient’s symptoms progressively worsened over the next 3 d, and 1 wk after the use of

**Table 1** Laboratory values

	AST	ALT	AP	GGT	Bilirubin	INR
One year before	22	18	78	33	0.4	1.0
D 1-3		Celecoxib (eight 200 mg-tablet)				
D 10	104	258	700	262	10.8	1.0
D 24	220	297	889	711	15	1.0
D 48	442	509	1427	895	35	1.0
D 51	152	167	1024	573	38	2.6
D 54		Orthotopic liver transplantation				
1 mo (post OLTx)	16	27	570	116	1.7	1.1
6 mo (post OLTx)	36	28	204	109	0.3	0.9

AST: Aspartate aminotransferase (17-59 IU/L); ALT: Alanine aminotransferase (21-72 IU/L); AP: Alkaline phosphatase (38-126 IU/L); GGT: Gamma glutamyl transpeptidase (0-65 IU/L); Bil: Total bilirubin (0.2-1.3 mg/dL); INR: International normalized ratio (0.8-1.2).

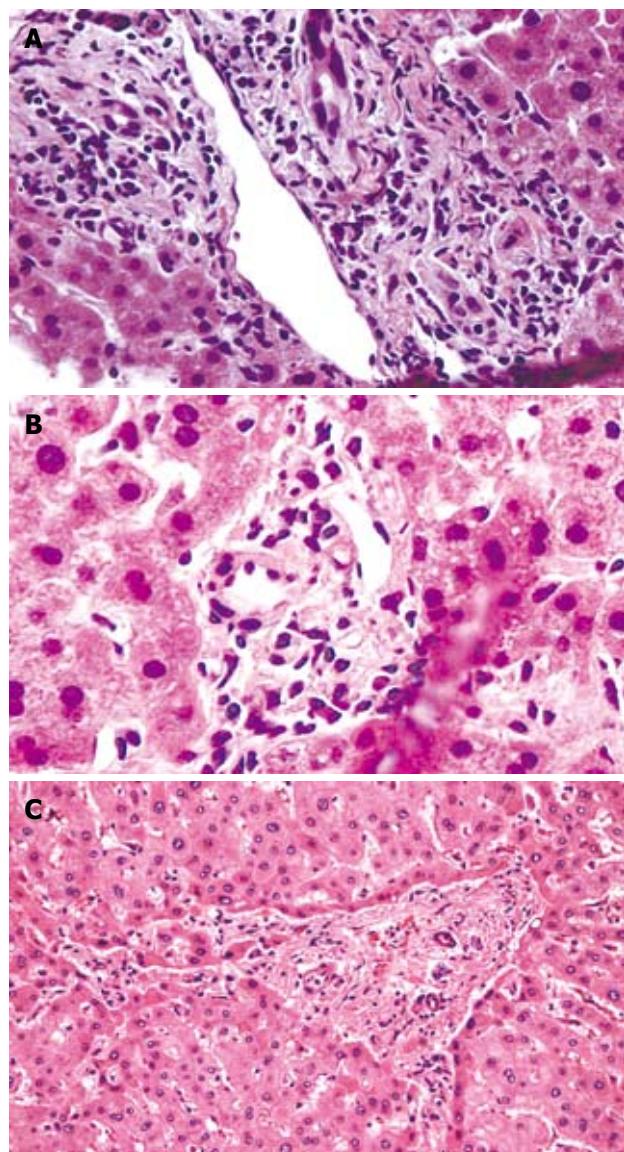
celecoxib, she presented to the local emergency room. She denied abdominal pain, nausea, vomiting, but did endorse a weight loss of five pounds.

The patient worked as a registered nurse. Past medical history was positive for a needle stick injury from an HIV/HCV co-infected patient one year earlier, for which she was evaluated at employee health. Her liver function tests (LFTs) were normal (Table 1), and the serologies for HIV, hepatitis B and C were negative at the time of needle stick and at subsequent follow-up visits at three, seven and 10 mo. The patient was single and not sexually active. She denied any alcohol intake, smoking, use of aspirin, or over the counter medications, herbals, and illicit drugs. She denied recent travel or sick contacts.

On physical exam, she was afebrile and jaundiced with mild right upper quadrant tenderness. Initial laboratory studies revealed abnormal LFTs with predominantly cholestatic pattern (Table 1). Serum urea nitrogen and creatinine levels were normal. Blood count differential was notable for peripheral eosinophilia with an absolute eosinophil count of 760/mL. Platelets count, INR and serum albumin were within normal limits.

Repeat testing at a 2-wk follow-up revealed worsening LFTs (Table 1). Antibodies to hepatitis A, B and C and Epstein-Barr virus, Cytomegalovirus and Herpes simplex virus were negative. Iron studies, an autoimmune panel (Anti-nuclear antibodies, anti-smooth muscle antibodies, liver-kidney-microsomal antibodies and immunoglobulins), anti-mitochondrial antibodies, alpha-one anti-trypsin, and ceruloplasmin were within normal limits. A CT scan of abdomen with intravenous contrast revealed normal liver morphology with no focal lesions and no biliary duct dilatation; the hepatic and portal veins were patent. Ultrasound guided liver biopsy showed ductopenia with lobular foam cell change and cholestasis along with periportal fibrosis and no evidence of bridging fibrosis (Figure 1A and B). The patient was started on 500 mg of ursodiol twice daily, oral fat-soluble vitamins, and prednisone 40 mg daily, tapered progressively over 2 wk for presumed drug induced liver injury (DILI).

On a return visit 2 wk later, the patient showed no improvement in her symptoms and was complaining of



**Figure 1** Hepatic histology. A: Relatively large portal tract containing bile ducts with increased nuclear to cytoplasmic ratio, eosinophilic transformation of the cytoplasm, nuclear hyperchromasia, and uneven nuclear spacing; B: Small portal tract containing the hepatic artery and portal vein, but there is no bile duct. Five of twelve interlobular portal tracts in this biopsy lacked bile ducts; C: Hepatectomy specimen: loss of interlobular bile ducts in most of the small portal tracts, more advanced portal and periportal fibrosis with short fibrous septa. (Haematoxylin & Eosin stain,  $\times 100$ ).

worsening pruritus and an additional 10-pound weight loss. Lab tests at this time revealed a total bilirubin of 35 mg/dL and acute renal failure with BUN of 89 and creatinine of 7.5 (Table 1). The patient was admitted to the medical intensive care unit. She was challenged with intravenous fluids with improvement in her creatinine to 3.9 mg/dL. However, the patient's clinical condition continued to deteriorate with a new development of lethargy. The INR increased to 3.4 despite subcutaneous injections of Vitamin K, and the total bilirubin peaked at 51 mg/dL. The patient underwent orthotopic liver transplantation, 54 d after the initial ingestion of celecoxib.

Sections through the hepatectomy specimen showed severe intrahepatic cholestasis, prominent ductopenia,

and degenerative changes of the duct epithelial cells involving most of the small bile duct branches; the large bile ducts showed little to no evidence of duct injury. More advanced portal and periportal fibrosis was noted (Figure 1C). The postoperative course was uneventful. On follow-up visits at 1 and 6 mo, the patient remained clinically stable and had normal LFTs (Table 1).

## DISCUSSION

Many NSAIDs and COX-2 inhibitors have been associated with hepatotoxicity varying from transient cholestatic<sup>[7-10]</sup> and hepatocellular injury<sup>[11,12]</sup> to fulminant hepatic failure<sup>[3-6]</sup>. The incidence of COX-2-associated hepatotoxicity is extremely difficult to estimate accurately and the current literature is based on case reports of side effects. Several cases of liver injury associated with celecoxib use have been reported. Self-limited cholestatic hepatitis was reported in most of the cases with resolution of symptoms after stopping celecoxib<sup>[7-10]</sup>.

Liver failure associated with COX-2 inhibitor use has been reported with Nimesulide and rofecoxib<sup>[3-6]</sup>. Fulminant liver failure requiring orthotopic liver transplantation after celecoxib use has been recently reported in one case<sup>[3]</sup>. To our knowledge, our case is the second documented case of celecoxib-induced liver failure requiring liver transplantation. Interestingly, our patient developed a liver injury after a 3-d course of celecoxib when compared to the 2-wk course in the other reported case of celecoxib-induced liver failure<sup>[3]</sup>.

Several factors support the diagnosis of drug-induced liver injury (DILI) secondary to celecoxib as the most likely etiology for our patient's liver failure. These include: the acute presentation following celecoxib use; the significant eosinophilia in the blood and on the liver biopsy; and finally, the exclusion of other etiologies for liver failure.

To further assess the likelihood of DILI, we used the Roussel Uclaf Causality Assessment Method/Council for International Organizations of Medical Sciences (RUCAM/CIOMS) scoring system<sup>[13]</sup>. The criteria for scoring include: time to onset of the reaction, course of the reaction, risk factor(s) for drug reaction, concomitant drug(s), non drug-related causes, previous information on the drug, and response to re-administration. Using this system, the causality of the drug in our case is classified as "probable", with a score of 8. A more recent DILI diagnostic scale uses temporal relationship between drug intake and the onset of clinical picture, exclusion of alternative causes, extrahepatic manifestations, intentional or accidental re-exposure to the drug, previous reports in the literature of cases of DILI associated with the drug<sup>[14]</sup>. On a scale of -6 to 20, the probability of the diagnosis of DILI is expressed as: definite  $\geq 17$ , probable = 14-17, possible = 10-13, unlikely = 6-9, and excluded  $\leq 6$ . Our patient scored 15, hence the likelihood of DILI is "probable".

The underlying mechanisms for NSAIDs-induced

liver injury are poorly understood. The adverse effects are due to either host dependent idiosyncratic reactions or dose dependent intrinsic reactions. Idiosyncratic reaction, which is the most common type, is mediated by either an immunological mechanism or abnormalities in drug metabolism<sup>[15]</sup>. The etiology of DILI in our patient is most likely an idiosyncratic reaction as she developed her symptoms after taking a relatively short course of celecoxib, well within its recommended daily adult dose.

In conclusion, we present a case of cholestatic hepatic injury following a short course of celecoxib progressing to liver failure and requiring liver transplantation. Clinicians should be aware that despite its better safety profile for gastrointestinal side effects compared to nonselective NSAIDs, celecoxib may be associated with severe hepatotoxicity.

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CASE REPORT

# Combined hepatocellular and cholangiocellular carcinoma presenting with radiological characteristics of focal nodular hyperplasia

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## INTRODUCTION

The two major subtypes of primary liver cancer are hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC). Combined hepatocellular and cholangiocellular carcinoma (cHCC-CC) is a rare tumor type containing unequivocal elements of both HCC and CC that are intimately mixed<sup>[1,2]</sup>. It accounts for 1.0%-14.2% of all primary liver carcinomas<sup>[3]</sup>. Initial misdiagnosis was reported in several patients. The main diagnostic techniques for cHCC-CC include serum markers, imaging and histological analysis.

## CASE REPORT

A 47-year-old woman, who was admitted to the radiology department of our hospital, presented with a 2 mo history of general illness, slight weight loss and a palpable mass in the right upper abdomen. Ultrasound and contrast-enhanced computed tomography (CT) of the abdomen were performed in another hospital. Ultrasound of the upper abdomen revealed a heterogeneous hypoechoic tumor in the liver. The CT scan showed a heterogeneous contrast-enhanced mass in segments 5 and 6 of the liver. Her past medical history included an appendectomy and hepatitis B positivity for more than 15 years. She was considered a so called healthy carrier of hepatitis B.

Magnetic Resonance Imaging (MRI) was performed in our hospital. On MRI a focal liver lesion inferior in the right lobe was visualized. The mass was homogeneous hypointense on T1 (Figure 1C and G) and slightly hyperintense on T2 (Figure 1A and B). The dynamic contrast-enhancement with gadolinium chelate (Magnevist®, Baeyer HealthCare Pharmaceuticals, Düsseldorf, Germany) established a relatively hypervasculature heterogeneous nature with late enhancement of the central scar (Figure 1D-H). The contrast-enhancement

## Abstract

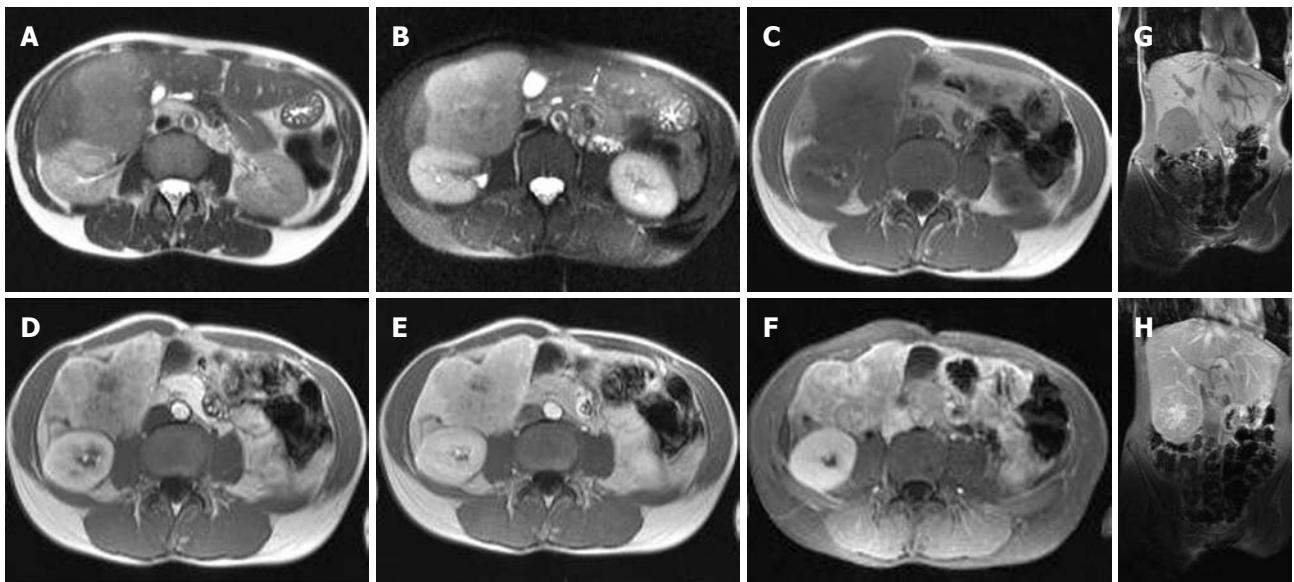
Combined hepatocellular and cholangiocellular carcinoma (cHCC-CC) is a rare tumor type containing unequivocal elements of both hepatocellular carcinoma and cholangiocarcinoma that are intimately mixed. Although these tumors are usually considered to be more related to hepatocellular carcinoma than to cholangiocarcinoma, they sometimes, in contrast to hepatocellular carcinoma, contain a significant amount of fibrous stroma. This might in some cases explain atypical radiological features. We report a case of a cHCC-CC in a 47-year-old female that resembled focal nodular hyperplasia on Magnetic Resonance Imaging. Correlation of imaging and serum levels of  $\alpha$ -fetoprotein and CA19.9 can help to make the correct diagnosis preoperatively.

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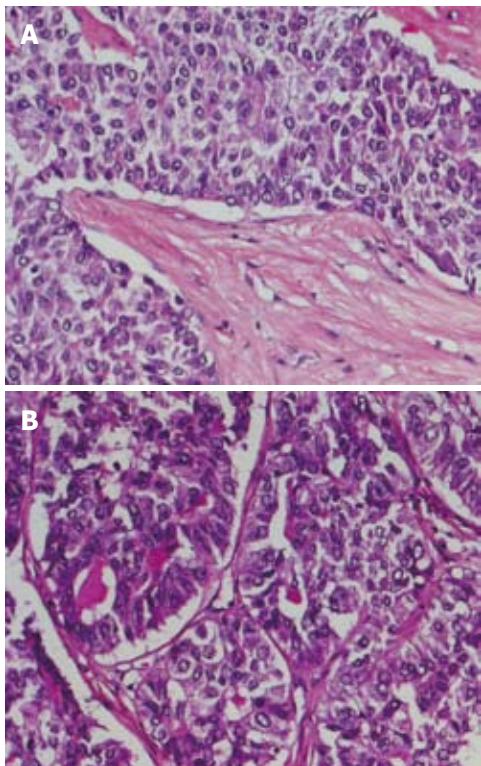
**Key words:** Combined hepatocellular; Cholangiocellular carcinoma; Magnetic resonance imaging

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**Figure 1 MRI images of combined hepatocellular and cholangiocellular carcinoma.** Axial T2 (A) and T2 fat saturated (B) MRI images showed a slightly well circumscribed lesion. The mass is hyperintense in comparison to the normal liver. Axial T1 (C) MRI images demonstrated a hypointense lesion. Early arterial (D), late arterial (E) and venous (F) MRI images displayed a hypervascular heterogeneous enhancement with central scar. The coronal T1 (G) image showed a hypointense lesion, while the coronal T1 MRI image after Gadolinium contrast (H) demonstrated a heterogeneous early enhancement of the lesion with delayed uptake in the scar.



**Figure 2 Histopathological images of combined hepatocellular and cholangiocellular carcinoma.** A: Nests of tumor cells with trabecular architecture, reminiscent of hepatocellular differentiation, separated by bands of fibrous tissue; B: Focally tumor cells formed glandular structures with mucinous secretory material, compatible with cholangiocellular differentiation.

and morphologies were consistent with focal nodular hyperplasia. Laboratory results, however, revealed an elevated  $\alpha$ -fetoprotein level of 154 000 ng/mL.

Right hepatectomy was performed. A white firm tumoral mass of 8.5 cm in diameter and one satellite

nodule of 1 cm were identified during macroscopic examination of the resection specimen. The non-tumoral liver did not display signs of cirrhosis macroscopically. Microscopically, the tumor consisted of nests of tumor cells separated by broad bands of fibrous tissue, focally resembling a fibrous scar (Figure 2A). In most tumor cell nests, tumor cells displayed a trabecular growth pattern. Trabecular structures were several cell layers thick, in contrast to normal liver tissue in which trabeculae are only 1 to 2 layers in thickness. Focally, however, glandular structures containing secretory material were observed (Figure 2B). Tumor cells arranged in trabecular structures displayed positivity for HepPar1 and  $\alpha$ -fetoprotein and immunohistochemistry with a polyclonal anti-CEA antibody revealed the presence of canalicular structures. These features are consistent with hepatocellular differentiation. Where tumor cells formed glandular structures, the presence of neutral mucin could be demonstrated. The secretory material displayed alcian blue positivity and was positive in a PAS-diastase reaction, compatible with cholangiocellular differentiation. Moreover, areas of glandular differentiation displayed positivity for CA19.9 and MUC1, while areas of hepatocellular differentiation were negative. In addition, positivity for CK7 and CK19 was observed in areas of glandular growth pattern, while areas with trabecular growth pattern displayed most often only positivity for CK8 and CK18. These histological features together with the immunohistochemical profile are consistent with a diagnosis of cHCC-CC.

## DISCUSSION

cHCC-CC is a rare primary liver tumor type containing unequivocal elements of both hepatocellular carcinoma and cholangiocarcinoma<sup>[1,2]</sup>. It accounts for 1.0%-14.2% of

all primary liver carcinomas<sup>[3]</sup>. In 1949, the first description of cHCC-CC was reported by Allen *et al*<sup>[4]</sup>. Some studies found that this tumor type is more frequently diagnosed in patients with chronic hepatitis and liver cirrhosis<sup>[5]</sup>, although this could not be confirmed in other studies<sup>[1]</sup>. Most studies report that patients with cHCC-CC show similar clinical and pathological features as hepatocellular carcinoma patients, including a mean age onset in the sixth decade, male predominance, a high incidence of hepatitis B virus infection, underlying chronic liver disease and elevated serum  $\alpha$ -fetoprotein levels<sup>[6]</sup>. Several reported patients were initially misdiagnosed<sup>[7]</sup>. Various factors, such as an atypical enhancing pattern on imaging, the small size of one of the components, and the presence of a tumor composed of intermediate tumor cells, were found to be the causes of misdiagnosis<sup>[8]</sup>. The tumor remains usually generally clinically silent and patients present with advanced illness<sup>[6]</sup>. A simultaneous elevation of serum levels of CA 19-9 and  $\alpha$ -fetoprotein in patients with a liver mass on imaging may suggest the presence of cHCC-CC<sup>[7]</sup>. Ultrasound usually demonstrates a round or ovoid hypoechoic mass with a central hyperechoic area<sup>[9]</sup>. On contrast-enhanced CT, cHCC-CC lesions are divided into 3 classes of enhancement patterns. The first group of lesions, namely cHCC-CC resembling hepatocellular carcinoma, shows hyperenhancement in the early phase and hypoenhancement in the late phase due to washout of contrast (Type I lesions). The second group, Type II lesions, demonstrates peripheral enhancement in the early as well as in the late phase. Type III lesions present an area of hyperenhancement in the early phase and an area of slight delayed enhancement in the late phase<sup>[10]</sup>. The cHCC-CC lesions resembling cholangiocarcinoma, however, enable low attenuation in the arterial phase and low, iso- or high attenuation in the venous phase<sup>[11]</sup>. Preoperatively cHCC-CC is, however, usually only considered when a liver tumor has CT features of both hepatocellular carcinoma and cholangiocellular carcinoma<sup>[12]</sup>. In addition, Toh *et al*<sup>[13]</sup> reported a hypodense tumor with vague contrast enhancement associated with elevation of  $\alpha$ -fetoprotein levels and multiple regional lymphadenopathies as a cHCC-CC. Finally, cHCC-CC has also been reported as presenting as a hypervascular tumor associated with increased serum CEA and CA 19-9<sup>[14]</sup>.

On MRI, cHCC-CC has been reported to show low signal intensities on T1-weighted images and high signal intensities on T2-weighted images<sup>[15]</sup>. In the arterial phase, it has been described that the T1-weighted images describe an enhancing mass nearly isointense to the surrounding parenchyma with a central necrotic non-enhancing zone<sup>[10]</sup>.

Final diagnosis of cHCC-CC can only be made by histological examination. Extensive sampling of tissue is required in order not to miss the diagnosis in case one of the tumor compounds represents only a minor component of the tumor. Surgery is the only effective treatment for this tumor. The median survival time following cHCC-CC surgery has been reported to be 48 mo<sup>[17]</sup>. Partial hepatectomy with lymph node dissection can result in 5-year survival rates exceeding 50% in

patients with early stage disease<sup>[18]</sup>. Prognostic factors of poor outcome of cHCC-CC are distant metastasis, major vascular branch invasion, regional lymph node involvement, bilobar distribution, multiple tumors, tumor necrosis and ascites<sup>[19]</sup>.

In conclusion, preoperative diagnosis of cHCC-CC remains difficult. It is of great importance to choose appropriate imaging, such as MRI, in combination with serum  $\alpha$ -fetoprotein and CA 19.9 levels. In addition, extensive tissue sampling is required in order to not miss this diagnosis in cases where one of the two components is of small size. Most studies show that this tumor type has similar geographical distribution and age and sex distribution as hepatocellular carcinoma. Some authors, however, have reported a worse prognosis as compared to patients with pure hepatocellular carcinoma.

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CASE REPORT

## Sustained virologic response following HCV eradication in two brothers with X-linked agammaglobulinaemia

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### Abstract

X-linked agammaglobulinaemia (XLA) is a humoral immunodeficiency syndrome characterized from childhood by the absence of circulating B lymphocytes, absent or reduced levels of serum immunoglobulin and recurrent bacterial infections. For many affected patients, regular treatment with immunoglobulin is life saving. Hepatitis C viral (HCV) infection acquired through contaminated blood products is widely described in this patient cohort. The natural history of HCV infection in patients with XLA tends to follow a more rapid and aggressive course compared to immunocompetent individuals. Furthermore, standard anti-viral therapy appears to be less efficacious in this patient cohort. Here we report the cases of two brothers with XLA who contracted HCV through contaminated blood products. They were treated with a six month course of Interferon alpha-2b and Ribavirin. We report a sustained virologic response five years after completing treatment.

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**Key words:** Hepatitis C virus; X-linked agammaglobulinaemia; Immunodeficiency; Viral hepatitis; Cirrhosis; Hepatocellular carcinoma

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### INTRODUCTION

Infection with the hepatitis C virus (HCV) is characterized by low grade hepatitis which may progress to cirrhosis and hepatocellular carcinoma over many years. The mortality associated with HCV infection is adversely affected by several factors including: the age of the patient at initial infection, ongoing alcohol consumption, intra-venous drug abuse and the viral genotype<sup>[1,2]</sup>. Furthermore, the natural history of HCV infection in immunocompromised patients appears to follow a more aggressive course leading to rapid development of cirrhosis and hepatocellular carcinoma.

X-linked agammaglobulinaemia (XLA) is an inherited immunodeficiency disease caused by mutations in the gene coding for Bruton's tyrosine kinase (BTK) and occurs with a frequency of 1 in 250 000 males<sup>[3]</sup>. Abnormal gene expression prevents B lymphocyte differentiation and maturation in the bone marrow leading to absence of circulating antibody-producing plasma cells<sup>[4]</sup>. Furthermore, abnormalities in T cell function have been demonstrated in patients with XLA<sup>[5]</sup>. The diagnosis of XLA may be made clinically when the following criteria are met: recurrent bacterial infections in a male infant, absence of circulating peripheral B cells and more than one male in the family affected in different generations.

Early reports of HCV infection in hypogammaglobulinaemic patients suggest a severe and rapidly progressive course<sup>[6-9]</sup>. Initial attempts at treatment with interferon alpha demonstrated poor efficacy in maintaining virologic response and made little impact on the mortality and morbidity of those with a rapidly progressive course. Here we report the cases (diagnosis, management and follow-up) of two brothers with XLA who acquired HCV infection through infected blood products.

### CASE REPORT

C.D. and J.D. are brother's aged 27 and 30 years,

respectively. They both suffered from recurrent lower respiratory tract infections as infants. Their younger brother died of pseudomonal meningitis and septicaemia as an infant. Their clinical history raised suspicion for an underlying immunodeficiency syndrome, which was confirmed when both brothers were found to be deficient in serum B cells and immunoglobulin in early childhood. Genotyping for BTK mutation was carried out and a diagnosis of XLA was established in each case. Subsequently, their cousin, who had a similar clinical history, was also diagnosed with XLA.

Both brothers were commenced on gammaglobulin infusions in 1985. This was comprised of fresh frozen plasma pooled from donors, which was not initially screened for viral contaminants. In 2002, routine biochemical analysis revealed raised liver function tests in J.D. and a full liver screen was carried out. Initial screening tests for anti-HCV including Ortho HCV ELISA Test System and Recombinant Immunoblot Assay-3 were negative. Subsequently, PCR for HCV RNA was carried out and was positive for HCV genotype 3 infection. C.D. was then screened and also found to be HCV antibody negative, but PCR positive for genotype 3 infection. The baseline viral loads were 1723102 copies/mL and 52352 copies/mL for J.D. and C.D., respectively. Neither brother had risk factors for HCV infection other than their previous treatment with intravenous gammaglobulin. The results of a full infectious, metabolic and auto-immune liver screen were otherwise negative in both patients. The baseline clinical data for each patient is summarized in Table 1.

Both patients underwent a liver biopsy, which showed chronic active hepatitis. J.D. had stage 1 fibrosis with mild to moderate inflammatory activity, while C.D. had stage 0 fibrosis with mild inflammation. Both brothers were treated with a 24-wk of Pegylated Interferon alpha-2b and Ribavirin which they completed in June, 2003. There were no significant complications during treatment. Both patients were treated with antibiotics for respiratory tract infections during treatment, but did not require admission to hospital. Their viral load became undetectable 4 wk into therapy and both patients remain PCR negative five years post treatment. J.D. currently receives 35 g of Flebogamma and C.D. receives 40 g, every 3 wk, which is well tolerated by both.

## DISCUSSION

Immunoglobulin therapy is widely used for the treatment of immune deficiency syndromes. Unfortunately, several outbreaks of HCV infection secondary to immunoglobulin replacement therapy have been documented in recent times. Although the true prevalence of HCV infection is difficult to estimate in this population, large studies estimate the incidence at approximately 8.3%, a number far greater than that in the immunocompetent population<sup>[10]</sup>. Unfortunately, the diagnosis of HCV in this high risk population remains problematic for several reasons. Firstly, the poor sensitivity of traditionally used screening tests including measurement of serum

**Table 1 Baseline clinical data for both patients**

	Age at diagnosis (yr)	Baseline viral load (copies/mL)	Baseline ALT (IU/L)	Liver histology	Treatment	Time to PCR negative (wk)
C.D.	27	52352	37 (< 42)	Mild inflammation	Peg-Interferon alpha-2b + Ribavirin	< 4
J.D.	30	1723102	87 (< 42)	Stage 1 fibrosis + moderate inflammation	Peg-Interferon alpha-2b + Ribavirin	< 4

aminotransferase and detection of anti-HCV antibody means that a diagnosis of HCV infection will be missed unless PCR for viral RNA is carried out. Secondly, as there is no set of international guidelines or recommendations for screening this patient cohort, there is wide variation in the screening tests employed between different centers. Finally, despite significant developments regarding viral safety for immunoglobulin preparations, clear product documentation is often absent, making early detection of HCV infection and identification and tracing of exposed individuals difficult.

The clinical course of HCV infection in patients with hypogammaglobulinaemia is unclear. Some reports suggest a rapidly progressive course with poor patient outcomes<sup>[8,11]</sup>, while others demonstrate the more usual, slowly progressive course, at least in the short term<sup>[12,13]</sup>. A less aggressive clinical course has been observed in patients with XLA compared with those with combined variable immunodeficiency<sup>[14]</sup>, although this remains a source of debate<sup>[15]</sup>. The variability between different trials remains unexplained. The duration of infection remains as an important predictor of patient outcome. Undoubtedly other co-morbidities including alcohol consumption, intra-venous drug abuse and co-infection with hepatitis B (HBV) adversely impact on the clinical course also. It is noteworthy that Italian patients appear to follow a more benign course following HCV infection than other populations<sup>[13,16]</sup>, suggesting a role of HCV viral load and genotype in the genesis of liver disease.

Early attempts to treat HCV infection with interferon- $\alpha$  monotherapy in patients with primary immunodeficiency have been disappointing<sup>[6,8]</sup>. However, more recent data demonstrates sustained virologic responses (SVRs) of 54%<sup>[13]</sup>, and between 35%-40% approximately<sup>[14,15]</sup>, in similar patients with acute and chronic HCV infection respectively. Due to the small number of patients involved, it is difficult to determine the influence of viral load and viral genotype on treatment response. Improved treatment outcomes are seen however in patients with a shorter duration of infection and when combination therapy (Interferon and Ribavirin) is used. The excellent treatment response seen in our patients may relate to multiple factors including a favourable genotype, the young age of each patient, minimal liver fibrosis and the absence of other co-morbidities. The duration of infection is unknown as the infected batch was never identified. Although patients are considered to have achieved SVR when they remain PCR

negative six months post completion of treatment, we delayed the reporting of our patients in case of late relapse.

In conclusion, our report raises a few important points. Firstly, all patients who received intravenous immunoglobulin during periods when routine screening was not carried out, should be tested for HCV infection. The importance of measuring HCV RNA rather than looking for HCV antibodies in this patient cohort can not be overstated. Indeed, serological tests failed to make a diagnosis of active HCV infection in both our patients. Secondly, the successful outcome of treatment in both patients highlights the limited role of humoral immunity in the mechanisms of HCV clearance. Finally, despite pessimistic initial reports, the diagnosis and treatment of HCV infection in patients with XLA can lead to excellent long-term outcomes.

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CASE REPORT

## Cavernous mesenteric lymphangiomatosis mimicking metastasis in a patient with rectal cancer: A case report

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### Abstract

Lymphangioma usually occurs in children and usually involves the skin. Mesenteric lymphangioma is extremely rare in adults. Typically, lymphangioma appears on computed tomography (CT) as a lower attenuation of a cystic mass, however, some cases appear to be a solid mass. We describe the CT and 18F-FDG positron emission tomography/CT appearance in a case of jejunal and mesenteric cavernous lymphangiomatosis mimicking metastasis in an adult patient with rectal cancer.

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**Key words:** Lymphangioma; Lymphangiomatosis; Mesentery; Small intestine; Computed tomography; 18F-FDG positron emission tomography/computed tomography

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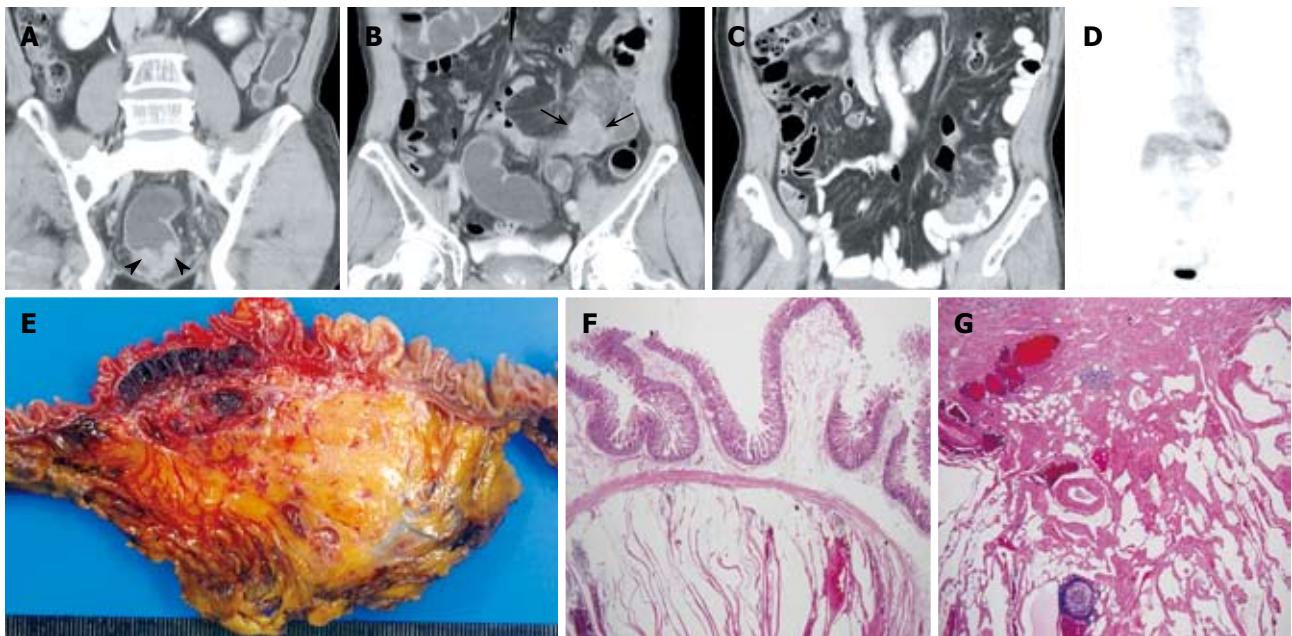
Hwang SS, Choi HJ, Park SY. Cavernous mesenteric lymphangiomatosis mimicking metastasis in a patient with rectal cancer: A case report. *World J Gastroenterol* 2009; 15(31): 3947-3949 Available from: URL: <http://www.wjgnet.com/1007-9327/15/3947.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.3947>

### INTRODUCTION

Gastrointestinal and mesenteric lymphangioma or lymphangiomatosis are extremely rare in adults<sup>[1-3]</sup>. A lymphangioma usually appears as a partially septated, cystic mass on imaging studies including ultrasound, computed tomography (CT), and magnetic resonance imaging (MRI). CT is a basic imaging modality for detecting and evaluating various malignancies. Occasionally, lymphangioma may appear as a solid or infiltrative soft tissue mass on CT because of its microcystic nature or if there is intracystic debris or hemorrhage. The non-cystic appearance of mesenteric lymphangioma may be confused on CT with metastasis or a malignant tumor, especially in patients with malignancy. 18F-FDG positron emission tomography (PET)/CT may have an important role for staging malignancy because hypermetabolic tumor cells actively uptake 18F-FDG<sup>[4]</sup>. This report describes the CT and 18F-FDG PET/CT appearance of a mesenteric and jejunal lymphangiomatosis mimicking metastasis in an adult patient with rectal cancer.

### CASE REPORT

A 71-year-old man was referred for evaluation of a rectal cancer detected on colonoscopy and CT during an evaluation of hematochezia. He had a 5-year history of taking medication for underlying angina. Rectal examination showed a hard mass in the rectum. The standard laboratory tests were normal. Rectal cancer was seen as segmental and irregular wall thickening in the distal rectum with several enlarged perirectal lymph nodes on contrast-enhanced CT (Figure 1A). The soft-tissue density of the nodular mass and hazy attenuations in the jejunal mesentery (Figure 1B) were overlooked initially on either CT scans or on surgery. The patient underwent laparoscopic abdominal transanal resection and ileostomy. Adjuvant chemotherapy and ileostomy closure were performed during the year following surgery. Follow-up, contrast-enhanced CT revealed nodular jejunal and adjacent mesenteric masses in contrast to the barium-filled jejunum; the masses measured approximately 8 cm × 6 cm in width (Figure 1C). Under the suspicion of mesenteric and jejunal wall metastasis from the rectal cancer, 18F-FDG PET/CT was performed, but no remarkable FDG uptake was seen (Figure 1D).



**Figure 1** A 71-year-old man with rectal cancer and mesenteric lymphangioma. A, B: Contrast-enhanced, coronal CT images show irregular and concentric rectal wall thickening (arrow heads), a nodular soft-tissue-density mass, and hazy strands in the jejunal mesentery (arrows); C: Follow-up, contrast-enhanced, coronal CT obtained one year after laparoscopic rectal cancer resection, reveals clear demarcation of the jejunal nodular lesions and infiltrative soft tissue masses in the adjacent mesentery. No remarkable jejunal obstruction was found; D: 18F-FDG PET shows no 18F-FDG uptake in the jejunum or mesenteric lesions; E: The cut section of the jejunum reveals a dark-red, multiloculated, cystic lesion measuring 8.0 cm × 5.0 cm in the mucosa to the subserosa; F, G: Histopathologic view of the tumor shows numerous, multiloculated, cystically dilated lymphatic spaces lined by attenuated endothelium in the entire jejunal wall (HE stain,  $\times 40$ ) and adjacent mesentery (HE,  $\times 20$ ).

Pathological examination of a resected segment of the jejunum revealed a macroscopic, dark red, multiloculated cystic lesion measuring 8.0 cm × 5.0 cm from the mucosa to the subserosa (Figure 1E). Microscopically, it showed numerous multiloculated, cystically dilated spaces lined by attenuated endothelium and involved mucosa to subserosa. In the focal area, ectatic spaces appeared to dissect through the muscularis propria of the small intestine. Proteinaceous, fluid-containing lymphocytes seen in the cystic spaces revealed that the channel originated in the lymphatic system. The stroma was composed of a delicate meshwork of collagen punctuated by lymphoid aggregates (Figure 1F). Immunohistochemical staining for CD34 showed positivity in the endothelial cells of the tumor (Figure 1G). The diagnosis of cavernous lymphangioma involving the jejunum and mesentery was established. The patient had an uneventful postoperative course and was discharged one week after surgery.

## DISCUSSION

Lymphangioma or lymphangiomatosis affect the skin, the covering of various organs and areas except of the brain. About 90% are diagnosed within the first two years of their existence<sup>[1,2]</sup>. In adults, gastrointestinal tract involvement of mesenteric lymphangioma is very rare, the distal ileal mesentery is most frequently involved<sup>[3]</sup>. The etiology of lymphangiomas is still unclear. They are considered to be a congenital dysplasia of lymphatic tissue and abnormal development of the lymphatic vessels during fetal life<sup>[5,6]</sup>. The macroscopic appearance of lymphangioma is a cystic mass with partial septations

and its histological characteristics are endothelial-lined, dilated, communicating lymphatic channels containing a variable amount of connective tissue and smooth muscle fiber<sup>[5,7]</sup>. Lymphangiomas are generally classified as simple capillary, cavernous, and cystic according to the size of lymphatic space and the nature of the lymphatic wall<sup>[8,9]</sup>. Cavernous lymphangioma is composed of dilated lymphatic vessels and lymphoid stroma and is connected with the adjacent normal lymphatics. Alternatively, cystic lymphangioma is composed of various-sized lymphatic spaces and has no connection with the adjacent normal lymphatics. However, as cystic lymphangiomas may have a cavernous area, clear differentiation between cystic and cavernous lymphangioma is not always possible<sup>[9,10]</sup>.

Most intra-abdominal lymphangiomas are of cystic form and generally appear as a thin-walled, multiseptated, cystic mass with or without intracystic debris. Although these lymphangioma characteristics may appear in typical, multiseptated, cystic masses on images including ultrasound and CT, some lymphangiomas may appear to be solid masses because they contain intracystic debris or hemorrhage or due to the microcystic nature of cavernous lymphangiomas. MRI is advantageous for detecting fluid-filled cystic lesions as it may reveal the cystic nature of cavernous lymphangiomas that appear as solid masses on CT<sup>[11]</sup>. In our patient with rectal cancer, multiple nodular mesenteric masses infiltrating into the jejunum and adjacent mesentery were found. Because we did not suspect the microcystic nature of cavernous lymphangiomas, MRI was not performed to differentiate a microcystic tumor mimicking a solid mass.

As 18F-FDG is an analogue of glucose, its uptake

within viable tumor cells is in proportion to the rate of glycolysis. Therefore, as 18F-FDG PET/CT can detect hypermetabolic tumor cells, it can be widely used for the detection, staging, and management of various malignant tumors<sup>[4]</sup>. In our patient, as 18F-FDG PET/CT revealed no remarkable 18F-FDG uptake within the solid, mass-like lesions in the jejunal and adjacent mesentery, we were able to exclude the possibility of metastasis from the underlying rectal cancer.

The clinical symptoms of gastrointestinal and mesenteric lymphangiomas vary from being asymptomatic to acute abdominal symptoms such as obstruction or bleeding, according to the size and the localization of the tumor<sup>[10,12,13]</sup>. The treatment of choice is complete surgical resection. Because lymphangioma is benign in nature, the prognosis is usually good despite the possibility of tumor recurrence. As Goh *et al*<sup>[14]</sup> reported a 100% recurrence rate, complete lymphangioma resection is important in order to prevent tumor recurrence.

In conclusion, rarely occurring, cavernous mesenteric lymphangioma in adults occasionally appears on CT as a solid mass and it may be confused with metastasis in patients with malignancies. Therefore, 18F-FDG PET/CT may be helpful in excluding the presence of metastasis.

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CASE REPORT

## Duodenal stenosis resulting from a preduodenal portal vein and an operation for scoliosis

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**Author contributions:** Masumoto K, Nakatsuji T, and Taguchi T took part in management of the treatment and analyzed the data together with other co-authors. All the authors discussed the cause of duodenal stenosis and the patient's outcome and commented on the manuscript.

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such as PDPV and duodenal stretching induced by previous spinal surgery.

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**Key words:** Preduodenal portal vein; Duodenum; Malnutrition; Gastrojejunostomy; Nutritional support; Stenosis

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## INTRODUCTION

Preduodenal portal vein (PDPV) is a rare congenital anomaly which was first described by Knight in 1921<sup>[1]</sup>. Clinically, 50% of the patients present with symptoms related to intestinal obstruction, but only in a few is the obstruction directly caused by the PDPV<sup>[2-4]</sup>. In the majority of cases, the obstruction is thought to be caused by associated anomalies such as duodenal diaphragm, intestinal malrotation, and annular pancreas. In the remaining 50%, PDPV is asymptomatic and diagnosed incidentally on preoperative investigation or during surgery for other diseases<sup>[2-4]</sup>. Patients with symptoms directly associated with PDPV or resulting from associated anomalies often require correction of the intestinal obstruction<sup>[2-5]</sup>.

Scoliosis is a well known anomaly in the pediatric orthopedic field. In patients with severe scoliosis, a surgical correction is often needed. The hallmark of surgical treatment is thought to be early intervention before the development of large curvatures<sup>[6]</sup>. Recent orthopedic surgery for scoliosis has evolved to include the routine use of spinal instrumentation to achieve a good result.

No reports have been published regarding patients

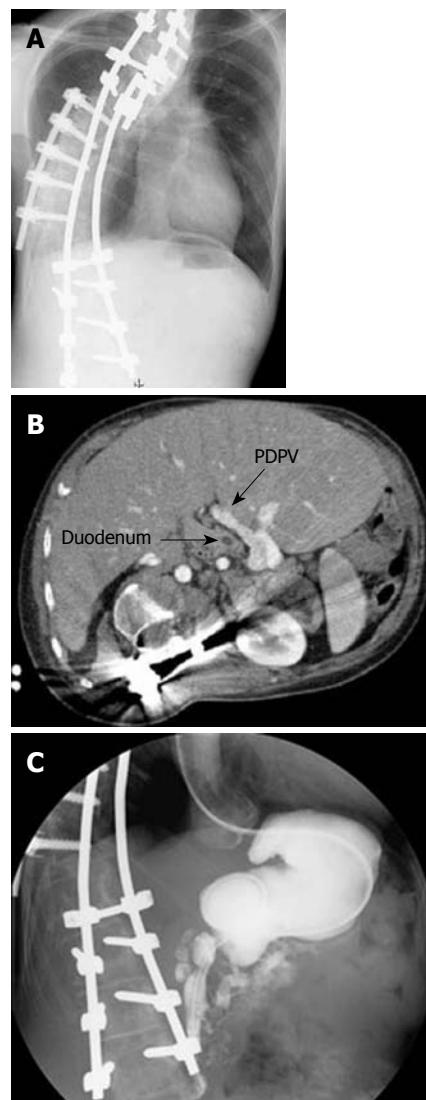
with PDPV associated with severe scoliosis. We experienced one male patient with malnutrition resulting from PDPV and a previously performed operation for scoliosis. An improvement in the quality of life (QOL) was found after being treated with a combination of a gastrojejunostomy, with both Braun's anastomosis and a side-to-side anastomosis between the duodenal second portion and jejunum, and nutritional support. Therefore, we herein report and discuss the clinical course of this patient and his treatment.

## CASE REPORT

A 22-year-old male patient with PDPV presented with duodenal stenosis, ranging from the first to the second portions. Frequent vomiting had occurred at 1 year of age. Therefore, fundoplication had been performed at 2 years of age in another hospital. However, he had occasionally suffered from vomiting since the fundoplication. In addition, he gradually developed severe scoliosis over time. Therefore, he underwent surgery twice for scoliosis at 21 and 22 years of age. After the orthopedic operations, he developed malnutrition because of severe vomiting during a 6-mo period. In another hospital, the cause of the vomiting was investigated, and found to be the result of widespread duodenal stenosis.

On admission to our department, an X-ray film showed a spine with a large curve to the right side although it was corrected by previous orthopedic surgery (Figure 1A). Abdominal computerized tomography (Figure 1B) and a gastrointestinal contrast study (Figure 1C) revealed that the stenosis was associated with both the PDPV and the previously performed operation for scoliosis. In addition, his nutritional condition at admission was extremely poor (body mass index: 11.4). Furthermore, he also had diabetes associated with dysfunction of Vater's sphincter. We therefore introduced nutritional support using both parenteral and enteral nutrition by jejunal feeding tube at first. Two months later, the blood sugar was well controlled by insulin therapy. During nutritional support with insulin therapy for 6 mo, his nutritional condition gradually improved (Figure 2). Six months later, his nutritional condition was observed to have returned to the normal level for his age (body mass index: 18.2, Figure 2).

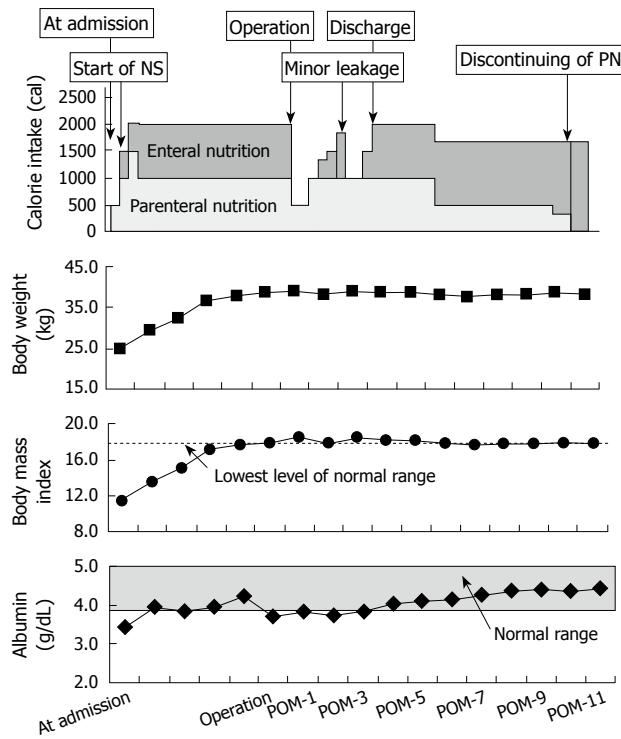
Thereafter, an operation was performed. The operative findings showed that the PVPD had induced the severe stenosis of the duodenal first portion, and that the stenosis widely ranging from the second to third portions was a result of stretching by the corrected spine (Figure 3A). No intestinal malrotation was found. The liver was confirmed to be a symmetrical liver. The head of the pancreas was found to be attached to the stenosis of the first duodenal portion. However, the pancreas did not surround any portions of the duodenum. Although the second portion was mildly dilated after performing mobilization of the duodenal second portion, neither the first nor third duodenal portion was dilated (Figure 3B). Therefore, a gastrojejunostomy with Braun's anastomosis was selected and performed (Figure 3B). In addition, a side-to-side anastomosis between the duodenal second



**Figure 1** Images at admission. A: Plain X-ray film. Although the spine was corrected by a previous orthopedic operation, a severe scoliosis with right side protrusion was found at admission; B: Abdominal CT scan. The preduodenal portal vein (PDPV) was found to be located in the anterior side of the duodenum; C: Contrast study of the upper gastrointestinal tract. A contrast study of the duodenum showed a stenosis ranging from the second to third portions. In addition, the contents flowed to both the dilated main pancreatic duct and common bile duct.

portion and jejunum was performed to allow smooth passage of the duodenal contents (Figure 3B).

The postoperative course was uneventful except for slight leakage at the site of anastomosis. Regarding his diabetes, he required insulin therapy for the control of blood sugar. The postoperative contrast study showed that duodenal passage in the second portion improved and that most of the contents in the stomach flowed smoothly into the connected jejunum through the gastrojejunostomy. Two months later, he could eat a normal volume of solid food by mouth (Figure 2). Three months later, he was discharged, using both feeding by mouth and home parenteral nutrition with insulin therapy, and was again able to attend university (Figure 2). Seven months after discharge, he no longer required home parenteral nutrition. Unfortunately, an improvement in his diabetes



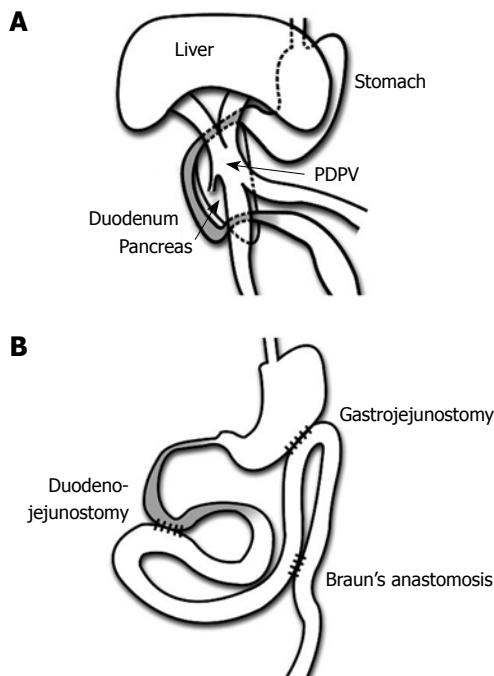
**Figure 2 Clinical course of our patient.** This figure shows the clinical course of our patient during the period from admission to the cessation of parenteral nutrition. After the preoperative nutritional support using both parenteral and enteral nutrition for 6 mo, an improvement in body weight and body mass index was seen. After the operation, the calorie intake from parenteral nutrition was gradually reduced. The postoperative anthropological markers were maintained. NS: Nutritional support; POM: Postoperative months.

was not observed after the operation and therefore he has needed to continue insulin therapy.

## DISCUSSION

In general, a PDPV without associated anomalies may be the cause of duodenal stenosis in the first or second portion if symptoms related to PDPV occur<sup>[1-5,7,8]</sup>. Therefore, in such patients, an operation including duodenoduodenostomy, is thought to be required for the smooth passage of duodenal contents<sup>[1-5,7,8]</sup>. However, in this patient with PDPV, the stenosis ranged from the first to the third portions of the duodenum. This may be why duodenal stenosis in this patient was also associated with severe scoliosis. He had needed an operation for severe scoliosis but the surgery was not thought to be good for duodenal passage because the duodenum was stretched longitudinally to the spinal axis. After the orthopedic operation, his symptoms had further deteriorated. The first portion of the duodenum was thought to be anchored to both the thick pedicle of the PDPV and the head of the pancreas, and stretched to the hepatic side before the orthopedic operation. Therefore, the orthopedic operation may have induced excessive stretching of the duodenum, resulting in the duodenal stenosis, ranging from the second to third portions, in addition to the stenosis of first portion arising from the PDPV.

In this patient, the long-term symptoms related to



**Figure 3 Operative findings and performed procedures in our patient.** A: Operative findings. The PDPV was found to induce the severe stenosis of the duodenal first portion and the stenosis ranging from the second to third portions was the result of stretching by spinal correction. No intestinal malrotation was found. The liver was confirmed to be a symmetrical liver. The head of the pancreas was found to be attached to the stenosis of the first duodenal portion. The duodenum is colored gray in this figure; B: Performed procedures. A gastrojejunostomy with Braun's anastomosis was performed for the stenosis in the first portion. In addition, a duodenojjunostomy between the duodenal second portion and jejunum was performed for smooth passage of the duodenal contents. The duodenum is also colored gray in this figure.

PDPV, including frequent vomiting, induced malnutrition. In addition, the duodenal stretching after the orthopedic operation also contributed to the malnutrition. In general, if the severe symptoms are thought to be related to PDPV, duodenoduodenostomy is performed first<sup>[1,7,8]</sup>. However, in this patient, we preceded surgery with the recovery of his nutritional condition using both parenteral and enteral nutrition, since his nutritional condition had been very severe at admission. Although the time required for the nutritional recovery was long, the operation for the duodenal stenosis was performed smoothly, leading to a good postoperative clinical course. Therefore, in a specific case with severe malnutrition as in this report, nutritional support may precede the operation for severe duodenal stenosis.

In cases where duodenoduodenostomy is performed for duodenal stenosis resulting from PDPV, recent studies recommend the loose overbridging duodenoduodenostomy in order not to compress the blood flow of the portal vein<sup>[9]</sup>. In this patient, we could not perform the loose overbridging duodenoduodenostomy for stenosis of the duodenal first portion because the stenosis was widespread. Therefore, a gastrojejunostomy with Braun's anastomosis was performed. In addition, a side-to-side anastomosis between the duodenal second portion and the jejunum was performed to allow smooth passage of the duodenal contents, including the pancreatic juice and

bile, because Kocher's duodenal mobilization did not result in enough improvement of the stenosis ranging from the second to third portions. As no patient with a clinical course similar to our patient had been reported in the literature, we were uncertain which operations were the best choice for this patient. However, based on the postoperative clinical course in our patient, the operations we performed resulted in an improvement in the patient's QOL. Therefore, such a combination of operations is thought to be one choice for severe widespread duodenal stenosis.

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S- Editor Li LF L- Editor Cant MR E- Editor Lin YP



CASE REPORT

## Jejunal small ectopic pancreas developing into jejunojejunal intussusception: A rare cause of ileus

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small ectopic pancreas developing into jejunojejunal intussusception: A rare cause of ileus. *World J Gastroenterol* 2009; 15(31): 3954-3956 Available from: URL: <http://www.wjgnet.com/1007-9327/15/3954.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.3954>

### INTRODUCTION

Ectopic pancreas is not an extremely rare pathological condition<sup>[1-3]</sup>. It is defined as the presence of pancreatic tissue lacking anatomical and vascular continuity with the pancreas<sup>[1]</sup>. It occurs most commonly in the stomach, duodenum, and jejunum and has been reported in other locations, including the ileum, Meckel's diverticulum, colon, gall bladder, umbilicus, fallopian tube, mediastinum, spleen, and liver<sup>[2]</sup>. The majority of the cases have been found incidentally at laparotomy performed for other abdominal diseases. When an ectopic pancreas is found incidentally during surgery for other abdominal conditions, resection should be considered because of the risk of late clinical problems. This disease occasionally develops symptoms such as bleeding, vomiting or abdominal pain due to pancreatitis<sup>[2,3]</sup>. Even if symptoms are present, the preoperative diagnosis of ectopic pancreas in the small bowel appears to be difficult. Malignant transformation can occur in the ectopic pancreas tissue as well as in the tissue of a normally located pancreas. However, the incidence of malignant change in the ectopic pancreas has been estimated to be less than that of the normal pancreas itself<sup>[3]</sup>. Ectopic pancreas rarely occurs as intestinal invagination leading to ileus in adults<sup>[4]</sup>. Here, we describe a rare case of small jejunal ectopic pancreas that led to jejunojejunal intussusception and ileus.

### CASE REPORT

The patient was a 62-year-old Japanese man. He was admitted to our hospital complaining of intermittent abdominal pain of several months' duration that was increasing in both magnitude and frequency. He had not had previous abdominal surgery and had been in good health. No specific family history was identified. His body temperature was 36.8°C, blood pressure was 132/76 mmHg, and radial pulse rate was 62 beats/min and regular. He had neither anemia nor jaundice. A neurological examination revealed no abnormal findings.

### Abstract

Intussusception is rare in adults. We describe a 62-year-old man with jejunal ectopic pancreas that led to jejunojejunal intussusception and ileus. The patient was admitted to our hospital because of intermittent abdominal pain. Plain abdominal radiography showed some intestinal gas and fluid levels. Abdominal CT scan demonstrated a target sign suggesting bowel intussusception. Jejunography using a naso-jejunal tube showed an oval-shaped mass about 15 mm in diameter with a smooth surface in the jejunum, which suggested a submucosal tumor (SMT), and edematous mucosa around the mass. Partial jejunal resection was carried out and the resected oval-shaped tumor, 14 mm × 11 mm in size, was found to be covered with normal jejunal mucosa. The tumor was histologically diagnosed as type III ectopic pancreas according to the classification proposed by Heinrich. Abdominal pain resolved postoperatively. This case reminds us that jejunal ectopic pancreas should be included in the differential diagnosis of intussusception caused by an SMT in the intestine.

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**Key words:** Jejunal submucosal tumor; Small intestine; Invagination; Aberrant pancreas; Surgery

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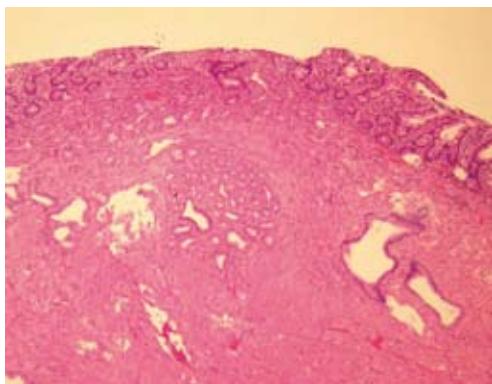
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**Figure 1** Jejunography using a naso-jejunal tube showed an oval-shaped mass in the jejunum (arrow) and edematous mucosa around the mass.



**Figure 2** Macroscopic findings of the tumor. A 14 mm x 11 mm oval-shaped submucosal tumor covered with normal mucosa was observed.



**Figure 3** Histological findings of the tumor. The localized tumor was composed of proliferating ducts and proliferation of smooth muscle bundles without mitotic figures. However, both exocrine acini and endocrine elements were lacking (HE,  $\times 100$ ).

Laboratory tests showed hemoglobin concentration of 14.6 g/dL, a red blood cell count of  $441 \times 10^4/\mu\text{L}$ , a white blood cell count of  $10300/\mu\text{L}$  (normal range:  $4000-8000/\mu\text{L}$ ), and a platelet count of  $19.5 \times 10^4/\mu\text{L}$ . Biochemical tests were within normal limits. Plain abdominal radiography showed some intestinal gas and fluid levels. Abdominal CT scan demonstrated a target sign suggesting bowel intussusception and dilated small bowel. Subsequent jejunography using a naso-jejunal tube showed an oval-shaped mass 15 mm in diameter with a smooth surface in the jejunum, which suggested a submucosal tumor (SMT), and edematous mucosa around the mass (Figure 1). Based on these findings, the patient was diagnosed with intussusception due to intestinal SMT. The patient underwent a laparotomy. At laparotomy, a small solid tumor that was 60 cm distal to the Treitz's ligament was palpable and the small bowel around the tumor was edematous. Liver and spleen were normal. There was no evidence of mesenteric or retroperitoneal lymphadenopathy, ascites or peritoneal disease. The resection of jejunum with 2 cm margins and an end-to-end anastomosis was performed. The resected oval shaped tumor, 14 mm x 11 mm in size, was covered with normal jejunal mucosa and no ulcer or erosion was seen on the mucosal surface (Figure 2). Opening of the rudimentary pancreatic duct was not detected. Histologically, the mass was distributed from submucosa to smooth muscle layer and was composed of proliferating ducts and proliferation of smooth muscle bundles without mitotic figures

(Figure 3). However, both exocrine acini and endocrine elements were lacking. Based on the above findings, this tumor was diagnosed as jejunal ectopic pancreas (type III ectopic pancreas according to the classification proposed by von Heinrich<sup>[5]</sup>). The postoperative course was uneventful. As a result of this treatment, the patient's abdominal pain resolved postoperatively.

## DISCUSSION

Ectopic pancreas is defined as the presence of pancreatic tissue lacking anatomical and vascular continuity with the pancreas<sup>[1]</sup>. It occurs most commonly in the stomach, duodenum, and jejunum and has been reported in other locations, including the ileum, Meckel's diverticulum, colon, gall bladder, umbilicus, fallopian tube, mediastinum, spleen, and liver<sup>[2]</sup>. Ectopic pancreas occurs in 0.25%-13.7% of patients based on both autopsy and surgical series; approximately 70% of all such tissues are found in the stomach, duodenum, and jejunum<sup>[2]</sup>. When an ectopic pancreas is found coincidentally during surgery for other abdominal conditions, resection should be considered because of the risk of late clinical problems. Although this disease occasionally develops symptoms such as bleeding, vomiting or abdominal pain due to pancreatitis<sup>[2,3,6,7]</sup>, intestinal obstruction or intussusception is rare<sup>[4,8,9]</sup>. No invagination case caused by ectopic pancreas had been observed in a series of 53 invaginations, reported by Ong *et al*<sup>[10]</sup>. Ectopic pancreas in the small intestine may rarely be fatal; however, Hitosugi *et al*<sup>[11]</sup> reported a sudden death case (11-year-old Japanese female) caused by intestinal obstruction due to jejunal ectopic pancreas (about 4 cm in diameter). Adult intussusception represents 5% of all cases of intussusception and accounts for only 1%-5% of intestinal obstructions in adults<sup>[12]</sup>. In contrast with intussusception in children, nearly all adult cases have primary causes such as a polyp, an SMT or a malignant tumor that needs to be resected<sup>[13,14]</sup>. In the present case, the small SMT (ectopic pancreas) in the jejunum caused ileus due to intussusception.

The preoperative diagnosis of ectopic pancreas in the small bowel appears to be difficult. However, there are some recent reports describing ectopic pancreas in the small bowel discovered by double-balloon enteroscopy or capsule endoscopy<sup>[15-17]</sup>. In spite of using double-balloon enteroscopy, endoscopic diagnosis is rarely

conclusive on endoscopic biopsies because the lesion locates mainly in the submucosal layer and the biopsy is often not deep enough to reach the submucosal tumor tissue. In the present case, the diagnosis could not be confirmed preoperatively although we knew that bowel intussusception due to small SMT might cause ileus. Because of the suspicion of ileus due to jejunal tumor, the patient underwent laparotomy; thus, double-balloon enteroscopy was not performed in the present case.

This disease is treated in principle by surgical resection if bowel intussusception or ileus occurs<sup>[4]</sup>. Moreover, physicians should be aware that ectopic pancreas in the small bowel may associate with endocrine tumor or carcinoma<sup>[18-21]</sup>. However, ectopic pancreas in the small intestine is usually benign. Ectopic pancreas in the small intestine is rarely fatal and patients remain asymptomatic in their daily lives, except for when bleeding, bowel intussusception, obstruction or pancreatitis occurs. Therefore, it is likely that there are patients with latent small bowel ectopic pancreas which may be incidentally discovered in the future, as a result of advances in diagnostic imaging, such as improved CT, MRI, capsule endoscopy and double-balloon enteroscopy.

In conclusion, we have reported a very rare case of jejunal ectopic pancreas that led to jejunoojejunal intussusception. Thus, it is necessary to be aware that a jejunal ectopic pancreas may cause jejunoojejunal intussusception even though the likelihood is small. This etiology should be suspected in a patient with chronic atypical abdominal pain.

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## Giant vesical diverticulum: A rare cause of defecation disturbance

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### INTRODUCTION

Vesical diverticula are herniations of the bladder mucosa and submucosa through the muscular wall of the bladder<sup>[1]</sup>. They are often asymptomatic and are discovered incidentally during an examination for other reasons<sup>[1,2]</sup>. Some patients present with urinary tract infections, obstruction, gallstones, or frequent voiding caused by diverticula, especially when they are large and empty poorly. The common causes of large bowel mechanical obstruction in adults are malignant tumors, diverticular disease, and volvulus. Extrinsic obstruction secondary to distension of a giant vesical diverticulum is rare. To our knowledge, this is the fourth report of a vesical diverticulum causing disrupted defecation or intestinal obstruction in the English medical literature since 1957<sup>[3-5]</sup>. We report an unusual case of large bowel obstruction caused by a giant bladder diverticulum. In addition, this is the largest vesical diverticulum reported in the literature.

### Abstract

Vesical diverticula frequently result from bladder outlet obstructions. However, giant vesical diverticula which cause acute abdomen or intestinal obstruction are very rare. Our review of the English medical literature found 3 cases of bladder diverticula which caused gastrointestinal symptoms. Here, we present a 57-year-old man with a giant diverticulum of the urinary bladder who complained of abdominal pain, nausea and vomiting, constipation, no passage of gas or feces, and abdominal distension for 3 d. A 20 cm × 15 cm diverticulum was observed upon laparotomy. The colonic obstruction was secondary to external compression of the rectum against the sacrum by a distended vesical diverticulum. We performed a diverticulectomy and primary closure. Twelve months postoperatively, the patient had no difficulty with voiding or defecation.

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**Key words:** Colonic obstruction; Defecation; Diverticulectomy; Urinary bladder; Vesical diverticulum

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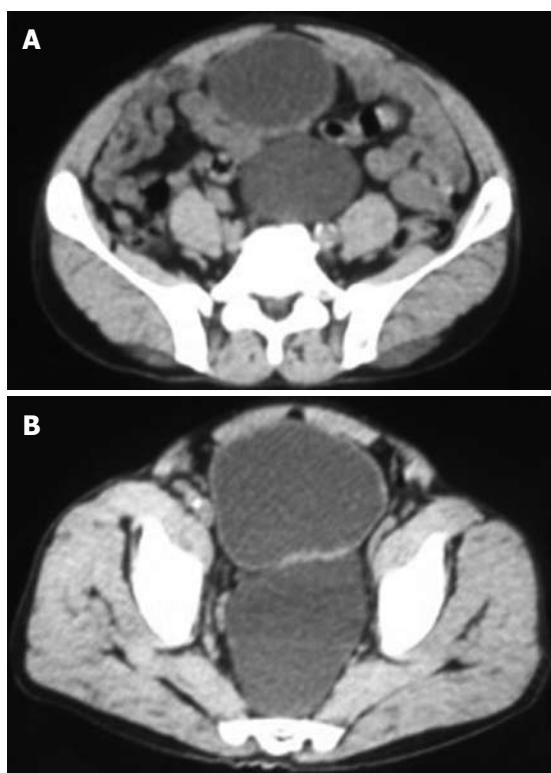
### CASE REPORT

A 57-year-old man was admitted to the general surgery department with abdominal pain, nausea and vomiting, constipation, no passage of gas or feces, and abdominal distension for 3 d. The relevant physical examination revealed a distended abdomen, decreased bowel sounds, diffuse sensitivity on palpation of the abdomen, and empty rectal ampulla. Laboratory investigations showed a blood urea nitrogen level of 31 mg/dL, a creatinine level of 1.1 mg/dL, and a C-reactive protein level of 35 mg/L. The blood cell count revealed leukocytosis at 16500/µL, a hemoglobin level of 13.5 g/dL, and a platelet count of 423000/µL. Other serum parameters, including prostate specific antigen (PSA, 1.9 ng/mL), were within normal limits. Computed tomography (CT) showed a 15 cm × 10 cm low density cystic lesion with smooth contours located in the presacral region, pushing the rectum to the right and the sigmoid colon and bladder superiorly (Figure 1A and B). The patient had a history of trauma because of a traffic accident 4 years previously, and

**Table 1 A summary of 12 cases of giant vesical diverticula reported in the English medical literature from 1957 to 2009 and description of the studies**

Ref.	Yr	Age	Sex	Medical history	Initial symptom	Diagnosis	Management	Complication
Kauffman <i>et al</i> <sup>[3]</sup>	1957	70	M	Not available	Constipation	X-ray films, intravenous urography	Diverticulectomy	Not found
Mirow <i>et al</i> <sup>[4]</sup>	2007	84	M	Sigmoid carcinoma	Abdominal pain, intestinal obstruction	Intraoperative	Diverticulectomy	Not found
Shaked <i>et al</i> <sup>[5]</sup>	2009	76	M	Hypertension, diabetes mellitus	Abdominal pain, constipation	CT		
Shukla <i>et al</i> <sup>[6]</sup>	2004	11	F	EDS	Infection, incomplete voiding	Cystogram	Diverticulectomy	Not found
		4 mo-3 yr	M	No medical history of voiding dysfunction	Decreasing urinary stream and urinary retention			
Burrows <i>et al</i> <sup>[7]</sup>	1998	16	M	EDS type 1	Outflow obstruction	Cystogram	Diverticulectomy	Not found
Suzuki <i>et al</i> <sup>[8]</sup>	2002	84	M	Bladder injury with bullet	Abdominal distension	CT, cystogram	Diverticulectomy	Not found
Farhi <i>et al</i> <sup>[9]</sup>	1991	31	F	Recurrent urinary infection	Ovarian cyst	USG, cystogram	Not available	Not available
Taha <i>et al</i> <sup>[10]</sup>	1987	65	M	Not available	Abdominal distension, slow stream of urine	Intravenous urography, CT	Reduction cystoplasty	Not found
Siddiqui <i>et al</i> <sup>[11]</sup>	2003	77	M	TUR-P was performed twice because of urinary retention	Acute urinary retention	Intravenous urography	Diverticulectomy	Not found

The first three references present cases of bladder diverticula which caused gastrointestinal symptoms. CT: Computed tomography; EDS: Ehlers-Danlos syndrome; TUR-P: Transurethral prostatectomy; USG: Ultrasonography.



**Figure 1** Computed tomography showed a 15 cm × 10 cm low density cystic lesion with smooth contours located in the presacral region, pushing the rectum to the right and the sigmoid colon and bladder superiorly (A and B).

because of this diagnostic laparotomy was performed and a few sutures were placed in the bladder. As the patient had symptoms of acute abdomen, laparotomy was performed.

During laparotomy, the mass was found to be a diverticulum originating from the posterosuperior region



**Figure 2** No significant pathology was observed on intravenous pyelography 3 mo postoperatively.

of the bladder. The colonic obstruction was secondary to external compression of the rectum against the sacrum by a distended vesical diverticulum. The diverticulum measured approximately 20 cm × 15 cm and was difficult to mobilize by dissecting its delicate attachments to the anterior rectal wall. The diverticular orifice was approximately 1-1.5 cm in diameter. A diverticulectomy and primary closure were performed. Postoperatively, the bladder was catheterized for 10 d. Three months postoperatively, the patient's urinary frequency and constipation had disappeared. An intravenous pyelography (IVP) showed no significant pathologic findings (Figure 2). The patient had no difficulty in voiding nor had constipation 12 mo postoperatively.

## DISCUSSION

We described a rare cause of a giant vesical diverticulum causing mechanical bowel obstruction in an adult patient. We summarized the characteristics of the 12 cases of "giant bladder diverticulum" which we found in the literature in the Table 1<sup>[3-11]</sup>. Three of the cases

caused intestinal obstruction<sup>[3-5]</sup>. The bladder is a hollow muscular organ that stores and evacuates urine. The normal bladder can store approximately 350-400 mL of urine. A diverticulum is an abnormal sac or pouch protruding from the wall of a hollow organ. Most bladder diverticula are primary, congenital, or secondary to outflow obstruction or neurogenic bladders<sup>[12-14]</sup>. Congenital diverticula usually occur in areas where there is insufficient muscle, typically at the ureterovesical junction, or between bundles of hypertrophied muscle. They are usually asymptomatic and are discovered incidentally. Occasionally, a diverticulum may produce urinary obstruction as a result of compression of the urethra, or urinary tract infection arising from retention of urine within the diverticulum<sup>[8,15]</sup>. In this case, only minimal obstruction caused by the pressure on the ureters was observed. No obstruction of the urethra developed. Although diverticula generally develop from the ureteral orifices in the bladder, in this case the diverticulum was located in the posterosuperior region of the bladder distant from the orifices.

In this patient, the diverticulum was in the same location as previous trauma. Because of this and since trauma is known to be one of the etiologic factors for acquired diverticula, we believed that this diverticula had occurred as a result of the previous trauma.

The indications for surgery are persistent or recurrent urinary infection, the presence of a stone or tumor in the diverticulum<sup>[16,17]</sup>, a vesicocutaneous fistula<sup>[18]</sup>, lower urinary tract symptoms, and voiding symptoms or vesicoureteral reflux<sup>[19]</sup> resulting from the diverticulum or ureteral obstruction<sup>[6,12]</sup>. In this case, laparotomy was indicated by the normal IVP results and CT scan showing a mass exerting pressure on the ureters and rectum. Vesical diverticula are a common pathology of the urinary bladder, generally secondary to cervico-urethral obstruction. Shaked *et al*<sup>[5]</sup> presented a case of bladder distention and diverticulum arising from obstruction caused by a prostate adenoma. In their case, they reported that colonic obstruction occurred because the colon was packed between the sacrum and bladder. In our case, although the bladder diverticulum was large, no obstruction was observed because the diverticulum developed from the posterosuperior part of the bladder. This was reflected in the PSA and transrectal ultrasonography results.

In conclusion, there are several reported cases of bladder distention and diverticula causing colonic obstruction. For this reason, although rare, bladder pathology should be considered in patients with obstruction of the rectosigmoid region.

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CASE REPORT

## Therapy of central pontine myelinolysis following living donor liver transplantation: Report of three cases

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## INTRODUCTION

With the increased number of living donor liver transplantation (LDLT) patients, some special complications have become apparent. Central pontine myelinolysis (CPM) is a severe nervous system complication following liver transplantation<sup>[1-3]</sup>. To date, no studies have been performed addressing CPM occurrence and treatment following LDLT. This study aimed to report the clinical manifestations and treatment of three patients diagnosed with CPM following LDLT from January 2005 to November 2007. The three donors in this study were lineal relatives of these patients. No organs were used from prisoners and no subjects were prisoners at the time of data collection.

## CASE REPORT

### Case one

This patient was a 51-year-old female diagnosed with chronic, type B hepatitis, hepatic decompensation, and hepatic encephalopathy. Her blood type was B/Rh (-). Pre-operative examination revealed the following parameters: total bilirubin (107.9 μmol/L), direct bilirubin (53.6 μmol/L), aspartate aminotransferase (595 IU/L), glutamate-pyruvate transaminase (600 IU/L), and serum cholesterol (0.18 mmol/L). Blood electrolytes, Child-Turcotte-Pugh score, and MELD score are shown in Table 1. In November 2005, the patient underwent adult-to-adult LDLT with a right lobe graft. The donor was the patient's son, whose blood type was B/Rh (+). Electrolytes were re-examined in the intensive care unit (Table 1). Post-operative immunosuppressive regimen was administered. The patient was treated with cyclosporine A (250 mg, twice daily), 0.75 g mycophenolic acid (oral)

## Abstract

We analyzed the clinical manifestations and experiences of diagnosing and treating central pontine myelinolysis following living donor liver transplantation. The clinical data of three patients with central pontine myelinolysis following living donor liver transplantation from January 2005 to November 2007 were retrospectively analyzed at the West China Hospital, Sichuan University, China. The three patients developed hyponatremia prior to surgery. Case 1 suffered locked-in syndrome following surgery, and received a large dose of gamma globulin, and subsequently recovered. Case 2 was in a coma for three days, and received hyperbaric chamber treatment. This patient remained in a mild coma for six months following surgery. Case 3 developed consciousness disturbances, gradually went into a coma following surgery, and died due to pulmonary infection. Central pontine myelinolysis is a severe complication in patients following living donor liver transplantation. Large-dose gamma globulin treatment, as well as hyperbaric oxygen, might be effective therapeutic methods.

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**Key words:** Cyclosporine; Hyponatremia; Liver transplantation; Myelinolysis; Pontine

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**Table 1** Blood electrolytes, Child-Turcotte-Pugh (CTP) score, and “Model for end-stage liver disease” (MELD) score

	<b>Case 1</b>	<b>Case 2</b>	<b>Case 3</b>
Pre-operative serum sodium (mmol/L)	124	119	119
Post-operative serum sodium (mmol/L)	151	153	141
Pre-operative serum magnesium (mmol/L)	0.52	0.47	0.64
Post-operative serum magnesium (mmol/L)	0.58	0.62	0.68
Pre-operative plasma osmotic pressure (mOsm/kg H <sub>2</sub> O)	238.71	232.47	258.45
Post-operative plasma osmotic pressure (mOsm/kg H <sub>2</sub> O)	295.36	288.93	291.92
Plasma cholesterol (mmol/L)	0.18	1.24	1.07
CTP score	B	C	C
MELD score	30	36	35
Cyclosporine A or FK506 concentration	Cyclosporine A 180-250 mg/L	FK506 6.5-8 ng/mL	No
Clinical manifestations	Locked-in syndrome	Coma	Coma
Special therapy	Gamma globulin (i.v.)	Hyperbaric oxygen treatment	No

twice daily, and methylprednisolone (iv). Subsequent, cyclosporine A blood concentration was shown to be 180-250 mg/L. At 6 h after surgery, the patient was conscious, and the tracheal cannula was removed. On day one after surgery, the patient maintained clarity, answered questions with short sentences, exhibited locomotor activities, and drank water. On day three after surgery, the patient presented with decreased spontaneous speech, slowed actions, could not answer a simple question correctly, had indistinct pronunciation, and bucked while drinking water. On day five after surgery, the patient had stable vital body signs, clear consciousness, spontaneous eye opening, ocular movement, but did not exhibit spontaneous speech, spontaneous limb activities, or reaction to pain stimulation. Physical examination showed increased tension of limb muscles, muscle force 0°, decreased deep and superficial sensations, bilateral Babinski sign (+), and an Expanded Disability Status Scale score of 9.5. Skull nuclear magnetic resonance was subsequently performed and the findings are displayed in Figure 1. The patient was diagnosed with “locked-in syndrome” induced by CPM, and was administered citicoline (0.5 g daily *via* intravenous drip) and a large dose of gamma globulin (0.5 g/kg daily for ten days, iv). Oral cyclosporine A was not administered. On day twelve after surgery, the patient pronounced monosyllables, performed light limb activities with grade III muscle force, but was still not able to swallow. On day fourteen after surgery, the patient was conscious, producing longer utterances, pronounced indistinctly and slowly, demonstrated increased autonomic activities, bucked during swallowing, and presented with an Expanded Disability Status Scale score of 6. Three months after surgery, the patient presented with normal liver function,

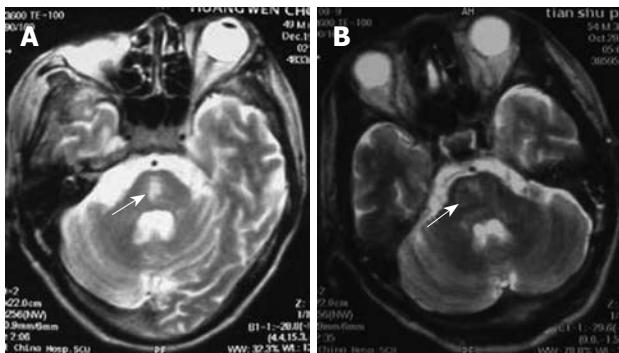
**Figure 1** Magnetic resonance imaging displayed high-signal intensity on T2-weighted images of the central pons 7 d after surgery (arrow).

clear consciousness, normal diet, self-care, slow speech rate, and slow motor reactions.

### Case two

This patient was a 54-year-old male diagnosed with chronic severe hepatitis (type B), hepatic encephalopathy, and hepatorenal syndrome. His blood type was O/Rh (+). Pre-operative examination revealed the following parameters: total bilirubin (280 μmol/L), direct bilirubin (158.6 μmol/L), aspartate aminotransferase (269 IU/L), glutamate-pyruvate transaminase (188 IU/L), serum cholesterol (1.24 mmol/L), and serum creatinine (263.7 μmol/L). Blood electrolytes, Child-Turcotte-Pugh score, and MELD score are presented in Table 1. In March 2006, the patient received adult-to-adult LDLT with a right lobe graft. The donor was the patient’s brother, whose blood type was O/Rh (+). Electrolytes were re-tested in the intensive care unit (Table 1).

Post-operative immunosuppressive regimen was administered. The patient received tacrolimus (FK506, 2 mg twice daily, oral), 0.75 g mycophenolic acid (oral, twice daily), and methylprednisolone (iv). Subsequent tacrolimus blood concentration was found to be 6.5-8 ng/mL. At 11 h after surgery, the patient was conscious, and the tracheal cannula was removed. The patient had stable vital body signs, poor mental state, and little spontaneous speech. On day two after surgery, the patient presented with lethargy, eye-opening reaction to powerful stimulation, and indistinct pronunciation. On day three after surgery, the bilateral pupils reacted poorly to light reflex and were equal in size and shape. The patient suffered from moderate coma, with decreased muscular tension, Babinski sign of the right side (+), Babinski sign of the left side (-), and a Glasgow-Pittsburgh score of 20. On day 6 after surgery, the patient received a tracheotomy, and respiration was supported by a respirator due to pulmonary infection. CPM was diagnosed with skull magnetic resonance imaging (Figure 2A). On day ten after surgery, the patient developed moderate coma, with good recovery of liver function. Following hyperbaric chamber treatment (2 h, once per day, for 14 d), the patient presented with good light reflex in both pupils, mild coma, increased autonomic activities, improved tension of limb muscles, and a Glasgow-Pittsburgh score of 26. At six months, the patient suffered from mild coma, but exhibited good liver function.



**Figure 2** Magnetic resonance imaging displayed high-signal intensity on T2-weighted images of the central pons 9 d (A) and 8 d (B) after surgery (arrows).

### Case three

This patient was a 47-year-old male diagnosed with chronic severe hepatitis (type B), upper gastrointestinal bleeding, and hepatorenal syndrome. His blood type was B/Rh (+). Pre-operative examination revealed the following parameters: total bilirubin (197.4 μmol/L), direct bilirubin (116.5 μmol/L), aspartate aminotransferase (141 IU/L), glutamate-pyruvate transaminase (107 IU/L), serum cholesterol (1.07 mmol/L), and serum creatinine (207.7 μmol/L). Blood electrolytes, Child-Turcotte-Pugh score, and MELD score are presented in Table 1. In August 2007, the patient received adult-to-adult LDLT with a right lobe graft. The donor was the patient's wife, whose blood type was B/Rh (+). Electrolytes were re-measured in the intensive care unit (Table 1). Post-operative immunosuppressive regimen was performed. The patient was treated with zenapax (containing daclizumab), 0.75 g mycophenolic acid (oral, twice daily), and methylprednisolone (iv). At 9 h following surgery, the patient was conscious, and the tracheal cannula was removed. The patient received hemofiltration due to worsened renal function. At 1 wk, the patient was conscious, displayed autonomic activities, reduced speech, low speech sound, and could provide correct answers to simple questions. Both pupils exhibited good light reflexes and normal ocular fundus, and were equal in size and shape. Tendon reflexes were symmetric and active, with pathological sign (-). Muscular tension of the upper limbs was increased, with flexion deformity and grade II muscle force, but muscular tension of the lower limbs was decreased. On day 8 after surgery, the patient went into a coma, opened eyes when powerfully stimulated, could not pronounce or perform autonomic limb activities, and presented with normal liver and kidney function. CPM was diagnosed using skull magnetic resonance imaging (Figure 2B). On day 14 after surgery, the patient suffered from mild coma and received a tracheotomy, his respiration was supported by a respirator due to pulmonary infection. Subsequently, the patient developed severe sepsis and multiple organ failure, and died at twenty-three days following surgery.

### DISCUSSION

CPM is a severe complication of the nervous system

following liver transplantation, with high mortality and disability rates<sup>[4-6]</sup>. CPM is difficult to diagnose, due to the various clinical manifestations, and precise epidemiological data are lacking. From January 2005 to December 2007, 3/184 patients at the Center for Liver Transplantation, West China Hospital, Sichuan University, China undergoing living donor liver transplantation were diagnosed with CPM, with an occurrence rate of 1.63%.

CPM following liver transplantation is due to multiple factors. (1) Severe hyponatremia, especially during rapid correction of chronic hyponatremia: patients who develop CPM following liver transplantation suffer from severe hyponatremia prior to transplantation. However, not all hyponatremia patients develop CPM<sup>[7]</sup>. Rapid correction of hyponatremia, as well as larger alterations in plasma osmotic pressure, influences CPM occurrence<sup>[1]</sup>. Increased serum sodium (> 15 mmol/L) within 24 h has also been closely associated with CPM occurrence<sup>[1]</sup>. (2) Neurotoxicity of cyclosporine A and tacrolimus can induce or aggravate CPM. Cyclosporine A-related motor aphasia patients were shown to develop CPM, as diagnosed by skull magnetic resonance imaging<sup>[8,9]</sup>. (3) Severe damage to liver function prior to surgery is associated with CPM occurrence, especially in patients with hepatic encephalopathy and hypcholesterolemia. (4) Hemorrhage, infection, vascular complications, and impaired graft function following liver transplantation can result in CPM occurrence. Immune factors might participate in CPM pathogenesis, such as myelin sheath antibody formation<sup>[10,11]</sup>. In the present study, these three cases developed severe hyponatremia prior to surgery. Serum sodium ion concentration increased by > 20 mmol/L, and plasma osmotic pressure was greatly altered 6-8 h after surgery. These factors were responsible for CPM occurrence. Living donor liver transplantation is an elective procedure. Thus, there should be ample opportunity to correct serum sodium before surgery. A large number of blood products and synthetic colloids should be infused into patients during the peri-operative period, such as erythrocyte suspension, fresh frozen plasma, platelets, cryoprecipitate, hetastarch, and succinylated gelatin. These liquids contain many sodium salts, which increase serum sodium within a short period of time. More attention should be paid to regulating serum sodium during the peri-operative period to avoid adverse outcomes due to rapid correction of hyponatremia. It is also important for anesthesiologists to correct serum sodium very slowly during surgery. In case 1, the post-operative immunosuppressive agent was cyclosporine A. The blood drug concentration ranged between 180-250 mg/L. In case 2, the post-operative immunosuppressive agent was tacrolimus, and the blood drug concentration ranged between 6.5-8 ng/mL. Although blood drug levels were not significantly increased, severe liver disease-induced metabolic disturbances could lead to brain glial cell damage. It seems that critically ill patients with end-stage liver disease may have an organic mental syndrome. Any insult such as calcinerin inhibitors under these circumstances may cause further damage resulting in CPM.

The clinical manifestations of CPM are multi-faceted following liver transplantation, and include

mental disorder, cortical blindness, pseudobulbar palsy, aphasia, ataxia, visual hallucination, epileptic seizure, locked-in syndrome, and coma<sup>[12-14]</sup>. Case 1 was diagnosed with locked-in syndrome, meaning that the bilateral corticospinal tract and corticobulbar tract were blocked surrounding the pontine abducent nucleus. The characteristics of this syndrome include quadriplegia and anarthria, with preservation of consciousness. Patients retain vertical eye movement, and can facilitate non-verbal communication. Few patients with this syndrome have been reported, especially in LDLT. Cases 2 and 3 suffered from coma. Magnetic resonance imaging has proven to be a good diagnostic method for CPM. Positive diagnosis has not been detected in many patients using magnetic resonance imaging following the onset of clinical symptoms. This was associated with abnormal imaging manifestations following clinical symptoms.

To date, there remains a lack of effective therapeutic methods for CPM. The present methods primarily attempt to nourish brain cells, improve cerebral metabolism, and reduce immunosuppressive concentrations. In addition, gamma globulin has been injected intravenously into CPM patients, resulting in improved scores on the Expanded Disability Status Scale<sup>[10,11]</sup>. In the present study, case 1 was treated with a large dose of gamma globulin and was tested according to the Expanded Disability Status Scale. Case 2 received hyperbaric oxygen treatment and underwent Glasgow-Pittsburgh Score testing. Case 1 presented with improved (> 36%) scores on the Expanded Disability Status Scale following treatment. This might be correlated to treatment with large-dose gamma globulin which destroys myelin sheath-related antibodies by decreasing toxic substances in the myelin sheath and by accelerating remyelination. Case 2 presented with improved (30%) scores on the Glasgow-Pittsburgh Score following treatment. This might be correlated to hyperbaric oxygen, which reduced myelin sheath destruction, accelerated cell regeneration, and decreased edema in the injured regions. However, we cannot thoroughly assess the outcomes of these two methods, due to the limited number of cases.

CPM, a life-threatening illness, is difficult to diagnose and treat, and presents with a poor prognosis following living donor liver transplantation. More attention should be directed to the risk factors for CPM to avoid clinical complications, and to continue the search for an effective therapeutic method.

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## Meetings

### Events Calendar 2009

January 12-15, 2009  
Hyatt Regency San Francisco, San Francisco, CA  
Mouse Models of Cancer

January 21-24, 2009  
Westin San Diego Hotel, San Diego, CA  
Advances in Prostate Cancer Research

February 3-6, 2009  
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)  
Second AACR Conference  
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009  
Hyatt Regency Boston, Boston, MA  
Translation of the Cancer Genome

February 8-11, 2009  
Westin New Orleans Canal Place, New Orleans, LA  
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009  
Hong Kong Convention and Exhibition Centre, Hong Kong, China  
19th Conference of the APASL  
<http://www.apasl2009hongkong.org/en/home.aspx>

February 27-28, 2009  
Orlando, Florida  
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009  
Vienna, Austria  
EASL/ AASLD Monothematic: Nuclear Receptors and Liver Disease  
[www.easl.ch/vienna2009](http://www.easl.ch/vienna2009)

March 13-14, 2009  
Phoenix, Arizona  
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009  
Marriott Wardman Park Hotel  
Washington, DC  
13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009  
Glasgow, Scotland  
British Society of Gastroenterology (BSC) Annual Meeting  
Email: bsg@mailbox.ulcc.ac.uk

April 8-9, 2009  
Silver Spring, Maryland  
2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009  
Colorado Convention Center, Denver, CO  
AACR 100th Annual Meeting 2009

April 22-26, 2009  
Copenhagen, Denmark  
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)  
<http://www.easl.ch/>

May 17-20, 2009  
Denver, Colorado, USA  
Digestive Disease Week 2009

May 29-June 2, 2009  
Orange County Convention Center Orlando, Florida  
45th ASCO Annual Meeting  
[www.asco.org/annualmeeting](http://www.asco.org/annualmeeting)

May 30, 2009  
Chicago, Illinois  
Endpoints Workshop: NASH

May 30-June 4, 2009  
McCormick Place, Chicago, IL  
DDW 2009  
<http://www.ddw.org>

June 17-19, 2009  
North Bethesda, MD  
Accelerating Anticancer Agent Development

June 20-26, 2009  
Flims, Switzerland  
Methods in Clinical Cancer Research (Europe)

June 24-27 2009  
Barcelona, Spain  
ESMO Conference: 11th World Congress on Gastrointestinal Cancer  
[www.worldgicancer.com](http://www.worldgicancer.com)

June 25-28, 2009  
Beijing International Convention Center (BICC), Beijing, China  
World Conference on Interventional Oncology  
<http://www.chinamed.com.cn/wcio2009/>

July 5-12, 2009  
Snowmass, CO, United States  
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009  
Aspen, CO, United States  
Molecular Biology in Clinical Oncology

August 1-7, 2009  
Vail Marriott Mountain Resort, Vail, CO, United States  
Methods in Clinical Cancer Research

August 14-16, 2009  
Bell Harbor Conference Center, Seattle, Washington, United States  
Practical Solutions for Successful Management  
<http://www.asge.org/index.aspx?id=5040>

September 23-26, 2009  
Beijing International Convention Center (BICC), Beijing, China  
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists(IASGO)  
<http://iasgo2009.org/en/index.shtml>

September 27-30, 2009  
Taipei, China  
Asian Pacific Digestive Week  
<http://www.apdwcongress.org/2009/index.shtml>

October 7-11, 2009  
Boston Park Plaza Hotel and Towers, Boston, MA, United States  
Frontiers in Basic Cancer Research

October 13-16, 2009  
Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States  
Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009  
Versailles, France  
Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009  
Boston, MA, United States  
The Liver Meeting

November 15-19, 2009  
John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States  
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009  
London, UK  
Gastro 2009 UEGW/World Congress of Gastroenterology  
[www.gastro2009.org](http://www.gastro2009.org)



### Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.



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- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

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- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc, assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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## Current clinical approach to achalasia

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## INTRODUCTION

Idiopathic achalasia is a rare primary motility disorder of the esophagus. The classical features are incomplete relaxation of a frequently hypertensive lower esophageal sphincter (LES) and a lack of peristalsis in the tubular esophagus. These motor abnormalities lead to dysphagia, stasis, regurgitation, weight loss, or secondary respiratory complications. Although major strides have been made in understanding the pathogenesis of this rare disorder, including a probable autoimmune mediated destruction of inhibitory neurons in response to an unknown insult in genetically susceptible individuals, a definite trigger has not been identified. The diagnosis of achalasia is suggested by clinical features and confirmed by further diagnostic tests, such as esophagogastroduodenoscopy (EGD), manometry or barium swallow. These studies are not only used to exclude pseudoachalasia, but also might help to categorize the disease by severity or clinical subtype. Recent advances in diagnostic methods, including high resolution manometry (HRM), might allow prediction of treatment responses. The primary treatments for achieving long-term symptom relief are surgery and endoscopic methods. Although limited high-quality data exist, it appears that laparoscopic Heller myotomy with partial fundoplication is superior to endoscopic methods in achieving long-term relief of symptoms in the majority of patients. However, the current clinical approach to achalasia will depend not only on patients' characteristics and clinical subtypes of the disease, but also on local expertise and patient preferences.

## Abstract

Idiopathic achalasia is a rare primary motility disorder of the esophagus. The classical features are incomplete relaxation of a frequently hypertensive lower esophageal sphincter (LES) and a lack of peristalsis in the tubular esophagus. These motor abnormalities lead to dysphagia, stasis, regurgitation, weight loss, or secondary respiratory complications. Although major strides have been made in understanding the pathogenesis of this rare disorder, including a probable autoimmune mediated destruction of inhibitory neurons in response to an unknown insult in genetically susceptible individuals, a definite trigger has not been identified. The diagnosis of achalasia is suggested by clinical features and confirmed by further diagnostic tests, such as esophagogastroduodenoscopy (EGD), manometry or barium swallow. These studies are not only used to exclude pseudoachalasia, but also might help to categorize the disease by severity or clinical subtype. Recent advances in diagnostic methods, including high resolution manometry (HRM), might allow prediction of treatment responses. The primary treatments for achieving long-term symptom relief are surgery and endoscopic methods. Although limited high-quality data exist, it appears that laparoscopic Heller myotomy with partial fundoplication is superior to endoscopic methods in achieving long-term relief of symptoms in the majority of patients. However, the current clinical approach to achalasia will depend not only on patients' characteristics and clinical subtypes of the disease, but also on local expertise and patient preferences.

## CLINICAL PRESENTATION

The hallmark of achalasia (Greek: failure to relax) is dysphagia for solids and liquids in up to 100% and 97% of patients, respectively<sup>[1-3]</sup>. As a result of stasis and retention of food and liquids in the esophagus, patients frequently experience weight loss (30%-91%), chest pain (17%-95%), regurgitation (59%-64%), and nocturnal cough (11%-46%)<sup>[1]</sup>. Difficulty with belching might result from alteration of the upper esophageal belch reflex<sup>[4]</sup>. Patients might frequently complain of heartburn. Although heartburn is the cardinal symptom

of gastroesophageal reflux, which is the antithesis of achalasia, it occurred with a frequency of 72% in one study, even after the onset of dysphagia<sup>[5]</sup>. The sensation of heartburn in patients with achalasia might be explained by retention of acidic or noxious food contents or by lactate production from bacterial fermentation within the esophagus<sup>[6]</sup>. Hiccups can also occur, and probably result from esophageal distention and stimulation of afferent vagal fibers<sup>[7]</sup>. The distribution of symptoms can differ in the population studied. Chest pain occurs predominantly in younger patients (mean age 40 years) and appears to improve over time<sup>[8]</sup>. However, neither manometric, nor radiographic findings predict the occurrence of retrosternal pain. Most symptoms do not appear to have a specific gender distribution, although in Iranian patients, chest pain appeared to be more common among females<sup>[9]</sup>. Physicians need to be aware of the spectrum of symptoms of achalasia, because diagnostic delays for years after the onset of symptoms appear to be due to misinterpretation of typical findings, rather than atypical presentations<sup>[10]</sup>.

The most common extraesophageal manifestations of achalasia are pulmonary complications. Structural or functional pulmonary abnormalities occur in more than half of patients and might be due to recurrent aspiration or tracheal compression from a dilated esophagus<sup>[11]</sup>. In cases of extreme dilation and distortion of the cervical esophagus, a “bull frog neck” appearance can develop, leading to tracheal obstruction above the larynx and associated stridor<sup>[2]</sup>. Although some investigators have observed delayed gastric emptying or gallbladder dysfunction in patients with achalasia<sup>[12-14]</sup>, others were unable to confirm these observations<sup>[15]</sup>, and it still remains elusive whether a selective defect of vagal ganglionic neurons might affect other parts of the gastrointestinal tract as well.

## DIAGNOSIS AND CLINICAL VARIANTS

A number of tests are available to confirm the diagnosis of achalasia, once the clinical suspicion arises. Radiographic studies, EGD and esophageal manometry are the primary tools of investigation. Although EGD appears normal in 44% of patients with achalasia<sup>[11]</sup>, it might show esophageal dilatation and retention of food or secretions. During inversion of the endoscope in the stomach, tight adherence of the distal esophagus with downward motion of the gastroesophageal junction upon endoscope advancement can sometimes be visualized. However, despite the commonly elevated LES pressure, the esophagogastric junction can usually be traversed easily, and firm resistance should raise the suspicion of neoplastic infiltration or other causes of pseudoachalasia. Pseudoachalasia can mimic all endoscopic, radiographic and manometric findings of achalasia and has a broad differential diagnosis of neoplastic and non-neoplastic causes, which have been recently reviewed<sup>[16,17]</sup>. Infiltration of the esophageal myenteric plexus by neoplastic cells or paraneoplastic processes have been suggested in patients with a

**Table 1 Manometric variants of achalasia**

Standard Manometry
Vigorous achalasia (high amplitude esophageal body contractions)
A short segment of esophageal body aperistalsis
Retained complete deglutitive LES relaxation with aperistalsis
Intact transient LES relaxation with aperistalsis
High resolution manometry (patients with impaired EGJ relaxation)
Type I : Minimal esophageal pressurization
Type II : Esophageal pressurization > 30 mmHg
Type III : Esophageal spasm

LES: Lower esophageal sphincter; EGJ: Esophagogastroduodenoscopy.

malignant etiology<sup>[16]</sup>. A shorter duration of symptoms and older age at presentation should raise a suspicion of pseudoachalasia, which often requires further testing with endoscopic ultrasound or CT scan to rule out malignancy<sup>[17]</sup>. In our view, a simple and non-invasive initial test to differentiate between primary and secondary achalasia is transabdominal ultrasonography, which often allows a clear visualization of the gastric cardia and its surrounding structures<sup>[18]</sup>. However, a negative ultrasound does not always exclude pseudoachalasia, and CT or other cross-sectional imaging should be added if clinical suspicion remains strong.

A barium esophagogram (barium swallow) is the most commonly used initial diagnostic study. It classically shows a typical smooth tapering of the distal esophagus (“bird’s beak”) with proximal dilation of the esophagus and lack of peristalsis during fluoroscopy. The value of obtaining a timed barium esophagogram in patients with achalasia lies in its potential to monitor the success of therapeutic interventions and to detect disease recurrence prior to the development of symptoms<sup>[19]</sup>. The timed barium swallow is performed by having the patient drink 100-250 mL of barium in an upright position and by taking radiographs one, two and five minutes after the last swallow. The distance from the distal oesophagus to the top of the barium column, as well as the maximal esophageal width, are measured for comparison before and after treatment.

Manometry remains the diagnostic modality with the highest sensitivity and should be part of the diagnostic evaluation in all patients with achalasia. Three cardinal features support the diagnosis of classic achalasia: Aperistalsis of the smooth muscle portion of the esophagus, incomplete LES relaxation and elevated LES resting pressure. As mentioned above, manometric variants of achalasia have been described (Table 1). Vigorous achalasia is a variant characterized by aperistaltic, simultaneous esophageal contractions with higher average amplitudes (> 37 mmHg)<sup>[20]</sup>. It has been suggested that vigorous achalasia might present an earlier form of achalasia, in which esophageal contractions against the outflow obstruction at the LES are still maintained. However, vigorous achalasia appears to be independent of age of onset and symptom duration, and is not associated with return to normal peristalsis after surgical myotomy<sup>[21,22]</sup>. Although it has been suggested that patients with vigorous achalasia might show better success with botulinum toxin injection than patients with classic

achalasia<sup>[23]</sup>, this has minor clinical relevance, because other treatment options are superior in most cases. Therefore, it remains unclear whether dividing patients into those with “vigorous achalasia” and “classic achalasia” has any clinical implications. Despite such reservations, physicians need to be aware that achalasia might present with a spectrum of manometric findings that might not meet all of the criteria specified above (Table 1)<sup>[24]</sup>. Their significance lies in the recognition that these sometimes confusing manometric findings are consistent with achalasia when combined with additional clinical data supportive of the diagnosis. As mentioned for the timed barium swallow, manometry also plays a role in monitoring treatment response and predicting treatment success of pneumatic dilatation, as discussed below.

The recent introduction of HRM with pressure topography plotting into the diagnostic armory has brought a renaissance to the classification of idiopathic achalasia into variants with possible clinical implications. A retrospective study by Pandolfino *et al*<sup>[25]</sup> described three distinct variants, with type I exhibiting minimal esophageal contractility without pressurization, type II with absent peristalsis but compartmentalized, pan-esophageal pressurization, and type III with lumen obliterating spasm. The authors showed that pan-esophageal pressurization (type II) had the best overall treatment response, whereas type III predicted a poor treatment response to all types of therapy. Further prospective studies are needed to confirm these interesting early results.

## TREATMENT

Treatment of idiopathic achalasia remains strictly palliative. In view of the suspected autoimmune mechanism of the disease, it appears surprising that no study has systematically addressed the use of immunosuppressive therapy in an attempt to prevent disease progression<sup>[26]</sup>. Therefore, current treatment modalities are primarily directed at relieving distal esophageal obstruction and consist of pharmacologic therapy, endoscopic treatment with pneumatic dilation or botulinum toxin injection, and surgery. The appropriate choice of therapeutic options depends on multiple factors, such as the patient’s characteristics, clinical presentation, local expertise and patient preferences, but should be based on the best available evidence.

Pharmacological therapy is directed at achieving a reduction of LES pressure by the use of smooth muscle relaxants, such as calcium channel blockers (e.g. nifedipine 10-30 mg sublingually 30-45 min before meals), nitrates (e.g. isosorbide dinitrate 5 mg sublingually 10-15 min before a meal) or phosphodiesterase 5 inhibitors<sup>[27,28]</sup>. The main limitations of these agents are their short duration of action, limited improvement of dysphagia despite documented LES relaxation, or the frequent occurrence of side effects, such as peripheral edema, headaches or hypotension, which especially occur with calcium channel blockers and nitrates. Their use is, therefore, limited to symptomatic relief in patients who have very early disease, or as a temporary measure

for patients who are awaiting a more definite treatment option, or are high risk for more invasive options<sup>[29]</sup>.

Endoscopic options of treatment include disruption of the LES by pneumatic dilation or botulinum toxin injection. Botulinum toxin is a potent neurotoxin that leads to a blockade of the release of acetylcholine from excitatory motor neurons. In a landmark study, Pasricha *et al*<sup>[30]</sup> showed that endoscopic injection of botulinum toxin into the area of the LES lead to symptomatic improvement in patients with achalasia, which was accompanied by reduced esophageal retention over a period of 6 mo. One randomized controlled trial (RCT) has shown that the two commercially available formulations of botulinum toxin are equally effective, but need to be given in different dosages because of variable potency<sup>[31]</sup>. The treatment effect of one of these formulations (Botox® Allergan Inc, Irvine, California, USA) might be maximized when a repeated injection of 100 IU is given one month after the first injection<sup>[32]</sup>. In contrast, a lack of an initial symptomatic response and residual LES pressure  $\geq 18$  mmHg after botulinum toxin are associated with a poor overall response<sup>[33]</sup>. The best results of botulinum toxin have been achieved in patients with vigorous achalasia, older patients and patients whose LES pressures do not exceed  $\geq 50\%$  of the upper limit of normal<sup>[23,34]</sup>. However, the use of botulinum toxin is limited by its lack of long-term efficacy with recurrence rates of approximately 50% after one year and universal symptomatic relapse at two years<sup>[35,36]</sup>. Two recent meta-analyses concluded that although botulinum toxin has an excellent safety profile, it seems slightly less effective than pneumatic dilatation in the short-term and is clearly inferior in the long-term for the treatment of achalasia<sup>[37,38]</sup>.

Pneumatic dilatation has been used for the treatment of patients with achalasia for more than half a century and is currently considered the most effective non-surgical treatment for achalasia<sup>[39,40]</sup>. A number of different pneumatic dilators with variable balloon compliance have been used in clinical trials. Currently, the low-compliance polyethylene pneumatic dilator (Rigiflex®, Boston Scientific, Boston, MA, USA) appears to be the most widely used. Although pneumatic dilators from other manufacturers are available (e.g. Cook Medical, Bloomington, IN; USA or Hobbs Medical, Stafford Springs CT, USA; HCDD, latex balloon, Rüsch Inc, Germany), only limited comparative data exist, which have not shown a difference in efficacy or safety<sup>[41,42]</sup>. Using a graded approach with the polyethylene balloon dilator, with increasing diameters from 3.0 to 4.0 cm, a 93% response rate was achieved over a mean follow up period of four years with a relatively low complication risk<sup>[43]</sup>. The most feared complication of pneumatic dilation is perforation, which occurred in 1.6% of patients in a meta-analysis with 1065 patients in experienced hands<sup>[39,40]</sup>. Studies assessing the long-term efficacy of pneumatic dilation have shown that a permanent treatment success can only be achieved in 40%-60% of patients after a follow up of  $\geq 15$  years<sup>[44-46]</sup>. Although one study showed that “on demand” repeat dilations may again lead to remission in the majority of patients<sup>[47]</sup>, others have shown that longer lasting treatment effects cannot be expected

from such therapy<sup>[48]</sup>.

Predictors of treatment failure with balloon dilation appear to be younger age (< 40 years), male gender, pulmonary symptoms and failed response to one or two initial dilations<sup>[49-52]</sup>. In contrast older age appears to be associated with favorable outcomes of pneumatic dilation. Manometric findings that predict poor outcome are high initial LES pressures (e.g. > 15-30 mmHg) or a reduction of LES pressure < 50% after the first dilation<sup>[50,52,53]</sup>. Manometry should, therefore, be routinely performed pre- and post-interventionally.

With the advent of minimally invasive laparoscopic approaches, surgery has evolved from an ancillary procedure, used when pneumatic dilation failed, to the favored primary approach by many surgeons and gastroenterologists in a majority of patients with achalasia<sup>[54]</sup>. The goal of surgery is to alleviate the esophageal obstruction by myotomy of the LES. To prevent secondary gastroesophageal reflux, the procedure has usually been combined with some type of fundoplication procedure. The superiority of surgical myotomy over pneumatic dilation was suggested by three recent meta-analyses in the English and Chinese literature that mostly considered retrospective cohort studies<sup>[38,39,55]</sup>. Although both pneumatic dilation and surgical myotomy have a substantial risk of subsequent need of interventions (repeated pneumatic dilation, surgical myotomy or esophagectomy) over a period of 10 years, the probability was significantly smaller in the latter group (56% vs 26%, respectively) in one study<sup>[56]</sup>. To date, only one RCT with data on long-term follow-up has been published by Csendes *et al*<sup>[57]</sup> comparing myotomy followed by 180° Dor fundoplication to pneumatic dilation with a Mosher bag. Although this study has been criticized because of potentially technique-related suboptimal results in the pneumatic dilation group, it still remains the best available evidence to date. The authors showed good response after a five year follow up period for 95% of surgically treated patients vs 65% of patients in the pneumatic dilation group. However, very late results in the surgical group showed that clinical deterioration occurs, reducing the surgical success rate to 75% after a mean follow up of 15.8 years<sup>[58]</sup>. Of the patients with poor surgical results, 92% resulted from complications of severe reflux disease and not from incomplete myotomy. A number of trials have, therefore, investigated the benefit of anti-reflux procedures in addition to myotomy. In a prospective RCT, reflux symptoms were reduced from 47.6% with laparoscopic Heller myotomy alone to 9.1% when a Dor fundoplication was added<sup>[59]</sup>. In another RCT, laparoscopic myotomy with Dor fundoplication was equally effective as a myotomy with “floppy” Nissen fundoplication in controlling reflux, but dysphagia rates were significantly higher in the latter group (2.8% vs 15%, respectively;  $P < 0.001$ )<sup>[60]</sup>. With success rates of 47%-82% at 10 years, laparoscopic Heller myotomy with partial fundoplication appears to have evolved into the surgical procedure of choice<sup>[39,61,62]</sup>. A recent single center RCT compared laparoscopic cardiomyotomy with partial Toupet fundoplication to pneumatic dilation in patients with newly diagnosed achalasia. Similar to

the Csendes study, it also showed significantly fewer treatment failures in the surgical arm after a period of 12 mo<sup>[63]</sup>. Another head to head multicenter RCT has been ongoing for a number of years, but publication is still pending<sup>[64]</sup>.

Predictors of a negative outcome with surgical myotomy were severe preoperative dysphagia, lower preoperative LES pressures of < 30-35 mmHg, progressive esophageal body dilation with flask type or sigmoid esophagus, and preoperative endoscopic treatment in some studies<sup>[61,65-68]</sup>. However, other studies showed treatment responses even in (selected) patients with dilated esophageal bodies or sigmoid esophagus, and in patients who previously failed pneumatic dilation<sup>[48,69,70]</sup>. The effect of surgical myotomy on chest pain remains controversial, and patients should be aware that this symptom might not reliably improve after either pneumatic dilation or surgery<sup>[8]</sup>. Occasionally, a temporary placement of self-expanding metal stents (SEMS) has been suggested as a possible means of dilation or as a bridge to surgery<sup>[71,72]</sup>. However, because no information with regard to its long-term effectiveness exist and complications might be frequent and potentially severe<sup>[73]</sup>, stent treatment for achalasia cannot be recommended at the present time.

Finally, it should not be forgotten that for patients not responding to any one of the above mentioned therapies, subtotal esophageal resection with gastric pull-up remains as a viable treatment option. Although such therapy is extremely invasive and associated with a high post-operative morbidity, favorable long-term results with significant improvement of symptoms can be achieved, even if endoscopic therapy or surgical myotomy have persistently remained unsuccessful<sup>[74]</sup>.

## COMPLICATIONS AND PROGNOSIS

Complications in patients with achalasia might occur from the natural course of the disease (e.g. aspiration, squamous cell carcinoma, and megaesophagus), from iatrogenic interventions (e.g. perforation after balloon dilation, or postoperative complications after myotomy), or from the late consequences of a successful intervention (e.g. reflux related complications, such as strictures or adenocarcinoma).

As a result of the natural course of the disease, structural parenchymal pulmonary disease occurs in 33% of patients with achalasia, probably from chronic microaspiration. Furthermore, delayed diagnosis, or ineffective intervention, might lead to progressive dilation of the esophagus and the development of a megaesophagus. This complication occurs in 10% of patients at a median of 18-21 years after the onset of symptoms and might require esophagectomy in the most severe cases<sup>[75,76]</sup>.

In addition, treatment modalities carry their own inherent risks. As previously mentioned, the main risk of pneumatic dilation is perforation, which occurs at a mean of 1.6% (range from 0%-8%), even in experienced hands<sup>[39]</sup>. The risk of perforation appears to be highest during initial dilation, as opposed to subsequent

**Table 2** Predictors of treatment response in achalasia

Treatment option	Positive predictors	Negative predictors
Botulinum toxin injection	Vigorous achalasia Older patients	Initial LES pressure $\geq 50\%$ of the upper limit of normal Lack of clinical response or residual LES pressure $\geq 18$ mmHg after initial botulinum toxin treatment
Pneumatic dilatation	Older patients	Male Gender Pulmonary symptoms Failed response to 1-2 initial dilations High initial LES pressure ( $> 15-30$ mmHg) <sup>1</sup> Reduction of LES pressure $< 50\%$ after the first dilation
Myotomy	Younger patients (< 40 yr)	Severe preoperative dysphagia Lower preoperative LES pressures of $< 30-35$ mmHg <sup>1</sup> Esophageal body dilation (flask type or sigmoid esophagus) Preoperative endoscopic treatment (in some studies)

<sup>1</sup>Pressure values show considerable inter-study variability, depending on techniques used by different authors. Therefore, the pressure values shown in the table only reflect estimates based on the available literature.

dilations<sup>[77]</sup>. Although the perforation risk of laparoscopic myotomy is smaller with 0.7% (range 0%-8%), the overall rate of postoperative complications is 6.3%, with a periprocedural mortality of 0.1%<sup>[39]</sup>. As a result of endoscopic or surgical treatment, reflux esophagitis occurred in approximately 10% of patients in our own prospective cohort, even though 43% of patients received acid suppressing medications<sup>[76]</sup>. Reflux esophagitis was more commonly observed after surgical myotomy with Dor fundoplication (14%) than after pneumatic dilation (5%), possibly indicating more effective disruption of the LES. Late reflux complications, such as esophageal stricture occurred in half of these patients.

The most feared complication of achalasia is esophageal cancer. A recent review of the available literature reported a mean prevalence of esophageal cancer of 3% in patients with achalasia, indicating a fifty-fold increased risk over the general population<sup>[78]</sup>. Squamous cell carcinoma appears to occur most commonly, and probably results from stasis, causing bacterial overgrowth and production of nitrosamines, which in turn lead to chronic inflammation, dysplasia and cancer<sup>[79]</sup>. In addition, adenocarcinoma may result from long-standing reflux after successful treatment<sup>[80,81]</sup>. Although insufficient data are available to make evidence-based surveillance recommendations, many experts support a strategy of surveillance for cancer or reflux complications. Accordingly, the latest ASGE guideline suggests that it would be reasonable to consider such a strategy after 15 years of symptoms<sup>[82]</sup>. Annual follow up surveillance intervals have been suggested at least by one author<sup>[77]</sup>. Patients should be kept on a liquid diet three to four days before the surveillance endoscopy and an esophageal lavage should be considered immediately before the procedure to optimize visualization. Despite the described cancer risk and frequent long-term complications, patients with achalasia do not appear to experience a significant compromise of their overall life expectancy<sup>[76]</sup>.

## CONCLUSIONS AND FUTURE PERSPECTIVES

Achalasia is an idiopathic disorder, likely caused by

autoimmune mediated destruction of inhibitory neurons in response to an unknown, possibly viral, insult in genetically susceptible individuals. Physicians should be aware of typical and atypical presentations of achalasia to avoid diagnostic delays. Standard diagnostic work-up should include an EGD, timed barium swallow and manometry. Additional testing may become necessary if pseudoachalasia is suspected. The appropriate choice of therapy depends on multiple factors, including local expertise, patient preferences, and known predictors of treatment failures (Table 2). Based on the current evidence, we prefer laparoscopic myotomy in combination with partial fundoplication in young patients (< 40 years) with low surgical risk as the primary treatment option. In older patients, or those who want to avoid surgery, pneumatic dilation produces good long-term results, unless the first one to two dilations are unsuccessful, or LES pressure is not adequately decreased. Botulinum toxin might be especially useful in very old patients, or those with major comorbidities, because of its excellent safety profile. Subsequent treatments should be based on symptom recurrence. Pharmacological therapy should be reserved for patients awaiting a more definite treatment option. For patients not responding to any one of the above mentioned therapies, or patients with megaesophagus, esophageal resection remains a viable option.

In the future, well designed prospective studies are needed to identify optimal treatment options for different subgroups of patients with idiopathic achalasia. The advent of new exciting diagnostic methods, such as HRM, may aid in predicting treatment responses and warrants further investigation. Finally, with growing insight into the pathophysiology of this disease, novel treatment options that aim at preventing the late stages of the disease might evolve.

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EDITORIAL

## Effect of perioperative blood transfusion on clinical outcomes in hepatic surgery for cancer

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## INTRODUCTION

Improvements in surgical techniques, in pre- and post-operative care, and increased experience have improved the safety of liver resections for hepatocellular carcinoma (HCC), and these procedures frequently can be carried out without blood transfusions<sup>[1-5]</sup>. By contrast, riskier hepatectomies, including posterior resections with reconstruction of the vena cava or resection of the caudate lobe, represent complex procedures which could require perioperative blood transfusion. Transfusion of allogenic blood has been reported to be associated with potentially devastating complications such as transmission of human immunodeficiency virus and hepatitis, transfusion reactions, increased postoperative infection rate, and increased incidence of recurrences for certain cancers<sup>[6]</sup>. Moreover, pulmonary oedemas occurring during or after a blood transfusion appear as the most frequent serious immediate incidents: they include transfusion-associated circulatory overload and transfusion-related acute lung injury (TRALI)<sup>[6]</sup>. Transfusion of allogenic whole blood products has been shown to induce variations in certain immune functions<sup>[7,8]</sup>, such as reduced NK cell activity, T lymphocyte blastogenesis, and increased suppressor T lymphocyte activity, which may be of great relevance for host resistance to infection and the spread of neoplastic cells. But, the adverse effects of allogeneic whole blood transfusion on cancer recurrence and survival rates<sup>[9-12]</sup>, regardless of innumerable published studies, continue to be debatable, since as many studies can be found that invalidate<sup>[13-18]</sup> as those that substantiate<sup>[19-27]</sup> this hypothesis.

Recent advances in surgical techniques to control blood loss and transfusion need<sup>[28-32]</sup>, and the growing vast experience with hepatic resections, have been

## Abstract

Allogeneic blood transfusion during liver resection for malignancies has been associated with an increased incidence of different types of complications: infectious complications, tumor recurrence, decreased survival. Even if there is clear evidence of transfusion-induced immunosuppression, it is difficult to demonstrate that transfusion is the only determinant factor that decisively affects the outcome. In any case there are several motivations to reduce the practice of blood transfusion. The advantages and drawbacks of different transfusion alternatives are reviewed here, emphasizing that surgeons and anesthetists who practice in centers with a high volume of liver resections, should be familiar with all the possible alternatives.

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**Key words:** Blood transfusion; Blood products; Allogeneic blood transfusion; Intraoperative autotransfusion; Preoperative autologous blood donation; Intraoperative isovolemic hemodilution; Infectious complications; Liver resection; Hepatocellular carcinoma

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responsible for a remarkable reduction in the use of blood and blood products during surgery. Despite these efforts, allogeneic blood transfusion rates during hepatic resections have been reported at 40% to 80% depending upon the magnitude of the resection<sup>[3]</sup>. Furthermore, even though the introduction of the hepatic inflow occlusion technique introduced by Pringle<sup>[33]</sup> and selective and/or intermittent inflow occlusion have been very effective at reducing blood loss during hepatic resection, back bleeding from the hepatic veins and their tributaries during the Pringle manoeuvre can still be unpredictable, severe, and unexpected<sup>[34]</sup>.

This paper outlines the current perspectives on blood transfusion in hepatic resection, focusing on allogeneic blood transfusion, intraoperative autotransfusion, preoperative autologous blood donation, and intraoperative isovolemic hemodilution.

## ALLOGENEIC BLOOD TRANSFUSION

New measures to reduce transfusion errors have recently been defined by Regan *et al*<sup>[35]</sup>. The incidence of allogeneic blood transfusion is high in patients with cirrhotic livers undergoing liver resections for HCC, and for that reason it is vital to determine whether these transfusions stimulate tumor recurrence. The postoperative recurrence of HCC associated with perioperative blood transfusion has been supported<sup>[36]</sup> and disputed<sup>[37]</sup>. Furthermore the relationship between perioperative allogeneic blood transfusions, recurrence free survival, and the immunologic profiles of patients with HCC who have undergone curative liver resections has been investigated<sup>[38]</sup>. These studies have shown that in transfused patients, the CD4 levels are decreased by 90 postoperative days, whereas the CD8 levels are elevated during 14-90 d after surgery, as compared with nontransfused patients. Postoperative levels of the CD57+NK-cell subset and PHA responses in the transfused group are elevated as compared with the nontransfused group, and the PHA response of the transfused patients is significantly increased at seven postoperative days. Recurrence free survival seems not to be affected by perioperative blood transfusions.

All these studies suggest the significance of perioperative blood transfusion as an independent prognostic variable in terms of recurrence, survival, complications, and death. Patients who need preoperative, intraoperative, or postoperative transfusions are generally those with large lesions that either require a tri-segmentectomy, or are too close to the vena cava. On the other side, patients who do not need blood transfusions tend to have smaller, more peripheral lesions that can be resected under close hemostatic control. This suggests that patients with large HCC (with poor prognosis) are more likely to receive blood, and possible other factors should be taken into consideration for a more accurate evaluation. In regard to survival, for instance, the margin of resection, evidence of metastatic disease, liver failure or other perioperative complications should always be reviewed.

## INTRAOPERATIVE AUTOTRANSFUSION

Intraoperative autotransfusion [also known as autologous blood salvage or intraoperative blood salvage (IBS)] is a medical procedure involving recovering blood lost during surgery and re-infusing it into the patient. Different medical devices have been developed to assist in salvaging the patient's own blood in the perioperative setting. IBS is widely used in a variety of surgical procedures, including cardiovascular, orthopedic, and gynecologic procedures, and emergency medical situations<sup>[39-41]</sup>, but IBS in oncologic patients has not been widely studied. IBS has been cited as a contraindication<sup>[42]</sup> because of the potential risk of disseminating metastasis. This concept was introduced firstly by Yaw *et al*<sup>[43]</sup> who demonstrated tumor cells in processed blood that passed through filters in the Bentley autotransfusion device. Other studies support that IBS can be safely used in patients with cancer<sup>[44-46]</sup>. Because the Haemonetics cell saver processes blood by centrifuge-based washing after filtration, the risk of reinfusion of malignant cells seems to be lower than by the Bentley system. Clinical evidence of dissemination of cancer cells caused by IBS has not been reported, and several studies show no correlation between the presence of malignant cells and their subsequent dissemination<sup>[47,48]</sup>. The haemonetics cell saver was employed by Fujimoto *et al*<sup>[49]</sup> as an intraoperative scavenger of blood in patients undergoing hepatectomy for HCC. In this study autotransfusion was shown to be safe and effective, and the pattern and frequency of recurrence suggest that autotransfusion is not responsible for recurrence or metastasis. Hashimoto *et al*<sup>[50]</sup> showed that IBS in living liver donors undergoing liver resection for graft procurement offered the advantage of reduced blood loss during parenchymal transection.

At the present time, the processes used to assist in salvaging the patient's own whole blood in the perioperative setting can be categorized into three general types: (1) Cell processors and salvage devices that wash and save red blood cells (RBCs), i.e. "cell washers" or RBC-savers; (2) Direct transfusion; (3) Ultrafiltration of whole blood. Cell processors are red cell washing devices that collect anticoagulated shed or recovered blood, wash and separate the RBCs by centrifugation, and reinfuse the RBCs. RBC washing devices can help remove byproducts in salvaged blood such as activated cytokines, anaphylatoxins, and other waste substances that may have been collected in the reservoir suctioned from the surgical field. However, they also remove viable platelets, clotting factors, and other plasma proteins essential for homeostasis. Direct transfusion is a blood salvaging method associated with cardiopulmonary bypass circuits or other extracorporeal circuits that are used in surgery such as coronary artery bypass grafts, valve replacement, or surgical repair of the great vessels. Hemofiltration or ultrafiltration devices constitute the third major type of blood salvage appearing in operating rooms. In general, ultrafiltration devices filter the patient's anticoagulated whole blood. The filtration process

removes unwanted, excess non-cellular plasma water, low molecular weight solutes, platelet inhibitors and some particulate matter through hemoconcentration, including activated cytokines, anaphylatoxins, and other waste substances making concentrated whole blood available for reinfusion. Hemofiltration devices return the patient's whole blood with all the blood elements and fractions including platelets, clotting factors, and plasma proteins with a substantial Hb level. Presently, the only whole blood ultrafiltration device in clinical use is the *Hemobag*.

Concerns about possible contamination of autologous RBC with cancer cells responsible for metastasis still continues to limit the use of IBS in cancer patients. This is despite the fact that no evidence has been reported showing an increase in metastasis or a decrease in patient survival, regardless of the obvious demonstration that salvaged blood is contaminated with viable tumor cells which are not washed out of the RBC layer during IBS. Total elimination of the risk of reinfusion of cancer cells by irradiation has been proposed by Hansen<sup>[51]</sup>, who has been able to show that IBS with blood irradiation is safe as it provides efficient elimination of contaminating cancer cells, does not compromise the quality of RBC, and is very effective in saving blood resources. The effectiveness of this procedure has been shown on a large number of oncologic patients<sup>[52]</sup>.

## PREOPERATIVE AUTOLOGOUS BLOOD DONATION

Evidence that allogeneic transfusion may lead to a potential risk of postoperative infections, and the increased demand for blood with a declining population of qualified, willing, and healthy donors, give reason for the current support for preoperative autologous transfusion (PAD)<sup>[53,54]</sup>. The overall benefits of PAD have been assessed in both randomized trials and cohort studies<sup>[55]</sup>. Assuming that the donor is not bacteremic at the time of donation and/or there are no clerical errors resulting in the accidental transfusion of the wrong unit of blood, the patient is also protected against hemolytic, febrile or allergic transfusion reactions; alloimmunization to erythrocyte, leukocyte, platelet or protein antigens; and graft-versus-host disease (GVHD). An additional benefit is that erythropoiesis may be stimulated by repeated phlebotomies, thereby enabling the patient to regenerate hemoglobin at an accelerated rate after surgery.

PAD programs are not without some disadvantages. Perhaps the most important is that autologous blood is considerably more expensive than allogeneic blood. This problem is compounded by the fact that current reimbursement programs of most of the National Health systems around the world either deny the medical necessity of PAD or ignore the well-documented increase in cost<sup>[56]</sup>. Moreover, the blood that is not transfused to the intended recipient (approximately 50% of donated blood) is generally wasted rather than being transfused to other patients<sup>[57]</sup>. This wastage of blood

and the costs of administering autologous programmes result in collection expenses that are higher than those for allogeneic transfusion.

Patients undergoing PAD may donate a unit (450 ± 45 mL) of blood as often as twice weekly, until 72 h before surgery. Under normal conditions, patients conventionally donate once weekly. Oral iron supplements are routinely prescribed. This iatrogenic blood loss is accompanied by a response in endogenous erythropoietin (EPO) levels that, although increased significantly over basal levels, remain within the normal range. The erythropoietic response that occurs under these conditions is therefore modest<sup>[58]</sup>. With routine PAD, erythropoiesis of 220-351 mL (11%-19% RBC expansion)<sup>[59,60]</sup> or the equivalent of 1-1.75 blood units, occurs in excess of basal erythropoiesis, which indicates the efficacy of this blood conservation practice.

The use of autologous blood deposits for cancer patients undergoing elective surgical procedures has been studied by Lichtiger<sup>[61]</sup>, who was able to show that the majority (132/182) of his patients (with head and neck, neurosurgical, gastrointestinal and colorectal, adrenal, gynecologic, soft tissue and bone, breast, and genitourinary tumors) underwent surgery using only autologous transfusions. Kajikawa *et al*<sup>[62]</sup> evaluated the benefit of autologous blood transfusion and the effect of recombinant human erythropoietin (rh-EPO) on preoperative autologous blood donation for hepatectomy in patients with cirrhosis. Their study shows that autologous blood transfusion yields clinically superior results for hepatectomy in patients with cirrhosis when compared with homologous transfusion. In addition preoperative rh-EPO administration minimizes presurgical decreases in hematocrit (HCT) caused by autologous blood donation<sup>[62]</sup>. Likewise preoperative autologous blood donation in combination with rh-EPO therapy markedly reduces the requirements for homologous blood transfusion during hepatic resections<sup>[63]</sup>.

Other studies on patients undergoing hepatic resection have shown that the predeposition of autologous blood decreased the need for homologous transfusions from 56% to 38%. A further reduction in the transfusion rate of 25% could have been possible if all patients had donated 2 U of autologous blood<sup>[64]</sup>.

To determine if predonation of autologous blood impacts upon transfusion practice and clinical outcome following liver resection, clinical records of 379 consecutive patients undergoing hepatic resection for metastases of colorectal cancer were identified from the prospective hepatobiliary database and reviewed by Chan *et al*<sup>[65]</sup>. No conclusion could be drawn from their data concerning the influence of allogeneic transfusion on tumor recurrence, since their study was not a randomized trial comparing allogeneic blood transfusion with autologous transfusion. Data from their study however demonstrated that PAD alone is insufficient to alter the rate of tumor recurrence or disease-specific survival. Furthermore major hepatic resections using current surgical techniques can be performed safely with low blood loss so that transfusion is required for only a minority

of patients. PAD may further reduce the need for allogeneic blood. Autologous blood transfusion is safe after storage and it has advantages if compared with homologous blood transfusion with regard to postoperative liver function and survival rate after hepatectomy for HCC<sup>[66]</sup>.

In a recent study, Hirano *et al*<sup>[67]</sup> have shown that their autologous blood program, with IBS and preoperative blood donation, reduces the volume of banked blood needed and improves the prognosis of patients undergoing hepatectomy for HCC.

## INTRAOPERATIVE ISOVOLEMIC HEMODILUTION

Acute isovolemic hemodilution (ANH) is another possible alternative to allogeneic blood transfusions, which was introduced in the early 1970s<sup>[68]</sup>. The procedure implies the removal of blood from the patient immediately before operation and the simultaneous replacement with appropriate volume of crystalloid or colloid fluids. ANH will reduce the HCT so that blood shed during the operative procedure will result in less RBC mass loss. The amount of blood removed varies between one and three units (450-500 mL constitutes 1 U), although larger volumes may be withdrawn safely in certain circumstances. The removed blood is then reinfused as autologous whole blood after the major blood loss portion of the procedure is completed. The blood withdrawn is anticoagulated and maintained at room temperature, in the operating room, for up to 8 h. It is reinfused into the patient as needed during, or after, the surgical procedure. ANH can be used as the only blood preservation technique, or it can be combined with preoperative autologous donation, blood salvage, or both.

Hemodilution could be classified according to the target HCT as mild ( $HCT \geq 30\%$ ), moderate ( $30\% < HCT \geq 20\%$ ), or severe ( $HCT < 20\%$ )<sup>[16]</sup>. The target HCT with ANH is variable but is often around 25%-30%. Severe hemodilution (e.g. 20%) is likely to be more efficacious with regards to blood conservation, but the risks are greater, particularly for patients with preexisting medical conditions such as coronary heart disease<sup>[69]</sup>.

ANH should be taken into consideration for patients with good initial HCTs who are assumed to be deprived of more than two units of blood (900-1000 mL) during surgery. This technique works better in healthy, young adults, but it has been successfully employed in children and elderly patients. ANH has been used in vascular, orthopedic, and in some general surgical procedures. In addition, Jehovah's Witnesses patients accept this technique with the modification that we keep the blood moving and in direct contact with the patient's vascular system. Some Jehovah's Witnesses will agree to ANH if the blood is maintained in a closed circuit continuous flow system<sup>[70]</sup>.

ANH is contraindicated in cardiac disease, since the main compensatory mechanism for the induced anemia is an increase in the cardiac output, when renal function

is impaired, since large amounts of infused fluids need to be excreted, and when baseline hemoglobin is below 110 mg/L (11 g/dL). Furthermore low concentrations of coagulation proteins, inadequate vascular access, and the absence of appropriate monitoring capability indicate that ANH should not be used<sup>[71]</sup>.

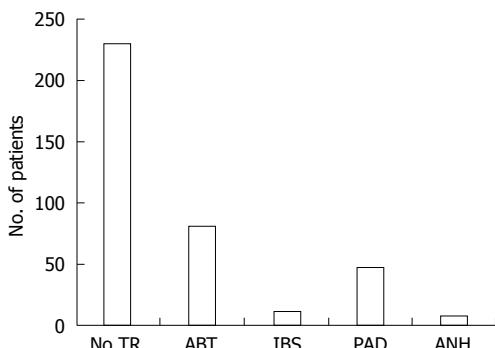
In the last 20 years several groups reported the use of ANH during major hepatic resections<sup>[72-76]</sup>, and the overall conclusion is that ANH, in selected patients, is a safe and effective technique that appears to reduce the number of patients requiring homologous blood transfusion as well as the number of units transfused per patient. Furthermore, Jehovah's Witnesses with hepatic tumors represent a major problem for liver surgeons to achieve good outcome, in fact these patients, because of their religious beliefs, refuse transfusion of blood and blood products. In order to avoid transfusion Barakat *et al*<sup>[75]</sup> have recently described the use of ANH in a Jehovah's Witness who underwent a combined left trisegmentectomy and caudate lobectomy to treat a large intrahepatic cholangiocarcinoma.

ANH is considered a simple and inexpensive procedure, and has the advantage that fresh autologous blood is readily available. Numerous studies of its efficacy, however, have produced conflicting results, perhaps because of the heterogeneity of the surgeries in which it was used, differences in study protocol, and differences in the definition of outcome variables<sup>[77,78]</sup>.

## DISCUSSION

Liver resection is still the mainstay of treatment for patient with HCC. Even though improved surgical techniques and anesthesia have remarkably decreased the mortality rates of liver resections, morbidity rates, remain high. One of the major risks of hepatectomy is large-volume blood loss, which necessitates perioperative blood transfusion. The possible consequences of homologous blood transfusion are well known and include noninfectious risks such as transfusion reactions, transient immunodeficiency, transfusion-associated GVHD, and TRALI<sup>[79-84]</sup>. Thus there are conclusive motivations to reduce blood loss during surgery and, as a consequence to lessen blood transfusion. It has been clearly shown that transfusion has a significant negative effect on perioperative mortality, complications, and length of hospital stay, even if it is difficult to demonstrate that transfusion is the only factor that decisively affects the outcome. The magnitude of the surgical procedure has always to be considered the most critical factor. It is intuitive that anterior, small, marginal atypical resections are quite different to complicated posterior large resections which include reconstruction of resected vena cava.

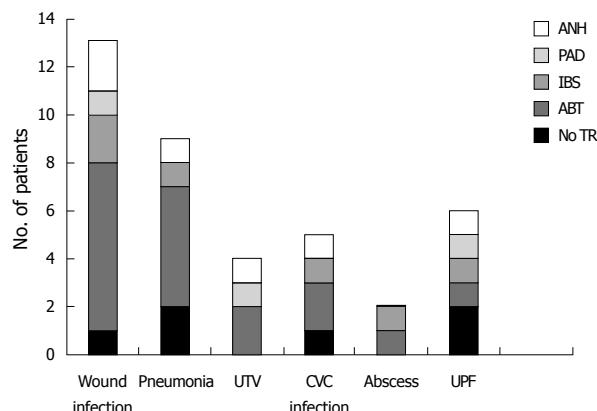
An association between transfusion and postoperative complications has been shown in preclinical models<sup>[85,86]</sup> and in clinical studies<sup>[87-91]</sup>. The review of 378 consecutive elective liver resections performed in our institution shows that 62% of the patients were not transfused, and the remaining 38% received blood products delivered with different procedures (Figure 1).



**Figure 1 Transfusion procedures in 378 patients undergoing liver resection.** No TR: Not transfused (62%); ABT: Autologous blood transfusion (21%); IBS: Intraoperative blood salvage (3%); PAD: Preoperative autologous blood donation (12%); ANH: Acute normovolemic hemodilution (2%, seven of the eight pts were Jehovah's Witnesses). Data from the Department of Surgical Sciences, University of Insubria, Varese, Italy.

Infectious complications (wound infections, pneumonia, urinary tract infections, central venous catheter infections, abscesses, and undiagnosed postoperative fever) have been more frequent in the transfused group of patients (33 vs 7). Most of the infections complications (18) have been recorded in the patients receiving autologous blood transfusions, the most frequent being wound infections (7) and pneumonia (5). Our results confirm the observation of Alfieri *et al*<sup>[92]</sup> who in a series of 254 liver resections found a significant association between blood transfusions and development of complications. More recently, Kooby *et al*<sup>[93]</sup> have been able to show that perioperative blood transfusion is a prognostic factor for the development of complications in univariate and multivariate analysis. Transfused patients had twice as high a chance of developing major complications and four times the risk of perioperative death. Transfused patients also had a higher incidence of infectious complications (17% vs 13%,  $P = 0.03$ )<sup>[93]</sup>.

Despite these results and studies, it is still debatable whether transfusion is the only and independent factor related to short term outcome, and specifically the only determinant of postoperative infectious complications. Is the transfusion itself and not the reason for the transfusion the cause of postoperative morbidity? Intraoperative hypotension, complexity of operation (extended hepatectomies vs lesser resections), duration of anesthesia, age, stage of the neoplastic lesion, degree of liver dysfunction, nutritional status, and possible neoadjuvant treatment, are all factors which could interfere with some aspects of the complex immunologic response. Furthermore, timing of the transfusion and the circumstances necessitating transfusions have been proposed as the real determinants of prognosis<sup>[94]</sup>. Today we are not able to conclude that transfusion is the factor producing the infectious complication, and the correlation we found of transfusion with complications should not be interpreted as a direct cause and effect relationship. The infectious complications are different in the transfused and non-transfused patients, but we cannot say for sure



**Figure 2 Details of postoperative infectious complications (44 pts, 11.6%) occurred in 378 patients undergoing liver resections and correlated to transfusion procedures.** UTV: Urinary tract infection; CVC: Central venous catheter; UPF: Undiagnosed post-operative fever. Data from the Department of Surgical Sciences, University of Insubria, Varese, Italy.

that immunologic irregularities are what produces the difference.

In recent years, we have had the occasion to carry out seven major liver resections on Jehovah's Witnesses with large tumors. The management of Jehovah's Witnesses with HCC, or any other type of liver tumor, entails a multidisciplinary, adapted plan in harmony with their religious beliefs to achieve good outcome<sup>[95]</sup>. This approach enabled us to perform the surgical procedure respecting their religious conviction, and authorized us to anticipate that ANH could be considered a safe alternative for use in selected cases in which allogeneic blood transfusion is considered of high risk. This approach, in our series, has been associated with a relative high incidence of infectious complications, if compared with other autologous blood transfusion procedures (Figure 2).

## CONCLUSION

A substantial discrepancy is apparent in transfusion practice for elective surgery, and even more so for liver resections<sup>[96]</sup>. Reducing unneeded exposure to blood components by blood saving measures is essential in patients undergoing elective surgery. A publication for anesthesiologists reviews good transfusion practices in surgical patients<sup>[97]</sup>.

Perioperative blood transfusion has been described as one of the risk factors for poor outcome after liver resection. This seems particularly verifiable for infectious complications. The postoperative recurrence of HCC associated with perioperative blood transfusion has been the subject of controversy due to conflicting results. Although allogeneic blood transfusion may have immunosuppressive effects, perioperative blood transfusions seem not to influence the cancer free survival rate in patients with HCC. Even if there is no evidence of one transfusion procedure which prevails over the others, surgeons who practice in Centers with high volume of liver resections should be familiar with

all the possible alternatives (ABT, IBS, PAD, ANH), since each of them, when blood products are needed, have a place depending upon the different clinical pattern.

Finally, maintaining a low central venous pressure has been shown recently to be effective in reducing blood loss during partial liver resections. Moreover antifibrinolytic drugs have proved to be effective in reducing blood loss during liver transplantation<sup>[98]</sup>.

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REVIEW

## Comparative genomics of *Helicobacter pylori*

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### INTRODUCTION

The gastric pathogen, *Helicobacter pylori* (*H pylori*), is a member of the epsilon-bacteria. This microaerophilic, Gram-negative bacterium colonizes the human stomach<sup>[1]</sup>. It is estimated that over half of the human population are infected by *H pylori*<sup>[2]</sup>. The infection causes mucosal inflammation, atrophy, ulceration and cancer<sup>[3,4]</sup>. Five strains of *H pylori* and a number of related bacteria have been sequenced. Genomics, evolutionary studies and population genetics have advanced our understanding of this bacterium.

### GENOMIC FEATURES

In 1997, *H pylori* strain 26695 was firstly sequenced<sup>[5]</sup>. It was isolated from an English patient with chronic gastritis. The chromosome of strain 26695 is circular and composed of 1667867 base pairs. The average GC content is approximately 39%. In the initial annotation, it has 1590 open reading frames that are possibly protein-coding<sup>[5]</sup>, in addition to the RNA coding genes (2 copies of 16S rRNA and 23S rRNA genes, 36 tRNA genes). Later analysis of the genome sequence suggested a smaller number of ORFs in strain 26695<sup>[6]</sup>. The ongoing studies have found genes that were neglected in the initial analysis. A general secretion machinery is widely present in bacteria, which functions in secretion of outer membrane proteins from the inner membrane and delivery of proteins to extracellular environments<sup>[7]</sup>. The initial annotation revealed a partial general secretion machinery because it lacked SecE in 26695<sup>[5]</sup>. A further analysis of the genome sequences with GeneMark, Glimmer and BlastX found a small open reading frame between nusG and rmpG (HP1203-HP1204)<sup>[8]</sup>. It has a high homology and structural similarity to the SecE protein in related bacteria. Therefore, strain 26695 has a complete general secretion machinery, which is consistent with the fact that the bacterium is capable of protein secretion. In addition, small RNA genes are universally present in bacteria<sup>[9]</sup>. The tmRNA gene (*ssrA*) has been found in *H pylori*, which encodes a functional RNA molecule and a small peptide that is involved in

### Abstract

Genomic sequences have been determined for a number of strains of *Helicobacter pylori* (*H pylori*) and related bacteria. With the development of microarray analysis and the wide use of subtractive hybridization techniques, comparative studies have been carried out with respect to the interstrain differences between *H pylori* and inter-species differences in the genome of related bacteria. It was found that the core genome of *H pylori* constitutes 1111 genes that are determinants of the species properties. A great pool of auxillary genes are mainly from the categories of *cag* pathogenicity islands, outer membrane proteins, restriction-modification system and hypothetical proteins of unknown function. Persistence of *H pylori* in the human stomach leads to the diversification of the genome. Comparative genomics suggest that a host jump has occurs from humans to felines. Candidate genes specific for the development of the gastric diseases were identified. With the aid of proteomics, population genetics and other molecular methods, future comparative genomic studies would dramatically promote our understanding of the evolution, pathogenesis and microbiology of *H pylori*.

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**Key words:** *Helicobacter pylori*; Genomics; Pathogenesis; Cancer

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quality control of translation<sup>[10]</sup>. In addition, *H pylori* also possesses a sRNA gene encoding the RNA component of Rnase P and the 4.5S RNA gene which is involved in secretion<sup>[11,12]</sup>.

In 1999, strain J99 was sequenced which was isolated from an American patient with a duodenal ulcer<sup>[6]</sup>. Compared to strain 26 695, it has a slightly smaller circular chromosome (1 643 831). The overall genomic organization, gene order and predicted proteomes of the sequenced strains are very similar. The predicted open reading frames are less in strain J99, amounting to 1495. There are 1406 genes shared by both strains, but 86 open reading frames are absent from strain 26 695. Both strains contain a complete *cag* pathogenicity island that codes for a type IV secretion system which delivers the CagA cytotoxin protein into gastric epithelial cells<sup>[13]</sup>. Comparison of the two genomes reveals the occurrence of translocation and inversion events. A 83 kb inversion contains most of the strain specific genes. This region was named a plasticity zone since it has a much lower GC content (35%) than the rest of the genome.

In 2006, a chronic atrophic gastritis *H pylori* strain, HPAG1, was sequenced<sup>[14]</sup>. It was isolated from an 80 year-old female patient who was enrolled in a Swedish case-control study of gastric cancer<sup>[15]</sup>. Similar to the sequenced strains 26 695 and J99, HPAG1 is a type 1 strain that contains *cagA* and a virulent allele of *vacA*<sup>[15]</sup>. The genome of HPAG1 (1 596 366 bp) is the smallest in the three sequenced strains. A total of 1536 open reading frames were predicted. Of these, 43 genes are only present in HPAG1. Analysis revealed that 29 genes that are found in both J99 and 26 695 were missing from HPAG1. If genes in a strain are absent from other strains, they are called strain-specific genes. The comparison of three sequenced *H pylori* strains shows that the majority of strain-specific genes are functionally unknown. Another group of strain-specific genes is composed of genes of the R-M system (restriction-modification). They encode proteins involved in DNA restriction or modification. Other strain-specific genes include those encoding outer membrane proteins and *cag* proteins.

*H pylori* strain G27 was sequenced recently<sup>[16]</sup>. It was originally isolated from an Italian patient<sup>[17]</sup> and has been used widely in *H pylori* research. This strain is naturally transformable<sup>[18]</sup>, capable of delivering CagA into epithelial cells in culture<sup>[19,20]</sup>, and capable of adapting to variable environments<sup>[21]</sup>.

The G27 genome has a similar size to the other three sequenced strains. It is 1 652 983 bp long and has a GC content of 38.9%. 1515 open reading frames was predicted. In addition, G27 also contains one 10032 bp AT rich (65.2%) plasmid resembling that found in strain HPAG1<sup>[14]</sup>. The plasmid encodes 11 genes. In agreement with the previous report<sup>[6]</sup>, the *cag* pathogenicity island of G27 is disrupted by a transposon. This, however, does not seem to interfere with any of the open reading frames or to the delivery of CagA into host cells. Unlike strain 26 695, there is a single plasticity region in G27 which contains a large number of *H pylori* specific genes.

It is predicted that strain G27 has 58 genes that are not found in 26 695, J99, or HPAG1.

The *H pylori* Shi470 genome has also been sequenced by Washington University Medical School. It is 1.61 Million bp long and contains approximately 1609 predicted genes. The sequences are available on the university website (<http://hpylori.ucsc.edu>).

The finding of strain-specific genes from the comparison of the sequenced strains is in agreement with the earlier studies which demonstrated the high diversity of the *H pylori* genome<sup>[22-24]</sup>. No identical strains of *H pylori* have been found in their genetic types unless they are isolated from a family<sup>[25-27]</sup>. *H pylori* has great mutation and recombination capacities. Analysis of the genomic sequences failed to identify a complete mismatch repair system controlling the confidentiality of replication, despite the presence of a homology of MutS<sup>[28,29]</sup>. This results in a high mutation rate of *H pylori*. Examination of 29 clinical isolates revealed that approximately 1/4 of them had mutator phenotype with higher mutation frequencies than Enterobacteriaceae mismatch-repair defective mutants<sup>[30]</sup>. In another study, examination of paired strains isolated from a patient at different times suggested a mutation rate of  $4.1 \times 10^{-5}$ , which is comparable to that of *E. coli* mutator<sup>[31]</sup>. *H pylori* is naturally competent for transformation<sup>[32]</sup>. Nonrandomly distributed repetitive sequences are found in the genome, which leads to frequent recombination events<sup>[33]</sup>. The recombination rate (recombination events starting at any particular nucleotide) is estimated to be  $6.9 \times 10^{-5}$ <sup>[34,35]</sup>. High levels of recombination and mutation could explain the observed genomic diversity in *H pylori*.

## DETERMINATION OF THE CORE GENOME OF *H PYLORI*

Inter-strain diversity, represented by variations in number and contents of genes, chromosomal rearrangements and allelic diversities, is not unique to *H pylori*<sup>[36]</sup>. This has been noted in a number of other bacterial species. For *H pylori*, each strain contains many strain-specific genes<sup>[7,14]</sup>. It has been proposed that a particular bacterial species contains a core set of genes and the auxiliary genes<sup>[37]</sup>. The core genome contains genes that are present in all or nearly all of the strains. It determines the properties that are characteristic of the species. The auxiliary genes are present in some of strains. They are determinants of the biological properties unique to some of the strains. Salama *et al*<sup>[38]</sup> firstly explored the core set of genes in *H pylori*. A total of 15 strains of *H pylori* mainly isolated from Western countries were examined using a microarray method<sup>[38]</sup>. It was found that 1281 genes were common to all the examined strains, therefore constituting the core genome of *H pylori*. Considering the limited number of strains examined and the fact that *H pylori* is highly prevalent in human, could these genes represent the actual core set of the *H pylori* species? Additionally, these strains were only isolated from Western individuals. In fact, molecular typing of global strains has found that the modern population of *H pylori* divides into five major

groups, hpEurope, HpAfrica1, hpafrica2, hpEastAsia and hpAsia2<sup>[39,40]</sup>. They are possibly derived from different ancestral groups. Gressmann *et al*<sup>[41]</sup> further examined 56 globally representative strains of *H pylori*. The number of the genes in the core set of *H pylori* was diminished to 1150. The author concluded through a calculation that the core genome of *H pylori* only consists of 1111 genes.

The auxiliary set of genes in *H pylori* amounts to 22%-27% of the genome<sup>[14,41]</sup>. In agreement with the findings from the whole genome sequencing of *H pylori* strains, the auxiliary genes consist of those coding for functionally unknown proteins, cag protein, outer membrane proteins and proteins of DNA metabolism<sup>[7]</sup>.

Candidate genes specific for development of gastric diseases.

The long term clinical outcomes of the *H pylori* infection are diverse. The infected gastric mucosa may develop inflammation, atrophy, intestinal metaplasia, ulceration, cancer and MALT lymphoma<sup>[1,3,4]</sup>. Genes in the auxiliary set are specific only for some strains. Do they play roles in the determination of the final outcome of an infected individual? *H pylori* broth culture filtrates cause the formation of intracellular vacuoles in mammalian cells<sup>[42]</sup>. The protein which has the vacuolation activity was purified and named VacA (Vacuolating cytotoxin). Despite of the universal presence of the *vacA* gene in *H pylori*, some strains do not cause the vacuolation of epithelial cells. This is attributed to the mosaic structure of *vacA*<sup>[42]</sup>. A signal region in the N-terminal and a mid region of *vacA* are polymorphic. The signal region affects the vacuolating activity of the cytotoxin: a 12 amino acid extension on the s2 form blocks the activity, although not all s1 forms have the cytotoxic activity<sup>[43]</sup>. The mid region is a determinant of the cell specificity of VacA<sup>[44]</sup>. There are three *vacA* genotypes, s1/m1, s1/m2 and s2/m2 in *H pylori*. The association of s1/m1 strains with severe diseases has been observed in some studies. A recent study found an intermediate region (i-region) of *vacA* between the signal region and the mid region that also contributes to the levels of vacuolating activity<sup>[45]</sup>. The genotype i1 was more frequently found in gastric cancer associated *H pylori* than the i2<sup>[45]</sup>. Strains possessing *vacA* i1 are strongly associated with peptic ulcer<sup>[46]</sup>. Another protein has been found to be co-present in almost all of the strains possessing the vacuolating activity<sup>[47]</sup>. The protein was named as cytotoxin-associated gene A (*cagA*) protein. The gene is present in the majority of strains. Those possessing the vacuolating activity and the CagA expression are called type I strains, or virulent strains<sup>[48]</sup>. The presence of *cagA* is generally the marker for a large DNA region called *cag* pathogenicity island<sup>[49]</sup>. Proteins produced by the *cag* island make up a type IV secretion system which delivers CagA into the epithelial cells<sup>[50,51]</sup>. The type IV secretion system locates across the inner and outer membrane and forms a pilus-like structure at the surface<sup>[51,52]</sup>. The CagL protein is a specialized adhesin that is targeted to the pilus surface<sup>[53]</sup>. Through an arginine-glycine-aspartate motif, it binds to and activates integrin  $\alpha 5\beta 1$  receptor on gastric epithelial cells. This interaction triggers CagA delivery into target cells<sup>[54]</sup> and activation of Src of gastric epithelial

cells<sup>[55]</sup>. Translocated CagA remains associated with the host membrane and becomes tyrosine phosphorylated on carboxyl-terminal repeat motifs (Glu-Pro-Ile-Tyr-Ala, or EPIYA motifs)<sup>[56,57]</sup> by members of the Src family of protein tyrosine kinases such as c-Src, Fyn, Lyn, and Yes<sup>[58]</sup>. Phosphorylated CagA interacts with SHP-2<sup>[59]</sup>, which thereafter activates a number of phosphatases inducing alteration of signaling pathways. This alters the spreading, migration, adhesion, polarity and cytoskeletal structures of epithelial cells<sup>[60-63]</sup>. A large European study, demonstrated that *cagA* positive strains are significantly associated with the development of gastric cancer<sup>[64]</sup>. The *cag* island is thus an important determinant of the clinical outcomes of the *H pylori* infection. Most *H pylori* strains, and almost all in certain geographical locations, however, are virulent (that is they expressing CagA and VacA). Are there any other genomic differences associated with the clinical outcomes?

Comparison of the genomic contents of different strains has found genes that are potentially disease-specific. Peptic ulcer disease frequently occurs in humans with severe, or even lethal complications. The disease may also affect children. Oleastro *et al*<sup>[65]</sup> reported the study of the genomic comparison of a *H pylori* strain isolated from a child presenting with duodenal ulcer and a strain from a sex and age matched child with gastritis. It was found that genes jhp0562 and jhp0870 are more frequently seen in children with peptic ulcer than in those with gastritis. Both genes are located in the plasticity zone. Jhp0562 encodes a putative LPS glycosyltransferase involved in LPS biosynthesis<sup>[66]</sup>, whereas jhp0870 codes for an outer membrane protein. LPS and outer membrane proteins play roles in the induction of an inflammatory response from the gastric mucosa<sup>[66,67]</sup>. Whether jhp0562 and jhp0870 contribute to the development of ulceration in children deserves further study. Other genomic comparison studies have found that the *cag* island and a 670 bp-long DNA fragment that is partially homologous to the hydrolase kinase gene are potentially associated with peptic ulcer diseases<sup>[68]</sup>. Gastric mucosa infected by *H pylori* develops inflammation, and gradually become atrophic. Mucosal atrophy is an important stage in stomach carcinogenesis. A thorough examination of the genome of 6 strains from atrophic gastritis found a set of 121 "ChAG-associated" (ChAG, chronic atrophic gastritis) genes<sup>[14,69]</sup>. They are universally present in these 6 strains but absent from 56 globally derived strains of *H pylori*<sup>[69]</sup>. Their putative roles in the development of atrophy and promotion of carcinogenesis are yet to be studied. Intestinal metaplasia of gastric mucosa is a precancerous lesion. *H pylori* in the patient with intestinal metaplasia is likely associated with progression into gastric cancer. Comparison of a cancer strain and a duodenal ulcer strain of *H pylori* found a novel sequence named Clone P32 with homology to GepA in *Dichelobacter nodosus*<sup>[70]</sup>. Examination of strains from diverse gastric diseases demonstrated that Clone P32 is inversely associated with intestinal metaplasia. Gastric B cell lymphoma of mucosa associated lymphoid tissue is highly associated with the *H pylori* infection<sup>[1]</sup>. Eradication of the bacterium leads to the alleviation of the disease. Comparing

the genome of a strain from gastric B cell lymphoma with that from gastritis revealed that jhp0950 encoding a *H pylori* specific protein of unknown function was potentially associated with the development of the disease<sup>[71]</sup>. It was present in about 3 quarters of strains from gastric lymphoma, but only present in about half of strains from gastritis, duodenal ulcer or gastric adenocarcinoma. If other virulent factors were taken into account, the odds of having gastric MZBL among patients harbouring JHP950, *iceA1* (coding for a restriction enzyme), and *sabA* (coding for a major adhesin) "on" strains were 10 times higher than for the others<sup>[72]</sup>. Although these genes are specific for strains from a specified disease, it is uncertain whether they are pathogenic for a particular disease. Actually, different gastric diseases greatly differ in a variety of environmental factors that have potential impacts on the biological behaviors and genetics of the bacterium. For example, secretion of gastric acid is varied in different diseases<sup>[73]</sup>. Therefore, further studies are required to say that a gene is specific for the pathogenesis of a particular disease.

## INTRA-HOST EVOLUTION OF THE *H PYLORI* GENOME

It is believed that the *H pylori* infection is usually acquired in childhood<sup>[74]</sup>. The bacterium is transmitted from parents to their children with a bias of mother to children transmission<sup>[75,76]</sup>. Once the infection is established, the bacterium persists in the host for decades unless eradicated with antibiotics. Transmission of bacteria to a new host is a major barrier for bacterial spreading. It may affect the bacterial genome contents. Four healthy adults were experimentally infected with *H pylori*. Examination of isolates form 15 d or 90 d postinfection demonstrated that their genomic contents were identical to the challenging strain<sup>[77]</sup>. A similar result was found in a study of experimental infected mice<sup>[78]</sup>. These findings suggest that for *H pylori*, transmission does not cause any alteration of the gene components of the genome, or, in the other words, the establishment of the *H pylori* infection does not require the involvement of novel genes. Further evidence supporting this conclusion comes from a study of the analysis of the strains from a mother and her three children<sup>[75]</sup>. Microarray analysis demonstrated that the genomic contents of isolates from the mother were identical to those from the children. *H pylori* persists in the human stomach for decades, probably from childhood. It may experience a variety of ecological alterations, which may in turn have large impacts on the genome of the organism. The output of gastric acid alters with aging and with infection by *H pylori*. Alterations of the constituents and the quantity of the gastric mucus underneath which the bacterium resides are observed during the course of the *H pylori* infection. The gastric epithelial cells may undergo metaplasia and changes in surface proteins, which affect adhesion and the supply of nutrients. The gastric mucosa may produce immune and inflammatory products against

the bacterium. The co-infection with other microbes is also frequent seen in the stomach. These alterations may affect genomic contents of *H pylori*. Kraft et al<sup>[79]</sup> examined paired strains of *H pylori* with respect to their genomic contents using the microarray method. Paired strains were isolated from the same patients at an interval from 3 to 36 mo. Of 21 pairs of strains examined, 4 pairs showed differences in their genomic contents, suggesting the occurrence of evolutionary events. These included a complete deletion and a partial loss of the *cag* pathogenicity island, a replacement of an open reading frame of unknown function with the restriction-modification system HpyAIV, an acquisition of 14 genes in the plasticity zone, a duplication of the *ceuE* genes (HP1561/HP1562) and a truncation of tandem arranged *ackA* and *pta* genes resulting in the formation of pseudogenes. A study has compared 2 pairs of strains obtained from the same patients at an interval of 4 years<sup>[69]</sup>. The patients had progressed from atrophic gastritis to cancer. Six genes were absent, including 3 genes involved in DNA repair, an outer membrane protein and two hypothetical proteins. Nine genes were gained, including a ligase, a metalloprotease, a tRNA formyltransferase, a putative ribonuclease, a restriction enzyme and four hypothetical genes. The comparison suggests that with the progression of the atrophy to cancer, the bacterium may have a propensity for losing its diversifying capacity. Findings from these studies demonstrate that *H pylori* may acquire or lose genes during the intra-host colonization<sup>[80]</sup>. The genes involved fall into the same categories as the strain specific genes. This was further supported by the results from the comparison of the sequenced strain J99 with isolates obtained 6 years later<sup>[81]</sup>. These comparative studies of the *H pylori* genome draw a picture of the genomic changes during the cycle of invasion, colonization and transmission to a new host. It appears that invasion into a new host has little effect on the gene composition of the genome. This indicates that the current genome of *H pylori* has sufficient capacities for permitting bacterial invasion into a human host or even into a host of different species under experimental conditions. Once the infection is established, the bacterium has to cope with the dynamic changes of the ecology during the long-term coexistence with the host. Genomic diversifications, or gain and/or loss of genes, occur in response to these changes. The diversifications involve genes that are mainly those strain specific genes observed from comparative studies of unrelated strains of *H pylori*. Intra-host evolution of *H pylori*, thus, results in the creation of a pool of genes that are generally needed by some strains. This pool of genes can be considered as the auxiliary set of genes of *H pylori*.

## COMPARATIVE STUDIES OF *H PYLORI* AND ITS RELATED BACTERIA

Since the isolation of *H pylori*, a number of closely related bacteria have been identified, constituting a

**Table 1** General genomic features of *helicobacters* and *campylobacters*

Species	<i>Helicobacter pylori</i>		<i>H. acinonychis</i>		<i>H. hepaticus</i>	<i>W. succinogenes</i>	<i>C. jejuni</i>	<i>C. coli</i>	<i>C. lari</i>	<i>C. upsaliensis</i>
Strain	26695	j99	HPAG1	Sheeba	ATCC51449	DSM1740	NCTC1168	RM2228	RM2100	RM3195
Origin	Clinical	Clinical	Clinical	Felines	Rodents	Cattle	Clinical	Chicken	Clinical	Clinical
Genome size (bp)	1667867	1643831	1596366	1553928	1799146	2110355	1641481			
GC content (%)	39.0	39.0	-	38.2	35.9	48.5	30.5	31.3	29.6	34.5
Coding sequences										
Predicted number	1590	1459	1536	1611	1875	2046	1634	1764	1554	1782
Coding area (%)	91.0	90.8	-	89.7	93.0	93.0	94.3	-	-	-
Average length (bp)	945	998	-	865	1082	964	948	-	-	-
Flexible genome pool										
Plasmids	None	None	pHPAG1	pHac1	None	None	None	pCC178	pCL46	pCU3, pCu110
Insertion elements	IS605, IS606	IS606	None	ISHa1675, ISHa1942, ISHa1152	None	ISWSu1302, ISWSu1203	None	IS605	-	-
Genomic islands	cag PAI	cag PAI	cag PAI	HacGI	HHGI1	WSUGI I and II	None	-	-	-

new bacterial genus named Helicobacter genus<sup>[82,83]</sup>. Bacteria within this genus have been shown to colonise the gastrointestinal tract of mammals. Many of these Helicobacter species are involved in the pathogenesis of gastrointestinal diseases<sup>[82,83]</sup>. Phylogenetic analysis has shown that the *helicobacters* can be separated into two clusters<sup>[84]</sup>. Gastric species that colonise the stomach of mammals form a cluster. Species that inhabit the intestine and biliary tracts cluster together to form the enterohepatic cluster. In addition to *H. pylori*, the genome sequences have been determined for several other *helicobacters*, including *H. mustelae* from ferret, *H. acinonychis* from large felines (cheetahs, lions and tigers)<sup>[85]</sup>, *H. hepaticus* from mice which causes hepatoma<sup>[86]</sup>, and *Wolinella succinogenes* from cattle<sup>[87]</sup>.

General genomic features of these *helicobacters* are listed in Table 1, which also includes information for several species of *campylobacter*<sup>[88-91]</sup>. Of these related bacteria, the size and GC content of *H. acinonychis* are most similar to those of *H. pylori*<sup>[85]</sup>. Comparison of 612 orthologues that are present in both *H. acinonychis* and *H. pylori* found that they differ at only few of their amino acids. The Blast scores against *H. pylori* of most coding sequences in *H. acinonychis* are very high. These findings lead to the conclusion that a host jump has occurred from human to felines<sup>[85]</sup>. This event probably occurred 100 000 years ago. More studies are required to confirm this conclusion considering that universally accepted criteria to identify a host jump event are currently unavailable. The study also found a set of fragmented genes and newly acquired genes in *H. acinonychis*. These genes include a set of genes encoding outer membrane proteins and a cluster of genes encoding proteins for sialylation of bacterial surface carbohydrates. It has been suggested that these genes are probably beneficial for the bacterium to evade host immune defenses<sup>[92]</sup>.

Information from comparative genomics has greatly enhanced our understanding of the microbiology, physiology, evolution and pathogenesis of *H. pylori*. Candidate genes specific for the development of the gastric disease, particularly gastric cancer have been identified. Considering the striking diversities in the

*H. pylori* genome which are intensified by intra-host evolution, further studies exploring these genes must take account of them.

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ORIGINAL ARTICLES

## MRI of gastric carcinoma: Results of T and N-staging in an *in vitro* study

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### Abstract

**AIM:** To determine the accuracy of 1.5-T magnetic resonance imaging (MRI) in the evaluation of gastric wall invasion and perigastric lymph node metastasis in gastric adenocarcinoma.

**METHODS:** Twenty resected gastric specimens containing 20 tumors were studied with a 1.5-T MR system using a commercial head surface coil. MR scanning was performed with a T1 weighted image (TR/TE = 500/20), and a T2 weighted image (TR/TE = 2500/90). MR findings were compared with pathologic findings.

**RESULTS:** A T1-weighted image demonstrated three layers in the normal gastric wall. All of the gastric tumors were well demonstrated by lesions and location. In a MRI findings of gastric wall invasion, there was 1 case of T1, 7 of T2, 11 of T3. Pathologic results of resected specimens included 3 cases of pT1, 4 of pT2, and 12 of pT3. The accuracy of T staging with MRI was 74% (14 of 19). MRI findings of lymph node me-

tastasis included 6 cases of N0, 13 cases of N1. The accuracy of the N staging with MRI was 47% (9 of 19).

**CONCLUSION:** MRI has a high diagnostic accuracy in the evaluation of the T staging of gastric cancer *in vitro* and thus potentially enables preoperative histopathologic staging.

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**Key words:** Gastric cancer; Magnetic resonance imaging; Neoplasm; Staging; Stomach; Depiction of wall layer

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### INTRODUCTION

The preoperative staging workup of gastric carcinoma is performed mainly with computed tomography (CT). CT has been a favored method for preoperative evaluation and staging in patients with gastric carcinoma<sup>[1-3]</sup>. Parallel advances in CT equipment and scanning techniques have reduced scanning time and decreased motion artifacts. Simultaneously, rapid IV contrast administration with an automatic power injector has improved contrast enhancement of the gastric wall and gastric cancer. Helical CT has advantages over conventional CT, including faster scanning time and fewer respiratory misregistration artifacts in a single breath-hold<sup>[4]</sup>. However, CT is limited, particularly in the diagnosis of lymph node metastasis, peritoneal metastasis, and small hematogenous metastasis<sup>[5,6]</sup>.

Endoscopic sonography has been reported to be the most accurate technique for the T staging of gastric carcinoma because it can define five layers of the gastric

wall. But this technique cannot evaluate other factors such as liver metastasis and peritoneal seeding<sup>[7]</sup>. In addition, endoscopic sonography is an invasive technique dependent on the operator.

Magnetic resonance imaging (MRI) has not become popular for staging because of a number of limitations, including motion artifacts, lack of a stable contrast medium, and the high cost<sup>[8,9]</sup>. However, continuous technical improvements have been made in MRI of the abdomen, thereby reducing motion artifacts and improving image quality. These improvements include breath-hold fast imaging techniques, placement of abdominal binders, administration of antiperistaltic agents, and the use of phased array coils<sup>[10]</sup>. *In vitro* studies using 1.4-7-T MR systems have shown that MRI allows the depiction of gastric wall layers and therefore, technically permits the evaluation of the local tumor stage of gastric carcinomas<sup>[11-13]</sup>.

The purpose of this study was to assess the accuracy of the evaluation of gastric carcinomas and lymph node metastasis *in vitro* by using gastrectomy specimens that were studied with 1.5-T MRI.

## MATERIALS AND METHODS

### Subjects

Over a period of 13 mo, a total number of 20 consecutive patients with histopathologically proven gastric carcinoma underwent subtotal or total gastrectomy. There were twenty resected gastric specimens that were obtained from the patients who were diagnosed with gastric carcinoma histologically by fiberoptic biopsy. The patients underwent subtotal or total gastrectomy. They consisted of fourteen men and six women [mean age, 53 years: 34-77 years (14 men, 6 women)]. Nineteen subtotal gastrectomy specimens and one total gastrectomy specimen was obtained and used in this study.

### Specimen preparation

We needed to distend the gastric specimens for MRI. During their operations, all the stomachs were not opened for the purpose of this study. To distend the stomach of the specimens, the duodenal resection border was sealed with a continuous suture before the specimen was filled with saline solution. The specimens were then placed in a plastic box that had been filled with 5-6 L of saline solution.

The proximal portion of the gastrectomy specimen was hanged at the cap by strings. The box was capped and placed in the head coil of a 1.5-T MRI (SMT 1.5 scanner, Shimazu Co., Tokyo, Japan). Then the MR examination was performed. The study protocol was approved by the Institutional Review Board, and agreement was obtained from each patient before surgery.

### MRI protocol

The MRI obtained in this study was based on the following multisection spin echo sequences for T1-weighted images, repetition time (TR) ms/echo time (TE) ms = 500/20, and for T2-weighted images, 2500/90.

Two numbers of excitation were applied in this scanning. The matrix size was 256 × 256. Slice thickness was 5 mm and the intersection gap was 1 mm. Field of view was 20 cm. MR scans of the gastrectomy specimen were taken along the axial and sagittal planes. A head coil was used for scanning.

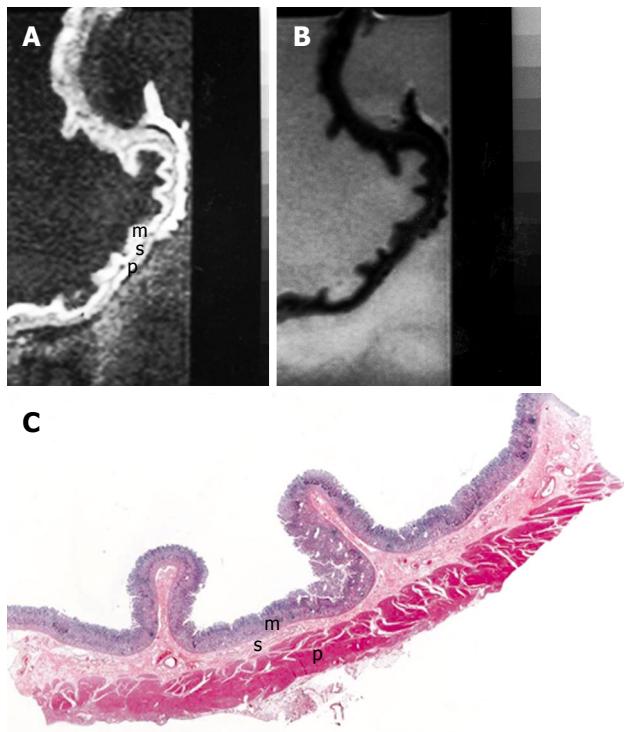
### Image analysis

The MR images of 20 resected gastric specimens were analyzed by two radiologists (H.S and S.K with 3 and 10 years of abdominal CT and MRI experience, respectively) in consensus before the results from the pathologic examination were available. The number of visible wall layers and their specific signal intensity (SI) characteristics were examined. Wall-layer correlation was made on the basis of the layer thickness of the visible layers in MRI compared with the ones visible in histology. The presence of a tumor, defined as destruction of the normal gastric wall layers, was noted. The tumors were examined for variations in SI. The depth of infiltration was evaluated according to earlier publications<sup>[12,13]</sup>. A normal gastric wall was identified as having 3 layers. In terms of scanning direction and degree of distention of the wall, a gastric wall that was more than 1 cm thick or that showed an abrupt change of pattern from normal to pathologic was considered abnormal. The location, gross appearance, size and degree of serosal invasion of tumors were evaluated. Location was classified according to four areas: antrum, body, body and antrum, and fundus. Gross appearance was classified into four categories by Bormann's classification for advanced gastric carcinoma<sup>[14]</sup>. T and N staging were based on the TNM system developed by the American Joint Committee on Cancer (AJCC)<sup>[15]</sup>. Early gastric cancer was evaluated according to the Japanese Research Society for Gastric Cancer<sup>[16]</sup>. The degree of tumor invasion in the gastric wall according to the T stage was measured as follows: T1 meant that MR showed obliteration of SI within the thickened mucosal layer and second submucosal layer, T2 meant that thickening of the gastric wall and obliteration of the third layer of muscularis propria, and T3 meant irregular SI in the outer margin of the third layer.

We counted the total number of lymph nodes which were located in the perigastric area. A lymph node of > 8 mm at the short axis was considered to be pathologic<sup>[17]</sup>. N staging of lymph nodes was performed. N0 is defined as no regional lymph node metastasis, N1 as metastasis in one to six regional lymph nodes, N2 as metastasis in seven to 15 regional lymph nodes, N3 as metastasis in more than 15 regional lymph nodes. Results of MR images were compared with findings in pathologic specimens and a report made by pathologists.

### Histologic preparation

Immediately after the MR examination of resected gastric specimens, the specimens were transferred to the department of pathology. The time interval between resection and fixation of the specimens was 2-3.5 h. The pathologist was not informed of the findings of



**Figure 1 MRI and histology of normal gastric wall.** A: T1-weighted (500/20) sagittal image of resected gastric wall showed three layers. The inner layer corresponds to the mucosa (m) and the middle layer to the submucosa (s). The outer layer basically consists of the muscularis propria (p) from which the serosa cannot be differentiated; B: T2-weighted (2500/90) MRI showed low SI on mucosa and muscularis propria and relatively high SI on submucosa; C: Light microscopic section of normal gastric wall obtained from the greater curvature site of stomach body showed three layers which are compatible with inner mucosal layer (m), middle submucosa layer (s) and outer muscularis propria and serosal layer (p) (HE stain; original magnification,  $\times 1$ ).

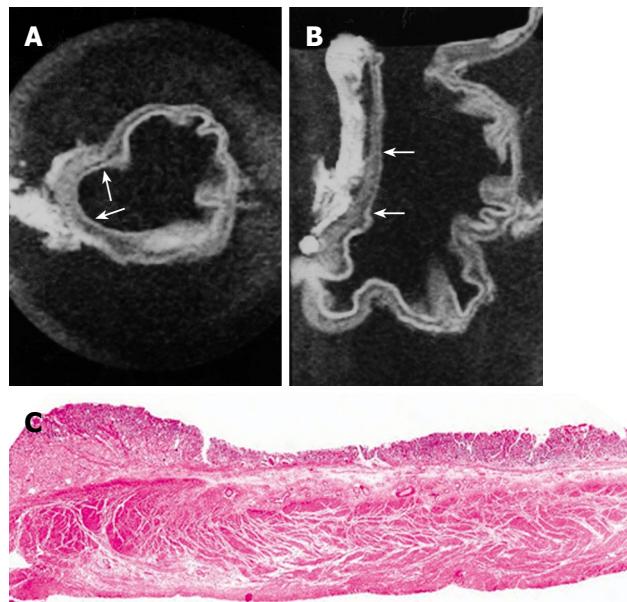
the MRI. The gastrectomy specimens were cut in planes corresponding to MRI imaging planes. The location, gross appearance, tumor size, and the histologic depth of invasion were determined for each specimen. The gastric carcinomas were staged according to the American Joint Committee on Cancer<sup>[15]</sup>. The diagnosed early gastric carcinomas (EGCs) were evaluated<sup>[16]</sup>. Finally, the histopathological staging of the specimens were compared with the staging by MRI. The depth of tumor invasion was decided according to the T factor of the TNM classification. Invasion of the mucosa or submucosa is classified as pT1, invasion of the muscularis propria as pT2, and tumor penetration of the serosa as pT3.

All the lymph nodes in the perigastric area of the specimens were counted and examined. Lymph nodes were stained with hematoxylin-eosin and examined by a light microscope for metastasis. Correlation between MR staging of lymph node metastasis and pathologic staging were performed by AJCC protocol<sup>[15]</sup>.

## RESULTS

### SI characteristics of normal gastric wall

On MRI, two to three layers with different SI in the normal gastric wall can be depicted. However, there was



**Figure 2 MRI and histology of early gastric cancer.** A: T1-weighted (500/20) axial image showed depression of gastric wall and obliteration of submucosal low SI (arrows); B: T1-weighted sagittal MRI showed depressed mucosa with tumor invasion to submucosa layer (arrows); C: Light microscopic section showed depressed mucosa with tumor invasion to submucosa (HE stain; original magnification,  $\times 1$ ).

a mainly three-layered structure (multilayered pattern) of the gastric wall by MRI. The inner layer showed an increase of SI and was 1–3 mm thick on the T1-weighted images. The second had a lower SI with thickness that varied at different sites in the same individual. The outer layer showed a slightly higher SI.

On T2-weighted images, the inner and outer layers regularly had a low SI, and the middle layer a high SI. On the basis of the comparison, the inner layer corresponds to the mucosa, the middle to the submucosa and the outer to the muscularis propria and serosal layers (Figure 1).

### Detection of primary tumor

MRI of gastric carcinoma on resected specimens showed as follows: two cases of Bormann's type 1 carcinoma (polypoid type), seven cases of Bormann's type 2 (ulcerative type), six cases of Bormann's type 3 (ulcerative type with infiltration), and four cases of Bormann's type 4 (infiltrating type). One case of early gastric carcinoma with type IIc was observed, whose lesion was seen as a depression of the mucosa with thinning of the gastric wall on axial and sagittal scannings (Figure 2). Gross pathologic findings showed tumor lesions as follows; two cases of Bormann's type 1, four of Bormann's type 2, nine of Bormann's type 3, four of Bormann's type 4. One case of early gastric carcinoma with type IIc was proved upon histologic examination.

In terms of the classification of gross appearance in the nineteen lesions detected as advanced gastric carcinoma, the accuracy of MRI in the Bormann's type classification was 89% (16 of 19). Differentiation between Bormann's type 2 and type 3 lesions was

**Table 1 Magnetic resonance imaging (MRI) and pathologic correlation of tumor invasion in gastric wall**

Diagnosis at histologic examination					
Diagnosis' at MR	pT0	pT1	pT2	pT3	Total
T0					
T1		1			1
T2		2	3	2	7
T3			1	10	11
	3	4	12		19

erroneous in three lesions.

The location of gastric carcinoma was also identified on the MR images. There were nine cases of gastric carcinoma involvement in the gastric antrum, three cases in the stomach body, seven cases in the antrum and the body, one case involving the entire stomach. Upon gross specimen examination, there was no difference between them and the MRIs.

#### Depth of tumor invasion

Degree of invasion was evaluated in the nineteen cases of advanced gastric carcinoma (Table 1). MRIs of gastric carcinoma in resected specimens showed various findings, including thickening of the gastric wall with irregularity in the mucosal SI obliteration, thickening of the gastric wall with first and second layer SI obliteration, diffuse thickening of the gastric wall with third layer SI obliteration and irregularity with ulceration as well.

T1-weighted images showed intermediate SI in regions affected by gastric carcinoma compared to the surrounding normal mucosa and muscularis propria SI. T2-weighted images showed low SI in the gastric carcinoma. Most tumors had a homogenous SI. However, in some cases necrosis and calcification caused an inhomogeneous SI. It is not possible to differentiate between the muscularis propria, subserosa, and serosa. The reason for this inability was that we considered the subserosa and serosa as being located on the outer border of the joint layer representing the muscularis propria, subserosa, and serosa. If an infiltration was visible, the tumor was classified as T2 as long as it did not reach the outer border. Penetration of the external margin meant at once infiltration of the serosa, and the tumor was staged as a T3 carcinoma. A tumor infiltrating the subserosa without penetrating the serosa was still considered T2, according to the AJCC<sup>[5]</sup>. The MRI findings of gastric wall invasion included 1 case of T1, 7 of T2 (Figure 3), and 11 of T3 (Figure 4). Pathologic results of resected specimens included 3 cases of pT1, 4 of pT2, and 12 of pT3. Differentiation between T1 and T2 classifications was not difficult in cases displaying a distinction between three layers. However, two cases of pT1 were over staged as T2. One case of pT2 was over staged as T3. Two cases of pT3 were understaged as T2. Differentiation between T2 and T3 lesions was difficult due to the outer muscularis propria and serosal layer's thinness and could not always be demonstrated by MRIs. The level of accuracy

**Table 2 MRI and pathologic correlation of lymph node metastasis**

Diagnosis at histologic examination				
Diagnosis at MR	pN0	pN1	pN2	Total
N0	2	3	1	6
N1	4	7	2	13
N2	6	10	3	19

in determining the T factor according to the TNM classification was 74% (14 of 19 lesions).

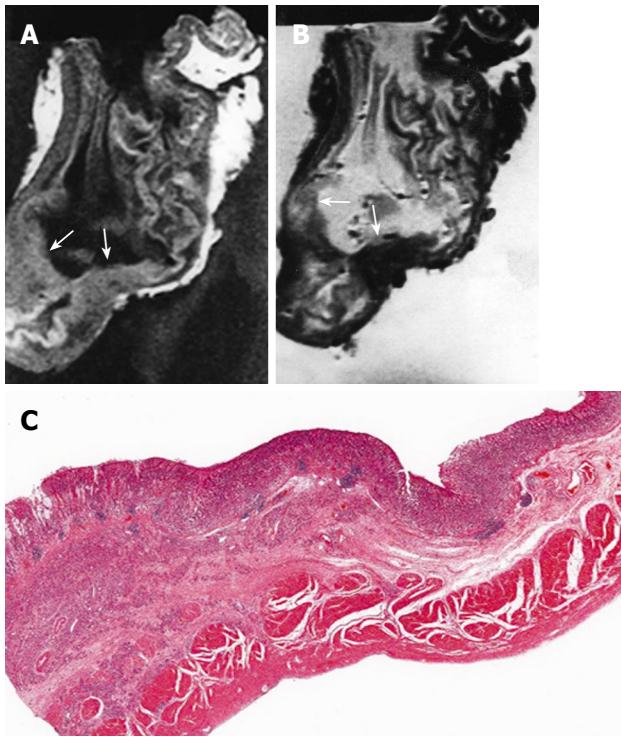
#### Regional lymph node metastasis

The lymph nodes presented with intermediate SI on T1-weighted images, intermediate SI on T2-weighted images. The sizes were measured as being from 0.35 cm to 3.5 cm. We counted 34 lymph nodes in the MRIs. Only 1 lymph node was measured as less than 0.8 cm on its short axis and the other 33 lymph nodes were measured as more than 0.8 cm on their short axis.

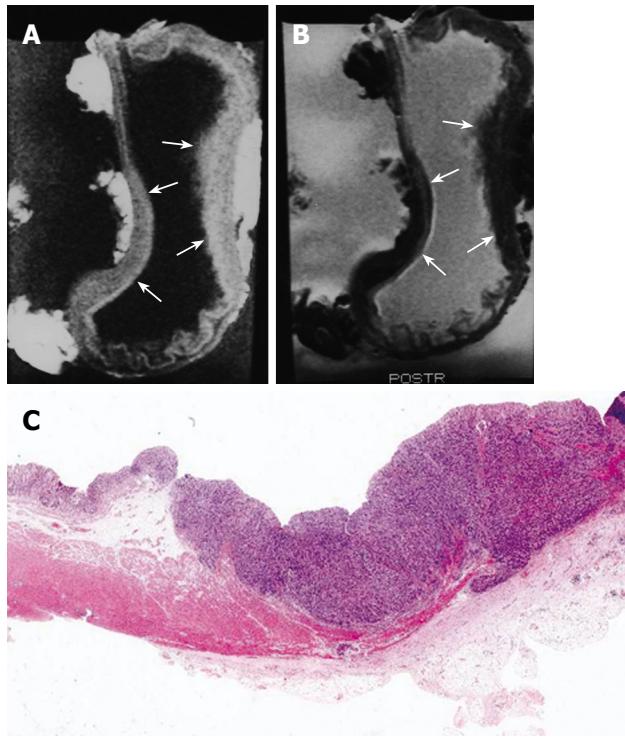
One hundred forty lymph nodes were removed from 19 cases of resected gastric specimens. The short axis of resected nodes proved malignant upon pathologic examination ranging from 0.3 to 3.5 cm. Overall metastasis was found in 60 lymph nodes. Degree of lymph node metastasis was also evaluated in 19 cases of gastric carcinoma (Table 2). MRI findings of lymph node metastasis included 6 cases of N0, and 13 of N1 (Figure 5). Pathologic findings of lymph node metastasis included 6 cases of pN0, 10 of pN1 and 3 of pN2 (Figure 6). Four cases of pN0 were over staged as N1 on the MR images. Three cases of pN1 were understaged as N0. Three cases of pN2 were understaged as N1 and N0. The accuracy of N staging by MRI was 47% (9 of 19).

## DISCUSSION

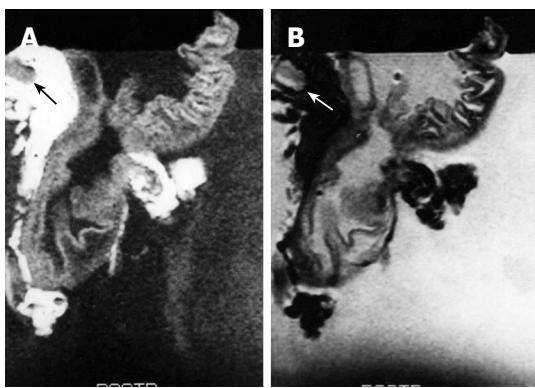
Imaging modalities, such as CT and endoscopic sonography are performed for the staging of gastric carcinoma. However, the accuracy of staging gastric carcinoma is still controversial in any diagnostic modality and a definitive diagnostic modality has not been established yet. Preoperative staging of gastric carcinoma is limited by the fact that available imaging modalities do not enable accurate evaluation of depth of infiltration of the gastric wall<sup>[18-21]</sup>. CT imaging is still evaluated for gastric wall invasion and staging of gastric carcinoma. However, the results of CT in local tumor staging are also insufficient, particularly because no reliable anatomic layer differentiation of the gastric wall can be achieved<sup>[20]</sup>. Without depiction of the wall layers, a secure distinction between T1, T2 and T3 tumors cannot be achieved. Results of early studies concerning CT diagnosis of gastric carcinoma were encouraging; however, findings in later articles were pessimistic about the ability of CT to enable staging of gastric carcinoma<sup>[7,9]</sup>. Recently, spiral CT has been founded to be more accurate than previous CT studies<sup>[22,23]</sup>.



**Figure 3 MRI and histology of T2 gastric cancer.** A: T1-weighted (500/20) sagittal image showed diffuse thickening of gastric wall with obliteration of mucosa, submucosa and muscularis propria SI in antrum and lower body, while preserved outer marginal SI (arrows); B: T2-weighted (2000/90) sagittal MRI showed ill defined lesion with minimal increased and same SI compared to surrounding normal gastric wall (arrows); C: Light microscopic section demonstrate proper muscle invasion of gastric cancer (HE stain; original magnification,  $\times 1$ ).

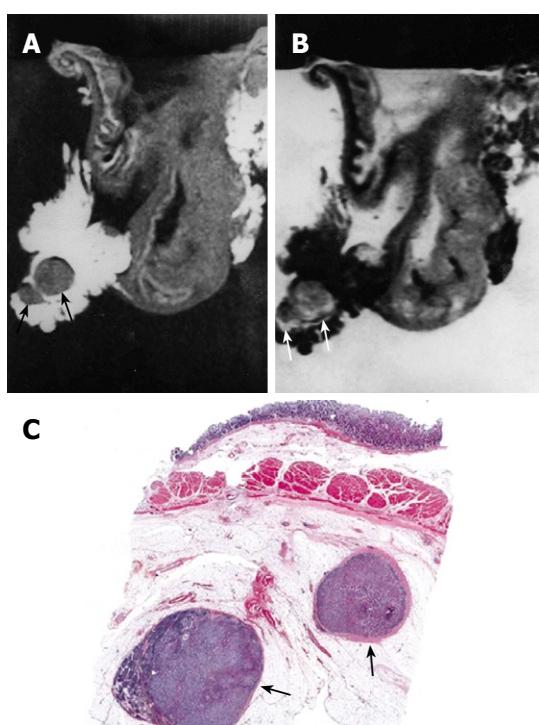


**Figure 4 MRI and histology of T3 gastric adenocarcinoma.** A: T1-weighted (500/20) sagittal image showed thickening of gastric wall with all three layer SI change in lesser and greater curvature site of stomach body (arrows); B: T2-weighted (2000/90) sagittal MRI showed minimal increase of SI on lesion site and poor delineation of gastric wall SI at outer layer margin compared to normal gastric wall (arrows); C: Light microscopic section showed extension of tumor invasion to serosal layer (HE stain; original magnification,  $\times 1$ ).



**Figure 5 MRI of N1 gastric adenocarcinoma.** A: T1-weighted (500/20) MR image showed single lymph node on lesser curvature site of stomach body (arrow); B: T2-weighted (2000/90) MRI showed intermediate signal SI of lymph node (arrow).

Endoscopic sonography is effective for detection of lymph node involvement in the perigastric area<sup>[24]</sup>. Moreover, Botet *et al*<sup>[7]</sup> reported the accuracy of the N factor and overall staging with endoscopic sonography to be 78% (39 of 50), which is significantly higher to that examined with the conventional dynamic CT technique. However, there are limitations to endoscopic sonography in the evaluation of distant perigastric lymph node metastasis. Another problem is that endoscopic sonography is invasive and the results are



**Figure 6 MRI and histology of N2 gastric adenocarcinoma.** A: T1-weighted (500/20) MRI showed two lymph nodes in lesser curvature site of stomach antrum (arrows). Eight lymph nodes are detected in total in perigastric area (not shown); B: T2-weighted (2000/90) MRI showed intermediate SI in the lymph nodes (arrows); C: Light microscopic section showed two lymph nodes in lesser curvature site of gastric antrum (arrows) (HE stain; original magnification,  $\times 1$ ).

highly operator-dependent.

Interest in the use of MRI for the staging of gastric carcinoma is increasing, but most clinical studies stage the local tumor situation without the differentiation of gastric wall layers<sup>[4,8-10]</sup>. Studies that use depiction of gastric wall layers as a basis for local tumor staging and lymph node metastasis are rare<sup>[13,25]</sup>.

The high quality of soft-tissue imaging of MR systems enables the depiction of anatomic wall layers. Auh *et al*<sup>[11]</sup> studied the gastric wall using an experimental 4.7-T system whereas Lubienski *et al*<sup>[12]</sup> used an experimental 2.4-T system. Both groups proved that the depiction of gastric-wall layers is technically possible. Auh *et al*<sup>[11]</sup> depicted 3 layers whereas Lubienski *et al*<sup>[12]</sup> was able to differentiate 4 layers and correlated them to the mucosa, lamina muscularis mucosa, submucosa and muscularis propria. Typically 3 gastric wall layers are visible. The inner layer corresponds to the mucosa and lamina muscularis mucosa and the middle layer to the submucosa. The outer layer showed the same SI as the muscularis propria in the study of Lubinski's *et al*<sup>[12]</sup> study and therefore mainly consisted of muscle tissue and serosal layers. Palmowski *et al*<sup>[13]</sup> demonstrated that a reliable depiction of gastric-wall layers can be achieved by a conventional 1-T MRI. As no subserosa and serosa could be depicted, it must be presumed that they were located on the outer side of the third layer. So the third layer represented the muscularis propria, subserosa, and serosa together<sup>[13]</sup>. We could demonstrate that the inner and outer layers as hyperintense and the middle layer as hypointense at 1.5-MRI. When the three layers were depicted in the gastric wall, the mucosa and the muscularis propria were clearly different from the intervening submucosal layer on T1-weighted images. The distinction among the layers is based mainly on the lower SI of the submucosa compared with that of the mucosa or muscularis propria. The difference between the three layers was also depicted in the T2-weighted images.

In this study, gastric carcinomas appeared as masses with destruction of the normal structure of the gastric wall or diffuse thickening of the gastric wall and showed intermediate SI compared to surrounding normal gastric walls on T1-weighted images and low SI on T2-weighted images. Both sequences were useful for tumor localization and complement each other because some carcinomas in the study could only be recognized by deviating signal behavior in one of the 2 sequences. In our study, signal characteristics of the carcinoma depending on the MR sequence were not analyzed. Palmowski *et al*<sup>[13]</sup> reported that carcinomas show an intermediate SI on T1-weighted images, a low SI on T2-weighted images and a high SI on opposed phase images. Opposed phase images were not obtained in our study, but Dux *et al*<sup>[25]</sup> demonstrated that opposed phase images show a very high SI in gastric tumors and insisted that this was useful for the staging of gastric carcinoma. In this study, the infiltration of gastric carcinoma was correctly defined in 74% of the cases. This was not different from that of CT images that had an accuracy rate of 50%-85% and that of MR images

that had an accuracy rate of 73%<sup>[4,7,26]</sup>. Yamada *et al*<sup>[27]</sup> reported that gastric specimens that were imaged after fixation in formalin and then MR imaged could also depict early gastric carcinoma. In this study, one case of early gastric carcinoma was depicted on MRI with a shallow depressed wall. This was made possible by adequate distention of the resected stomach with saline. To our knowledge, this is the first MRI depiction of early gastric carcinoma using gastric specimens without fixation in formalin. In this study, unfortunately, cases with pathologic T4 were not included because most patients who were diagnosed as T4 on preoperative imaging studies did not undergo surgery.

The evaluation of lymph node metastasis on MRIs had some limitation in this study, since the size criteria was used only on MRIs and there was no trial of contrast enhancement because of *in vitro* study of gastric carcinoma. Lymph node borders and signal intensity were not also evaluated for diagnosis of lymph node metastasis. But some cases of lymph nodes showed intermediate SI on T1 and T2-weighted images in the tumor infiltration region and this was correctly correlated with the histology. One-to-one pathologic-to-radiologic correlation on each lymph node was not performed in our study. According to Dux *et al*<sup>[25]</sup> study, lymph nodes showed a high SI on opposed phased images. MRI had low rate in depicting lymph node metastasis, with an accuracy of 47%. However, the result was similar to the other reports<sup>[25,27]</sup>. Further study is needed to increase accuracy in the finding of lymph node metastasis in gastric carcinoma.

In conclusion, the present study demonstrated that MRI can reliably depict several anatomical layers of the gastric wall and also MRI of gastric carcinoma could enable accurate diagnosis of location, gross appearance, degree of gastric wall invasion of the tumors and delineation of regional lymph node metastasis. A clear image of the tumor can be achieved. Therefore, an evaluation of the local tumor stage of gastric carcinoma and perigastric lymph node metastasis based on morphologic criteria is technically possible. This study, using a conventional 1.5-T MRI in combination with standard sequences, demonstrated the potential of MRI in the staging of gastric carcinomas. Although the result obtained in N-staging was not acceptable, it should be explored further. However, we were able to show not only MR findings of gastric wall invasion but also perigastric lymph node involvement in the gastric carcinoma. The results of our study cannot, at this time, be transferred to clinical practice, because conventional imaging acquisition techniques do not provide an image quality comparable to that of those taken of *in vitro* specimens. Advances in the variation of sequence techniques, as well as application of ultrafast imaging techniques, may in the future allow preoperative staging of gastric carcinomas by MRI.

## COMMENTS

### Background

The preoperative staging workup of gastric cancer is performed mainly with

computed tomography (CT). Magnetic resonance imaging (MRI) has not become popular for staging because of a number of limitations, including motion artifacts, lack of a suitable oral contrast medium, and the high cost. However, continuous technical improvements have been made in MRI of the abdomen, thereby reducing motion artifacts and improving image quality.

### Research frontiers

MRI has not yet reached clinical importance because of some limitations. However, *in vitro* studies using experimental 2.4-4.7-T MR systems have shown that MRI allows the depiction of gastric wall layers and therefore technically permits the evaluation of the local tumor stage of gastric cancer.

### Innovations and breakthroughs

MRI in the staging of gastric cancer is not usually applied in clinical practice. Its results are the same or inferior to CT in accuracy. There are many MRI *in vitro* and clinical imaging gastric cancer studies. *In vitro* MRI systems demonstrate well gastric wall layers and tumor invasion well and MRI has shown tumor invasion and lymph node metastasis in some clinical studies as well. However, normal gastric wall depiction and tumor invasion of the gastric wall are important to tumor staging, as are demonstrations of perigastric lymph node metastasis. The authors studied resected gastric specimens for the accurate depiction of normal gastric walls, tumor invasion and lymph node metastasis using 1.5-T MRI.

### Applications

The study results suggest that MRI could be useful in gastric wall invasion in the staging of gastric cancer. However, further study in the staging of lymph node metastasis is still needed.

### Peer review

The authors successfully demonstrated normal gastric wall layers and tumor invasion in their *in vitro* study using 1.5-T MRI. The results suggest that MRI is a potential diagnostic tool in the staging of gastric cancer.

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# Efficacy of intramuscular diclofenac and fluid replacement in prevention of post-ERCP pancreatitis

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## Abstract

**AIM:** To assess the efficacy of intramuscular diclofenac and fluid replacement for prevention of post-endoscopic retrograde cholangiopancreatography (ERCP) pancreatitis.

**METHODS:** A prospective, placebo-controlled study was conducted in 80 patients who underwent ERCP. Patients were randomized to receive parenteral diclofenac at a loading dose of 75 mg followed by the infusion of 5-10 mL/kg per hour isotonic saline over 4 h after the procedure, or the infusion of 500 mL isotonic saline as placebo. Patients were evaluated clinically, and serum amylase levels were measured 4, 8 and 24 h after the procedure.

**RESULTS:** The two groups were matched for age, sex, underlying disease, ERCP findings, and type of treatment. The overall incidence of pancreatitis was 7.5% in the diclofenac group and 17.5% in the placebo group (12.5% in total). There were no significant differences in the incidence of pancreatitis and other variables between the two groups. In the subgroup analysis, the frequency of pancreatitis in the patients without sphincter of Oddi dysfunction (SOD) was significantly lower in the diclofenac group than in the control group ( $P = 0.047$ ).

**CONCLUSION:** Intramuscular diclofenac and fluid replacement lowered the rate of pancreatitis in patients without SOD.

## INTRODUCTION

Pancreatitis is the most common complication of endoscopic retrograde cholangiopancreatography (ERCP), with a reported incidence of 1%-10% in most prospective studies<sup>[1-9]</sup>. The generally accepted criteria for the diagnosis of post-ERCP pancreatitis were proposed in 1991 during a consensus workshop. These criteria include new onset of pancreatic-type abdominal pain associated with at least a threefold increase in serum amylase or lipase occurring within 24 h after ERCP, and the pain symptoms need to be sufficiently severe to require admission to the hospital or to extend the length of stay of patients who are already hospitalized<sup>[1,10]</sup>.

There have been numerous theories about the mechanisms of pancreatitis. The most widely accepted theory is that mechanical trauma to the papilla or pancreatic sphincter, caused during instrumentation, creates transient obstruction of outflow of pancreatic juice<sup>[1]</sup>. Another theory suggests that the increased hydrostatic pressures in the pancreatic duct caused by injection of contrast or saline could cause injury to the pancreatic duct or parenchyma<sup>[1]</sup>.

Risk factors reported for ERCP-induced pancreatitis include a history of pancreatitis<sup>[11]</sup>, difficult cannulation<sup>[2]</sup>, repeated injection of the pancreatic duct<sup>[11]</sup>, pancreatic acinar opacification<sup>[12]</sup>, sphincter of Oddi dysfunction (SOD)<sup>[3,13]</sup> and precut or needle-knife endoscopic sphincterotomy<sup>[9,13,14]</sup>.

Cellular events that lead to pancreatitis involve an inflammatory process with premature activation of trypsin in acinar cells<sup>[15,16]</sup>. Phospholipase A<sub>2</sub> is believed to play a critical role in the initial inflammatory cascade of acute pancreatitis by regulating a number of pro-inflammatory mediators, including arachidonic acid products and platelet-activating factors<sup>[17]</sup>. Prevention or interruption of this cascade may prevent development of pancreatitis and its consequences. Although drug development has been impressive, the availability of effective drugs in the prevention and management of pancreatitis remains limited<sup>[15]</sup>.

Chemoprevention of pancreatitis still remains a debated question. Pharmacological prevention of pancreatitis after ERCP has been the topic of several investigations in recent years.

Diclofenac, a potent inhibitor of phospholipase A<sub>2</sub> activity, administered immediately after the procedure, is effective at preventing pancreatitis<sup>[9,18]</sup>. Advantages of this prophylaxis are the low cost and the possibility of "on-demand" treatment. Addition of non-steroidal anti-inflammatory drugs (NSAIDs) has also been shown to have beneficial effects in experimental acute pancreatitis<sup>[19]</sup>.

The aim of this study was to evaluate the efficacy of intramuscular (IM) diclofenac and fluid replacement for the prevention of pancreatitis in all eligible patients who underwent ERCP at our medical center.

## MATERIALS AND METHODS

The study described in this report was approved by the ethics committee of Suleyman Demirel University School of Medicine, Isparta, Turkey. Between August 2006 and April 2008, 91 patients fulfilled the inclusion criteria, 80 of whom were included in the final analysis. Patients were excluded from study participation if they had a contraindication for diclofenac, including patients with recently diagnosed peptic ulcer disease, renal failure, those who had taken an NSAID during the preceding week, those who developed acute pancreatitis during the 2 wk before ERCP, those with a history of chronic pancreatitis, and those who did not agree to participate in the study. Entry to the study was restricted to patients advised to have endoscopic retrograde cholangiography with or without pancreatography for extrahepatic cholestasis and/or impaired liver function tests.

A prospective, placebo-controlled trial was conducted in 80 patients who underwent ERCP. The patients received 75 mg IM diclofenac and intravenous (IV) isotonic saline at a rate of 5-10 mL/kg per hour for 4 h or an inert placebo (500 mL IV isotonic saline) immediately after ERCP. At the end of each procedure, the researchers recorded the details of the maneuvers performed, including the total time of the procedure, the number of attempts at cannulation, the number of pancreatic duct cannulations, the final diagnosis, and whether a sphincterotomy, a needle-knife papillotomy, or stent placement were performed. We did not use pancreatic duct stenting for prevention of pancreatitis.

Patients were sedated with IV midazolam. Xylocaine spray was used as a local anesthetic.

Serum amylase was determined 4 h after ERCP. If the 4-h serum amylase level was < 3 times the upper normal limit and there was no clinical evidence of acute pancreatitis at that time, patients were allowed free oral fluids and a diet. If the 4-h serum amylase level was > 3 times the upper normal limit and the patient exhibited pain or nausea and vomiting, then the patient was kept fasting and IV crystalloid fluids with opiate analgesics were prescribed. The following 8 h and 24 h blood tests were repeated for serum amylase and the patients were interviewed and examined for clinical evidence of acute pancreatitis. Acute pancreatitis was defined as serum amylase > 3 times the upper limit of normal associated with epigastric pain, back pain, and epigastric tenderness. Patients with persistent signs and symptoms of pancreatitis after 48 h underwent contrast-enhanced computed tomography.

Pancreatitis was graded as mild, moderate, or severe. Sphincter of Oddi dysfunction (SOD) was defined according to the Milwaukee Biliary Group Classification<sup>[20]</sup>.

The instruments used were cannula, sphincterotome, guidewire, and stone basket (Boston Scientific, Natick, MA, USA).

## Statistical analysis

Data were summarized by descriptive statistics. The  $\chi^2$  square and Fischer's exact tests were used to compare categorical patient data. The Mann-Whitney U test and Student's *t* test were used to compare continuous variables. Two-tailed *P* < 0.05 were considered to indicate significance.

## RESULTS

A total of 80 patients were eligible for the study. Forty patients received 75 mg diclofenac and isotonic saline replacement (diclofenac group), and 40 received inert parenteral fluid replacement (control group). No patients discontinued the study medication because of adverse effects. Overall, the baseline characteristics were consistent across all treatment groups (Table 1). The mean ages of patients in the diclofenac and control groups were  $60.3 \pm 16.1$  years and  $59.3 \pm 14.4$  years, respectively. There were 15 women in the diclofenac group and 22 in the control group. Similarly, there were no statistically significant differences between the groups considering the procedures, and factors that might increase the risk of pancreatitis, including single or repeated pancreatic duct injection, SOD, younger age, female sex and precut endoscopic sphincterotomy (Table 2). Although the frequency of pancreatitis in the patients with SOD did not differ between the diclofenac and control groups, it was statistically significant between groups when the patients with SOD was excluded (*P* = 0.047). The most frequent indication for ERCP was bile duct stone in the diclofenac (57.5%) and control (27.5%) group. Post-endoscopic bleeding

**Table 1 Characteristics of patients in the diclofenac and control groups<sup>1</sup>**

	Diclofenac group (n = 40)	Control group (n = 40)	P
Variables			
Age (yr) <sup>2</sup>	60.3 ± 16.1	59.3 ± 14.5	0.766
Female sex	15 (37.5)	22 (55.0)	0.116
Bile duct cannulation time (min) <sup>3</sup>	5.0 (3.0-5.0)	5.0 (3.0-10.0)	0.601
Operation time (min) <sup>3</sup>	15 (15-20)	15 (10-25)	0.904
Sphincterotomy			
Precut	10 (25)	12 (30)	0.617
Pancreatic duct injection	19 (47.5)	18 (45.0)	0.823
Pancreatic duct cannulation, twice or more	8 (20.5)	6 (15.0)	0.556
Post-ERCP pancreatitis	3 (7.5)	7 (17.5)	0.176
Final diagnosis			
BD stone	23 (57.5)	11 (27.5)	
SOD	3 (7.5)	10 (25.0)	0.082
Biliopancreatic tumors	5 (12.5)	9 (22.5)	
Others <sup>4</sup>	9 (22.5)	10 (25.0)	

<sup>1</sup>All data were presented as [n (%)] and comparisons were made with  $\chi^2$  or Fisher's exact test unless otherwise stated. <sup>2</sup>Data was presented as (mean ± SD) and comparison was made with Student's *t* test. <sup>3</sup>Data were presented as [median (interquartile range)] and comparisons were made with Mann-Whitney *U* test. <sup>4</sup>Primary sclerosing cholangitis, hydatid cysts communicating with the bile ducts, Mirizzi syndrome, and postoperative complications. Post-ERCP: Post-endoscopic retrograde cholangiopancreatography; SOD: Sphincter of Oddi dysfunction.

because of sphincterotomy was observed in three of 75 sphincterotomy patients (3.75%). All the bleeding was seen during the procedure. No case of delayed bleeding occurred. Two of three bleeding episodes in the control group and one in the diclofenac group were self-limited and stopped during endoscopy without intervention.

Pancreatitis occurred in 10/80 patients (12.5%), three of whom (7.5%) belonged to the diclofenac group and seven (17.5%) belonged to the control group (Table 1). Four and eight hours after endoscopy, the mean ± SE serum amylase level was 283.15 ± 82.74 IU/L and 308.34 ± 96 IU/L in the control group and 223.95 ± 35.45 IU/L and 218.39 ± 35.44 IU/L in the diclofenac group. Twenty-four hours after endoscopy, the mean ± SE serum amylase level was 231.56 ± 57.73 IU/L in the control group and 161.82 ± 31.03 IU/L in the diclofenac group (Table 3). In the diclofenac group, the mean values of amylase were low but the statistical difference was not significant ( $P > 0.01$ ).

## DISCUSSION

The number of ERCP procedures performed annually worldwide has increased dramatically over the past 25 years. Pancreatitis occurs in 1%-10% of patients but may approach ≥ 25% depending on the presence of other risk factors<sup>[15]</sup>.

Several mechanical and pharmacological interventions have been evaluated in the prevention of pancreatitis. The availability of effective drugs and strategy of chemoprevention are unresolved issues in the pharmacological prophylaxis of pancreatitis. Previous

**Table 2 Incidence of post-ERCP pancreatitis in different subgroups<sup>1</sup>**

	Diclofenac group (n = 40)	Control group (n = 40)	P
Variables			
Age group (yr)			
> 60	2/23	4/20	0.300
≤ 60	1/17	3/20	0.420
Sex			
Male	0/25	2/18	0.850
Female	3/15	5/22	0.092
Pre-cut sphincterotomy			
Yes	1/10	4/12	0.210
No	2/30	3/28	0.590
Pancreatic duct injection			
Yes	2/19	4/18	0.350
No	1/21	3/22	0.330
Pancreatic duct cannulation, twice or more	2/8	1/6	0.730
SOD			
Yes	2/3	2/10	0.150
No	1/37	5/30	0.047 <sup>1</sup>

<sup>1</sup>All data were comparisons with the  $\chi^2$  test. There were no statistically significant differences except in the patients without SOD.

**Table 3 Serum amylase level (mean ± SE, IU/L) following ERCP in diclofenac and control groups**

Group	Amylase 4 h	Amylase 8 h	Amylase 24 h
Diclofenac	223.95 ± 33.45	218.39 ± 35.44	161.82 ± 31.03
Control	283.15 ± 82.74	308.34 ± 96	231.56 ± 57.73

There were no statistically significant differences.

studies on reducing the incidence of pancreatitis have targeted reduction of pancreatic secretion, prevention of intra-acinar trypsinogen activation, interruption of the inflammatory cascades, relaxation of the sphincter of Oddi, and prevention of infection<sup>[21]</sup>.

An ideal agent is highly effective in reducing pancreatitis, is safe for the patient, well tolerated, relatively affordable, and does not have a prolonged administration time<sup>[1]</sup>. Various pharmacological agents (such as nifedipine, glucagon, calcitonin, lidocaine, nitroglycerine, antibiotics, steroids, allopurinol, interleukin-10, and heparin) have been tried, but have met with disappointing results in preventing pancreatitis in randomized controlled trials<sup>[1,22-34]</sup>.

Only two agents seem to offer any clinical benefit: the protease inhibitor gabexate mesilate<sup>[35-37]</sup> and the antisecretory agent somatostatin may be efficacious in preventing pancreatitis when given by continuous IV infusion<sup>[34,38,39]</sup>. Since these agents require continuous and prolonged IV infusion, they are not suited for same-day outpatient ERCP<sup>[34]</sup>.

Several prospective randomized studies have shown that pancreatic stents have a beneficial role for prevention of pancreatitis in high-risk patients, including biliary and pancreatic sphincterotomy for SOD<sup>[40-42]</sup>, biliary balloon dilation for stone<sup>[43]</sup> and precut biliary sphincterotomy<sup>[21]</sup>. Although pancreatic stenting is

often beneficial, the down sides include the difficulty of stent insertion in patients with small or tortuous ducts and the follow-up required for stent removal. A simple prophylactic medication would be highly desirable<sup>[21]</sup>.

NSAIDs may prevent pancreatitis by inhibiting prostaglandin synthesis and interrupting the inflammatory cascade of pancreatitis<sup>[34]</sup>.

In the report by Sotoudehmanesh *et al*<sup>[15]</sup>, eligible patients undergoing ERCP ( $n = 490$ ) were randomized to receive a 100-mg indomethacin rectal suppository ( $n = 245$ ) or placebo ( $n = 245$ ) just prior to ERCP, and rates of post-procedure pancreatitis were assessed. Pancreatitis occurred in 7/221 (3.2%) patients in the indomethacin group and in 15/221 (6.8%) of those receiving placebo ( $P = 0.06$ ), with an overall pancreatitis rate of 5% (22/442).

Montaño Loza *et al*<sup>[45]</sup> have reported a randomized prospective clinical trial that compared indomethacin with placebo in the prevention of pancreatitis. They enrolled patients undergoing ERCP for suspected bile duct obstruction rather than selecting for a high-risk cohort. Rectal indomethacin (100 mg) or placebo was administered prior to ERCP. Seventy-five patients were randomized to each group, a sample size that was calculated a priori to detect a 15% reduction in pancreatitis. The overall incidence of pancreatitis was 10.7%. The incidence of pancreatitis was 16% (12/75) in the placebo group and 5.3% (4/75) in the indomethacin group. This difference was statistically significant, with a  $P$  value of 0.034. All pancreatitis cases in both groups were categorized as mild<sup>[44,45]</sup>.

Diclofenac, an NSAID, inhibits phospholipase A<sub>2</sub>, which is thought to play a critical role in the early inflammatory cascade. In addition, it strongly inhibits neutrophil/endothelial attachment, thus preventing accumulation of neutrophils at the site of tissue damage<sup>[46]</sup>, and inhibits the expression of nitric oxide synthase, an enzyme associated with inflammation and cell damage<sup>[47]</sup>. It is a cheap, widely available agent with a short, easy method of administration.

Murray *et al*<sup>[9]</sup> have conducted a single-center, prospective, randomized, double-blind, placebo-controlled study to determine if a single dose of rectally administered 100 mg diclofenac, given after ERCP, reduced the incidence of pancreatitis. Of 220 patients, 110 received rectal diclofenac, and the others, an inert placebo. Pancreatitis occurred in 6.4% of patients in the diclofenac group and in 15.5% of those receiving placebo ( $P = 0.049$ ). This difference was statistically significant. Also, the drug was not effective in the subgroup of patients with SOD, the very group at highest risk<sup>[9]</sup>.

Khoshbaten *et al*<sup>[18]</sup> have reported a randomized controlled study that compared 100 mg rectal diclofenac with placebo in 100 patients who underwent high-risk ERCP. To select high-risk cases, only those undergoing pancreatography (with or without cholangiography) were enrolled. The study drug or placebo was administered on arrival in the recovery area. The overall incidence of pancreatitis was 15%. The incidence of pancreatitis

in the placebo group was 26% (13/50), whereas the incidence of pancreatitis in the diclofenac group was 4% (2/50). This difference was statistically significant, with  $P < 0.01$ . No patients in this clinical trial developed necrotizing pancreatitis or required surgical intervention<sup>[18,44]</sup>.

We showed that, in the diclofenac group, pancreatitis was seen less than in the control group, but this was not statistically significant. The absence of the statistical difference may have been caused by the small number of patients.

A number of risk factors for post-ERCP pancreatitis have been identified by a multitude of studies that have different study designs, have examined different candidate predictor variables, and have taken place in a variety of settings<sup>[15]</sup>. The impact of some of these associations has been supported by large, multicenter prospective trials, while others have been suggested in smaller series and by clinical experience. Risk factors that have been recognized as independent predictors in more than one study include: younger age, female sex, pancreas divisum, SOD, prior ERCP-induced pancreatitis, difficulty of cannulation, and pancreatic duct injection<sup>[15]</sup>. None of the patients in our study had pancreas divisum or prior ERCP-induced pancreatitis. Also, there was no significant difference in the incidence of pancreatitis when comparing diclofenac with placebo, in patients with younger age, female sex, SOD, pre-cut sphincterotomy and twice or more pancreatic duct cannulation ( $P > 0.01$ ). In our study, subgroup analysis showed that diclofenac significantly decreased the frequency of pancreatitis only in the patients without SOD. All the cases of pancreatitis were mild and the patients were discharged from the hospital within several days without any complication.

Acute pancreatitis is an unstable disease that causes intravascular fluid loss because of local and systemic inflammation. Clinical improvement can be achieved by fluid infusion. Fluid resuscitation is the most important treatment during the first 72 h after onset of acute pancreatitis. Therefore, the two goals of early phase fluid resuscitation are amelioration of tissue hypoxia and prevention of complications<sup>[48]</sup>.

In our study, IV isotonic saline was given to the diclofenac group in the initial 4 h (5-10 mL/kg per hour) after ERCP. Five hundred milliliters isotonic saline was given to the control group to keep the IV line open. Although the importance of fluid management in acute pancreatitis is known, there have not been so many studies about the prophylactic effects of this approach.

In conclusion, our study showed that parenteral diclofenac and hydration tended to prevent post-ERCP pancreatitis, but the finding was not statistically significant. In the whole group, diclofenac did not prevent the occurrence of pancreatitis but, according to the subgroup analysis, in patients without SOD, it significantly prevented pancreatitis. For this reason, further studies are required on the efficacy of this treatment with other doses and combinations of diclofenac and hydration that might prevent pancreatitis.

## COMMENTS

### Background

Pancreatitis is the most common complication of endoscopic retrograde cholangiopancreatography (ERCP). Pharmacological prevention of pancreatitis after ERCP has been the topic of several investigations in recent years.

### Research frontiers

Various pharmacological agents have been tried but have met with disappointing results in preventing pancreatitis in randomized controlled trials. Non-steroidal anti-inflammatory drugs may prevent pancreatitis by inhibiting prostaglandin synthesis and interrupting the inflammatory cascade of pancreatitis. The importance of fluid management in acute pancreatitis is known, but there have not been many studies about the prophylactic effects of this approach.

### Innovations and breakthroughs

The overall results of this study showed that parenteral diclofenac and fluid replacement had no beneficial effect on the prevention of pancreatitis. Although diclofenac and fluid replacement did not prevent the occurrence of post-ERCP pancreatitis, the rate of pancreatitis was lower in those patients without sphincter of Oddi dysfunction (SOD) who received diclofenac. To prevent post-ERCP pancreatitis, further studies should be carried with the other doses and combinations of diclofenac and hydration in a larger group of patients.

### Applications

This study was designed to evaluate the efficacy of prophylactic intramuscular diclofenac and fluid replacement for the prevention of post-ERCP pancreatitis.

### Peer review

This study aimed to find a pharmacological way for preventing post-ERCP pancreatitis. The results show that prophylactic intramuscular diclofenac and fluid replacement has no benefit except in patients without SOD.

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## Reaching proficiency in laparoscopic splenectomy

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### Abstract

**AIM:** To investigate the proficiency level reached in laparoscopic splenectomy using the learning curve method.

**METHODS:** All patients in need of splenectomy for benign causes in whom laparoscopic splenectomy was attempted by a single surgeon during a time period of 6 years were included in the study ( $n = 33$ ). Besides demographics, operation-related variables and the response to surgery were recorded. The patients were allocated to groups of five, ranked according to the date of the operation. Operation duration, complications, postoperative length of stay, conversion to laparotomy and splenic weight were then compared between these groups.

**RESULTS:** There was a significant difference regarding operation times between the groups ( $P = 0.001$ ). An improvement was observed after the first 5 cases. The learning curve was flat up to the 25th case. Following the 25th case the operation times decreased still further. There was no difference between the groups regarding the other parameters.

**CONCLUSION:** Unlike the widely accepted "L" shape, the learning curve for laparoscopic splenectomy is a horizontal lazy "S" with two distinct slopes. Privileges may be granted after the first 5 cases. However proficiency seems to require 25 cases.

### INTRODUCTION

Laparoscopic splenectomy has become the gold standard intervention for the removal of the spleen, especially for benign causes. However, the organ's high anatomic location, fragility and generous blood supply makes the procedure an advanced laparoscopic operation<sup>[1,2]</sup>. Furthermore, unlike patients with gall bladder stones, patients who need splenectomy for benign disorders are rare. These factors may prohibit the laparoscopic surgeon from becoming proficient in laparoscopic splenectomy. Measuring the expertise and setting a minimum number of procedures to be performed in order to be accepted as proficient in this rather rare operation has proved difficult.

One method for quantifying the level of expertise is to split the study population arbitrarily into two, as early and late experience<sup>[3,4]</sup>. Several variables are then compared between the two groups. A more sensitive method is to depict the learning curve. The learning curve may be briefly defined as the repetition of the procedure until it is learned<sup>[5]</sup>. In this study, we aimed to define the learning curve for laparoscopic splenectomy based on the experience of a single surgeon.

### MATERIALS AND METHODS

All patients with laparoscopically attempted splenectomy, operated on by a single surgeon (TZN), were included in this study. The time period was from November 2002 to January 2008. During this time span 33 patients (23 female) with a mean  $\pm$  SD age of  $43.4 \pm 18.4$  years were operated. Operative indications were immune thrombocy-

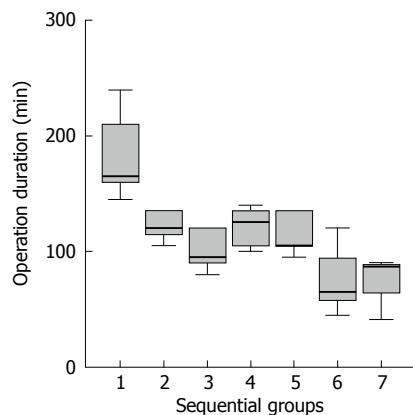
topenic purpura (ITP) ( $n = 27$ ), hemolytic anemia ( $n = 3$ ), thalassemia ( $n = 2$ ), and hydatid cyst ( $n = 1$ ). Besides demographics, associated disorders, the size of the spleen (long axis) as measured with preoperative ultrasonography, duration of surgery, the volume of gas used, additional procedures during operation, conversions to laparotomy, amount of intra/postoperative blood loss and blood products transfused, presence of accessory splenic tissue, complications, morsellated splenic weight, length of hospital stay, early and late success in hematological disorders and thrombocytosis in the long term were recorded.

Patients with hematological diseases were referred from the hematology clinic with resistant or persistent disease. The hydatid cyst patient had a type 3, 7-cm cyst in the spleen. He had percutaneous treatment for type 1 and 2 cysts located in the liver.

All patients received vaccination against encapsulated bacteria within 2 wk prior to operation. For ITP cases, the patients received pulse steroid or intravenous immunoglobulin therapy before the operation in order to increase the thrombocyte count to at least  $50\,000/\text{mm}^3$ . General anesthesia was used. A nasogastric tube was placed routinely. A lateral approach was used for the operation<sup>[6-8]</sup>. The patient was placed in a 70-80° right lateral position with reverse Trendelenburg for the table. The table was flexed 20-25° at the kidney rest to increase the left flank space. Following skin preparation and draping, the table was tilted to the left in order to obtain an approximately supine position for the initial insufflation. Four trocars, each 5 cm apart, were placed along an imaginary left subcostal incision with the most medial on the midline and most lateral on the left anterior axillary line. The table was again tilted to the original position. The 30° optic was introduced through the middle of the lateral 3 trocars. The stomach was manipulated through the most medial 4th trocar. The dissection was started from the inferior pole using clips and ultrasonic dissector for vascular control. Hilar vessels were controlled in the same manner, sometimes utilizing intracorporeal silk ligatures for the large-sized vessels. A vascular stapler was not used routinely: it was deemed necessary for only one patient with large-sized vessels. Lastly, short gastric vessels were controlled. After separating the lateral connections, the spleen was placed in a retrieval bag and was morsellated and removed through the lateral 10 mm trocar. The piecemeal specimen was weighed and sent for pathological assessment.

Patients were usually started on oral feeding the next day and discharged or transferred to the hematology clinic the following day. Hemoglobin and thrombocyte counts were studied on the first postoperative week and 3rd postoperative month to assess the early and late response to splenectomy for hematological diseases. Response to the surgery was defined as hemoglobin level greater than 100 g/L for hemolytic anemia and thrombocyte count of more than  $150\,000/\text{mm}^3$  for ITP. Thrombocytosis is defined as a thrombocyte count of more than  $600\,000/\text{mm}^3$ .

General descriptive characteristics of the group



**Figure 1** The learning curve for laparoscopic splenectomy based on the operation times. Groups are formed according to the date of the operation.

were expressed as mean  $\pm$  SD and percentages where necessary. In order to define the learning curve, patients were divided into groups of five ranked according to the date of operation (the last group consisted of 3 patients). Dichotomous variables such as rate of complications and conversion to laparotomy were compared between groups by  $\chi^2$  analysis. Continuous variables such as the duration of operation, the amount of gas used, splenic weight and length of hospital stay between ranked groups were compared using the ANOVA technique.  $P < 0.05$  was accepted as significant. All analyses were performed using SPSS version 13.0 (SPSS, Chicago, Illinois).

## RESULTS

In 18 (54.5%) patients the size of the spleen was less than 12 cm. The size was in the range of 12-20 cm in 14 (42.4%) patients and over 20 cm in one patient (3.0%). One third of the patients ( $n = 10$ ) had additional systemic diseases, diabetes mellitus ( $n = 4$ ) being the most common.

The immediate preoperative thrombocyte count for ITP cases was  $60\,960 \pm 77\,440/\text{mm}^3$ . Hemoglobin level for hemolytic anemia and thalassemia cases was  $92 \pm 38\text{ g/L}$ .

Only one patient (3%) was converted to laparotomy. In this patient (12th case, 3rd group) the spleen was large, over 20 cm and weighed 1 040 g. Hemorrhage could not be controlled and the case was converted at the 105th min. Erythrocyte suspension (5 units) was transfused to 3 hemolytic anemia patients (9.1%) during the perioperative period. Thrombocyte transfusion was not needed.

The mean operation duration for the whole group was  $120.2 \pm 46.2$  min. There was a significant difference between ranked groups regarding operation time ( $P = 0.001$ ). The last two groups' operation time, i.e. after the 25th case, was less than the first 5 cases according to the Bonferroni post hoc test (Figure 1).

Accessory spleens were detected and removed in 5 patients (15.2%). The average amount of CO<sub>2</sub> used was  $137.5 \pm 80.7\text{ L}$ . There was no difference between ranked groups regarding CO<sub>2</sub> use ( $P = 0.119$ ). Postoperative complications were observed in 3 patients (one atelectasis,

one wound infection and one suture reaction). Early and late success rates were 83.9% and 81.0%, respectively, and were not significantly different between the groups. Thrombocytosis was observed in 5 patients (15.2%). Antiaggregant therapy with salicylic acid was instituted for these patients.

## DISCUSSION

There has been a rush for minimal access surgery with the introduction of laparoscopic cholecystectomy in the 1980s. However, the surgical community was not technically prepared for this uncontrolled demand. Although weekend courses, seminars and hands-on courses were held worldwide, minimal access surgery education was less than optimal<sup>[9-11]</sup>. Surgical societies, not wishing to repeat these mistakes, started regular courses for both basic and advanced laparoscopic procedures. Universities and teaching hospitals included these procedures in their curricula. Several societies advocated a minimum number of procedures to be set to grant privileges to perform an operation<sup>[12]</sup>.

It is difficult to define a point when mastery of a procedure is reached. Subjective assessment in this regard is unreliable and several objective methods have been devised for this task. One simple method is to compare the early with late experience. For this, a chronologically-ranked patient population is divided in two<sup>[4]</sup>. Markers of expertise such as operation time, complication rate, conversion rates (in case of laparoscopy), time to oral feed and cost are then compared between these arbitrarily separated groups. The learning curve, which is more sensitive, usually is a graphic representation of the expertise level reached for a particular procedure/process. The cases are individually ranked on a chronological basis and are placed on one axis of the graph. The above-mentioned variables are depicted on the other axis. The initial slope of the curve is usually steep where the learning process is fast and with each case performance increases. After a while, the slope flattens and no major improvement can be observed following a certain number of the procedures. The point, the nth case, where the curve starts to flatten is accepted as the instance where the procedure is learnt. Even after this point minor improvement in performance is detected, albeit the difference is usually not significant. A variation of this technique is to allocate patients into small groups and compare these groups with regard to determined variables. Similarly there is a point when a significant difference cannot be observed, which is accepted as the number of procedures needed to perform to reach proficiency.

Since the first report of laparoscopic splenectomy by Delaitre, this procedure has become the preferred treatment for the removal of the spleen<sup>[13-15]</sup>. However, this being one of the advanced laparoscopic procedures and due to the relatively rare occurrence of the conditions requiring splenectomy, the procedure is difficult to master.

Several studies have defined the minimum number of laparoscopic splenectomies to be performed for proficiency. Operation time is the most used variable

as it is easier to calculate and validate. In the study by Rege and Joehl, these authors suggested that an improvement occurred with the first 15-25 patients<sup>[3]</sup>. In a review generally comparing the results of initial and late experience for laparoscopic procedures, it was determined that 20 laparoscopic splenectomies were sufficient for mastery<sup>[5]</sup>. In the study by Peters *et al*<sup>[16]</sup>, a minimum of 20 laparoscopic splenectomies were deemed necessary for ITP surgery proficiency. Regarding pediatric surgery, 20 cases, similarly, were declared to be the threshold for proficiency<sup>[17]</sup>.

However, there are some design limitations of the above-mentioned studies. In the reports by Rege and Chan the patient populations were split in two; an initial first 15, and those that came later<sup>[3,4]</sup>. The cut-off point was chosen arbitrarily and compared only two groups, i.e. the initial and late experience, which may decrease the sensitivity as stated previously. Further, in order to document an accurate progress and exclude personal variations, the performance of a single surgeon should be recorded. If other surgeons' experiences are included, the investigated parameters may differ according to the individual's skill. Among the above-mentioned studies, the experience of a single surgeon was assessed in only one report<sup>[17]</sup>. Among the rest of the reports, either senior residents gradually started to perform the operations or different hospitals/surgeons were included in the analyses<sup>[3,16,18]</sup>. The concern about inclusion of low-volume surgeons in such assessments has also been previously stated<sup>[19,20]</sup>. Although Cordera *et al*<sup>[20]</sup> have attempted some 42 laparoscopic splenectomies, rightfully they have not calculated a learning curve due to inclusion of several surgeons. Another potential source of bias is the introduction of new devices or technology during the study period. For instance, an ultrasonic dissector was introduced during the latter course of the study in one report<sup>[4]</sup>.

In our study the experience of a single surgeon was assessed to exclude personal variations in skill. In order to eliminate the possible confounding effects of the mechanical set-up, standard techniques and instruments were used during the course of the study. Instead of comparing the arbitrary initial and late experience we have assessed the differences between sequential multiple groups. Twenty to twenty-five laparoscopic splenectomies were cited to be the minimum number for mastery of the procedure in the literature. However, it is common knowledge that privileges are granted with far less experience of this relatively rare operation. Usually 3 to 5 laparoscopic splenectomies, arbitrarily defined, are accepted to be sufficient<sup>[3]</sup>. However, there is no evidence in the literature supporting this figure. We provide the first evidence that 5 laparoscopic splenectomies could be accepted as the minimum number for the general curricula. There is a dual curve in our study of the learning process (Figure 1). Progress is rapid with the first 5 cases and then the curve steadies up to the 25th case. At that point there is again a significant increase in the performance i.e. a decrease in the operation time.

We have shown that the learning curve for laparo-

scopic splenectomy is not a smooth "L" but has two distinct slopes. With the first 5 cases the novice may be granted privileges for performing laparoscopic splenectomy on an individual basis. Past the 25th case the surgeon could be accepted as an expert in the field. Although unlikely, it is yet to be shown that any further improvement could be observed after this level.

## COMMENTS

### Background

Laparoscopic revolution resulted in some controversies regarding the education in advanced laparoscopic surgery. In order to assess the level of expertise and to grant privileges in surgery, a learning curve of the procedure is used.

### Research frontiers

Laparoscopic splenectomy has become the gold standard intervention for the removal of the spleen, especially for benign causes. In this study, the authors have investigated the proficiency level reached in laparoscopic splenectomy using the learning curve method.

### Innovations and breakthroughs

Unlike the widely accepted "L" shape, the learning curve for laparoscopic splenectomy is a horizontal lazy "S" with two distinct slopes. Privileges may be granted after the first 5 cases. However, proficiency seems to require 25 cases.

### Applications

The authors have shown that the learning curve for laparoscopic splenectomy is not a smooth "L" but has two distinct slopes. With the first 5 cases the novice may be granted privileges for performing laparoscopic splenectomy on an individual basis. Past the 25th case the surgeon could be accepted as an expert in the field.

### Terminology

The learning curve is a graphic representation of the expertise level reached for a particular procedure. This is briefly defined as the repetition of the procedure until it is learned. Reaching proficiency is defined by measuring the expertise and setting a minimum number of procedures to be performed in order to be accepted as proficient.

### Peer review

It is an interesting topic for the readers. It should be accepted for publication.

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## Splenectomy with chemotherapy vs surgery alone as initial treatment for splenic marginal zone lymphoma

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### Abstract

**AIM:** To evaluate the clinical characteristics of splenic marginal-zone lymphoma (SMZL) following antigen expression and the influence of therapeutic approaches on clinical outcome and overall survival (OS).

**METHODS:** A total of 30 patients with typical histological and immunohistochemical SMZL patterns were examined. Splenectomy plus chemotherapy was applied in 20 patients, while splenectomy as a single treatment-option was performed in 10 patients. Prognostic factor and overall survival rate were analyzed.

**RESULTS:** Complete remission (CR) was achieved in 20 (66.7%), partial remission (PR) in seven (23.3%), and lethal outcome due to disease progression occurred in three (10.0%) patients. Median survival

of patients with a splenectomy was 93.0 mo and for patients with splenectomy plus chemotherapy it was 107.5 mo (Log rank = 0.056,  $P > 0.05$ ). Time from onset of first symptoms to the beginning of the treatment (mean 9.4 mo) was influenced by spleen dimensions, as measured by computerized tomography and ultra-sound ( $t = 2.558$ ,  $P = 0.018$ ). Strong positivity (++) of CD20 antigen expression in splenic tissue had a positive influence on OS (Log rank = 5.244,  $P < 0.05$ ). The analysis of factors interfering with survival (by the Kaplan-Meier method) revealed that gender, general symptoms, clinical stage, and spleen infiltration type (nodular vs diffuse) had no significant ( $P > 0.05$ ) effects on the OS. The expression of other antigens (immunohistochemistry) also had no effect on survival-rate, as measured by a  $\chi^2$  test ( $P > 0.05$ ).

**CONCLUSION:** Initial splenectomy combined with chemotherapy has been shown to be beneficial due to its advanced remission rate/duration; however, a larger controlled clinical study is required to confirm our findings.

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**Key words:** Splenic marginal zone lymphoma; Chemotherapy; Splenectomy; Clinical outcome

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### INTRODUCTION

Splenic marginal-zone lymphoma (SMZL) is an indolent B-cell lymphoma, generally presented with splenomegaly, and frequent involvement of the bone marrow and

peripheral blood. The typical immunophenotypic profile is: IgM+, IgD+/-, cytoplasmic Ig-/+; pan B antigens+, CD5-, CD10-, CD23-, CD43-/+; and cyclin D1-. It is characterized by micronodular infiltration of the spleen with marginal-zone differentiation<sup>[1-4]</sup>. An SMZL variant with villous lymphocytes (SMZL + VL) has been previously described as "splenic lymphoma with villous lymphocytes", and included into the FAB classification of chronic B-cell leukemias<sup>[5]</sup>. Lymphomas of the marginal zone are recognized as separate clinical phenomena amongst B-cell NHL in the REAL classification. Thus, SMZL was divided from other marginal zone lymphomas, such as mucosa associated lymphoid tissue (MALT) lymphoma or nodal B-type marginal zone lymphoma, and accepted by WHO classification in 2001 as a distinct clinical and pathohistological (PH) entity<sup>[6-9]</sup>. According to more recent data, SMZL and SMZL with or without villous lymphocytes (SMZL ± VL) are two phases (tissue and leukemic) of the same disease<sup>[10,11]</sup>.

Typical genetic abnormalities in SMZL are deletions at 7q22-7q32<sup>[8]</sup>. The majority of SMZL patients have good long-time survival. Standard prognostic factors cannot differentiate patients into groups with poor or high-quality clinical outcomes. However, several immune-mediated events, such as hemolytic anemia and thrombocytopenia, as well as the presence of the serum monoclonal component, could be predictive factors for survival-rate. Splenectomy is considered the first-line treatment for SMZL patients<sup>[12,13]</sup>. Even if this therapy results just in partial remission (PR), surgery-response is usually sufficient to correct (pan) cytopenia and also to improve the patient's quality of life and overall survival-rate. The presence of SMZL in peripheral lymph nodes and extranodal locations is uncommon. Thus, the spleen is considered the site of lymphoma origin, even if there were regional enlargements of the lymph nodes<sup>[9-11]</sup>. The frequency of mutation in the 5' non-coding region of the *bcl-6* gene has been used as a marker of germinal center derivation, which might be used to establish the molecular heterogeneity<sup>[14-16]</sup>. Therefore, SMZL is a primary disease of the spleen, with subsequent bone marrow (BM) and peripheral blood (PB) involvement<sup>[17-19]</sup>. The diagnosis is based on the spleen PH, in accordance with clinical data. Incorporation of immunophenotypic profiles and molecular characteristics into BM and PB morphology, improves the diagnostic validity. SMZL is an indolent lymphoma, although there is a small subset of patients with an aggressive clinical course<sup>[20-22]</sup>. Studies on this entity have been aggravated by the fact that the disease is very rare<sup>[23]</sup>.

The purpose of this pilot study is to show PH features, as well as clinical data and follow-up in splenectomy with chemotherapy vs surgery alone treated SMZL ± VL patients.

## MATERIALS AND METHODS

### **Patients**

The study group included 30 patients with SMZL ± VL. Splenectomy plus chemotherapy was applied to 20 patients, while splenectomy as a single treatment-option

was performed for ten patients. The follow-up time was 12 years (1994-2006). Diagnosis was established and confirmed after initial splenectomy with consecutive PH and immunohistochemical (IHC) analysis, only upon consensus of two independent hematopathologists. Criterion of SMZL + VL was more than 20% of such lymphocytes in the peripheral blood<sup>[1]</sup>. Clinical stage (CS) was determined according to the Ann Arbor staging classification<sup>[24]</sup>. The following clinical characteristics were analyzed: sex, age, constitutional "B" symptoms, CS, and time from the onset of first symptoms to the beginning of treatment. Thereafter, complete blood count and standard biochemical analyses were done as follows: serum iron (sFe), ferritin, lymphoma activity parameters: lactate dehydrogenase (LDH) and beta-2-microglobulin (β-2M), serum paraprotein presence (M-component), virological analysis (hepatitis B, C and HIV markers). The size of the spleen was determined by ultrasound (US) or computerized tomography (CT). Its weight in grams was also measured after spleen removal.

### **Histology and immunohistochemical analyses**

Diagnosis was based on analysis of tissue samples (spleen, lymph node, and BM) according to criteria of WHO classification system. All tissue samples were fixed in B5, processed by standard methods, embedded in paraffin, cut by a microtome (4 μm sections) and stained by classical staining methods: hematoxylin eosin (HE), Giemsa and reticulin (Gordon-Sweet).

Immunostaining was performed using a labeled streptavidin-biotin procedure with monoclonal antibodies (DAKO, Glostrup, Denmark). The relevant antibodies used in routine diagnosis were: LCA, EMA, IgD, IgM, CD20, CD79-α, CD5, CD23, CD43, CD10, bcl-2, CD3, Cyclin D-1, and Ki-67.

The strength of CD20 antigen (Ag) expression was graduated semi-quantitatively as strong (+++), moderate (++) or weak (+) according to cell positivity, almost in all cells, more than 50%, and in less than 50% of cells, respectively. For other antigens, expression was graded as, positive (+) or negative (-), whilst Ki-67 expression was evaluated numerically as a percentage of positive cells. Splenic marginal zone areas were selected, focusing in all cases on the areas with the highest growth fraction. Preparations of spleen, lymph nodes and BM were analyzed by standard light microscopy.

### **Drug-treatment**

The initial treatment in all (30) patients was splenectomy. The CHOP (cyclophosphamide, hydrodoxorubicine, vincristin, and prednisolone) protocol was applied in nine (45.0%) cases. Fludarabine containing regimens (FMD - fludarabine, mitoxantrone, dexamseone; and FMC - fludarabine, mitoxantrone, cyclophosphamide) were used in 11 (55.0%) cases. The decision to give additional chemotherapy was made according to the presence of constitutional "B" symptoms at presentation and lymphadenopathy. Two types of applied treatment (splenectomy alone or splenectomy with chemotherapy) in a 3-year follow-up period were analyzed for every patient.

### Statistical analysis

Among descriptive analysis, the arithmetical mean and standard deviation were used for parametric data and the median was used for description of non-parametric data. For the parametric analytical model, we applied Student's *t*-test. For non-parametric analytical models, we used Pearson's  $\chi^2$  tests, Fisher's exact test, Kolmogorov-Smirnov's test, and Kaplan-Maier's method for analysis of OS. For significance,  $\alpha$  errors of 0.05 were chosen in all methods. SPSS version 6.0 for Windows was used to create a database. Statistical analyses were completed within the statistics package of the Institute of Medical Statistics and Information Technology, School of Medicine, Belgrade.

## RESULTS

### Patient's characteristics

There were 11/30 (36.7%) males and 19/30 females (63.3%). The mean age was 58 years (range, 33-76). Performance status according to the Eastern Cooperative Oncology Group (ECOG) scale was 0 = 18%, 1 = 56%, and 2 = 26%. SMZL was diagnosed in 18 patients (60.0%), and SMZL + VL was found in 12 (40.0%) cases. The patients' general clinical features and hematological characteristics included in this study are summarized in Table 1.

Furthermore, HIV 1 and 2 antibodies were all negative (100%). Hepatitis B antibodies were also negative in all patients. Hepatitis C antibodies were positive in only one case (3.3%).

The mean sFe level was 7.24  $\mu\text{g/L}$  (range, 2.20-16.2). Decreased values below absolute in relation to gender were recorded in 19 (63.3%) patients, and normal were reported in 11 (36.7%) subjects. Relative iron deficiency might be explained in two ways. Firstly, iron resorption was reduced due to loss of appetite with an increase of passive intestinal hyperemia on account of splenomegaly. Secondly, more intensive loss of the same oligo-element was stimulated by hemorrhage, (most often occult). Ferritin levels were within normal limits in relation to gender in all subjects.

### Immunohistochemical findings

A total of 30 (100%) patients with typical histological and immunohistochemical splenic marginal zone lymphoma pattern were examined (Figure 1).

Immunohistochemical analysis revealed that none of the positive expressing antigens had a significant influence on disease outcome, as assessed using a  $\chi^2$  test with the following confirmed results: CD79-alfa ( $\chi^2 = 5.074$ ,  $P > 0.05$ ), CD20 ( $\chi^2 = 4.046$ ,  $P > 0.05$ ), CD43 ( $\chi^2 = 0.910$ ,  $P > 0.05$ ), IgD ( $\chi^2 = 2.503$ ,  $P > 0.05$ ), IgM ( $\chi^2 = 1.147$ ,  $P > 0.05$ ), bcl-2 ( $\chi^2 = 3.667$ ,  $P > 0.05$ ), and Ki-67 ( $\chi^2 = 2.503$ ,  $P > 0.05$ ). Moreover, Ki-67 positivity varied from 5%-35%, with following distributions: 5%-15%, 15%-25%, and 25%-35% in nine (30.0%), 14 (46.7%) and seven patients (23.3%), respectively.

Using the long rank test in the Kaplan Mayer method of survival showed that among all antigens, only CD20 antigen had some impact on OS. Namely, among three groups of patients according to CD20 positivity (strong,

**Table 1 Clinical and hematological characteristics of SMZL ± VL patients**

Clinical stage	n (%)
I E A+B	4 (13.3)
II A+B	0
III A+B	0
IV A+B	24 (80.0)
V A+B	2 (6.7)
Spleen-specific data	
US length of spleen (cm)	Range 6-32 Mean 22.72
US width of spleen (cm)	7-18 10.00
CT length of spleen (cm)	7-27 22.00
CT width of spleen (cm)	6-17 10.00
Spleen weight after surgery (g)	50-8000 2236
Spleen infiltration: nodular vs diffuse	18 (60.0) vs 12 (40.0)
Hb (g/L)	79-131 110
Anemia (Hb < 100 g/L)	27 (90.0)
Leukocytosis (WBC > 10 $\times 10^9/\text{L}$ )	10 (33.3)
Leukocytopenia (WBC < 4 $\times 10^9/\text{L}$ )	9 (30.0)
Neutropenia (Ne < 1.5 $\times 10^9/\text{L}$ )	16 (53.3)
Lymphocytosis (Ly > 5 $\times 10^9/\text{L}$ )	17 (56.7)
Thrombocytopenia (Plt < 150 $\times 10^9/\text{L}$ )	18 (60.0)
Thrombocytosis (Plt > 400 $\times 10^9/\text{L}$ )	5 (16.7)
Serum paraprotein (determined)	23 (76.7)
IgG lambda (found)	1 (3.3)
LDH elevated (> 320 U/L)	8 (26.7)
$\beta$ -2M elevated (> 1.8 $\mu\text{g/L}$ )	25 (83.3)

Hb: Hemoglobin; WBC: White blood cell; Ne: Neutrophil granulocyte; Ly: Lymphocyte; Plt: Platelet; LDH: Lactate dehydrogenase;  $\beta$ -2M:  $\beta$ -2-microglobulin.

moderate, weak), median survival was 113 mo, 83 mo, and only 43 mo, respectively (Log rank = 5.244,  $P < 0.05$ ).

### Prognostic factor analysis and overall survival

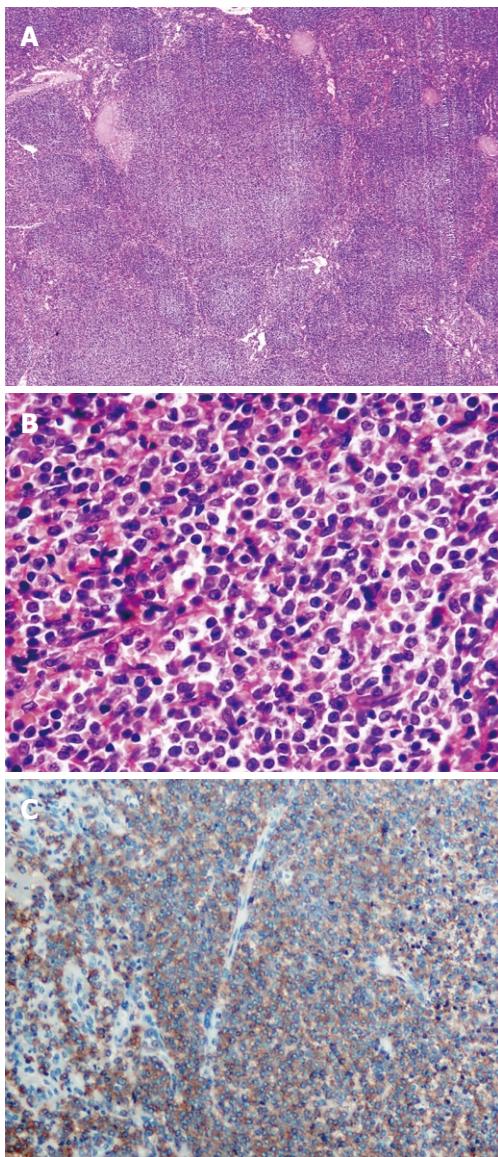
In this study, all the patients were splenectomized at the beginning of treatment. Therapeutic splenectomy, i.e. as the only curative modality in 10 (33.3%) patients was performed. Splenectomy with chemotherapy (different protocols) was done in the remaining 20 (66.7%) patients. After a mean three-year follow-up, the outcome was as follows: 20 (66.7%) patients had complete remission (CR), seven (23.3%) had partial remission (PR), and three (10.0%) died due to disease progression.

Out of 10 patients having undergone splenectomy only, eight (80.0%) had CR and two (20.0%) had significant PR (76 mo). In addition, all CR were stable after five years (60 mo). No patient with spleen rupture, a rare but possible complication, was reported.

Out of those receiving chemotherapy after splenectomy (20 patients), CR was achieved in 11 (55.0%) patients. The CHOP protocol was used in nine (45.0%) cases, and FMD or FMC were used in 11 (55.0%) cases. Ultimately, the mode of treatment was not a factor interfering with OS of our patients. Median survival of patients with splenectomy only was 93.0 mo and in patients with splenectomy and chemotherapy it was 107.5 mo (Log rank = 0.056,  $P > 0.05$ ).

The cumulative survival of patients included in this study is presented in Figure 2.

As shown, after 120 mo of follow-up, the incidence of survival was constant (about 77%). Time from onset

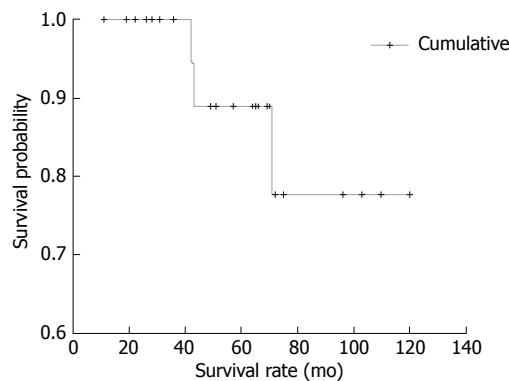


**Figure 1 SMZL-splenic tissue.** A: Nodular infiltration (HE,  $\times 40$ ); B: Neoplastic cells with monocyte-like morphology (HE,  $\times 400$ ); C: Immunohistochemistry for CD20 $^{+}$ , brown staining in lymphoma cells (streptavidin-biotin,  $\times 200$ ).

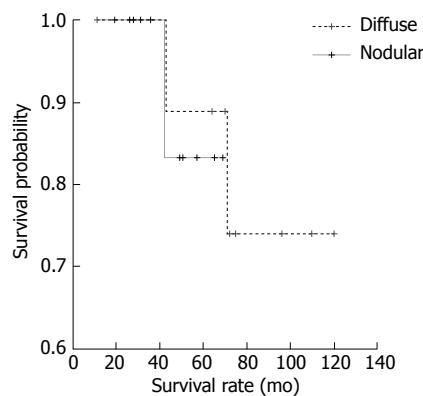
of first symptoms to the beginning of treatment (mean 9.4 mo, range 1-84) was influenced by spleen dimension, as measured by CT and US, and was significantly shorter in patients with higher spleen dimensions ( $t = 2.558$ ,  $P = 0.018$ ). The highest mean values of segmented neutrophil percentage were found in subjects who reached CR, and conversely, the lymphocyte percentage mean values were lowest in those who achieved CR ( $P = 0.026$ ).

Spleen dimensions measured by CT also correlated with clinical stage ( $P = 0.034$ ).

The analysis of factors interfering with survival, as measured by the Kaplan-Meier method, revealed that gender was not a factor affecting the length of survival (Log rank = 0.643,  $P > 0.05$ ). Further analysis of such factors showed that the presence of "B" symptoms also had no effect on survival (Log rank = 0.141,  $P > 0.05$ ). Moreover, CS was not a factor affecting patient survival (Log rank = 0.560,  $P > 0.05$ ). Similarly, the type of spleen infiltration (nodular vs diffuse) was not a factor



**Figure 2** Cumulative survival of patients investigated (with constant incidence of survival after 120 mo).



**Figure 3** Patients' survival with different type of spleen infiltration (nodular vs diffuse).

**Table 2** Results of Cox regression method and significance of compared factors of treatment outcome in our patients

Variables	Score	df	P
Gender	0.244	1	0.621
Age	0.657	1	0.418
Time from the onset of B symptoms to treatment	13.906	10	0.177
CS	0.557	2	0.757
Spleen infiltration (nodular vs diffuse)	0.009	1	0.923
Mode of treatment	0.089	1	0.900

affecting the survival of patients (Log rank = 0.021,  $P > 0.05$ ), as shown in Figure 3.

Finally, the analysis of lethal outcome predictors by the Cox regression model included the role of factors that could have any effect on such an outcome (Table 2). As illustrated, none of the studied factors appeared to be significant for predicting treatment outcome in our group of patients.

## DISCUSSION

Splenic marginal zone lymphoma is a relatively rare entity with a slight male predominance. In our group of patients there was higher incidence of female - 19 (63.3%), in relation to male - 11 (36.7%), as compared to other studies<sup>[25,26]</sup>. The mean patients' age was 58.2 years, which agreed with the fact that splenic lymphoma is a

disease of older age<sup>[1,7]</sup>.

A majority of the SMZL cases studied carried a non-mutated bcl-6 gene<sup>[14]</sup>. The frequency of these mutations in normal spleen confirms previous findings on the hypermutation IgVH process in normal B-cell populations<sup>[27,28]</sup>. This data supported the existence of molecular heterogeneity in this entity. It also favored the hypothesis that, in spite of initial morphological observations, a significant proportion of SMZL cases could derive from an non-mutated naive precursor, which is different from those of the marginal zone, and possibly located in the mantle zone of splenic lymphoid follicles. Thus, the marginal zone differentiation of these tumors could be related more to the splenic microenvironment than to the histogenetic characteristics of the tumor<sup>[20]</sup>.

Considering PH findings, in our group there were 18 patients (60.0%) with SMZL, and 12 (40.0%) with SMZL + VL. The largest number of patients had advanced disease in clinical stage IV + V (86.7%). The shortest time of the onset of first symptoms to the beginning of treatment was one month, and the longest was 84 mo. The average was 9.4 mo it was correlated with spleen dimension, as measured by CT and US. It was significantly shorter in patients with higher spleen dimensions. SMZL is a slow-course disease and is detected in progressive phase in the highest percentage<sup>[7,20]</sup>. In accordance with this, Thieblemont found BM infiltration in 95% of patients<sup>[11]</sup>. Almost all (97%) patients had clinical stage III and IV, as reported in the large cohort of patients published by Arcaini<sup>[25]</sup>, with a similar proportion of about 90% of BM infiltration in a series of 129 patients<sup>[30]</sup>. It was apparent that the majority of patients in our study were in clinical stage IV.

Splenomegaly lasted approximately 9.4 mo before diagnosis (interval of 1-36 mo), referring to a study in 1999<sup>[26]</sup>, which is similar with our results. Another study on 18 patients established an average pre-treatment presence of symptoms to be four months (two-six month interval)<sup>[31]</sup>. Similarly, a series of 17 patients reported pre-treatment duration of symptoms varying from several days to four months with a mean time of 2.1 mo<sup>[9]</sup>.

The finding that spleen dimension correlated with clinical stage, as well as with the time from onset of first symptoms to the beginning of treatment, is rational since the dominant tumor mass is in the spleen. The maximal weight of the spleen measured intraoperatively was 8000 g and minimal weight was 450 g (mean = 2235.7 g). Such a finding suggested that there was at least one a case with a huge splenomegaly, rarely seen in foreign literature<sup>[23,26]</sup>. The pattern of spleen infiltration (nodular vs diffuse) was not significant for OS in our group of patients. According to the literature, 85% of patients with relapsed or progressive disease have nodal involvement, with a relatively low frequency of nodal involvement at initial diagnosis<sup>[25]</sup>. This emphasizes the care necessary in sorting nodal involvement of SMZL from primary nodal MZL, and the necessity of differential diagnosis with additional molecular markers.

We found that 27 (90.0%) patients manifested a lower or higher degree of anemia. Anemia is strongly characteristic of spleen lymphoma. It occurs due to

hypersplenism rather than BM infiltration<sup>[1,7]</sup>. In a series of 81 patients reported by Thieblemont, 44 with spleen lymphoma had anemia and of these, 13 had Coombs-positive hemolytic anemia<sup>[11]</sup>. Similar results, with about half of the patients being anemic, were published for a large group of 309 patients<sup>[25]</sup>.

Despite the presence of anemia (Hb ≤ 110 g/L) in about 30% of patients with SMZL ± VL with mean value of hemoglobin 118 g/L<sup>[30]</sup>, the percentage of anemic cases in our patients was significant higher, due to the predominance of advanced clinical stage. Leukocytosis and leukocytopenia were found in about a third of the patients, whilst half of the patients had neutropenia and lymphocytosis. Such percentage of cells, rather than platelet count, in our patients was of no prognostic value regarding the survival period. A higher rate of achieving CR was found in patients with higher initial segmented neutrophil count and with a lower level of lymphocytes at presentation.

The distribution of serum paraprotein has varied from 8%<sup>[25]</sup>, which was comparable to our results, to as much as 46%<sup>[11]</sup>. In a third large series of 129 patients with spleen lymphoma, paraprotein was present in 22% of patients, with the highest concentration of 25 g/L, but without any prognostic value<sup>[30]</sup>. Contrastingly, Arcaini reported that the presence of paraprotein had prognostic value in terms of shorter time to disease progression<sup>[32]</sup>.

The established positivity of hepatitis C virus antibodies varied from 1%<sup>[11]</sup>, which was similar to our results, to 19%, while hepatitis B virus antibodies were detected in 5% of cases<sup>[25]</sup>.

Serum LDH is an important activity parameter of aggressive disease, while in low-aggressive ones, its increase can designate the transformation of disease to more severe form<sup>[18]</sup>. In our study group, this parameter was within referential limits in 72% of patients, which suggested low-grade disease status. On the other hand, Chacon reported about 60% patients with significantly elevated LDH<sup>[23]</sup>.

In our patients, β-2M was predominantly elevated (83.3%), ranging from 2.38 to 7.4 μg/L. The results are in accordance with the established fact that this parameter is increased in SMZL as a negative prognostic marker<sup>[33,34]</sup>.

Regarding the type and outcome of treatment, our results indicated that the use of adjuvant chemotherapy following the splenectomy had no influence on OS rate, as reported in published series so far<sup>[1,7,23]</sup>. This finding is in accordance with the fact that the use of alkylating drug therapy yields a rate of response of about 44%, but complete remissions are rare, probably due to the existing BM infiltration<sup>[1,7]</sup>. Until now, the treatment of SMZL has been controversial. In all large series, a significant group of patients received no therapy. These patients do not seem to have worst outcome than those initially treated. For these reasons, and assuming that SMZL is an indolent disease, some authors recommend a “watch and wait” approach<sup>[23]</sup>. Although, in general SMZL behaves as an indolent disease, there is a significant group of patients who died from the disease in a relatively short time period. The role of chemotherapy is still a matter of debate. One

of the striking findings is the relatively low percentage of patients who attain CR after chemotherapy, because of the presence of BM involvement after chemotherapy<sup>[23]</sup>. The literature presents diverse chemotherapy regimens for SMZL. Generally, splenectomy leads to somatic compensation of patients, rendering it impossible for local relapse in the spleen, prevents continuous dissemination from the primary tumor site, and mostly corrects cytopenias, creating better conditions for chemotherapy<sup>[35]</sup>.

SMZL is a relatively indolent disease, but in some cases it displays more aggressive behavior, which should stimulate the search for predictive biologic factors and alternative therapies. Early splenectomy combined with chemotherapy in properly stratified patients at presentation has been shown to be beneficial because of improvement in remission rate/duration and superior OS. However, there was no statistical significance, probably due to the limited number of patients. Thus, the evaluation of these therapeutic approaches requires larger, randomized, and controlled clinical studies.

## COMMENTS

### Background

Splenic marginal-zone lymphoma (SMZL) is a relative uncommon low-grade lymphoma and primary disease of the spleen, with bone marrow and peripheral blood involvement. The existence of molecular heterogeneity in this entity gave additional results in favor of the hypothesis that, in spite of initial morphological observations, most SMZL cases could derive from a non-mutated naive precursor, different from those of the marginal zone, and possibly located in the mantle zone of splenic lymphoid follicles. Thus the marginal zone differentiation could be related more to the splenic microenvironment than it is to the histogenetic characteristics of the tumor. Until now, standard prognostic factors could not differentiate patients into groups with poor or favorable clinical outcome.

### Research frontiers

Despite the evaluation of different prognostic factors, the treatment of SMZL is still controversial. However, several immune-mediated events, such as hemolytic anemia and thrombocytopenia, as well as the presence of the serum monoclonal component, could be predictive factors for survival-rate. Splenectomy is considered the first-line treatment. Even when this therapy results in partial remission, the response to the surgery is usually sufficient to correct cytopenia and also to improve the patient's quality of life, as well as overall survival-rate.

### Innovations and breakthroughs

SMZL is an indolent lymphoma, although there is a small subset of patients with an aggressive clinical course. Despite this fact, there have been significant groups of patients who have received no therapy, with only a "watch and wait" approach being adopted to their indolent clinical course. Early splenectomy combined with consecutive chemotherapy leads to somatic compensation of patients, renders it impossible for local relapse in the spleen, prevents continuous dissemination from the primary tumor site, commonly corrects cytopenias, and improves the survival-rate.

### Applications

This data shows that early splenectomy combined with chemotherapy at presentation leads to some improvements in duration of overall survival, although with no statistical significance, probably due to the limited number of patients. Therefore, future approaches will need well controlled and larger clinical studies.

### Peer review

In this manuscript, the authors delivered some interesting data on clinical-pathological features and clinical outcomes of 30 SMZL patients. They confirm that early splenectomy, at the beginning of treatment, combined with chemotherapy is helpful to prevent disease recurrence. The data presented might be beneficial for clinical treatment of patients with SMZL.

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BRIEF ARTICLES

## Dysregulation of gastric H,K-ATPase by cigarette smoke extract

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**CONCLUSION:** Administration of cigarette smoke extract is associated with an increase in the amount and activity of H,K-ATPase and hence, smokers are susceptible to development of peptic ulcer.

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**Key words:** Proton pump; H,K-ATPase; Parietal cell; Gastric gland; Oxytic mucosa; Cigarette smoke extract; Smoking

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### Abstract

**AIM:** To test whether the expression and activity of H,K-ATPase in parietal cells would be affected by cigarette smoke extract.

**METHODS:** Extracts of cigarette smoke were administered into mice by gastric gavage (5 mg/kg body weight/day) for 3 d or in drinking water for 7 or 14 d. For the latter, each day a mouse consumed 5 mL water containing extracts of two cigarettes, on average. Control littermate mice received only vehicle. To compare the amount of H,K-ATPase in control and smoke-treated mice, the stomach was processed for Western blotting and immunohistochemical analysis using monoclonal antibodies specific for  $\alpha$ - or  $\beta$ -subunits of H,K-ATPase. The *p*-nitrophenylphosphatase activity assay was used as a measurement for K-dependent H,K-ATPase activity.

**RESULTS:** Probed transblots showed an increase in the amount of H,K-ATPase in smoke-treated mice which was confirmed by immunohistochemistry and was found to be due to increased amounts of protein per parietal cell rather than an increased parietal cell number. The increase in the amount of H,K-ATPase was associated with an enhancement of its enzymatic activity. K-dependent activity in control and smoke-treated mice was significantly different (respectively, 0.12  $\mu$ mol/mg vs 0.27  $\mu$ mol/mg per minute,  $P < 0.05$ ).

### INTRODUCTION

Cigarette smoking is a major worldwide health problem. According to the World Health Organization, smoking is the largest preventable cause of premature death worldwide. It is a common habit among teenagers, adults, and even health professionals<sup>[1]</sup>. In addition to the well known adverse effects of smoking on cardiovascular and respiratory systems, some studies have shown that smoking is a major risk factor for some gastrointestinal diseases<sup>[2-4]</sup>. Several clinical and epidemiological studies have provided evidence suggesting that smokers are more susceptible to peptic ulcer disease and respond to anti-ulcer drugs less efficiently than non-smokers<sup>[4-9]</sup>. In experimental animals, some studies have shown that cigarette smoking potentiates the damaging effects of ethanol or corticosteroids on the gastric mucosa<sup>[10,11]</sup>.

The mechanisms by which cigarette smoking adversely affects the gastric mucosa have not been fully elucidated. It has been shown that free radical production, infiltration of neutrophils, stimulation of angiotensin II production, down-regulation of epidermal growth factor and reduction of gastric blood flow play an important role in the harmful effects of smoking<sup>[7,12-14]</sup>. However, it is not

known whether cigarette smoking or nicotine adversely affects some other factors that may influence the integrity of the gastric mucosa. The hydrochloric acid secreted by parietal cells is one of the main aggressive factors that play an important role in gastric mucosal damage and the pathogenesis of peptic ulcer disease<sup>[15,16]</sup>. The major protein involved in this process of acid secretion is the proton pump or H,K-ATPase. It is not known whether the H,K-ATPase of gastric parietal cells is altered by cigarette smoking.

In the stomachs of rodents and humans, parietal cells are scattered throughout the gastric glands, made of pit, isthmus, neck and base regions (Figure 1). They develop from epithelial progenitors which are anchored in the isthmus region<sup>[17-19]</sup>. During their development, parietal cells concomitantly synthesize the catalytic  $\alpha$ - and regulatory  $\beta$ -subunits of H,K-ATPase<sup>[20]</sup>. Following their maturation in the isthmus, parietal cells bidirectionally migrate to become scattered throughout the glandular regions of the gastric epithelium. In mice, the turnover time of parietal cells averages 54 d. Old parietal cells undergo progressive physiological deterioration and eventually die and are eliminated at the luminal surface by extrusion into the gastric lumen or deep at the gland bottom by phagocytosis via a neighboring healthier glandular cell or an invasive connective tissue macrophage<sup>[21]</sup>.

In the cytoplasm of parietal cells, both  $\alpha$ - and  $\beta$ -subunits of H,K-ATPase are targeted to the membranes of tubulovesicles. Upon stimulation by histamine, acetylcholine, or gastrin, the tubulovesicles translocate from the cytoplasm to the apical and canalicular membranes of the parietal cell. Therefore, expansion of the canalicular system and elongation of the microvilli are features of a stimulated parietal cell. H,K-ATPase of the apical and canalicular membranes of stimulated parietal cells utilizes ATP generated by the numerous large mitochondria to pump protons into the lumina of canaluli and gastric glands in exchange for potassium<sup>[22]</sup>. While much research is directed to discover new inhibitors of gastric H,K-ATPase, little is known about the extrinsic factors involved in its dysregulation.

Since smokers are more susceptible than nonsmokers to developing peptic ulcer disease and more likely to experience delays in ulcer healing, and since parietal cells are considered a key player during the pathogenesis and healing of this disease, we hypothesized that smoking alters H,K-ATPase, the major protein of the parietal cell which is responsible for acid secretion. Therefore, the aim of this study was to test whether the expression and activity of H,K-ATPase of parietal cells would be affected in animals administered with cigarette smoke extract.

## MATERIALS AND METHODS

### Preparation of cigarette smoke extracts

The method of Shin *et al*<sup>[23]</sup> was slightly modified to prepare ethanol and aqueous extracts of cigarette smoke. The smoke of burning red Marlboro cigarettes (Philip Morris, Inc., Richmond, VA, USA) was bubbled into ethanol or water by using a vacuum system. The ethanol

extract was allowed to evaporate and the precipitate was dissolved in 0.1% dimethyl sulfoxide (DMSO).

### Animals and experimental design

The procedures follow experiment in this study were in accordance with the guidelines for the care and use of laboratory animals and were approved by the Animal Research Ethics Committee of the Faculty of Medicine and Health Sciences, UAE University. In this study, C57BL mice of both sexes were used at two different age groups. (1) Young adult 8-wk-old mice ( $n = 12$ ) received the ethanol smoke extract, 5 mg/kg body weight/day, *via* oro-gastric gavage needle on 3 consecutive days. (2) Weaning-age mice (3-wk-old,  $n = 32$ ) received the aqueous smoke extract in their drinking bottles which were made freely accessible for 7 or 14 continuous days. Fresh extract-containing water was used daily. It was estimated that every day, on average, each weaned mouse consumed 5 mL of water containing smoke extract of two cigarettes. For both age groups of smoke-treated mice, weight- and sex-matched littermate (8- or 3-wk-old) mice were used as controls and received only vehicle (0.1% DMSO or water, respectively). One day after treatment, each pair of smoke-treated and control littermate mice was killed by an overdose of ether. The stomachs were immediately removed and processed for biochemical and immunohistochemical analyses.

### Mucosal homogenate preparation

Gastric mucosal homogenates were prepared as previously described<sup>[20,24]</sup>. Part of the oxytic mucosa of the stomach was scraped, minced and then homogenized on ice-cold hypotonic buffer (pH 6.7) containing 113 mmol/L mannitol, 37 mmol/L sucrose, 0.4 mmol/L EDTA, 5 mmol/L piperazine-N,N'-bis(2-ethanesulfonic acid)-tris (hydroxymethyl) aminomethane. The crude homogenate was centrifuged at a low speed (35 g) for 5 min to remove unbroken cells and tissues. To obtain pellets enriched in H,K-ATPase, some solubilized crude homogenates of control and smoke-treated mice were centrifuged at a higher speed (25000 g) for 2 h. Portions of the low-speed supernatants and the re-suspended high-speed pellets of control and smoke-treated mice were processed for measurement of protein concentration using Bradford's method and then quantification of H,K-ATPase using Western blotting.

### Western blotting analysis

Portions of the homogenates or re-suspended pellets of control and smoke-treated mice were solubilized in buffer containing 1% SDS, 0.5 mol/L urea, 5% 2-mercaptoethanol, 0.25 mmol/L EDTA, 10% glycerol, 0.0025% bromophenol blue, and 30 mmol/L Tris-HCl, pH 6.8 and run through 8% or 10% acrylamide<sup>[20]</sup>. Proteins were subsequently electro-transferred onto nitrocellulose membranes (Schleicher & Schuell Bioscience, Dassel, Germany) and probed with mouse monoclonal antibodies specific for the  $\alpha$ - (97 kDa) or  $\beta$ - (60-80 kDa) subunits of H,K-ATPase (Medical & Biological Laboratories Co., Woburn, MA, USA) at a

dilution of 1:2000. After washing, blots were incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Ig) G (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) at 1:10 000 dilution. To control equal loading of proteins in both smoke-treated and control mice, anti- $\beta$ -actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at 1:2000 dilution. Bands of protein-antibody complexes were visualized using SuperSignal West Pico chemiluminescence detection kit (Thermo Fisher Scientific, Rockford, IL, USA) and their relative intensities were quantified by densitometry using the Scion Imaging program for Windows (version  $\beta$  4.0.3.2; developed by Scion, Frederick, MD, USA). Data were expressed as means in arbitrary units and the level of significance of differences between control and smoke-treated groups were determined by using the Student's *t* test.  $P < 0.05$  was taken as significant.

#### **Light microscopic and immunohistochemical analyses**

Pieces of the oxytic regions of the stomachs of control and smoke-treated mice were fixed in Bouin's solution and embedded in paraffin. For general histology, some sections (5  $\mu\text{m}$ ) were stained with hematoxylin-eosin or periodic acid Schiff technique. Adjacent sections were used for immunohistochemical analysis using antibodies specific for the  $\alpha$ - or  $\beta$ -subunits of the gastric H,K-ATPase. Antigen-antibody binding sites were visualized by using fluorescein isothiocyanate (FITC)-labeled donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA).

To measure the intensity of H,K-ATPase immunolabeling of the cells, image analysis was performed on probed gastric mucosal tissue sections of some control ( $n = 4$ ) and smoke-treated littermate mice ( $n = 4$ ) using the Scion Imaging program as previously described<sup>[20]</sup>. Probed sections were examined at 40  $\times$  magnification with an Olympus microscope and photographed with a DP-70 digital camera with the option of fixed manual exposure to ensure equal exposure of control and smoke-treated tissue sections. Digitalized TIFF images of immunolabeled parietal cells were stored at a resolution of 300 dpi. Labeled parietal cells cut through their nuclei were only considered for measurement by using the freehand tool and drawing a line around the periphery of the cell. The number of cells examined per animal ranged from 20 to 35. Following measurement of the immunostaining intensity of various cells in one section, the background intensity was subtracted. Quantitative results of the optical density were reported in arbitrary units corresponding to immunostaining intensity which is indicative of the amount of H,K-ATPase in the sectioned cells. For each stained cell examined, the area was also measured and expressed in arbitrary units. Data are presented as mean  $\pm$  SE.

#### **Enzymatic activity assay**

In some experiments, the gastric mucosa of control and smoke-treated mice were homogenized and briefly centrifuged at low speed as mentioned above. Some of

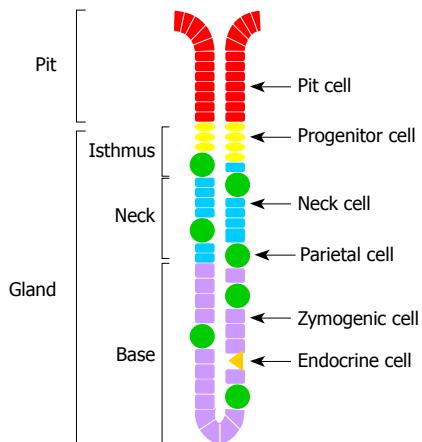
the supernatant was processed by the Bradford method for measurement of protein concentration and the remainder for K<sup>+</sup>-dependent  $p$ -nitrophenylphosphatase ( $p$ NPPase) activity assay which was used as an index of H,K-ATPase activity<sup>[20]</sup>. The  $p$ NPPase activity was measured at 37°C in buffer containing 7.5 mmol/L Tris-HCl (pH 7.5), 3.5 mmol/L MgSO<sub>4</sub>, 30 mmol/L sucrose, and 0.02 mmol/L EDTA. To eliminate the contribution of Na,K-ATPase, 0.1 mmol/L ouabain was included in the incubation buffer. The K-dependent and Na-dependent  $p$ NPPase activity was assayed with 20 mmol/L KCl and NaCl in the buffer, respectively. The reaction was initiated by the addition of 5 mmol/L sodium  $p$ -nitrophenyl phosphate and terminated by 1.5 mL of 0.5 mol/L NaOH. Liberated  $p$ -nitrophenol was read at 410 nm by a Beckman Du 70 spectrophotometer. The K-dependent  $p$ NPPase activity was obtained from the difference of the values obtained with and without 20 mmol/L KCl. The enzymatic activity was expressed in micromoles per milligram protein per minute. Student's *t* test was used to compare values in control *vs* smoke-treated mice.

## **RESULTS**

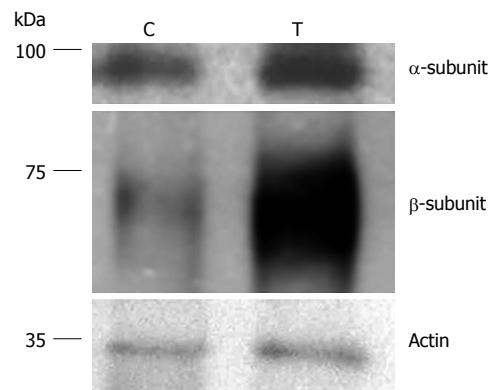
In the present study, extracts of cigarette smoke were administered to adult and weaned mice in two different modes: (1) aqueous extract in drinking water, and (2) ethanol extract *via* orogastric gavage needle. These two different modes of administration showed more or less similar effects on gastric H,K-ATPase. However, when the smoke extract was introduced with the drinking water, a duration-dependent effect was noted. The 7-d exposure of the gastric mucosa to smoke extract in the drinking water produced no significant difference between control and smoke-treated mice. When the duration of administration of smoke-containing drinking water was doubled, a significant difference was noted in the H,K-ATPase of smoke-treated *vs* control mice.

#### **Effect of cigarette smoke extract on the amount of H,K-ATPase**

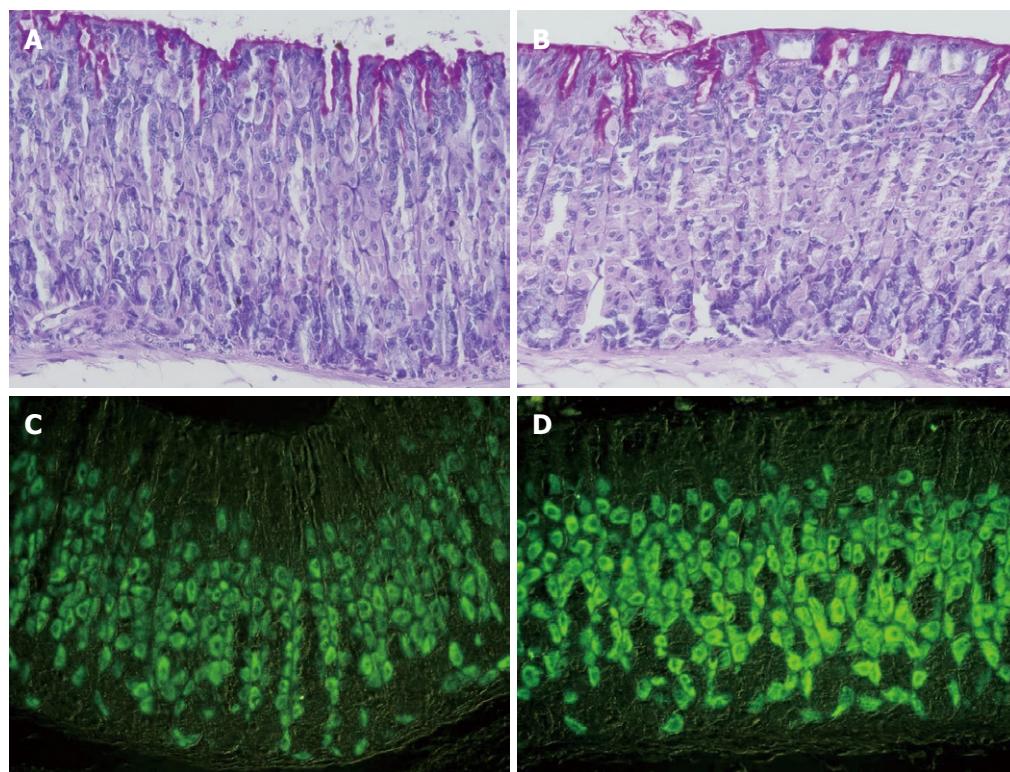
Proteins of the mucosal homogenates of mice treated with ethanol or aqueous extracts of cigarette smoke and their control littermates were separated on polyacrylamide gels and transblotted on nitrocellulose membranes. Probing of the membranes with antibodies specific for the  $\alpha$ - or the  $\beta$ -subunits of H,K-ATPase revealed an increase in the amount of this protein in smoke-treated mice (Figure 2). Densitometric analysis of the protein bands of H,K-ATPase showed that the amounts of both the  $\alpha$ - and  $\beta$ -subunits were significantly increased. It was estimated that the percent increase of the  $\alpha$  subunit averaged 220% and the percent increase of the  $\beta$  subunit averaged 350%. However, it should be noted that in the case of aqueous extract-treated mice, there was a dose/duration-dependent effect on H,K-ATPase expression. While the 7-d-treatment showed no significant difference as compared with control littermate mice (data not shown), 14 d of treatment showed a significant increase in the amount of H,K-ATPase ( $P < 0.05$ ).



**Figure 1** Schematic drawing of the structural unit of the gastric epithelium showing the pit and three glandular regions and the scattered H,K-ATPase-containing parietal cells.



**Figure 2** Representative transblots showing protein expression of the  $\alpha$ - and  $\beta$ -subunits of H,K-ATPase in control (C) and smoke-treated (T) mice. Note the differences in the intensity of the protein bands in control vs smoke-treated samples. Ten micrograms of proteins were loaded per lane. Beta actin was detected by a mouse monoclonal antibody and used as a loading control.



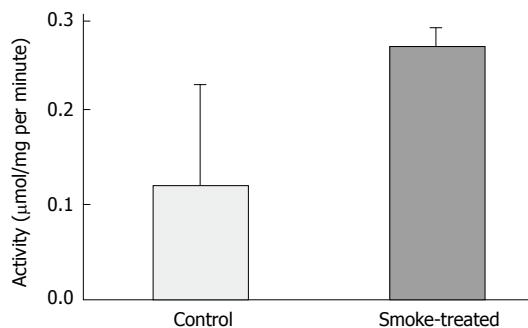
**Figure 3** Gastric mucosal tissue sections of control (A, C) and smoke-treated (B, D) mice. A and B demonstrate the gastric mucosae of control and treated mice stained with periodic acid-Schiff and hematoxylin. No difference is noted in parietal cells of control and treated tissues. Immunohistochemical labeling of parietal cells in the gastric mucosa of control (C) and smoke-treated (D) mice with antibodies specific for H,K-ATPase  $\beta$ -subunit. Labeled parietal cells are distributed throughout the gastric glands. Note the difference in the labeling intensity of parietal cells in control vs smoke-treated mice,  $\times 400$ .

#### **Effect of cigarette smoke extract on the immuno-labeling of parietal cells**

To test whether the increase in the amount of H,K-ATPase is due to an increase in parietal cell number or due to an increase in the amount of protein per cell, oxytic mucosal tissue sections of control and smoke-treated mice were probed with antibodies specific for the  $\alpha$ - or  $\beta$ -subunit of H,K-ATPase. To eliminate the possible variations in immunostaining conditions, stomach tissues obtained from smoke-treated and their littermate sex and weight-matched control mice were

processed simultaneously and embedded in the same paraffin blocks. Tissue sections of control and treated mice were de-waxed and immuno-probed together on the same slides.

Microscopic examination demonstrated the usual pattern of distribution of labeled parietal cells in both control and smoke-treated tissues (Figure 3). Parietal cells were scattered throughout the mucosa of all tissues examined. Counts of parietal cells in control and smoke-treated tissues showed no significant difference. The number of H,K-ATPase-labeled parietal cells averaged



**Figure 4 Analysis of the enzymatic activity of the H,K-ATPase of gastric mucosae in control and smoke-treated mice.** Note the increased activity in the cigarette smoke-treated homogenate.

12.3 cells per gland in control mice and 11.7 cells in the gland of smoke-treated mice. However, when the intensity of the immuno-labeling was compared in control and smoke-treated tissues, an apparent difference was noted. In general, immuno-stained cells of smoke-treated tissues appeared darker than in control tissues (Figure 3). This difference reflected an increase in the amount of H,K-ATPase per cell after treatment with cigarette smoke extract. Quantification of the intensity of H,K-ATPase immunostaining confirmed this difference. Measurements of parietal cell density in each pair of control and smoke-treated mice showed that the percentages of increase in staining intensity after smoke treatment are highly significant ( $P < 0.001$ ) and varied from 150% to 200%. In all mice examined, a similar staining pattern was obtained with antibodies specific for the  $\alpha$ - and  $\beta$ -subunits.

#### **Effect of cigarette smoke extract on the activity of the proton pump**

To test whether the increase in the expression of H,K-ATPase protein is associated with a change in its enzymatic activity, some of the mucosal homogenates were processed for *p*NNPase activity assay. The results indicated that while the enzymatic activity in control tissues averaged 0.12  $\mu\text{mol}/\text{mg}$  per minute, smoke-treated tissues showed more than 2-fold increased activity, 0.27  $\mu\text{mol}/\text{mg}$  per minute ( $P < 0.05$ , Figure 4).

## **DISCUSSION**

The present study demonstrates that cigarette smoke extract enhances the expression and activity of H,K-ATPase in the oxyntic mucosa of the mouse stomach which may explain the susceptibility of smokers to development of peptic ulcer disease.

The pathogenesis of peptic ulcer disease involves several factors including enhanced acid secretion, which is regarded as one of the major ulcerogenic factors. Acid secretion is also considered the primary target of contemporary drug therapy for peptic ulcer disease.

The available data concerning the effects of cigarette smoking on gastric acid secretion are controversial. Clinical studies showed that smoking may stimulate<sup>[25-28]</sup>, inhibit<sup>[29,30]</sup> or have no effect<sup>[31,32]</sup> on gastric acid secretion. Such disparity between studies may be explained, in part,

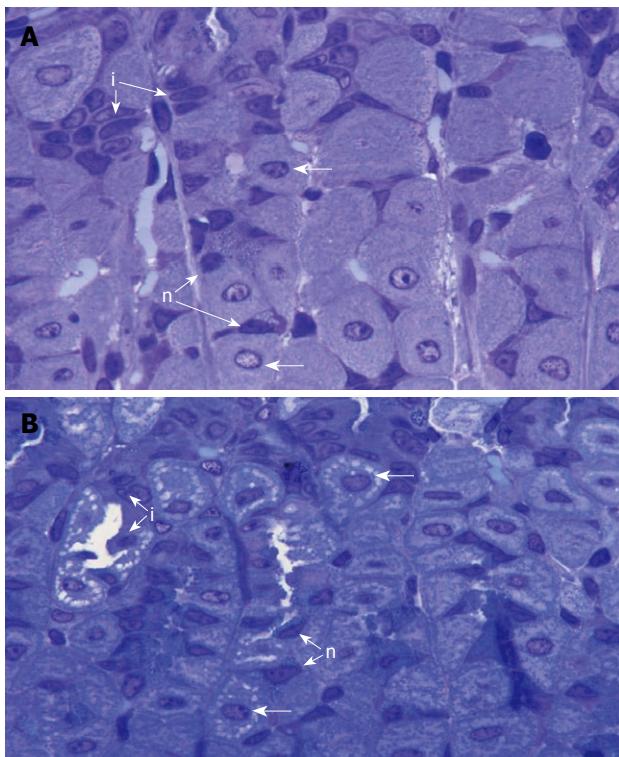
by the differences in nicotine content and number of cigarettes used, and by the lack of adequate controls due to marked individual variability of basal gastric secretion. In the current study, we did not intend to measure acid secretion, but the plan was to dissect the event down stream and examine the major enzyme of the parietal cell, H,K-ATPase, which is responsible for acid secretion.

Nicotine is one of the main constituents of cigarette smoke responsible for the adverse effects of smoking. The effect of nicotine on gastric acid secretion is also controversial. Several lines of evidence have suggested that it has a stimulating effect. Nicotine administered to rats for 10 d caused an increase in gastric secretory volume and acid output. This finding was attributed to increased muscarinic receptor sensitivity, and consequently, basal acid secretion<sup>[33]</sup>. Lindell *et al*<sup>[34]</sup> also noted that nicotine administration enhances gastric acidity and impairs postprandial gastric neutralization in humans. Likewise, nicotine treatment abolishes in a dose-dependent manner the depressing effect of ethanol on acid secretion in rats<sup>[35]</sup> and significantly stimulates basal gastric acid output in cats<sup>[36]</sup>. *In vitro* study demonstrated that nicotine could exert direct stimulatory effects on parietal cells and potentiate the histamine-mediated response in the isolated cell model<sup>[36]</sup>.

Some other studies showed contrasting findings. In one study, the gastric acid secretion stimulated by intravenous pentagastrin was completely inhibited by nicotine<sup>[37]</sup>. Another study also showed that acid output together with gastric secretory volume one hour after vagal stimulation induced by modified sham feeding was lower in human subjects on smoking than non-smoking days<sup>[38]</sup>. Based on the contrasting nature of evidence, the effect of nicotine or smoking on gastric acid secretion remained elusive.

The present study supports the view that smoking enhances gastric acid secretion by up-regulating the expression and activity of H,K-ATPase. Based on previous studies, the mechanism by which the smoke extract affected the acid secreting parietal cells could be a combination of receptor mediated and direct effects on the parietal cell.

Therefore, the present study provides an answer to some questions which were raised by some epidemiological studies. Why are cigarette smokers more susceptible to development of peptic ulcer disease? Why is recurrence of peptic ulcer more common in smokers than non-smokers? Why is the healing of peptic ulcer disease delayed in smokers? As an answer to these questions, we hypothesized that H,K-ATPase in the parietal cells is more aggressive in smokers than non-smokers. Then we tested this hypothesis by using an ethanol or aqueous extract of cigarette smoke, which were previously found to be rich in nicotine by various chromatography techniques<sup>[39]</sup>. The extract was administered to mice orally and three methods were carried out to characterize the anticipated alteration of the gastric H,K-ATPase. First, Western blotting analysis of homogenates obtained from the gastric mucosae of control and smoke-treated mice showed an increase in the amount of H,K-ATPase in the 3-d-treated adult mice and 14-d-treated weaning-age mice. However, when the



**Figure 5** Semithin (0.5-micron-thick) sections of the gastric mucosae of control (A) and smoke-treated (B) mice stained with toluidine blue to demonstrate the isthmus and neck regions of the gastric glands. The large numerous parietal cells (horizontal arrows) are separated by progenitor or isthmal cells (i) and neck cells (n). Note that, in the smoke-treated tissue but not control tissue, there are pale areas in the cytoplasm of parietal cells which represent expanded lumen of the intracellular canalicular system,  $\times 1000$ .

weaned mice were treated for only 7 d with half the dose, there was no significant change in the amount of H,K-ATPase as compared to their control littermates. Therefore, there is a duration- and dose-dependent effect of cigarette smoke extract in weaning-age mice. During this age, the developing gastric glands undergo compartmentalization into isthmus, neck and base regions<sup>[40]</sup>. In these developing glands, parietal cells might not have acquired all the machinery to respond to an extrinsic stimulus and therefore a longer duration of treatment with cigarette smoke extract is needed. Second, immunohistochemical analysis of gastric parietal cells using antibodies specific for their H,K-ATPase revealed an enhancement in the immunostaining of parietal cells of the smoke-treated mice. Since tissues of these treated mice and their weight- and sex-matched littermate control mice were processed together, embedded in the same tissue blocks and immuno-probed simultaneously on the same slides, the differences in immunostaining intensity were taken to represent an increase in the amount of H,K-ATPase per parietal cell. Third, the proton pump activity, measured by *p*NPPase assay was enhanced in the gastric mucosal homogenates of smoke-treated mice compared to control mice.

The question was raised whether the increased amount and activity of H,K-ATPase was associated with the translocation of tubulovesicles into the canalicular membranes. Some gastric mucosal tissues obtained from three pairs of smoke-treated and control mice were fixed in a Kar-

novesky's solution and processed for Araldite embedding and semithin sectioning. Microscopic examination of parietal cells located in the isthmus and neck regions of the gastric glands (known to be involved in acid secretion<sup>[41]</sup>) revealed that those of the smoke treated mice tend to acquire stimulated morphology with expanded canalicular system as compared to those of control mice (Figure 5).

In conclusion, the present study demonstrates a possible explanation for the susceptibility of smokers to develop peptic ulcer disease. Therefore, we propose the following scenario. It seems that smokers develop parietal cells with an increased amount and activity of H,K-ATPase and tendency to acquire an extended canalicular system. Accordingly, parietal cells of smokers are enhanced to produce much acid upon stimulation and their gastric and duodenal mucosae become more vulnerable to development of peptic ulcer disease.

## COMMENTS

### Background

Clinical, epidemiological and experimental studies have shown that smokers are more susceptible to peptic ulcer disease and respond to anti-ulcer drugs less efficiently than non-smokers. Parietal cells are targets for anti-ulcer drugs and their inhibition is an important modality for peptic ulcer treatment.

### Research frontiers

Parietal cell activation may be associated with gastric mucosal injury and peptic ulcer disease. Since smoking is a predisposing factor to peptic ulcer, it is hypothesized that cigarette smoke extract may cause activation of gastric parietal cells. In this study, the authors demonstrated that over-expression of the gastric proton pump could be a possible mechanism for the susceptibility of smokers to peptic ulcer disease.

### Innovations and breakthroughs

Previous reports have demonstrated the role of epidermal growth factors, blood flow, neutrophils, angiotensin II, and free radicals in the adverse effects of smoking on the gastrointestinal mucosa. In this study, the authors have demonstrated an additional possible effect of smoking on the expression and activity of the gastric proton pump.

### Applications

By understanding how smoking may alter the biological features of the gastric mucosa, this study may help in improving the current preventive and therapeutic modalities of peptic ulcer disease.

### Terminology

The proton pump or H,K-ATPase is the major protein of parietal cells responsible for acid secretion. It is the main target for anti-ulcer drugs. Better understanding of the biological features of these cells and defining factors responsible for the regulation or dysregulation of their proton pump is important for designing new modalities for prevention and treatment of peptic ulcer disease.

### Peer review

The authors used a smoking extract to study parietal cell H,K-ATPase expression and activity. It is a very interesting study.

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# Risk factors for rebleeding after angiographically negative acute gastrointestinal bleeding

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## INTRODUCTION

Acute non-variceal gastrointestinal bleeding accounts for approximately 20% of emergency room visits and 5% of admissions<sup>[1]</sup>. Although endoscopy (including the use of upper endoscopy and colonoscopy) has been used as a first-line treatment option in patients with gastrointestinal bleeding<sup>[2,3]</sup>, angiographic intervention can be used as a safe diagnostic and treatment method in patients with gastrointestinal bleeding that is refractory to endoscopic treatment<sup>[4-6]</sup>.

Angiography requires a bleeding rate of 0.5-1 mL/min for detection. When neither extravasation nor vascular abnormality such as pseudoaneurysm is found, the bleeding site cannot be embolized selectively. Thus, intermittent bleeding is likely to result in a negative angiographic study<sup>[7,8]</sup>. The incidence of rebleeding in patients with negative initial angiography has been reported in up to 60% of cases<sup>[9]</sup>. However, little is known about the predictive factors for rebleeding, to determine if further investigations should be performed. The aim of this retrospective study was to identify the factors related to rebleeding in patients with gastrointestinal bleeding and normal angiographic findings.

## MATERIALS AND METHODS

From January 2000 to July 2007, 341 patients with acute non-variceal gastrointestinal bleeding were referred to the angiography unit of our institution for possible transcatheter arterial embolization. We excluded 193 patients as they had active bleeding detected by angiography, and these patients received selective or empirical embolization. Among 148 patients with negative findings upon initial angiography, 20 were

## Abstract

**AIM:** To identify possible predictive factors for rebleeding after angiographically negative findings in patients with acute non-variceal gastrointestinal bleeding.

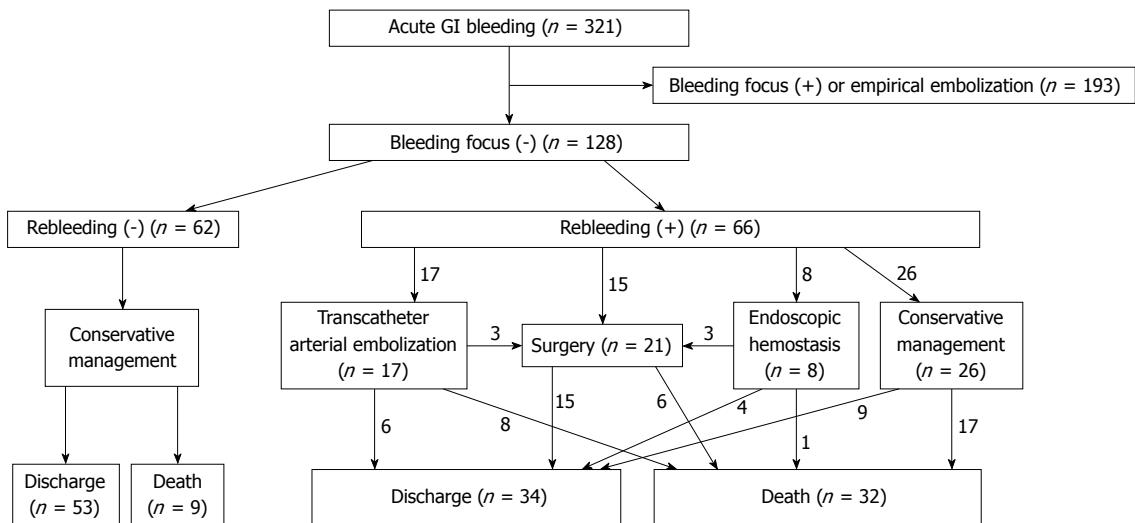
**METHODS:** From January 2000 to July 2007, 128 patients with acute non-variceal gastrointestinal bleeding had negative findings after initial angiography. Clinical and laboratory parameters were analyzed retrospectively.

**RESULTS:** Among 128 patients, 62 had no recurrent gastrointestinal bleeding and 66 had recurrent gastrointestinal bleeding within 30 d. As determined by the use of multivariate analysis, an underlying malignancy, liver cirrhosis and hematemesis were significant factors related to recurrent gastrointestinal bleeding.

**CONCLUSION:** Clinical factors including underlying malignancy, liver cirrhosis, and hematemesis are important predictors for rebleeding after angiographically negative findings in patients with acute non-variceal gastrointestinal bleeding.

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**Key words:** Angiography; Gastrointestinal hemorrhage; Predictive factor



**Figure 1** Flow diagram of patient outcome.

excluded because of limited medical records or suspected variceal gastrointestinal bleeding. A total of 128 patients (94 male, 34 female; age range, 18–85 years, mean age, 57.8 years) who had no active bleeding detected by initial angiography were included in this study. Approval was obtained from the ethical board committee of Seoul National University Hospital and patient informed consent was waived because of the retrospective nature of the study.

Clinical and laboratory parameters were reviewed retrospectively. The variables assessed included the following: patient age, sex, history of hematemesis, hematochezia or melena, shock, hemoglobin level, platelet count, prothrombin time, partial thromboplastin time, recent surgery, potential bleeding diatheses (liver cirrhosis or chronic renal failure), underlying malignancy, use of nonsteroidal anti-inflammatory drugs, and use of other antiplatelet agents or anticoagulants at the time of evaluation. Rebleeding was confirmed by endoscopy or surgery, and was clinically defined as: (1) fresh hematemesis; (2) fresh melena with systolic blood pressure < 100 mmHg; (3) a decrease in hemoglobin level of > 4 g/dL within 24 h; and (4) a requirement of two more red blood cell transfusions within 24 h.

The univariate association between clinical and laboratory variables and rebleeding was examined using Fisher's exact test for categorical variables and the *t* test for continuous variables. Variables with *P* < 0.25 as determined by univariate analysis were subjected to multiple logistic regression analysis with the use of a backward stepwise method. For univariate and multiple logistic regression analysis, *P* < 0.05 was regarded as statistically significant. Statistical analyses were performed using commercially available software (SPSS for Windows version 10.0 (Chicago, IL USA). All reported *P* values were two tailed.

## RESULTS

Before the initial angiography examination, endoscopy

was performed in 97 patients, endoscopic hemostasis was attempted in 18 patients, tagged red blood cell scintigraphy was performed in 21 patients, and a computed tomography (CT) scan was obtained for 40 patients. After initial angiography with negative findings, endoscopy was performed in 81 patients, endoscopic hemostasis was attempted in eight patients, tagged red blood cell scintigraphy was performed in 18 patients, a CT scan was obtained for 18 patients, and surgical treatment was performed in 21 patients.

The location of bleeding was the esophagus (*n* = 3), stomach (*n* = 31), duodenum (*n* = 25), small intestine (*n* = 12), colon (*n* = 13) and unknown (*n* = 44). The cause of bleeding was a benign ulcer (*n* = 50), gastrointestinal tumor (*n* = 7), ischemic enteritis (*n* = 3), radiation colitis (*n* = 2), iatrogenic injury (*n* = 2), angiodysplasia (*n* = 2) and undetermined (*n* = 62).

Among 128 patients who had no active bleeding detected by angiography, 62 had no recurrent gastrointestinal bleeding and 66 had recurrent gastrointestinal bleeding. Figure 1 documents the clinical course of the 128 patients. For the 66 patients with rebleeding, 40 received interventions including surgery, transcatheter arterial embolization and endoscopic hemostasis. The 4-wk mortality rate was 48% (32/66) for patients with rebleeding and 15% (9/62) for those without rebleeding. Among 41 expired patients, the cause of death was uncontrolled bleeding in 11, pneumonia or sepsis in 13, aggravation of the underlying malignancy in 10, cardiac failure in one, hepatic failure in one, and unknown in five.

Based on univariate analysis, the hemoglobin level, partial thromboplastin time, use of antiplatelet medication, underlying malignancy, presence of liver cirrhosis and shock were significant factors related to recurrent gastrointestinal bleeding. Based on multivariate analysis, underlying malignancy (*P* = 0.002, OR = 3.81), liver cirrhosis (*P* = 0.017, OR = 4.81) and hematemesis (*P* = 0.042, OR = 2.59) were significant factors related to recurrent gastrointestinal bleeding (Table 1).

**Table 1** Prediction of rebleeding in patients with angiographically negative gastrointestinal bleeding

Variable	Rebleeding		P value at univariate analysis	Multiple logistic regression		
	Absent	Present		P value	OR	CI
Age (yr)	59.2 ± 15.1	56.9 ± 15.9	0.414			
Hemoglobin (g/dL)	8.2 ± 1.9	7.3 ± 1.9	0.009			
Platelet ( $\times 1000/\text{mm}^3$ )	173.5 ± 98	151 ± 140	0.301			
PT (INR)	1.25 ± 0.31	1.38 ± 0.66	0.063			
aPTT (s)	38.7 ± 9.9	48.6 ± 31.8	0.027			
Sex						
Female	17	18	1.000			
Male	45	48				
NSAID						
No use	59	64	0.673			
Use	3	2				
Anticoagulation						
No	56	64	0.155			
Yes	6	2				
Antiplatelet therapy						
No	47	61	0.014			
Yes	15	5				
Malignancy						
Absent	42	26	0.002	0.002	3.81	1.63-8.91
Present	20	40				
GI tumor bleeding						
Absent	57	56	0.275			
Present	5	10				
Recent surgery						
Absent	44	41	0.350			
Present	18	25				
Recent GI surgery						
Absent	54	52	0.247			
Present	8	14				
Past GI bleeding history						
Absent	47	51	1.000			
Present	15	15				
Chronic renal failure						
Absent	54	58	1.000			
Present	8	8				
Liver cirrhosis						
Absent	56	47	0.007	0.017	4.81	1.32-17.54
Present	6	19				
Hematemesis						
Absent	46	38	0.063	0.042	2.59	1.04-6.07
Present	16	28				
Hematochezia						
Absent	32	34	1.000			
Present	30	32				
Melena						
Absent	40	43	1.000			
Present	22	23				
Shock						
Absent	37	24	0.013			
Present	25	42				

PT: Prothrombin time; aPTT: Activated partial thromboplastin time; NSAID: Non-steroidal anti-inflammatory drug.

## DISCUSSION

Acute non-variceal gastrointestinal bleeding is one of the common emergency conditions for inpatients as well as outpatients<sup>[1]</sup>. Superselective angiography and transcatheter embolization have been used widely for upper and lower gastrointestinal bleeding refractory to endoscopic therapy<sup>[4,5]</sup>. In the case of failure of endoscopic management caused by a large number of blood clots or poor bowel preparation, angiography may be the choice of diagnostic or therapeutic method<sup>[4]</sup>. The angiographic procedure can provide accurate localization of the bleeding focus and immediate hemostasis, and localization of the bleeding site prior to surgery can prevent “blind” bowel resection. In addition, the use of angiography is less invasive than surgery, and is a good option for poor surgical candidates<sup>[10]</sup>. Recently, the use of improved techniques and instruments has decreased the number of complications such as bowel ischemia within an acceptable range<sup>[5,6]</sup>.

Unfortunately, blood extravasation is not always visualized. In recent reviews of angiographic findings, blood extravasation or intraluminal blush was seen in 40%-60% of angiographic cases of non-variceal upper

gastrointestinal bleeding<sup>[1,7,8]</sup>. There have been many studies of patients with normal angiograms, but a gold standard for management has not been determined. Some suggest that, in the case of negative angiographic findings in patients with intermittent or slow flow bleeding, use of nuclear scintigraphy seems reasonable to help confirm and localize the lesion<sup>[11,12]</sup>. The use of CT angiography may add to the detection of intermittent bleeding with possible better localization of the source and etiology of the bleeding. Ettorre *et al*<sup>[13]</sup> have shown a detection rate of 72% in patients with obscure gastrointestinal bleeding, in whom endoscopic and nuclear imaging failed to localize the bleeding site. The use of angiography has been reported with intra-arterial or intravenous injection of vasodilators, heparin, and even thrombolytic drugs to improve the rate of positive angiographic findings in occult lower gastrointestinal bleeding, although these modifications have been considered provocative<sup>[14-16]</sup>. Some investigators have suggested that blind embolization of the left gastric artery after endoscopic localization can show a decrease in the rebleeding rate<sup>[17]</sup>. As a result of the safety of the procedure, empiric embolization of the upper gastrointestinal tract for acute bleeding has been recommended when guided by endoscopic findings.

We expect that determination of the predictive factors for rebleeding may help in the selection of patients for further work-up or treatment, and consequently, may increase the success rate and decrease the rate of complications. Several studies have demonstrated clinical and endoscopic factors including liver cirrhosis, recent surgery, hypovolemic shock, hematemesis, large ulcer size, non-bleeding visible vessel, and the presence of an adherent clot on an ulcer base as significant predictive factors for the recurrence of hemorrhage in patients with peptic ulcer<sup>[18,19]</sup>. In our study, underlying malignancy, liver cirrhosis and hematemesis were significant factors related to recurrent gastrointestinal bleeding. As we did not perform endoscopy in all patients, and the study population was heterogeneous, including upper and lower gastrointestinal bleeding, endoscopic factors were not included in the analysis.

Rebleeding rates reported in the literature vary from 7% to 25% in patients with peptic ulcer or lower gastrointestinal bleeding<sup>[18-20]</sup>. In our study, the incidence of rebleeding within 1 mo was 52% (66/128). We think that the rebleeding rate was high because many severely ill patients were included in our study population.

The mortality rate in our study was 48% for patients with rebleeding and 15% for those without. Since there are many variables, we cannot state that the rebleeding itself affected mortality. However, prediction of rebleeding seems to have a relation to the prediction of prognosis.

This study had some limitations. First, the variable diagnostic and therapeutic modalities were performed without a settled sequence or principle. Most of the patients (120/128) received variable transfusions of red blood cells, fresh frozen plasma or platelet concentrate before angiography. Tagged red blood cell scintigraphy was performed in 21 patients and CT angiography in 40. Endoscopy was performed in 97 patients and 18 underwent endoscopic treatment. The selection of endoscopy or angiography in acute gastrointestinal bleeding is not well established. In our retrospective review, in cases in which postoperative CT showed an active bleeding focus, or the condition of the patient was inappropriate for endoscopy, angiography was performed as the first-choice method for diagnosis and treatment of acute gastrointestinal bleeding. Second, patients were enrolled in the study from only a single referral hospital. Many of the patients were elderly and had numerous medical problems. These conditions may have influenced the relatively high rebleeding rate and high mortality rate. Third, although the clinical features of upper gastrointestinal bleeding are quite different from lower gastrointestinal bleeding, both upper and lower gastrointestinal bleeding were included in this study population, as we could not determine the location of the bleeding site in 44 of 128 patients.

In conclusion, clinical factors including underlying malignancy, liver cirrhosis, and hematemesis are important predictors of recurrent bleeding after negative angiographic findings in patients with acute non-variceal gastrointestinal bleeding.

## COMMENTS

### Background

Acute non-variceal gastrointestinal bleeding is a common emergency condition. Superselective angiography and transcatheter embolization have been used widely for gastrointestinal bleeding. Unfortunately, blood extravasation is not always visualized.

### Research frontiers

To predict the risk of gastrointestinal rebleeding after negative angiography may be important clinically. However, little is known about the predictive factors for rebleeding, to determine if further investigations should be performed.

### Innovations and breakthroughs

This study is believed to be the first to establish predictive factors for rebleeding after angiographically negative gastrointestinal bleeding.

### Applications

Determination of the predictive factors for rebleeding may help in the selection of patients for further work-up or treatment, and consequently, might increase the success rate and decrease the rate of complications.

### Peer review

This study evaluated retrospectively the risk factors for gastrointestinal rebleeding after negative angiography.

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BRIEF ARTICLES

## Hepatitis B virus subgenotypes and basal core promoter mutations in Indonesia

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### Abstract

**AIM:** To identify the distribution of hepatitis B virus (HBV) subgenotype and basal core promoter (BCP) mutations among patients with HBV-associated liver disease in Indonesia.

**METHODS:** Patients with chronic hepatitis (CH, n =

61), liver cirrhosis (LC, n = 62), and hepatocellular carcinoma (HCC, n = 48) were included in this study. HBV subgenotype was identified based on S or preS gene sequence, and mutations in the HBx gene including the overlapping BCP region were examined by direct sequencing.

**RESULTS:** HBV genotype B (subgenotypes B2, B3, B4, B5 and B7) the major genotype in the samples, accounted for 75.4%, 71.0% and 75.0% of CH, LC and HCC patients, respectively, while the genotype C (subgenotypes C1, C2 and C3) was detected in 24.6%, 29.0%, and 25.0% of CH, LC, and HCC patients, respectively. Subgenotypes B3 (84.9%) and C1 (82.2%) were the main subgenotype in HBV genotype B and C, respectively. Serotype adw2 (84.9%) and adrq+ (89.4%) were the most prevalent in HBV genotype B and C, respectively. Double mutation (A1762T/G1764A) in the BCP was significantly higher in LC (59.7%) and HCC (54.2%) than in CH (19.7%), suggesting that this mutation was associated with severity of liver disease. The T1753V was also higher in LC (46.8%), but lower in HCC (22.9%) and CH (18.0%), suggesting that this mutation may be an indicator of cirrhosis.

**CONCLUSION:** HBV genotype B/B3 and C/C1 are the major genotypes in Indonesia. Mutations in BCP, such as A1762T/G1764A and T1753V, might have an association with manifestations of liver disease.

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**Key words:** Basal core promoter mutation; Hepatitis B virus; Indonesia; Liver disease; Subgenotype

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## INTRODUCTION

Hepatitis B virus (HBV) infection is associated with a diverse clinical spectrum of liver damage ranging from asymptomatic carriers, chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC)<sup>[1]</sup>. HBV, a member of the *hepadnaviridae*, is a relaxed circular double-stranded DNA virus, and is currently classified into 8 genotypes (A to H), which reflect its geographical distribution<sup>[2,3]</sup>. For instance, HBV genotype A is prevalent in Europe, Africa, and India<sup>[4]</sup>. HBV genotypes B and C are predominant in most parts of Asia, including China, Japan, and Indonesia<sup>[4-10]</sup>. Genotype D is common in the Mediterranean area, the Middle East and India, whereas genotype E is localized in sub-Saharan Africa<sup>[4,11-13]</sup>. Genotype F and H are only identified in Central and South America<sup>[4,14,15]</sup>. Genotype G has been found in France, Germany, and the United States<sup>[4,16-18]</sup>.

Besides the differences in geographical distribution, there is growing evidence that the HBV genotype may also influence the clinical outcomes of liver disease. Among Asian patients who constitute approximately 75% of HBV carriers worldwide, it has been shown that HBV genotype C is more commonly associated with severe liver disease and the development of cirrhosis and HCC than HBV genotype B<sup>[19-23]</sup>. However, most of these studies were carried out in Taiwan and Japan, thus can not be generalized even for Asian countries.

In addition to HBV genotype, mutations in the core promoter, precore or HBx gene have been shown to have an association with severe liver disease. For instance, many studies have revealed that the double mutation in BCP (A1762T/G1764A) is associated with an increased risk of severe liver disease including HCC, and can be used as a prediagnostic biomarker of HCC<sup>[24-28]</sup>. The predominant mutation in the precore region of HBV which involved a G-to-A change at nucleotide 1896, and resulted in a premature stop codon at codon 28, was proved to be associated with increased HCC risk<sup>[23,28-30]</sup>. In addition, among HBV carriers, the A1762T/G1764A mutation is more frequently found in genotype C than genotype B<sup>[19,31]</sup>. However, an independent study on a comparison of HBV genotype C from Vietnam and Japan showed mutations at different positions in the core promoter/precore region of HBV<sup>[32]</sup>, indicating that the effect of mutation on liver carcinogenesis may not be universal. In addition, some mutations in HBx protein, in particular for HBV genotype C, have been shown to be significantly associated with HCC. A Serine-to-Alanine mutation at codon 31 (S31A) in HBx protein<sup>[33]</sup>, a Proline-to-Serine mutation at codon 38 (P38S) in HBx protein of HBV genotype C<sup>[34]</sup>, and some other particular mutations in HBx protein were found to be associated with increased risk of HCC<sup>[35]</sup>. Those studies, however, were independently carried out in different

countries (China Taiwan, Japan, and Korea), and resulted in three different results.

Despite various reports about the effect of HBV genotype and/or mutations on liver disease progression, the virological significance on liver carcinogenesis is not yet fully elucidated. In particular for Indonesia, some reports had been published regarding the distribution of HBV genotype<sup>[7-10,36]</sup>, and only one study reported samples from CH, LC, and HCC<sup>[8]</sup>. Moreover, to the best of our knowledge there is no report on the distribution of BCP mutations and their possible association with clinical manifestations of liver disease. Thus, the aims of the present study were to identify the distribution of HBV genotype/subgenotype and BCP mutations in patients with different clinical status, and to investigate the association of HBV genotype/subgenotype or BCP mutations and liver disease progression in Indonesia.

## MATERIALS AND METHODS

### Samples

Serum samples were obtained from 171 patients with HBV-associated liver disease, comprising 61 CH patients (mean age  $37.8 \pm 13.0$  years; male/female: 40/21), 62 LC patients (mean age  $50.2 \pm 11.6$  years; male/female: 44/18), and 48 HCC patients (mean age  $49.6 \pm 10.4$  years; male/female: 43/5). CH was defined as persistent seropositivity for HBsAg for at least 6 months. LC was diagnosed by liver function tests and ultrasonography. The diagnosis of HCC was on the basis of ultrasonography as well as an elevated serum  $\alpha$ -fetoprotein (AFP) level ( $\geq 200$  ng/mL), or liver biopsy samples by needle aspiration for samples in which the AFP level was low. Sera of CH, LC, and HCC patients were collected from Cipto Mangunkusumo Hospital, Gatot Soebroto Hospital, Klinik Hati, Jakarta, Siloam Hospital Lippo Karawaci, Tangerang, Mataram General Hospital, Mataram, and Wahidin Sudirohusodo Hospital, Makassar, from May 2006 until November 2008. All sera were hepatitis B surface antigen (HBsAg)-positive as determined by a commercially available enzyme-linked immunosorbent assay kit (Abbott Laboratories, Chicago, IL, USA). Blood samples were collected from each patient at the time of their clinical evaluation, separated into sera and stored at -70°C until viral DNA extraction. The study was approved by the Institutional Ethic Committee and informed consent was obtained from each patient.

### Viral DNA extraction and PCR amplification

HBV DNA was extracted from 200  $\mu$ L serum using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and 80  $\mu$ L of eluted DNA was stored at -70°C until use. Full S gene was amplified by PCR with primers Fgp2 and Rgp2 (Table 1). The following cycling parameters were used for 40 cycles of PCR: denaturation at 95°C (30 s), annealing at 55°C (45 s) and elongation at 72°C (2 min). When the PCR amplification was negative, a nested PCR was carried out to amplify the preS region.

**Table 1** Primers used in this study

Primer	Nucleotide sequence (5'→3')	Position	Polarity	Reference
Full S				
Fgp2	CGCCATGGGAGGTTGGTCTTCAAACCTCG	2848-2873	Forward	This study
Rgp2	GACAAGCTTAATGTATACCAAAGACAAAAGAAAATTGG	803-835	Reverse	
PreS (nested PCR)				
HBPr94	GGTAAAAAGGGACTCACGATG	775-795	Reverse	[2]
HBPr134	TGCTGCTATGCCCATCTTC	414-433	Forward	
HBPr135	CAAAGACAAAAGAAAAATTGC	803-822	Reverse	
HBx				
Fgp3	CGCCATGGCTGCTAGGCTGTGCTGCCAAC	1374-1398	Forward	This study
Rgp3	CGCTCGAGGGCAGAGGGAAAAAGTIGCATGGT	1811-1838	Reverse	
HBx (nested PCR)				
HB1	GCCAAGTGTGCTGACGC	1175-1193	Forward	[37]
HB2	CCATACTGCGGAACCTCTAG	1266-1285	Forward	
HB3	AAAGTTGCATGGTGTGGTG	1804-1823	Reverse	

Primers HBPr134 and HBPr135 (Table 1) were used as previously described for the first-round 35 cycles of PCR by the following cycling parameters<sup>[2]</sup>: denaturation at 95°C (1 min), annealing at 48°C (30 s) and elongation at 72°C (1 min). The second-round PCR was then performed using primers HBPr94 and HBPr134 (Table 1) with the same conditions as the first-round PCR except for annealing at 56°C (30 s). Similarly, HBx gene was amplified using primers Fgp3 and Rgp3 (Table 1). The cycling parameters were the same as that for S gene amplification, except with an elongation time of 1 min. A nested PCR was performed for PCR negative samples using primers HB1 and HB3 for the first round PCR [35 cycles: denaturation at 95°C (1 min), annealing at 48°C (30 s) and elongation at 72°C (1 min)] and using primers HB2 and HB3 for the second round PCR with the same parameters as the first-round PCR, but the annealing temperature was 46°C, as described previously<sup>[37]</sup>. Both sets of primers could amplify the full HBx gene. All PCR reactions were carried out by the PCR Core System (Promega, Madison, WI, USA). The PCR products were visualized on 1% agarose gel stained with ethidium bromide and purified using Wizard® SV Gel and the PCR Clean-Up System (Promega, Madison, WI, USA).

#### Analysis of HBV genotype/subgenotype, serotype and HBx mutations

Nucleotide sequences of the PCR fragments were determined with the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and the appropriate primers, and sequenced with 3130xl DNA sequencer (Applied Biosystems). All HBs and HBx gene sequences were edited manually and were aligned with reference sequences retrieved from GenBank, using the ClustalW program incorporated in Bioedit v7.0. HBV genotypes/subgenotypes were determined based on the homology in the S or preS gene. Phylogenetic trees were constructed by the neighbor-joining method. HBV serotypes were deduced on the basis of predicted amino acid sequences of HBsAg<sup>[3,38,39]</sup>.

#### Statistical analysis

All statistical analyses were performed using SPSS 15.0

software for Windows (SPSS Inc., Chicago, IL, USA) and MedCalc® version 10.1.0.0 for Windows (MedCalc Software, Broekstraat, Mariakerke, Belgium). Significance differentiations for continuous variables were analyzed using *t*-test analysis. While the categorical variables were analyzed using the Fisher's exact test and chi-square test. *P* < 0.05 were considered significant.

## RESULTS

#### **HBV genotypes/subgenotypes distribution and clinical diagnosis**

Only HBV genotype B and C were detected in the samples, which were respectively distributed in 73.7% and 26.3% of the samples (Table 2). Among HBV genotype B, subgenotypes B2, B3, B4, B5 and B7 were identified, although subgenotype B3 was the major subgenotype identified (84.9% of all genotype B or 62.6% of total samples). HBV subgenotypes B2 and B4 were only found in CH and LC, respectively. HBV subgenotype B5 was found in LC and HCC, while subgenotype B7 was detected in all different clinical diagnoses of the samples. On the other hand, among HBV genotype C, subgenotypes C1, C2, and C3 were found, but subgenotype C1 was dominant (82.62% of all genotype C or 21.6% of total samples). HBV subgenotype C1 was distributed in all samples, but subgenotype C2 and C3 were not detected in HCC samples. Based on statistical analysis, there was no significant association between HBV genotype/subgenotype and a clinical diagnosis of liver disease (Table 2). Serotype distribution demonstrated that adw2 was the major serotype (62.6%) in the samples, followed by adrq+ (24.6%) (Table 2). Other serotypes such as adw, adw3, ayw, ayw1, and ayr were also found in a small number of the samples. Similar to genotype results, no association between serotype and clinical status of the liver disease was observed (Table 2).

#### **HBx and basal core promoter mutations**

Initially, amino acid sequences of HBx from the samples were aligned and compared with reference sequences of amino acids retrieved from GenBank (accession no. BAA23459 and BAD86602 for HBV genotype B and C,

**Table 2** HBV genotype and serotype distribution in samples with different clinical diagnosis

Characteristics	n (%) in each clinical diagnosis							P			
	HCC				Total (n = 171)	CH vs LC	CH vs All HCC	LC vs All HCC	CH vs LC vs All HCC		
	CH (n = 61)	LC (n = 62)	With LC (n = 12)	Without LC (n = 36)							
Genotype and subgenotype											
B B2	5 (8.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (2.9)	NS	NS	NA	0.007	
B3	38 (62.3)	41 (66.1)	8 (66.7)	20 (55.6)	28 (58.3)	107 (62.6)	NS	NS	NS	NS	
B4	0 (0.0)	1 (1.6)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.6)	NS	NA	NS	NS	
B5	0 (0.0)	0 (0.0)	2 (16.7)	3 (8.3)	5 (10.4)	5 (2.9)	NA	0.034	0.032	0.002	
B7	3 (4.9)	2 (3.2)	0 (0.0)	3 (8.3)	3 (6.3)	8 (4.7)	NS	NS	NS	NS	
Total genotype B	46 (75.4)	44 (71.0)	10 (83.3)	26 (72.2)	36 (75.0)	126 (73.7)	NS	NS	NS	NS	
C C1	12 (19.7)	13 (21.0)	2 (16.7)	10 (27.8)	12 (25.0)	37 (21.6)	NS	NS	NS	NS	
C2	1 (1.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.6)	NS	NS	NA	NS	
C3	2 (3.3)	5 (8.1)	0 (0.0)	0 (0.0)	0 (0.0)	7 (4.1)	NS	NS	NS	NS	
Total genotype C	15 (24.6)	18 (29.0)	2 (16.7)	10 (27.8)	12 (25.0)	45 (26.3)	NS	NS	NS	NS	
No. HBV genotype	46/15 (75.4)	44/18 (71.0)	10/2 (83.3)	26/10 (72.2)	36/12 (75.0)	126/45 (73.7)	NS	NS	NS	NS	
B/C (%-B)											
Genotype and serotype											
B adw	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.8)	1 (2.1)	1 (0.6)	NA	NS	NS	NS	
adw2	38 (62.3)	39 (62.9)	9 (75.0)	21 (58.3)	30 (62.5)	107 (62.6)	NS	NS	NS	NS	
adw3	3 (4.9)	1 (1.6)	0 (0.0)	0 (0.0)	0 (0.0)	4 (2.3)	NS	NS	NS	NS	
ayw	0 (0.0)	1 (1.6)	0 (0.0)	1 (2.8)	1 (2.1)	2 (1.2)	NS	NS	NS	NS	
ayw1	5 (8.2)	3 (4.8)	0 (0.0)	4 (11.1)	4 (8.3)	12 (7.0)	NS	NS	NS	NS	
C adrq+	14 (23.0)	17 (27.4)	2 (16.7)	9 (25.0)	11 (22.9)	42 (24.6)	NS	NS	NS	NS	
adw2	0 (0.0)	1 (1.6)	0 (0.0)	1 (2.8)	1 (2.1)	2 (1.2)	NS	NS	NS	NS	
ayr	1 (1.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.6)	NS	NS	NA	NS	

CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; NS: Not significant; NA: Not applicable; HBV: Hepatitis B virus.

**Table 3** Frequencies of some HBx mutations in HBV genotype B according to different clinical diagnosis

Amino acid substitutions	n (%) in each clinical diagnosis							P			
	HCC				Total (n = 126)	CH vs LC	CH vs All HCC	LC vs All HCC	CH vs LC vs All HCC		
	CH (n = 46)	LC (n = 44)	With LC (n = 10)	Without LC (n = 26)							
T118N	23 (50.0)	6 (13.6)	4 (40.0)	7 (26.9)	11 (30.6)	40 (31.7)	< 0.001	NS	NS	0.028	
I127N/T/S	5 (10.9)	16 (36.4)	2 (20.0)	2 (7.7)	4 (11.1)	25 (19.8)	0.009	NS	0.019	NS	
K130M	8 (17.4)	23 (52.3)	5 (50.0)	11 (42.3)	16 (44.4)	47 (37.3)	0.001	0.015	NS	0.006	
V131I	8 (17.4)	22 (50.0)	4 (40.0)	11 (42.3)	15 (41.6)	45 (35.7)	0.002	0.030	NS	0.012	

respectively). Several amino acid changes were observed in both HBV genotype B and C. The prevalence of four amino acid substitutions (T118N, I127N/T/S, K130M and V131I) in HBV genotype B were significantly different between CH and LC, and three of them (T118N, K130M and V131I) showed a significant difference in prevalence between CH, LC and HCC, but none of them was significantly different between LC and HCC (Figure 1, Table 3). In contrast, none of the amino acid substitutions showed any significant difference in prevalence in the different clinical status in HBV genotype C (data not shown).

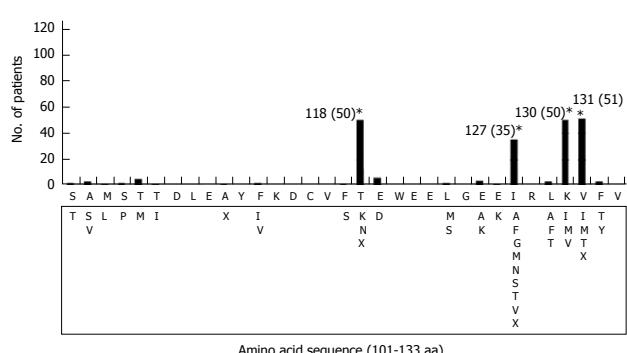
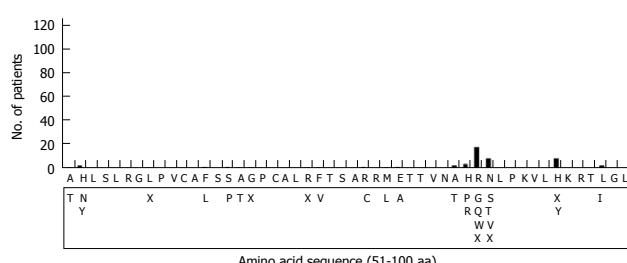
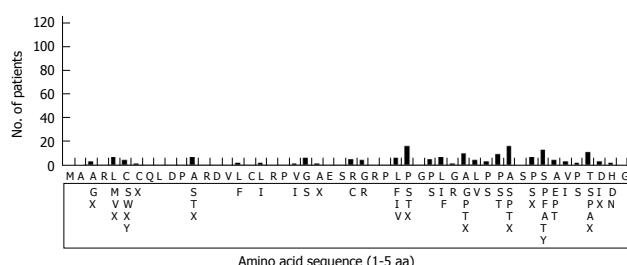
The four substituted amino acids located in BCP region, the corresponding nucleotides (C1726A/T1727(C/T) corresponding to T118N, T1753V corresponding to I127N/T/S, and A1762T/G1764A corresponding to K130M and V131I) were analyzed (Table 4). Mutations at positions 1762 and 1764 (corresponding to K130M

and V131I amino acid substitutions), either as a double mutation or an independent mutation, were significantly higher in LC and HCC than CH. Particularly, the double mutation (A1762T/G1764A) which was found in 19.7%, 59.7% and 54.2% of CH, LC and HCC, respectively ( $P < 0.001$ ). There was no significant difference in the prevalence of the double mutation between HCC with and without cirrhosis (41.7% and 58.3%). Analysis of the nucleotide at position 1753 showed that a T-to-V (A/G/C) mutation (corresponding to I127N/T/S amino acid substitutions) was significantly higher in LC (46.8%) compared with CH (18.0%) and HCC (22.9%) ( $P = 0.004$ ), suggesting that this mutation could be an indicator of liver cirrhosis. Moreover, the prevalence of T1753V mutation was also not significantly different between HCC with cirrhosis (16.7%) and that without cirrhosis (25.0%) (data not shown).

In addition, C1726A/T1727 (C/T) mutations

**Table 4** Prevalence of HBx and core promoter mutations in samples with different clinical diagnosis

Characteristics	n (%) in each clinical diagnosis						P	
	HCC		With LC (n = 12)	Without LC (n = 36)	All HCC (n = 48)	Total (n = 171)		
	CH (n = 61)	LC (n = 62)						
Genotype B/C (%)	46/15 (75.4)	44/18 (71.0)	10/2 (83.3)	26/10 (72.2)	36/12 (75.0)	126/45 (73.7)	NS	
BCP mutations							NS	
C1726A/T1727(C/T)	24 (39.3)	8 (12.9)	4 (33.3)	7 (19.4)	11 (22.9)	43 (34.1)	0.002	
T1753V	11 (18.0)	29 (46.8)	2 (16.7)	9 (25.0)	11 (22.9)	51 (40.5)	0.015	
A1762T	12 (19.7)	38 (61.3)	6 (50.0)	21 (58.3)	27 (56.3)	77 (61.1)	< 0.001	
G1764A	13 (21.3)	38 (61.3)	5 (41.7)	21 (58.3)	26 (54.2)	77 (61.1)	< 0.001	
C1766T	1 (1.6)	3 (4.8)	1 (8.3)	1 (2.8)	2 (4.2)	6 (4.8)	NS	
T1768A	1 (1.6)	2 (3.2)	1 (8.3)	2 (5.6)	3 (6.3)	6 (4.8)	NS	
A1762T/G1764A	12 (19.7)	37 (59.7)	5 (41.7)	21 (58.3)	26 (54.2)	75 (59.5)	< 0.001	



**Figure 1** Distribution and frequencies of the amino acid mutations in the 133 amino acids of HBx protein of HBV genotype B observed in the present study. Reference sequence of HBV genotype B (accession no. BAA23459) is shown at the top and mutations are shown below the reference sequence. Stars indicate the major substitutions observed and values in parentheses are number of patients with respective mutation.

(corresponding to T118N substitution) were significantly higher in CH (39.3%) than in LC (12.9%) and HCC (22.9%). These results suggested that these mutations were reversely associated with severity of liver disease. In another words, single nucleotide polymorphisms (SNPs) in C1726/T1727 have an association with liver disease manifestations. The distribution of SNPs in 1726/1727

is shown in Table 5. In HBV genotype B, most of nucleotides in 1726 were A or C. The percentage of 1726A was significantly higher in CH (52.2%) than in LC (20.5%) and HCC (33.3%) ( $P = 0.009$ ), while 1726C was more prevalent in LC (79.5%) and HCC (66.7%) than in CH (41.3%) ( $P = 0.001$ ). On the other hand, most of the nucleotides in 1727 were T, however there was no significant difference in the percentage of 1727T in CH (95.7%), LC (88.6%), and HCC (97.2%) ( $P = 0.279$ ). These results suggested that SNP in 1726, but not in 1727, of HBV genotype B was associated with the development of liver disease. In contrast, no association between SNP in the same positions in HBV genotype C and progression of liver disease was observed (Table 5).

### Comparison of BCP and HBx mutations between genotype B and C

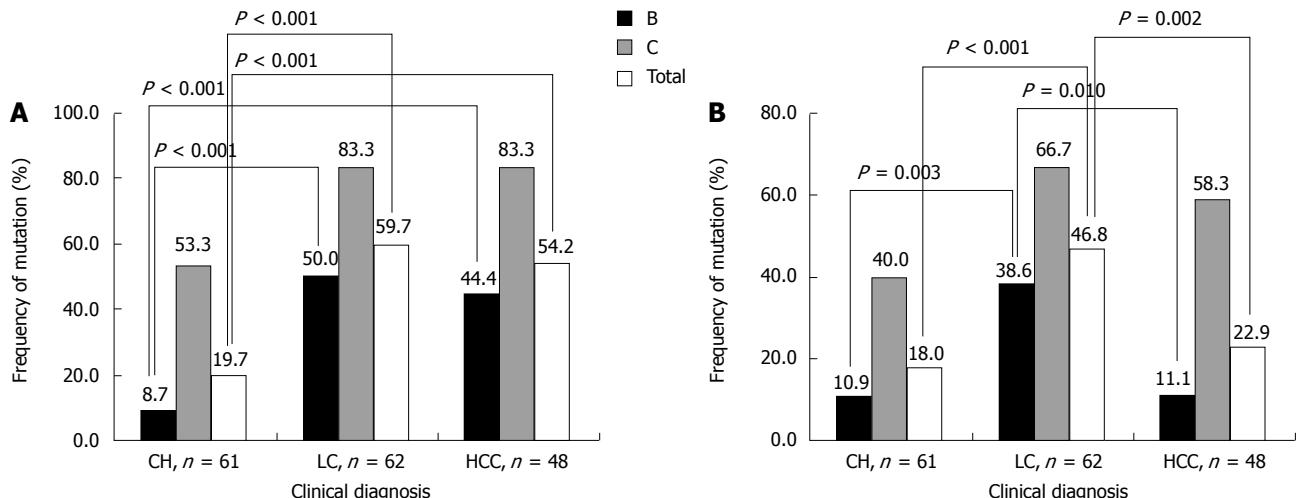
The percentage of cases with A1762T/G1764A mutation was significantly higher in genotype C than genotype B, regardless of clinical status: 53.3% vs 8.7% in CH, 83.3% vs 50.0% in LC, and 83.3% vs 45.5% in HCC (Figure 2A). From an analysis of total samples of these two genotypes, it was shown that the percentage of A1762T/G1764A mutation in genotype C was higher than that of genotype B (73.3% vs 33.3%,  $P < 0.001$ ). Similar to the results of A1762T/G1764A mutation, T1753V mutation also showed a significantly different distribution between genotypes C (55.6%) and B (20.6%) with  $P < 0.001$  (Table 6 and Figure 2B). When T1753V mutation was observed in each clinical status, its prevalence in HBV genotype C and B were 40.0% vs 10.9% in CH, 66.7% vs 38.6% in LC, and 58.3% vs 11.1% in HCC. In contrast, C1726A/T1727(C/T) mutation was more frequent in HBV genotype B (31.7%) than genotype C (6.7%) ( $P = 0.002$ ).

## DISCUSSION

Identification of viral as well as host factors associated with the development of severe liver disease including HCC may have important clinical implications in the management of patients with HBV infection. Many studies have suggested that HBV genotype might play

**Table 5** Single nucleotide polymorphisms in 1726/1727 of HBV Genotype B and C

SNP	n (%) in each HBV genotype and clinical diagnosis											
	Genotype B						Genotype C					
	HCC						HCC					
	CH (n = 46)	LC (n = 44)	With LC (n = 10)	Without LC (n = 26)	All HCC (n = 36)	P	CH (n = 15)	LC (n = 18)	With LC (n = 2)	Without LC (n = 10)	All HCC (n = 12)	P
1726A	24 (52.2)	9 (20.5)	4 (40.0)	8 (30.8)	11 (33.3)	0.009	13 (86.7)	13 (72.2)	2 (100.0)	7 (70.0)	9 (75.0)	NS
1726C	19 (41.3)	35 (79.5)	6 (60.0)	18 (69.2)	24 (66.7)	0.001	2 (13.3)	5 (27.8)	0 (0.0)	3 (30.0)	4 (33.3)	NS
1726T	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	NS	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	NA
1727A	1 (2.2)	2 (4.5)	0 (0.0)	0 (0.0)	0 (0.0)	NS	8 (53.3)	11 (61.1)	2 (100.0)	2 (20.0)	4 (33.3)	NS
1727C	0 (0.0)	1 (2.3)	0 (0.0)	0 (0.0)	0 (0.0)	NS	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	NA
1727G	1 (2.2)	2 (4.5)	0 (0.0)	1 (3.8)	1 (2.8)	NS	4 (26.7)	2 (11.1)	0 (0.0)	5 (50.0)	5 (41.7)	NS
1727T	44 (95.7)	39 (88.6)	10 (100.0)	25 (96.2)	35 (97.2)	NS	3 (20.0)	5 (27.8)	0 (0.0)	3 (30.0)	3 (25.0)	NS
1726A/1727A	1 (2.2)	1 (2.3)	0 (0.0)	0 (0.0)	0 (0.0)	NS	7 (46.7)	10 (55.6)	2 (100.0)	2 (20.0)	4 (33.3)	NS
1726A/1727G	1 (2.2)	1 (2.3)	0 (0.0)	1 (3.8)	1 (2.8)	NS	4 (26.7)	2 (11.1)	0 (0.0)	5 (50.0)	5 (41.7)	NS
1726A/1727T	22 (47.8)	7 (15.9)	4 (40.0)	7 (26.9)	11 (30.6)	0.007	2 (13.3)	1 (5.6)	0 (0.0)	0 (0.0)	0 (0.0)	NS
1726C/1727A	0 (0.0)	1 (2.3)	0 (0.0)	0 (0.0)	0 (0.0)	NS	1 (6.7)	1 (5.6)	0 (0.0)	0 (0.0)	0 (0.0)	NS
1726C/1727G	0 (0.0)	1 (2.3)	0 (0.0)	0 (0.0)	0 (0.0)	NS	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	NA
1726C/1727T	19 (41.3)	32 (72.7)	6 (60.0)	18 (69.2)	24 (66.7)	0.006	1 (6.7)	4 (22.2)	0 (0.0)	3 (30.0)	3 (25.0)	NS

**Figure 2** The prevalence of A1762T/G1764A (A) and T1753V (B) mutations in the samples with different clinical diagnoses. The number on each histogram represents the percentage of each mutation in each group. P values between the groups are shown respectively.

an important role in the development of severe liver diseases. However, it is also widely accepted that HBV genotypes appear to show varying geographic patterns in their distribution which means the association between HBV genotype and the severity of liver disease may differ from one region to another. For instance, studies from Taiwan and Japan demonstrated that HBV genotype C is associated more with severe liver disease than HBV genotype B<sup>[19-23]</sup>. Since HBV genotype B and C are the main genotypes transmitted in these areas, the investigators compared between genotype B and C. However, another study from Alaska showed that there was a significant association between HBV genotype F and the development of HCC among native Alaskan people<sup>[40]</sup>. This means that the association between HBV genotype and severity of liver disease could be different, depending on the area and the genotype of HBV transmitted in that particular area. From this study, a cross-sectional analysis of subjects from several

different centers in Indonesia, it was found that HBV genotype B was the major genotype and no association between HBV genotype as well as serotype and clinical status of liver disease was observed. Analysis of data from a prospective cohort study, however, is needed to further elucidate the association between HBV genotype and manifestations of liver disease in Indonesian HBV carriers.

Many studies have demonstrated that virus mutations, including mutations of HBx protein, BCP, and precore are linked to the severity and outcome of HBV infection. A study from Taiwan reported that the amino acid substitution at codon 31 of HBx protein (S31A) was frequently found in HCC patients and was predicted to have an association with HCC development<sup>[33]</sup>. A Japanese group also reported that a mutation at codon 38 (P38S) of HBV genotype C was associated with HCC development<sup>[34]</sup>. Recently, it was reported that mutations in HBx protein (V5M/L, P38S, H94Y, I127T/N, K130M

**Table 6 Comparison of core promoter mutations in HBV genotype B and C**

Characteristics	HBV genotype			P
	B	C	All	
No. (%) of patients	126 (73.7)	45 (26.3)	171 (100.0)	< 0.001
Age (mean ± SD)	44.3 ± 12.8	49.4 ± 13.3	45.6 ± 13.1	0.024
Male/Female (%Male)	95/31 (75.4)	32/13 (71.1)	127/44 (74.3)	NS
No. (%) of A1762T/ G1764A	42 (33.3)	33 (73.3)	75 (43.9)	< 0.001
No. (%) of T1753V	26 (20.6)	25 (55.6)	51 (29.8)	< 0.001
No. (%) of C1726A/ T1727 (C/T)	40 (31.7)	3 (6.7)	43 (25.1)	0.002

and V131I) from Korean patients are linked with severity of liver disease<sup>[35]</sup>. HBx protein analysis of samples in the present study showed that I127N/T/S, K130M and V131I amino acid substitutions are associated with severe liver disease, especially with liver cirrhosis (Table 3). However, no association between S31A as well as P38S mutations and liver disease progression was found, which is different from previous studies in Taiwan, Japan, and Korea<sup>[33-35]</sup>.

It is well known that the double mutation (A1762T/G1764A) in BCP is associated with an increased risk of liver disease. For instance, the frequency of double mutation (A1762T/G1764A) increased with advancing clinical status in Taiwanese patients [3%, 11%, 32% and 64% in asymptomatic carriers (AC), LC, CH, and HCC groups, respectively]<sup>[24]</sup>. A recent report from China has also demonstrated that the incidence of double mutation increased along with the progression of liver disease; the percentage of the double mutation was 33%, 56% and 85% in CH, LC, and HCC groups, respectively<sup>[31]</sup>. In Indonesian patients, however, the A1762T/G1764A double mutation was increased in CH from 19.7% to 59.7% in LC and was slightly decreased in HCC (54.2%) (Table 4). These results suggest that the double mutation is associated with severe liver disease. In addition, analysis of the nucleotide at position 1753 showed that a T-to-V (A/G/C) mutation increased to 46.8% in LC from 18.0% in CH, but dramatically decreased in HCC (22.9%) (Table 4), suggesting that this mutation is associated with liver cirrhosis rather than HCC. In contrast, analysis of sera or plasma from Japanese subjects with AC, CH, LC and HCC infected with HBV genotype C showed that the percentage of T1753V mutation increased with progression of liver disease<sup>[41]</sup>. It is also reported that T1753V mutation was higher in HCC (53.2%) compared with LC (18.8%) and CH (9.8%)<sup>[31]</sup>. These results were inconsistent with the present study, particularly in LC and HCC. These discrepancies might be associated with HCC status; most of HCC cases in the present study were without cirrhosis. Another possibility is that most of the samples analyzed in the previous reports were HBV genotype C, whereas most of samples in the present study were HBV genotype B.

The most interesting finding of the present study is the association of SNP at position 1726 of HBV

genotype B, but not genotype C, and severity of liver disease. Since HBV genotype B is the major genotype in Indonesia, this finding is important for the management and prevention of HBV carriers from developing more advanced disease such as liver cirrhosis and HCC in Indonesia. This association, however, has to be confirmed by analyzing more samples. A comparison of mutation prevalence between HBV genotype B and C showed that the percentage of T1753V and A1762T/G1764A mutations were higher in genotype C than in genotype B (Table 6). These results are in accordance with previous findings from Taiwan and China<sup>[20,31]</sup>.

In summary, the present study demonstrated that HBV genotype B and C were detected among HBV-associated liver disease patients in Indonesia, and genotype B was predominant. It was found that HBV genotype, as well as the serotype, might not be associated with an increased risk of HCC. The A1762T/G1764A and T1753V mutations in BCP can be used as an indicator for progression of liver disease in Indonesian patients.

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## COMMENTS

### Background

Hepatitis B virus (HBV) genotype, mutations in the core promoter, precore or HBx gene have been shown to have an association with severe liver disease. The aims of the study were to identify the distribution of HBV subgenotype and basal core promoter (BCP) mutations among patients with HBV-associated liver disease in Indonesia, and analyze the possible association between HBV genotype and/or BCP mutations and severity of liver disease among Indonesian patients.

### Research frontiers

Although there were some reports on the distribution of HBV genotype in Indonesia, the association between HBV genotype and/or BCP mutations and liver disease progression has not been investigated. Therefore it is important to have information not only related to the distribution of HBV genotype/subgenotype and BCP mutations in patients with different clinical status, but also the association of HBV genotype/subgenotype and/or BCP mutations and liver disease progression in Indonesia.

### Innovations and breakthroughs

The present study demonstrated that only HBV genotype B and C were detected among HBV-associated liver disease patients in Indonesia, and genotype B was predominant. It was found that HBV genotype, as well as the serotype, might not be associated with an increased risk of hepatocellular carcinoma (HCC). The double mutation (A1762T/G1764A) was associated with progression of liver disease, while T1753V mutation could be used as an indicator of liver cirrhosis rather than HCC. In addition, SNP in 1726 has an association with manifestations of liver disease.

### Applications

The double mutation (A1762T/G1764A) can be used for the prediction of severe liver disease including cirrhosis and HCC, whereas the T1753V mutation is a predictor of liver cirrhosis in Indonesian patients. In addition, SNP in 1726 can also be used for the prediction of liver disease severity.

### Terminology

HBs; HBs gene encode the surface protein of HBV that consist of preS1, preS2, and S. HBx; HBx gene encode functional X protein. BCP; BCP can be

considered a part of HBx gene that regulate the core gene expression.

#### Peer review

The study provides a identify HBV subgenotype and basal core promoter (BCP) mutations distribution among HBV-associated liver disease patients in Indonesia. The work is of theoretical and practical importance.

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# Imaging features of intraductal papillary mucinous neoplasms of the pancreas in multi-detector row computed tomography

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cystic lesions and main pancreatic ducts (MPDs) were significantly larger in malignant IPMNs compared with those of the benign IPMNs ( $P < 0.05$ ). The combined-type IPMNs had a higher rate of malignancy than the other two types of IPMNs ( $P < 0.05$ ). Tumors with mural nodules and thick septa had a significantly higher incidence of malignancy than tumors without these features ( $P < 0.05$ ). Communication of side-branch IPMNs with the MPD was present in nine cases at pathologic examination. Seven of them were identified from CTA and MPVR or CR images. From comparison with the pathological diagnosis, the sensitivity, specificity, and accuracy of MDCT in characterizing the malignancy of IPMN of the pancreas were determined to be 100%, 87.5% and 95%, respectively.

**CONCLUSION:** MDCT with CTA and MPVR or CR techniques can elucidate the imaging features of IPMNs and help predict the malignancy of these tumors.

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**Key words:** Computed tomography; Diagnostic imaging; Intraductal papillary mucinous neoplasm; Pancreatic neoplasms

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## Abstract

**AIM:** To retrospectively evaluate the imaging features of pancreatic intraductal papillary mucinous neoplasms (IPMNs) in multi-detector row computed tomography (MDCT).

**METHODS:** A total of 20 patients with pathologically-confirmed intraductal papillary mucinous neoplasms (IPMNs) were included in this study. Axial MDCT images combined with CT angiography (CTA) and multiplanar volume reformations (MPVR) or curved reformations (CR) were preoperatively acquired. Two radiologists (Tan L and Wang DB) reviewed all the images in consensus using an interactive picture archiving and communication system. The disputes in readings were resolved through consultation with a third experienced radiologist (Chen KM). Finally, the findings and diagnoses were compared with the pathologic results.

**RESULTS:** The pathological study revealed 12 malignant IPMNs and eight benign IPMNs. The diameters of the

## INTRODUCTION

Intraductal papillary mucinous neoplasms (IPMNs) of the pancreas, defined by the World Health Organization (WHO), are a broad spectrum of neoplasms arising from the pancreatic duct epithelia and characterized by cystic dilatation of the main and/or branch pancreatic ducts, and occasionally by the presence of mural nodules (papillary excrescence or protuberances)<sup>[1-6]</sup>. According to

the site and extent of involvement, IPMNs are classified into three subtypes: main duct type, branch duct type, and combined-type (mixed type) with both main and branch duct lesions<sup>[2,7,8]</sup>. Although an IPMN is a rare tumor, the increasingly common use of imaging techniques has contributed to the identification of an increasing number of the lesions, even in entirely asymptomatic cases<sup>[9-13]</sup>.

According to the International Guidelines for management of IPMN<sup>[2]</sup>, magnetic resonance imaging (MRI) along with magnetic resonance cholangiopancreatography (MRCP) is referred to as the best imaging modality to outline the gross appearance of IPMN. However, computed tomography (CT) is still the mainstay in the evaluation of patients with IPMNs of the pancreas. Improvements in CT such as the evolution of post-processing techniques in multi-detector row CT (MDCT), have enhanced the capability of CT in the evaluation of abnormalities of the pancreatic parenchyma and the pancreatic ducts in patients with IPMNs<sup>[10]</sup>. Axial CT images combined with multiplanar volume reformations (MPVR) or curved reformations (CR) and CT angiography (CTA) with maximum intensity projection should display the imaging features of IPMNs in more detail. Recent studies assessed the capability of MDCT in defining the invasiveness of the IPMN and in evaluating the resectability of IPMNs<sup>[14,15]</sup>. The IPMN can evolve through all biological stages, from slight dysplasia to carcinoma. As one of the few surgically curable pancreatic tumors, accurate preoperative prediction of malignancy remains one of the major issues in the optimal treatment of IPMNs, and it also influences the outcome of the resection<sup>[16,17]</sup>.

The present study retrospectively assessed the imaging findings of IPMNs with MDCT and evaluated the capabilities of MDCT with emphasis on the postprocessing techniques to predict the malignancy of IPMNs.

## MATERIALS AND METHODS

### Patient population

Between December 2005 and March 2008, IPMN of the pancreas was diagnosed in 31 cases at our institution based on surgico-pathological examination. Of these 31 cases, 20 patients [11 male, 9 female; mean age, 62 years (range, 41-81 years)] who underwent MDCT within a month prior to surgery were recruited into this study. At our hospital, surgical planning requires that most patients in whom IPMN of the pancreas is suspected undergo MDCT including CTA and MPVR or CR processing rather than dynamic MRI with MRCP for evaluation of the extent of the disease and the relationship between affected parts of the pancreas and surrounding vessels and organs. The lesions revealed by the subsequent pathological examinations were divided into three subtypes: benign adenoma, noninvasive carcinoma including borderline malignancy and carcinoma *in situ*, and invasive carcinoma in accordance with the WHO classification. A borderline lesion was defined as a tumor which was not overtly malignant but had some foci of severe cellular atypia, indicating that it should be treated as malignant. Thus, an IPMN considered at imaging to be malignant could include a borderline malignancy, carcinoma *in situ*, and invasive carcinoma on pathology

in this study. The mean interval between the imaging and surgery was 22 d (range, 10-31 d). Institutional Review Board approval and waiver of informed consent for this retrospective study were obtained.

### CT techniques

All CT examinations were performed with a 4- or 16-slice MDCT scanner (Lightspeed QX/I or Lightspeed 16; GE Medical Systems, Milwaukee, Wis). The parameters applied in the CT scan were as follows: a tube voltage of 120 kVp, a tube current of 280 mA, and tube rotation time of 0.5-0.8 s. Prior to administration of contrast agents, non-enhanced CT of the upper abdomen was performed with 10 mm slice thickness and 10 mm spacing. Non-ionic contrast materials with an iodine concentration of 300 mgI/mL were injected into the antecubital vein at a rate of 3-4 mL/s with an 18-20-gauge cannula for all the patients. In addition, 40 mL dextrose was administered at the same rate immediately after the administration of contrast agents. The pancreatic phase of dual phased CT was started at 35 s after the initiation of contrast material injection, whereas the venous phase was performed afterwards with a delayed time of 65-70 s. For the pancreatic phase, a slice thickness of 1.25 mm and a table speed of 11.25 mm/s were utilized while a slice thickness of 5 mm and a table speed of 18.75 mm/s were employed for the venous phase. Axial images were retrospectively reconstructed at a 50% overlap, using a 1.25 mm slice thickness, and 0.625 mm spacing.

In all patients, CTA and 2-dimensional (2D) MPVR or CR images were generated from the source axial images at a commercially available dedicated workstation (ADW 4; GE Medical Systems) by a radiologist (Zhao YE).

### Image analysis

Two radiologists (Tan L and Wang DB) reviewed all the images in consensus at an interactive picture archiving and communication system workstation. The readers were aware of the diagnosis of an IPMN but were blinded to the findings of surgery and pathological examination. In cases of interobserver disagreement, final decisions were made through consultation with a third experienced radiologist (Chen KM).

Each reader recorded the following items: (1) the largest diameter of the cystic lesion; (2) the largest diameter of the main pancreatic duct (MPD); (3) classification of the tumors as main duct type, branch duct type, and combined type (mixed type). The main duct type was diagnosed when dilatation of the MPD had increased its diameter to more than 5 mm. The presence of one or multiple cystic lesions in the pancreatic parenchyma, without dilatation of the MPD, indicated that a branch duct-type tumor was present. The combined type was diagnosed when the pancreas contained one or more cystic lesions and the diameter of the dilated MPD was more than 5 mm; (4) locations of the lesions: in the head or uncinate process, the body, or the tail of the pancreas. Diffuse involvement was denoted when the lesion involved the entire gland; (5) internal solid structures: presence or absence of mural nodules and thick septa; (6) other findings including vascular involvement, lymph node enlargement,

**Table 1 Relationship between imaging findings on MDCT and surgico-pathological diagnosis of pancreatic IPMNs**

MDCT findings	Surgical and pathological results		<i>P</i>
	Benign ( <i>n</i> = 8)	Malignant ( <i>n</i> = 12)	
Largest diameter of cystic lesion	21.6 ± 10.3 mm	43.5 ± 16.5 mm	< 0.05
Caliber of main pancreatic duct	3.3 ± 1.6 mm	7.5 ± 5.5 mm	< 0.05
Morphologic type			< 0.05
Branch duct	4	1	
Combined	2	10	
Main duct	2	1	
Location of cystic lesion			> 0.05
Head and uncinate	4	10	
Body	0	1	
Tail	2	0	
Diffuse	2	1	
Solid structures inside lesion			< 0.05
Mural nodules and thick septa	2	10	
No mural nodules or thick septa	4	1	

MDCT: Multi-detector row computed tomography; IPMNs: Intraductal papillary mucinous neoplasms.

and duodenal involvement, as well as dilatation of the bile duct and distant metastasis. All the imaging diagnoses were compared with the surgico-pathological outcomes.

### Statistical analysis

The unpaired Student's *t*-test was used to assess the differences in the largest diameters of the cystic lesion and MPDs between the benign and malignant groups. Fisher's exact test was used to evaluate the differences in classification, location, and internal solid structures between the benign and malignant tumors confirmed by pathology. The sensitivity, specificity, and accuracy of the MDCT diagnosis for IPMNs of the pancreas were calculated. *P* < 0.05 was considered statistically significant. Statistical analysis was performed with SPSS 13.0 computer software (Chicago, IL, USA).

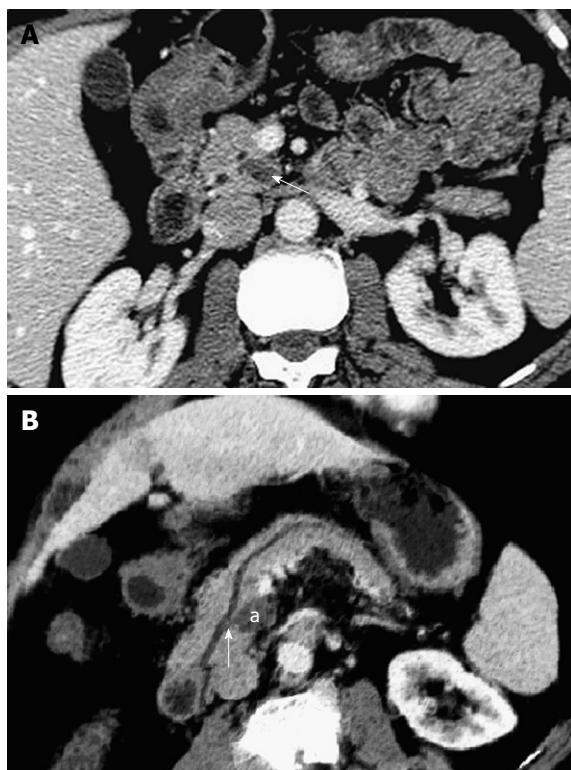
## RESULTS

### Pathologic results

Five patients (5 of 20, 25%) had invasive carcinoma, whereas seven patients (7 of 20, 35%) had a noninvasive carcinoma (including three borderline lesions and four carcinoma *in situ*). Both of these lesions were regarded as malignant in this study. The lesions in the other eight patients (8 of 20, 40%) were classified as benign IPMNs by pathology. Among the 12 malignant IPMNs, two patients had duodenal involvement with one also having vascular involvement, another two patients had regional lymph node metastasis on surgico-pathological examination, and one patient only had vascular involvement.

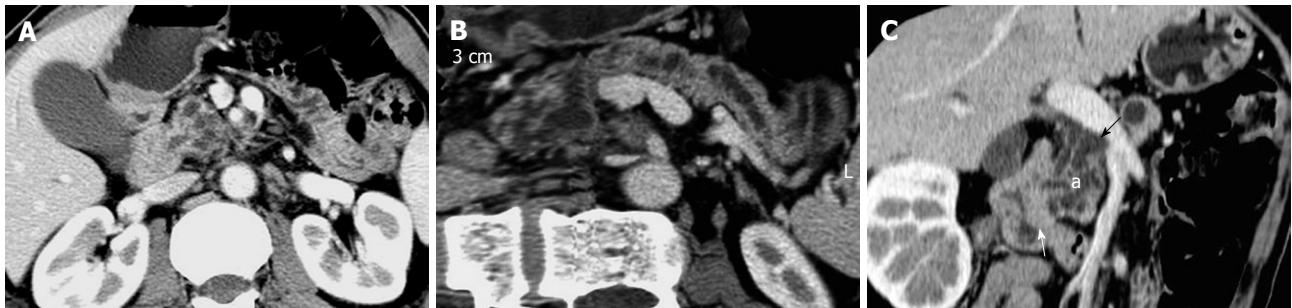
### Imaging features of IPMNs compared with surgico-pathological results

The imaging features of IPMNs at MDCT are summa-

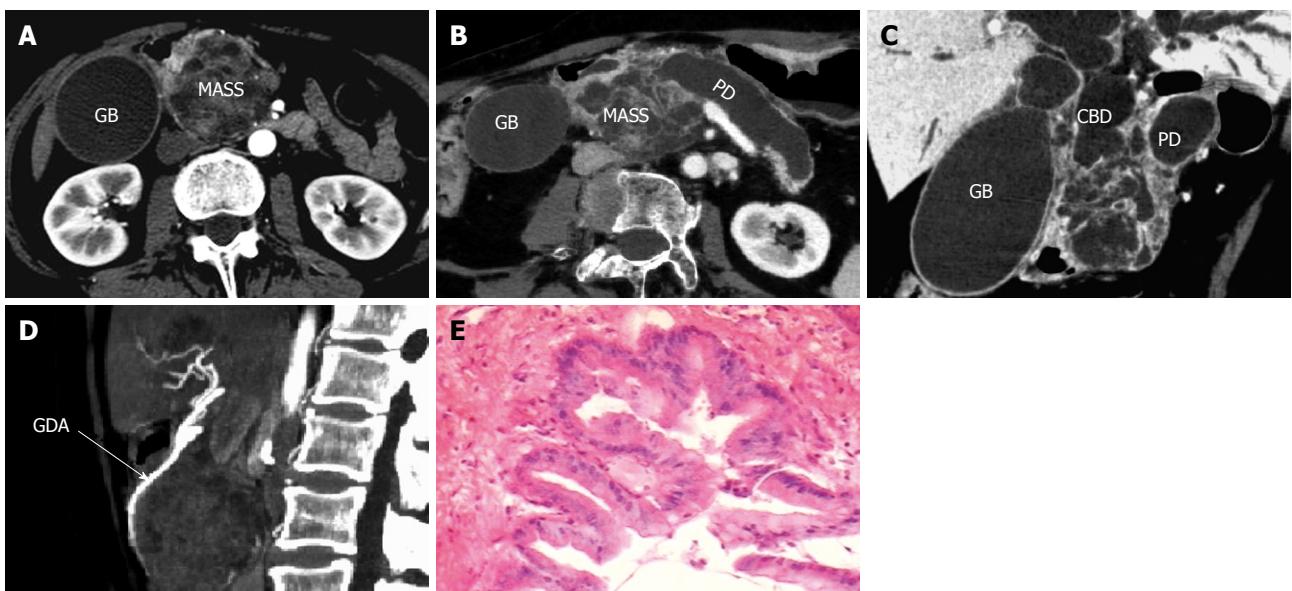


**Figure 1 Pathologically confirmed benign branch-duct-type IPMN in a 47-year-old woman with abdominal discomfort for about 6 mo. A:** There was a 2-cm cystic mass (white arrow) in the uncinate process of the pancreas at the axial abdominal MDCT image; **B:** The cystic mass (a) in the posterior pancreatic parenchyma was demonstrated with a communication (white arrow) between the mass and the main pancreatic duct which was slightly dilated in the curved reformed (CR) image.

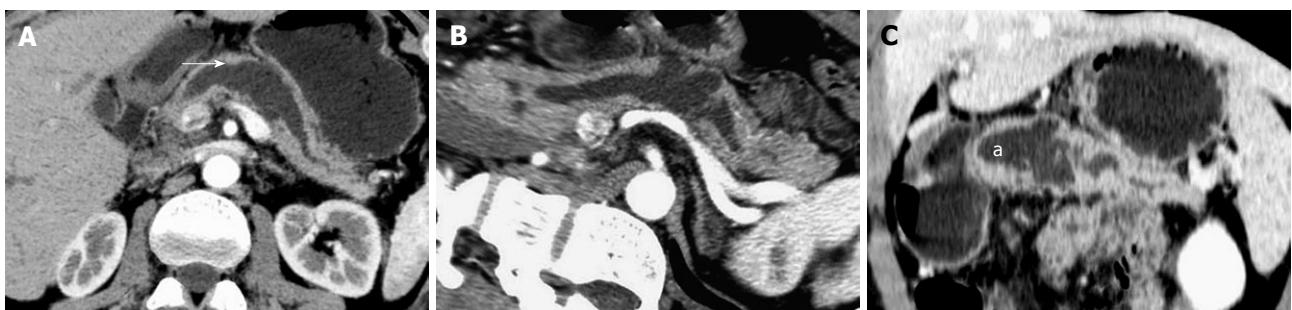
rized in Table 1. The largest diameters of the cystic lesions were 21.6 ± 10.3 mm and 43.5 ± 16.5 mm for benign and malignant IPMNs, respectively. A statistically significant difference has been demonstrated between the sizes of the lesions of benign and malignant IPMNs (*P* < 0.05). The mean diameter of most dilated segments of MPD was 3.3 ± 1.6 mm in patients with benign IPMNs, and 7.5 ± 5.5 mm in patients with malignant IPMNs. The diameter of the MPD was significantly larger in patients with malignant IPMNs compared with that of benign cases (*P* < 0.05) (Figure 1). Based on MDCT findings, five patients (5 of 20, 25%) were classified as branch duct type, three patients (3 of 20, 15%) as main duct type, and 12 patients (12 of 20, 60%) as combined type. With pathognomonic correlation, malignant IPMNs presented in one of the five patients with branch duct type, one of three patients with main duct type, and 10 of the 12 patients with combined type. Significant correlation was shown between the type of ductal involvement and the pathological results. The combined type also had a higher rate of malignancy (*P* < 0.05) (Figure 2). Three combined-type cases had accompanying marked dilatation of the biliary tracts including extrahepatic and intrahepatic bile ducts (Figure 3). The pancreatic head and uncinate process were the most common locations of IPMNs, accounting for 70% (14 of 20). Malignant IPMNs presented in 10 of these 14 patients with an incidence of 83.3% (10 of 12) among all the malignant cases. Only one malignant IPMN identified



**Figure 2 Pathologically confirmed malignant combined-type IPMN in a 41-year-old man with abdominal and back pain for about 2 years.** A: A 4 cm cystic mass with multiple septa arising from the pancreatic head was revealed in the axial MDCT image; B: Besides the cystic mass in the pancreatic head, the profile of the main pancreatic duct, which was severely dilated, was depicted on the CR image; C: The cystic lesion (a) in the pancreatic head invaded the duodenum and main portal vein resulting in the duodenal wall thickening (white arrow) with marked enhancement and irregular narrowing (dark arrow) of the vessel in the multiplanar volume reformation (MPVR) image.



**Figure 3 Pathologically confirmed malignant combined-type IPMN in a 65-year-old man with jaundice and abdominal pain for about 1 year.** A: An 8 cm cystic and solid mass (MASS) was seen in the axial arterio-phased MDCT image with contrast agents. The gallbladder (GB) was distended; B: The heterogeneous mass was shown with severe dilatation of the main pancreatic duct (PD) and the gallbladder (GB) in the CR image; C: The profile of the dilatation of pancreatobiliary system (CBD, common bile duct) was entirely depicted in the MPVR image; D: The gastroduodenal artery (GDA) showed irregularity as a result of infiltration of the tumor; E: The tumor consisted of papillary proliferations of tall columnar mucin-producing epithelium. Atypical epithelium characterized by enlarged nuclei (HE,  $\times 150$ ).



**Figure 4 Pathologically confirmed malignant combined-type IPMN in a 55-year-old man with abdominal pain for 1.5 years.** A: A 3 cm  $\times$  10 cm longitudinally cystic mass in the pancreatic body was shown with a mural nodule (white arrow) in the axial MDCT images; B: The classification of combined type for this case was accurately defined by the CR image; C: The profile of the cystic mass (a) and dilatation of the branch duct and the upstream pancreatic duct were identified in the MPVR images.

by MDCT was located at the body of the pancreas (Figure 4), whereas the IPMNs arising from the pancreatic tail in two cases were both benign and verified by pathology. The other three (3 of 20, 15%) cases had diffuse lesions,

and one of these (33.3%) was malignant. Among 17 branch duct-type and combined-type lesions, mural nodules and thick septa were seen in 12 cases (Figure 4). Ten of these 12 were referred to as malignant IPMNs on pa-

thology. Tumors with mural nodules and thick septa had a significantly higher incidence of malignancy than tumors without them ( $P < 0.05$ ).

No distant metastasis was revealed on MDCT in any of the patients. Vascular invasion was revealed in two patients from CTA images (Figure 2), and was proven by surgico-pathological studies. Lymph node enlargement was seen in three patients on axial CT images, but one was a false-positive when compared with the results of pathological examination. Two patients had duodenal involvement depicted by MPVR images and confirmed by pathological examination (Figure 2), and one of these had vascular involvement. Vascular involvement was also detected in another case. Communication of side-branch IPMNs with the MPD was present in nine cases at pathological examination. Seven of these (77.7%, 7 of 9) were identified from CTA and MPVR images (Figure 1).

From a comparison with the pathological outcomes, the sensitivity, specificity, and accuracy of MDCT in characterizing the malignancy of IPMN of the pancreas were determined to be 100%, 87.5% and 95%, respectively.

## DISCUSSION

After the first report of IPMN in 1982, this tumor entity has been increasingly recognized over the past decades as a result of the markedly improved imaging modalities<sup>[18,19]</sup>. IPMNs mainly occur in the 6th to 7th decades of life, affecting males slightly more frequently than females. IPMNs account for 0.5% of all pancreatic neoplasms found at autopsy, 7.5% of clinically diagnosed pancreatic neoplasms, and 16.3% of surgically resected pancreatic neoplasms<sup>[20]</sup>. The IPMN was histologically defined by the WHO as “intraductal mucin-producing neoplasm with tall columnar mucin-containing epithelium with or without papillary projections, involving the MPD and/or major side branches”<sup>[21]</sup>. The IPMN is also believed by some to follow the so-called “adenoma-carcinoma” sequence<sup>[19]</sup>. Sohn et al<sup>[22]</sup> reported that there appears to be a lag time of approximately 5 years in the progression from adenoma to invasive carcinoma and that progression to invasive carcinoma occurs relatively quickly once moderate dysplasia is found. Kawai et al<sup>[17]</sup> reported that the malignancy rate of IPMNs was 48% while it was 67% in the study conducted by Lopez Hänninen et al<sup>[23]</sup>. In our study, the malignancy rate was 60% (12 of 20). The treatment decision with regard to IPMN is often based on the patient’s age at presentation, the lesion location in the pancreas, the extent of ductal involvement, and also the presence or absence of malignant features<sup>[24]</sup>. Since most of the main duct IPMNs will progress into invasive carcinomas, the resection of main duct IPMNs and mixed variant IPMNs is recommended if the patient is a good surgical candidate with a reasonable life expectancy<sup>[25,26]</sup>. Currently, the cross-sectional imaging studies including MDCT and MRI with MRCP play a crucial role in structuring the treatment protocol for the patients with IPMNs.

Since the MDCT is widely used in clinical practice, more and more IPMNs are detected. Because of the overproduction of mucus, MPD dilatation can occur both proximal and distal to the tumor. In our study,

most (14 of 20) of the IPMNs were located at the head and uncinate process of the pancreas, but there was no statistically significant differences in the distribution of the lesion locations because of the bias resulting from the small size of the patient population in this group. However, the cystic lesions in the pancreatic parenchyma and the diameter of the MPD in malignant IPMNs presented with significantly larger sizes than the benign IPMNs ( $P < 0.05$ ). More combined-type IPMNs were malignant than the other two subtypes ( $P < 0.05$ ). The MDCT features most specific for a malignant IPMN were mural nodules and thick septa inside the lesion (10 of 20) in this group as indicated in the literature<sup>[27,28]</sup>. Thinner-slice reformed CTA and MPVR or CR images showed other malignant signs clearly, including vascular invasion, lymph node enlargement, and duodenal involvement, as well as dilatation of the common bile duct and common hepatic duct (Figure 3). Ogawa et al<sup>[15]</sup> reported that the sensitivity, specificity, and accuracy for identifying malignancy were 83%, 81% and 82% and for identifying pancreatic parenchymal invasion were 90%, 88% and 89%, respectively. According to the International Consensus Guidelines for management of IPMN, the branch duct IPMNs are benign more frequently than the main duct IPMNs<sup>[2]</sup>. The data in this study also complied with this rule. However, compared with the published series from Europe, Japan, and the USA<sup>[9,29-32]</sup>, the main duct IPMN had a lower rate of malignancy (1 of 3) in the present study, whereas the combined-type IPMNs demonstrated malignancy in more cases (10 of 12) on pathology. The sensitivity, specificity, and accuracy of MDCT in characterizing the malignancy of IPMNs were 100%, 87.5% and 95%, respectively. They were a little bit higher than the data in the published literature<sup>[2,15]</sup>. This could be explained possibly by the bias generated by the small number of purely main duct IPMNs and the limited size of the entire patient population in this group. Moreover, because of the limited cases of IPMN recruited in the present study, we considered the noninvasive carcinoma including borderline malignancy and carcinoma *in situ*, and invasive carcinoma together as a malignancy for analysis. On the other hand, this strategy without separation of the noninvasive and invasive carcinoma should be quite reasonable in the clinical context since progression from noninvasive malignancy to invasive carcinoma occurs relatively quickly once moderate dysplasia is found<sup>[22]</sup>. To some extent, the noninvasive carcinoma including borderline malignancy and carcinoma *in situ* should attract as much attention as invasive carcinoma in this setting where even the benign adenoma can progress to carcinoma in only 5 years<sup>[22]</sup>. Therefore, more prospective studies with a big IPMN population are required in order to reveal the more accurate profile of Chinese patients in the future.

Compared with the combined type IPMNs, patients with the branch-duct type IPMN without malignant features can be managed by follow-up examinations instead of surgery, particularly when the patient refuses surgery, or when the patient is in poor condition with other severe concomitant disease<sup>[33]</sup>. Thus, the imaging

modalities such as MDCT could be valuable in deciding whether the patient should undergo surgery or follow-up in most cases. The presence of communication of the pancreatic cystic lesion with the MPD is one of the most reliable findings for the diagnosis of branch duct IPMN<sup>[34]</sup>. With routine transverse CT scanning of the pancreas, the communicating duct is not easily seen. MPVR or CR images can markedly increase the chance of identification of the interaction.

Normally, IPMNs have a better outcome and prognosis compared to pancreatic ductal adenocarcinomas. The overall 5-year survival rate has been reported to exceed 80% for noninvasive IPMNs and 50% for the invasive malignant IPMNs<sup>[19]</sup>. Therefore, correct evaluation of IPMN is extremely important as a recent analysis has suggested that this entity is one of the few surgically curable pancreatic neoplasms<sup>[18]</sup>. Prior investigations showed MRI with MRCP was useful for assessment of IPMN as a noninvasive approach<sup>[23,35,36]</sup>. However, its spatial resolution is not as high as that of CT. Moreover, MDCT scanning is fast enough to acquire all necessary imaging data during a single breath-hold in which much thinner sliced images can be generated. It is less subject to respiratory motion and partial-volume effects than MRI<sup>[37,38]</sup>. Since most of the patients are elderly, the abovementioned characteristics of MDCT seem to be important for patients with IPMNs. With CTA and MPVR or CR images using 3D or 2D modes in different planar directions, MDCT can show the lesion itself, the surrounding structures, and the nearby vessels, as well as the bile duct during one set of CT scans. The reformed images of bile ducts were similar to the MRCP image (Figure 3). Dilatation of the bile ducts and the enlarged gallbladder could be clearly depicted. With the postprocessing techniques, CTA and MPVR or CR images can be generated with more details about the IPMN and the surrounding parenchyma or adjacent vessels.

In conclusion, MDCT scanning with CTA and MPVR or CR techniques can help predict malignant IPMN by differentiating the various types of ductal involvement and demonstrating the mural nodules and thick septa of the lesion, the MPD dilatation of the combined subtypes, the size of the cystic lesion as well as the involvement of surrounding structures. MDCT can be referred to as the diagnostic tool of choice for accurate evaluation of IPMN before treatment.

## COMMENTS

### Background

Intraductal papillary mucinous neoplasm (IPMN) mainly occurs in the 6th to 7th decades of life, affecting males slightly more frequently than females. IPMN accounts for 0.5% of all pancreatic neoplasms found at autopsy, 7.5% of clinically diagnosed pancreatic neoplasms, and 16.3% of surgically resected pancreatic neoplasms. The increasingly common use of imaging techniques has contributed to the identification of an increasing number of the lesions, even in entirely asymptomatic cases.

### Research frontiers

Accurate preoperative evaluation of an IPMN is extremely important for the clinician involved in the diagnosis and further evaluation and intervention, as it is one of the few surgically curable pancreatic neoplasms. However, main duct and combined type IPMNs are more likely to be malignant with biological aggressiveness. A noninvasive imaging approach is the tool of choice for

assessing an IPMN of the pancreas preoperatively and can provide accurate information for planning treatment protocols. Although magnetic resonance imaging with magnetic resonance cholangiopancreatography is referred to as the best imaging modality to outline the gross appearance of IPMNs, computed tomography (CT) is still the mainstay in evaluation of patients with IPMNs of the pancreas. Moreover, multi-detector row CT (MDCT) scanning is fast enough to acquire all the necessary imaging data during one single breath-hold in which much thinner sliced images can be generated for reformations.

### Innovations and breakthroughs

There is limited published literature concerning the prediction of malignancy of IPMNs of the pancreas. In this series, the combined-type IPMNs had a higher rate of malignancy than the other two types of IPMNs ( $P < 0.05$ ). The diameters of the cystic lesion and main pancreatic duct (MPD) were significantly larger in malignant tumors compared with those of benign IPMNs ( $P < 0.05$ ). Tumors with mural nodules and thick septa had a significantly higher incidence of malignancy than tumors without these features ( $P < 0.05$ ). In comparing with the pathological results, the sensitivity, specificity, and accuracy of MDCT in characterizing the malignancy of IPMN of the pancreas were determined as 100%, 87.5% and 95%, respectively. The imaging findings were predictive of the malignancy of the IPMNs.

### Applications

Based on their research, MDCT scanning with CT angiography (CTA) and MPVR or CR techniques can help predict malignant IPMN by differentiating the various types of ductal involvement and demonstrating the mural nodules and thick septa of the lesion, the MPD dilatation of the combined subtypes, the size of the cystic lesion as well as the involvement of surrounding structures. MDCT can be referred to as the diagnostic tool of choice in accurate evaluation of IPMN before treatment.

### Terminology

IPMN: histologically defined by the World Health Organization as an "intraductal mucin-producing neoplasm with tall columnar mucin-containing epithelium with or without papillary projections, involving the MPD and/or major side branches"; MDCT: multiple detectors applied to CT. This modality can improve the scanning speed and spatial resolution dramatically. Furthermore, MDCT is intrinsically suitable for CTA scanning.

### Peer review

The authors evaluated the predictive factors for the presence of malignancy associated with IPMN based on their own data. It is well organized and an overall theoretical analysis is given. The conclusions are scientifically reliable and valuable.

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BRIEF ARTICLES

## Conservative resection for benign tumors of the proximal pancreas

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### Abstract

**AIM:** To evaluate the safety and long-term prognosis of conservative resection (CR) for benign or borderline tumor of the proximal pancreas.

**METHODS:** We retrospectively analyzed 20 patients who underwent CR at the Second Affiliated Hospital of Zhejiang University School of Medicine between April 2000 and October 2008. For pancreaticojejunostomy, a modified invagination method, continuous circular invaginated pancreaticojejunostomy (CCI-PJ) was used. Modified continuous closed lavage (MCCL) was performed for patients with pancreatic fistula.

**RESULTS:** The indications were: serous cystadenomas in eight patients, insulinomas in six, non-functional islet cell tumors in three and solid pseudopapillary tumors in three. Perioperative mortality was zero and morbidity was 25%. Overall, pancreatic fistula was present in 25% of patients. At a mean follow up of 42.7 mo, all patients were alive with no recurrence and no new-onset diabetes mellitus or exocrine dysfunction.

**CONCLUSION:** CR is a safe and effective procedure for patients with benign tumors in the proximal pancreas, with careful CCI-PJ and postoperative MCCL.

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**Key words:** Adenoma; Islet cell; Conservative

resection; Cystadenoma; Enucleation; Insulinoma; Pancreatectomy; Pancreatic fistula; Pancreatic neoplasms

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### INTRODUCTION

Pancreaticoduodenectomy (PD) and distal pancreatectomy (DP) are standard operations for tumors located in the proximal portion of the pancreas. However, these standard techniques are associated with a significant risk of long-term endocrine and exocrine impairment. Recently, there has been increased interest in conservative surgery in an attempt to preserve as much as possible the pancreatic parenchyma and integrated upper digestive system anatomy. For patients with benign or low-grade malignant tumors, conservative resection (CR) such as central pancreatectomy or enucleation has been investigated to maintain the normal upper digestive system anatomy, and to reduce the risk of development of exocrine and endocrine functional insufficiency. Although CR of the proximal pancreas may be more appropriate and less time-consuming, it has not been used widely previously because of its high morbidity, especially pancreatic fistula. The focus of this study was to evaluate the safety and outcomes of CR for benign or borderline tumors of the head, neck and proximal part of the pancreas.

### MATERIALS AND METHODS

Twenty patients with benign or borderline tumors

localized in the head, neck and proximal part of the pancreas, who were treated with central pancreatectomy ( $n = 11$ ) and enucleation ( $n = 9$ ), between April 2000 and October 2008, were analyzed retrospectively. Data on preoperative, intraoperative and postoperative care were collected and maintained on a secure database. Preoperative parameters included demographics, clinical presentation, and exocrine and endocrine evaluation. Preoperative imaging modalities such as abdominal spiral contrast CT and ultrasound imaging were used to evaluate the suitability for surgical resection in all cases. Intraoperative details including operative time, total blood loss, transfusion and the method of surgery were recorded. Postoperative events and clinical outcomes such as surgical complications, mortality, pathological data and long-term follow-up were recorded carefully.

Postoperative pancreatic fistula was defined as drainage of  $> 50$  mL per 24 h of fluid, with amylase content  $> 3$  times serum amylase activity for  $> 10$  d after operation<sup>[1,2]</sup>. Perioperative mortality was defined as death in the hospital or within 30 d. Delayed gastric emptying (DGE) was defined to be present when nasogastric intubation was maintained for  $\geq 10$  d, combined with at least one of the following: vomiting after removal of the nasogastric tube, reinsertion of nasogastric tube, or failure to restore oral feeding<sup>[3]</sup>. Fasting glucose blood level was used for the diagnosis of new-onset diabetes<sup>[4]</sup>. Exocrine insufficiency was defined as steatorrhea and weight loss requiring pancreatic enzymes supplementation.

### Indications for CR of the pancreas

CR was indicated for benign or low-grade malignant lesions localized in the proximal pancreas, especially in young patients. Simple enucleation was used when the tumor was near or at the surface of the pancreas. Great care had to be taken to avoid the main pancreatic duct injury and obtain complete excision during surgery, otherwise central pancreatectomy was chosen. Central pancreatectomy was indicated when the tumor was deeply embedded in the pancreatic parenchyma without clear margin, and was not suitable for enucleation. The distal remnant of the pancreas was kept at least 5 cm in length.

### Surgical technique for central pancreatectomy

Central pancreatectomy was performed as reported previously<sup>[5-7]</sup>. After transecting the pancreas by electrocautery to the left and right of the tumor, the main pancreatic duct was ligated using a 3-0 silk suture, and the proximal pancreatic stump was oversewn with 3-0 polypropylene in a continuous running fashion. Reconstruction of the distal pancreatic remnant was accomplished with a retrocolic Roux-en-Y pancreaticojejunostomy. For pancreaticojejunostomy, a modified invagination method, continuous circular invaginated pancreaticojejunostomy (CCI-PJ), was used. The remnant of the pancreas was dissected for about 1-3 cm from the cut edge, and several small veins running between the pancreas and the splenic vein

had to be divided and ligated. The pancreatic stump was invaginated into the jejunum, and a single-layer continuous circular anastomosis with 3-0 polypropylene was performed between the full thickness of the resected jejunum and the body of the pancreas. The largest silicon stent that could be passed into the main pancreatic duct was used in all these patients. Finally, four 24-F drainage catheters were placed near the pancreatic anastomosis.

### Postoperative management

Antibiotics were used prophylactically for 5 d after operation, but octreotide was not used routinely. Parenteral nutrition and early enteral feeding were administered. Enteral feeding usually began gradually on postoperative day 3. The fluid from the drain tube placed near the pancreatic anastomosis was monitored routinely for volume and amylase level. For those patients with pancreatic fistulas, modified continuous closed lavage (MCCL) was performed as reported in our previous study<sup>[8]</sup>. A long 6/8-F silicon tube was inserted into each of the 24-F drainage catheters and pulled out from the lateral part of the catheter about 3-5 cm to the external end. The drainage catheter was connected with a drainage pack. A high volume (20-50 L/d) of normal saline was infused through the silicon tube and eventually ran out into the drainage pack through the same rubber catheter. The lavage volume and duration was adjusted according to the appearance and quality of the outflowing liquid.

### Statistical analysis

Results are presented as mean  $\pm$  SD. Statistical analysis was performed using SPSS version 15.0 statistical software. Student's *t* test was used for comparison of two independent samples. Categorical variables were compared using the  $\chi^2$  or Fisher exact test.  $P < 0.05$  was considered statistically significant.

## RESULTS

The characteristics of CR patients are summarized in Table 1. Eight patients had serous cystadenoma, six had insulinoma, three had solid pseudopapillary tumors, and three had non-functional islet cell tumors. Each tumor was resected with clear margins, as determined by intraoperative frozen section and confirmed by final pathological examination. Mean tumor diameter was 2.9 cm (range 0.8-10.0 cm). Mean operating time was 236.5 min (range 75-405 min). The mean intraoperative blood loss was 350 mL (range 100-1200 mL). Three patients (15%) required intraoperative blood transfusion. The mortality was zero and morbidity was 25%. Overall occurrence of pancreatic fistula was 25% and DGE was 20%. Compared with 12 cases of PD for benign tumors in the proximal pancreas during the same period in our hospital, the characteristics did not differ significantly between the two groups. The mortality was zero and the morbidity was 25% in both groups.

It should be pointed out that, among five cases of

**Table 1 Characteristics of the 20 patients who underwent CR**

Patient No.	Age (yr)	Sex	Pathological description	Size (cm)	Lesion location	Operation	Complication	Follow up (mo)
1	75	F	Serous cystadenoma	2.0	Neck	Central pancreatectomy	Pancreatic fistula (significant case) Hemorrhage DGE	100
2	66	M	Non-functional islet cell tumor	2.6	Neck	Enucleation	No	89
3	38	F	Insulinoma	1.0	Proximal body	Enucleation	No	76
4	39	M	Insulinoma	0.8	Uncinate process	Enucleation	No	75
5	62	M	Non-functional islet cell tumor	2.5	Proximal body	Central pancreatectomy	Pancreatic fistula (significant case), perianastomotic fluid collection with infection, DGE	62
6	45	F	Serous cystadenoma	2.8	Proximal body	Central pancreatectomy	No	54
7	12	F	Solid pseudopapillary tumor	3.0	Neck	Enucleation	No	46
8	28	F	Solid pseudopapillary tumor	10.0	Head	Enucleation	Pancreatic fistula (the largest daily volume > 1000 mL in the early postoperative period)	46
9	40	F	Insulinoma	2.0	Proximal body	Central pancreatectomy	No	45
10	61	F	Solid pseudopapillary tumor	1.8	Neck	Central pancreatectomy	No	45
11	39	M	Insulinoma	1.7	Proximal body	Enucleation	No	43
12	62	F	Non-functional islet cell tumor	2.0	Neck	Central pancreatectomy	No	34
13	47	F	Serous cystadenoma	3.0	Neck	Central pancreatectomy	No	34
14	29	F	Insulinoma	2.0	Neck	Central pancreatectomy	No	24
15	29	M	Insulinoma	1.5	Head	Enucleation	No	23
16	55	F	Serous cystadenoma	2.0	Neck	Central pancreatectomy	Pancreatic fistula, DGE	21
17	32	M	Serous cystadenoma	4.0	Proximal body	Central pancreatectomy	No	13
18	39	F	Serous cystadenoma	5.0	Neck	Enucleation	Pancreatic fistula, DGE	10
19	66	M	Serous cystadenoma	4.0	Proximal body	Central pancreatectomy	No	7
20	42	M	Serous cystadenoma	5.0	Head	Enucleation	No	6

CR: Conservative resection; DGE: Delayed gastric emptying.

fistula from 20 CR operations, there were two from nine enucleations and three from 11 central pancreatectomies. Fistula after enucleation usually healed within 7-15 d, by multiple drainage without special intervention. However, in one case of enucleation for a 10-cm solid pseudopapillary tumor located at the head of the pancreas, a small rupture in the main pancreatic duct was found during surgery, and it was repaired by primary suturing with 5-0 polypropylene. The volume of drainage came up to > 1 L/d at the early stage after operation. With MCCL, the fistula healed 15 d later. In the three fistulas from 11 central pancreatectomies, two cases presented with clinically significant pancreatic fistulas that required further medical intervention (Table 1).

After a postoperative follow up of 42.7 mo (range 6-100 mo), all patients were alive without tumor recurrence. No patients developed new-onset diabetes mellitus. However, two cases of new-onset diabetes developed in the PD group. None experienced clinical exocrine insufficiency or required pancreatic enzyme supplements, and three patients needed exocrine substitution in the PD group. Exocrine function of the pancreas was better preserved in the CR than PD group ( $P < 0.05$ ).

## DISCUSSION

For tumors located in the head, neck and proximal pancreas, standard or extended pancreatectomy, such as PD, DP and extended DP, which involves resection of a notable amount of normal parenchyma, has been indicated for benign lesions. It has been reported that the rates of new-onset diabetes mellitus and exocrine insufficiency after PD were 10%-40% and 22%-60% respectively<sup>[7,9-12]</sup>. Previous reports have shown that 72% of patients became insulin dependent after subtotal left pancreatectomy, whereas 85%-95% resection caused diabetes in all patients<sup>[13,14]</sup>. Besides pancreatic parenchyma, the integrated upper digestive and biliary anatomy also plays a key role in maintaining consequent digestive, immunological and coagulative function and neurohormonal regulation of insulin activity<sup>[15]</sup>. In addition, Reid-Lombardo *et al*<sup>[16]</sup> have reported that the 5- and 10-year cumulative probability of biliary stricture after PD for benign lesions was 8% and 13%; that is why, in recent years, CR, such as central pancreatectomy and enucleation, has been investigated with great interest. In the present study, with a mean of 42.7 mo follow-up, there was no new-onset diabetes mellitus

**Table 2 Postoperative morbidity and mortality rates of CR for benign tumors in the proximal pancreas: summary of cases in the literature**

Author	Year	No. of cases	Morbidity rate (%)	Pancreatic fistula (%)	Mortality rate (%)
Sperti et al <sup>[21]</sup>	2000	10 <sup>1</sup>	40	30	0
Balzano et al <sup>[6]</sup>	2003	46 <sup>2</sup>	51	39	0
Efron et al <sup>[24]</sup>	2004	14 <sup>1</sup>	50	36	0
Roggan et al <sup>[23]</sup>	2006	10 <sup>1</sup>	60	30	0
Brown et al <sup>[25]</sup>	2006	10 <sup>1</sup>	60	40	0
Christein et al <sup>[22]</sup>	2006	8 <sup>1</sup>	63	63	0
Falconi et al <sup>[7]</sup>	2007	36 <sup>3</sup>	44	31	0
Present series	2007	20	25	25	0

<sup>1</sup>Central pancreatectomy; <sup>2</sup>Thirty two central pancreatectomies and 14 enucleations; <sup>3</sup>Twenty one central pancreatectomies and 15 enucleations.

and pancreatic exocrine insufficiency, which is the same as previously reported<sup>[17-23]</sup>. Moreover, in our study, the mean age of patients was only 45.3 years old. For these young patients with benign pancreatic disease, CR might be much more significant in achieving good quality of life, because CR can avoid long-term anastomotic complications and pancreatic insufficiency.

However, conservative pancreatectomy has not been used widely to date because of its high morbidity rate, especially pancreatic fistula. It has been reported that the morbidity rate of CR ranged from 35% to 63% (Table 2), which is much higher than that for PD and DP<sup>[6,7,22-25]</sup>. In our study, the morbidity rate of CR was 25%. Overall pancreatic fistula rate was 25%, and DGE rate was 20%.

The pancreatic fistula rate in our study was a little better than that reported since 2000 (Table 2), which has ranged from 30% to 63%<sup>[6,7,22-25]</sup>. In our study, although fistula developed in five of 20 patients, only two with clinically significant fistula required further intervention. According to our experience here, CCI-PJ and efficient MCCL might be responsible for lower morbidity in CR. With CCI-PJ, the stump of the pancreas and the cut edge of the jejunum could be connected closely with polypropylene sutures, which were used for anastomosis of the blood vessels. After postoperative pancreatic fistula has been diagnosed clinically, postoperative MCCL with a high volume of normal saline (20-50 L/d) was necessary to control the pancreatic fistula effectively. The advantage of MCCL compared with general lavage is that there was no concern about the imbalance between ingoing and outgoing fluids because the small silicon tube for irrigation was placed at the tip of the large catheters for outgoing fluid. With MCCL, most patients with pancreatic fistula recover without further surgical intervention.

In conclusion, CR for benign or borderline tumors of the proximal pancreas could be performed safely with careful CCI-PJ and postoperative MCCL. It is useful for preserving long-term pancreatic function.

## COMMENTS

### Background

In recent years, there has been a marked increase of incidentally discovered benign or borderline tumor of the pancreas using advanced diagnostic imaging techniques. The resection of benign lesions located in the proximal portion of

the pancreas traditionally has been accomplished by pancreaticoduodenectomy and distal pancreatectomy. These extended resections result in removal of normal pancreatic tissue, which increased the risk of loss of exocrine and endocrine function.

### Research frontiers

Conservative pancreatic resection including pancreatic enucleation and central pancreatectomy has evolved as a means of preserving as much as possible the pancreatic parenchyma and integrated upper digestive system anatomy. In the present study, the authors evaluated the safety and long-term prognosis of patients who underwent conservative resection (CR) for benign or borderline tumors of the proximal pancreas.

### Innovations and breakthroughs

CR for benign or borderline tumors of the proximal pancreas could be performed safely with careful continuous circular invaginated pancreaticojejunostomy and postoperative modified continuous closed lavage (MCCL). It is effective for preserving long-term pancreatic function.

### Applications

CR is a safe and reasonable technique for benign tumors or lesions of low malignant potential in the proximal pancreas. To obtain good results, careful patient selection and experience in pancreatic surgery are of paramount importance in this setting.

### Terminology

Central pancreatectomy, also known as middle pancreatectomy, is a segmental resection of the pancreas. Dagradis and Serio performed the first central pancreatectomy with an oncological indication in 1984. The main advantage of this operation is that it permits preservation of most of the pancreatic parenchyma, extrahepatic bile duct, duodenum, and spleen.

### Peer review

The article is well written and demonstrates the results of CR of benign tumor of the proximal pancreas. CR is a procedure that offers benefits for benign and low-grade malignant pancreatic tumors, because it allows preservation of endocrine and exocrine functions. A lower risk of typical postoperative complications following pancreatectomy is especially important in young patients, which is stressed by authors of this study. A presented method (MCCL) of management in patients with pancreatic fistula may be useful for other researchers.

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# Stability of a rat model of prehepatic portal hypertension caused by partial ligation of the portal vein

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change in liver pathology. The volume of the spleen was enlarged 2-fold after ligation, and the sinus of the spleen was enlarged due to congestion. Significant sinus endothelial cell proliferation was observed, but no evidence of hypersplenia was found on hemogram and biochemical examination.

**CONCLUSION:** These findings suggest that a satisfactory prehepatic PHT rat model can be obtained by partial ligation of the portal vein, and this PHT rat model was stable for at least 10 wk.

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**Key words:** Ligation; Portal hypertension; Portal vein; Rat

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Wen Z, Zhang JZ, Xia HM, Yang CX, Chen YJ. Stability of a rat model of prehepatic portal hypertension caused by partial ligation of the portal vein. *World J Gastroenterol* 2009; 15(32): 4049-4054 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4049.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4049>

## Abstract

**AIM:** To study the stability of portal hypertension (PHT) caused by partial ligation of the portal vein ligation (PVL) in a rat model.

**METHODS:** Thirty male adult Wistar rats were divided into two groups: 10 in Group I received a sham operation; and 20 in Group II received partial PVL. Portal vein pressure (PVP) was measured at four time periods: before ligation, 2 wk, 6 wk and 10 wk post-surgery. Portal venography, blood sampling and liver and spleen pathological examinations were conducted at 10 wk after surgery.

**RESULTS:** The PVP was  $9.15 \pm 0.58$  cmH<sub>2</sub>O before ligation, and increased to  $17.32 \pm 0.63$  cmH<sub>2</sub>O 2 wk after PVL. By repeat measurement of the PVP in each rat, it was shown to remain elevated for 10 wk. There were no significant differences in the pressure measurements at 2 wk, 6 wk and 10 wk. Varices were found mainly in the mesenteric vein 2 wk after PVL, which were more obvious later, while these manifestations were similar at week 6 and week 10. Portal venography demonstrated the varices and collaterals. There was no significant

## INTRODUCTION

A rat model of prehepatic portal hypertension (PHT) produced by partial portal vein ligation (PVL) is an important tool in studying PHT. The PVL model has been used to study alterations in the splanchnic circulation and in the pathophysiology of the hyperdynamic circulation after ligation<sup>[1,2]</sup>. However, from the literature almost all of these studies were carried out around 2 wk after PVL; and are rarely reported beyond this time period. It is suspected that elevated portal vein pressure (PVP) can last for a longer period of time. The aim of this study was to determine the stability of artificially elevated PVP using rigidly controlled experiments. Furthermore, we used this

model to evaluate the effect of a newly designed splenohepatopexy in PHT treatment.

## MATERIALS AND METHODS

Thirty male Wistar rats weighing 280–320 g (average 302 g) were kept under temperature controlled conditions and an artificial 12-h light-dark cycle. They were allowed standard chow and water *ad libitum*. All animals and procedures were approved by the Ethics Committee of the Animal Experiment Center of Capital Medical University.

Animals were randomized into two groups: Ten rats in Group I had a sham operation; and twenty rats in Group II had partial portal-vein ligation. PVP was measured at four time periods: before ligation, 2 wk, 6 wk, and 10 wk after surgery. Portal venography was performed in rats at 10 wk. Other parameters measured included: weight and volume of spleen, hemogram, biochemical parameters and pathologic examination of the liver and spleen.

### Model establishment

Animals were anesthetized with intramuscular administration of 2% pentobarbital solution. The model was established according to Harvoldsen and Myking<sup>[3,4]</sup>. A 1.5 cm midline incision was made and the portal vein was exposed. The diameter of the portal vein was measured by Vernier calipers at the point where the ligation would be carried out. A No.4 silk thread was placed around the portal vein together with a pre-placed 21G blunt-tipped needle lying along the portal vein. By tying the ligature snugly beyond the left gastric vein, and then pulling out the needle, a stenosis of the portal vein with a constriction corresponding to the thickness of the pre-placed needle was left behind.

### PVP measurement

After anesthesia, a midline incision was made. A segment of the mesenteric branch vein was cannulated with a 24-g cannula needle, and the tip of the cannula was advanced just into the trunk of the superior mesenteric vein. The PVP was recorded, *via* a pressure transducer, by the BL-420E+ biophysical function experiment system (manufactured by Taimeng Technology Limited Company, Chengdu, China). Pressure measurement lasted for 3 min, and the average value was regarded as the PVP. To maintain stable anesthesia, the measurement was started 20 min after the injection of pentobarbital.

### Portal venography

A midline incision was made after anesthesia; the ileocolic vein was cannulated with a 20-g cannula, and the tip of the cannula was advanced into the lower part of the portal vein just above the entrance to the splenic vein. Seventy-six percent meglumine diatrizoate was injected at a speed of 2 mL/5 s, and the intrahepatic and extrahepatic portal vein system could be seen.

### Blood samples and liver, spleen pathological examination

At week ten, blood samples from the rats were obtained

**Table 1 PVP changes (cmH<sub>2</sub>O), (mean ± SD)**

Groups	wk-0	wk-2	wk-6	wk-10
G- I (SO, n = 10)	9.15 ± 0.58	9.22 ± 0.49	9.27 ± 0.43	9.21 ± 0.49
G- II (PVL, n = 16)	9.21 ± 0.63	17.32 ± 1.77 <sup>a</sup>	17.36 ± 1.93 <sup>a,b</sup>	16.82 ± 2.20 <sup>a,b</sup>

Portal vein pressure (PVP) changes at four time periods were compared. <sup>a</sup>P < 0.001 vs value of G- I ; <sup>b</sup>P < 0.001 vs value of wk-2 in G- II. wk-0: before ligation; wk-2, wk-6, wk-10: 2 weeks, 6 weeks and 10 weeks post-surgery, respectively.

for hemogram and biochemical examination. Both liver and spleen biopsy were taken for pathological examination, (using Hematoxylin-eosin staining). The rats were sacrificed at the end of the experiment.

### Statistical analysis

The data were expressed as mean ± SD. Repeated analysis of variance was used to compare the differences between groups by SPSS 11.5 software. Values of P < 0.05 were considered significant.

## RESULTS

### Constriction rate of the portal vein

The average diameter of the portal vein before ligation was 2.40 ± 0.18 mm, and the outer diameter of the 21-g needle was 0.80 mm. According to the formula, the constriction rate = (1 -  $\Delta r^2 / \Delta R^2$ ) × 100%<sup>[5]</sup> = 88.9%.

### Survival rate of animals

None of the rats in Group I died, while 4 rats in Group II died after PVL. The survival rate was 80% (16/20). Three rats died within 24 h of ligation, the other rat died between 24 and 48 h after surgery. Autopsy found severe congestion of the mesenteric and splenic veins, as well as marked cyanosis of the gut and spleen. No mortality occurred after this period.

### PVP

Partial ligation of the portal vein resulted in an immediate increase in PVP up to 25–30 cmH<sub>2</sub>O. Two weeks after surgery, PVP dropped to about 17.32 cmH<sub>2</sub>O, which was about twice the value of the control (P < 0.01). The pressure at week 6 and week 10 was maintained at a similar level, with no significant difference compared with the value for week 2. PVP in Group II was significantly higher than that in Group I. (Table 1 and Figure 1).

### Varices and collateral circulation after PVL

**Gross observations:** In Group I, in the mesenteric vein, there was no distortion, no varices and no visible collaterals. In Group II, in the mesenteric and gastric veins, varices were found mainly in the mesenteric vein 2 wk after PVL, and collaterals could be seen mainly between the spleen and left kidney. The left adrenal vein was markedly engorged. Collaterals could also be seen between the inferior mesenteric vein and the posterior peritoneum (Figures 2 and 3). Collateral

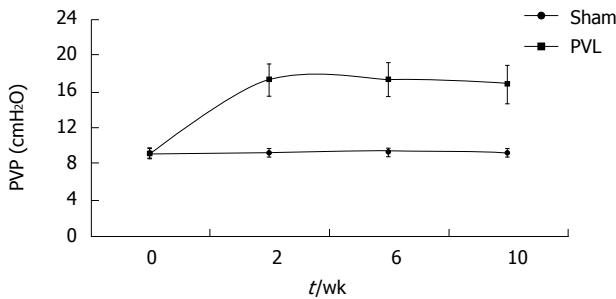


Figure 1 Pressure tendency of PVP after PVL.

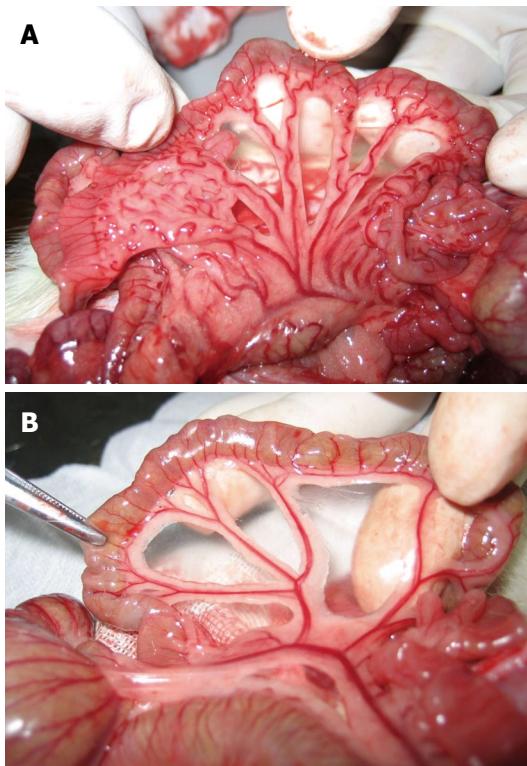


Figure 2 Changes in the mesenteric vein. A: There was no distortion, no varices, no visible collaterals in the mesenteric vein before portal vein ligation; B: Varices were found mainly in the mesenteric vein 2 wk after portal vein ligation.

vessels were found in the porta hepatis in some rats. These manifestations were more obvious later, and were similar at week 6 and week 10. The spleen was markedly enlarged 2 wk after surgery with some white fibrin deposits on the splenic capsule.

#### Portal venography

**Group I:** An angiographic study revealed normal mesenteric and portal vein image patterns in control rats. The intrahepatic portal vein was shown as tree twig branches. There was only a little contrast medium in the mesenteric vein and the splenic vein. After ligation of the portal vein, images of the superior and inferior mesenteric veins and the splenic vein appeared simultaneously, but no collateral circulation could be seen (Figure 4).

**Group II:** At week 10, portal venography showed the mesenteric vein with varices and collaterals, and a lot of

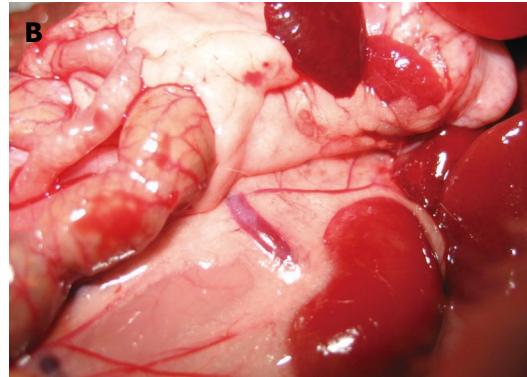
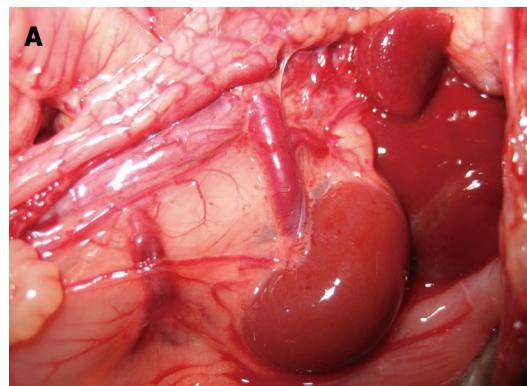


Figure 3 Collaterals after PVL. A: In the control rat, the left renal vein was thin and of normal size, no collaterals could be seen between the spleen and the left kidney; B: Collaterals could be seen mainly between the spleen and the left kidney 10 wk after PVL. The left adrenal vein was markedly engorged. The collaterals could also be seen between the inferior mesenteric vein and the posterior peritoneum.

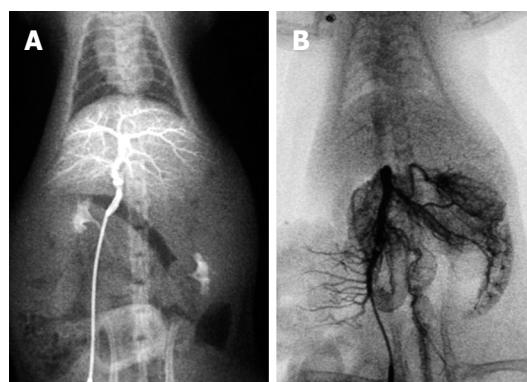
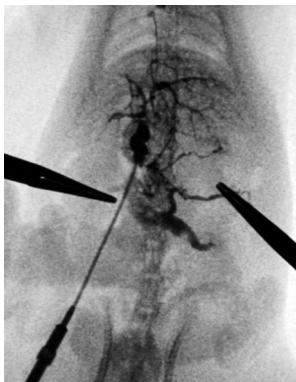
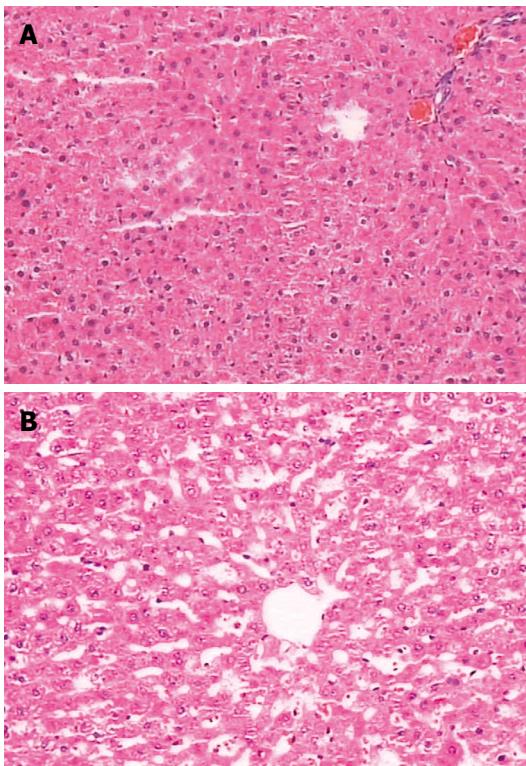


Figure 4 Portal venography in control rat. A: The intrahepatic portal vein was shown as tree twig branches. There was little contrast medium in the mesenteric vein and the splenic vein; B: After ligation of the portal vein, images of the superior and inferior mesenteric vein and the splenic vein appeared simultaneously, but no collateral circulation could be seen.

the contrast medium was seen in the vena cava, which indicated the establishment of the collateral circulation. These observations were not seen in the control rats. Following PVL, the left adrenal vein was clearly seen and had an enlarged diameter, while this vein was not seen in the control group. On continuous observation, some contrast medium was seen in the portal vein system which diffused via the left adrenal vein into the left renal vein and the vena cava. Additionally, collateral vessels between the inferior mesenteric vein and the



**Figure 5** At week 10, portal venography showed the mesenteric vein with varices and collaterals. A lot of contrast medium was seen in the vena cava, which indicated the establishment of the collateral circulation. These observations were not seen in the control rats. The left adrenal vein after PVL was clearly shown with an enlarged diameter, while this vein was not seen in the control group.



**Figure 6** There were no significant changes in liver pathology. A: Pathologic image of rat in Group- I ; B: Image of Group- II .

posterior peritoneum were also seen in the angiograph. No obvious esophageal varices were found. Contrast medium in the liver decreased significantly (Figure 5).

#### Splenic volume

The splenic volume was slightly increased when the weight of the animal increased in Group I , and was almost twice the original size in Group II after PVL. The difference in volume in Group II was significantly different from that in Group I ( $P < 0.01$ ) (Table 2).

#### Hemogram and biochemical parameters

There were no significant differences between Group I and II in hemogram and biochemical parameters. No evidence of hypersplenia was found.

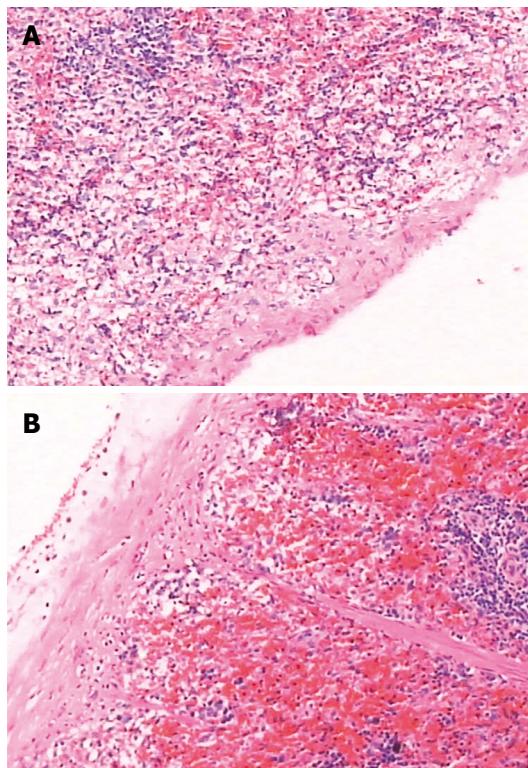
#### Pathology study

There was no significant change in liver pathology. The

**Table 2** Splenic volume changes ( $\text{cm}^3$ ), (mean  $\pm$  SD)

Groups	wk-0	wk-2	wk-6	wk-10
G- I (SO, $n = 10$ )	$1.90 \pm 0.28$	$1.93 \pm 0.24$	$2.03 \pm 0.23$	$2.04 \pm 0.25$
G- II (PVL, $n = 16$ )	$2.07 \pm 0.40$	$3.94 \pm 1.33^{a,b}$	$4.07 \pm 1.37^{a,b}$	$4.17 \pm 1.44^{a,b}$

<sup>a</sup> $P < 0.01$  vs value of G- I ; <sup>b</sup> $P < 0.01$  vs value of wk-0 in G- II . wk-0: before ligation; wk-2, wk-6, wk-10: 2 weeks, 6 weeks and 10 weeks post-surgery, respectively.



**Figure 7** Pathologic image of the spleen. A: Group- I ; B: Group- II , the capsule was thickened, the spleen sinus was enlarged due to congestion, there was significant sinus endothelial cell proliferation, and the spleen trabeculae were widened. The white pulp and germinal center had significant atrophy.

spleen capsule was thickened, the sinus of the spleen was enlarged due to congestion, there was significant sinus endothelial cell proliferation, and the spleen trabeculae were widened. The white pulp and germinal center had significant atrophy (Figures 6 and 7).

## DISCUSSION

The rat model of PHT produced by partial PVL is an important tool in studying PHT. It is fast, economic, simple and repeatable. Some authors have applied the model to the study of alterations in splanchnic circulation and the pathophysiology of the hyperdynamic circulation<sup>[1]</sup>. However, most of these studies were short-term experiments, which were carried out 2-3 wk after ligation. There are few reports of long-term experiments, two months or more after ligation. Some authors even doubt the long-term stability of the model, and are concerned that PVP will drop to the normal level due to establishment of the collateral circulation. For experiments on the surgical treatment of PHT,

such as spleno-hepatopexy, a long-term stable model is needed. The aims of the present study were to prove the stability of the PHT rat model under rigidly controlled experimental conditions, and to prove that this model is suitable for evaluating the effect of our newly designed spleno-hepatopexy for PHT treatment. For spleno-hepatopexy, a peripheral cut on the surface of the spleen and the same on the liver are made which adhere together, it is hoped that the high pressure blood in the spleen will diffuse into the liver through the collateral circulation instead of porto-systemic venous shunting to allow detoxification of the liver.

In our experiment, the PVP value before ligation and in control animals was within the same range as that found by other investigators<sup>[4,6,7]</sup>. The establishment of a partial PVL resulted in instant congestion in the portal vein system, and the PVP immediately increased to 25-30 cmH<sub>2</sub>O. At week 2 after surgery, following compensation by the animal itself, the pressure dropped to about 17 cmH<sub>2</sub>O. In subsequent observations from week 2 to week 10, the elevated PVP value was maintained at the increased level of about 17 cmH<sub>2</sub>O, which was about twice the level before ligation.

Several factors affected the results of these experiments. The portal vein constriction rate is a critical factor in rat survival in the PHT model. After complete portal vein ligation, the portal vein system was suddenly blocked, and this resulted in a serious blood deficiency in the circulation, and the rat died within one hour. For partial ligation, the survival rate following PVL was positively related to the constriction rate<sup>[4]</sup>. We obtained different constriction rates by using different-sized needles. With a 26-g (diameter = 0.45 mm) or a 23-g needle (diameter = 0.6 mm), all the rats died within 1-2 h after ligation. Using a 22-g needle (diameter = 0.7 mm), mortality was about 80%, which was obviously not acceptable for these experiments. Using a 21-g needle (diameter = 0.8 mm), the constriction rate was 88.9%, and the mortality dropped to 20%-30%, and was acceptable for this model of PTH. By decreasing the constriction rate using a 19-g needle (diameter = 1.0 mm), the survival rate was increased, but the PVP did not reach an ideal level to meet the experimental demand.

Constriction of the portal vein resulted in increased resistance in the portal vein, and therefore increased PVP. The resistance of the portal vein was mainly determined by the constriction rate of the portal vein in the experiment. Other factors such as the thickness of the thread were also important. We tried different sizes of thread in our preliminary experiments, including 3-0, 1-0 and No.1, No.4 and No.7 silk. For thin thread less than No.1, the PVP was unstable, and the pressure was usually low, because the resistance of a tube is positively related to the obstructive length. However, for thread thicker than No.7 silk, it was difficult to make a tight knot on such a small portal vein of nearly 1.0 mm. Eventually, we selected the No.4 thread and obtained satisfactory results. Some authors use triple-ligation on the portal vein to obtain the PVL model. Additionally, right ligation against the portal vein axis is also quite

important. The strength used for ligation is important and should be the same in every experiment. Too much strength could damage the portal vein, which may result in thrombosis, while too little strength would be too loose and inaccurate for standard constriction. Cleaning the surrounding tissue next to the portal vein is also useful to reduce errors in constriction diameter.

There have been different reports on the PVP level after PVL. Orda *et al*<sup>[8]</sup> induced PVL in a rat model with an elevated pressure for 2-3 wk, which then dropped to normal. This was thought to be due to a large amount of collateral circulation formation. Cantly<sup>[9]</sup> summarized the previous experience and thought that simple ligation or application of a meroid constrictor to the portal vein could only maintain a high pressure for 4-6 wk. He designed a method of using the ameroid constrictor accompanied by portal lymph node ligation, and hence a high portal pressure could be maintained for about 12 months. Myking *et al*<sup>[3]</sup> and Halvorsen *et al*<sup>[7]</sup> performed serial experiments on the PVL rat model, and successfully elevated PVP for 12 mo after simple ligation, and was considered a stable and repeatable rat model.

The difference in results in the literature might be due to multiple factors, e.g. the animal strain and body weight. In a very low weight rat, a stable elevated pressure is difficult to maintain<sup>[3]</sup>. With regard to the constriction rate, this might be influenced by the size of the needle, the thickness of the thread, and the design of the experiments.

In the PVL model of Myking and Halvorsen<sup>[3,7]</sup>, only one pressure measurement was carried out for each rat. In our experiment, four measurements were carried out for each rat. By this method, we could observe the PVP changes in every rat, which was much more accurate and reliable than a single measurement. Following surgery, there was little adhesion in the rat abdominal cavity except at the local area of ligation or puncture. Because the mesenteric vein branch can be used repeatedly, multiple measurements were possible for each rat.

The peak PVP level after PVL was usually 24 h post-surgery<sup>[10]</sup>. Under natural compensation, the collateral circulation is established to release the high pressure. It was proved that the collateral circulation could be observed two days after PVL, and was fully established 3-4 wk post-surgery<sup>[10]</sup>. The diverted volume of portal vein blood flow was about 95%<sup>[3]</sup>. In our experiments, we also observed that varices appeared in the mesenteric vein 2 wk after surgery. The collateral vessels between the spleen and left kidney, between the inferior mesenteric vein and the posterior peritoneum, and at the site of porta hepatis were also observed at this time, but were more apparent at 6 wk after ligation. The manifestations at week 10 were similar to those at week 6. Portal venography further proved the establishment of the collateral circulation. The left adrenal vein was significantly enlarged and became an important shunting vessel between the splanchnic circulation and the systemic circulation.

Some researchers<sup>[1]</sup> have studied the splanchnic and

systemic hemodynamics in the PVL rat model. They found that, after 2 wk of PVL, the rats with portal hypertension and greater than 93% portal-systemic shunting had an increase in portal venous inflow of 50%, and a concomitant 40% decrease in splanchnic arteriolar resistance. Cardiac index was elevated by 50%, and total peripheral resistance was decreased by 40%. The resistance to portal blood flow in portal vein-constricted rats was similar to that in control rats, indicating that the hyperdynamic portal venous inflow, and not resistance, was the mainstay of the elevated portal venous pressure. Some researchers<sup>[10]</sup> have suggested that many vessel-activated substances which accumulate in the systemic system, without inactivation by the liver, were the direct cause of the hyperdynamic circulation. Therefore, it is considered that, in the early period of PVL, portal vein obstruction is the direct cause of elevated PVP, but with the subsequent establishment of the collateral circulation, the hyperdynamic circulation becomes the important factor in maintaining the elevated pressure. Although portal-systemic shunting can reach to more than 90%, the PVP can still maintain an elevated level.

Other features of the PVL portal hypertensive rat model were noted: Hepatic function remained normal, which is similar to the clinical manifestation of PHT. Although the spleen volume increased almost 2-fold due to enlargement and congestion of splenic sinusoids, proliferation of sinus endothelial cells, hypersplenism was not shown by hemogram, which was similar to findings in the dog PHT model<sup>[5]</sup>. The reason for this may be due to species and anatomy differences to that in humans, or may be due to the short observation period.

In conclusion, portal vein partial ligation increases PVP to around twice the normal value and can be maintained for more than 10 wk. This method can provide a satisfactory model for investigating the surgical treatment of PHT.

## COMMENTS

### Background

Portal hypertension (PHT) is a serious disease in children. In about half of children with PHT it is prehepatic. However, the results of surgery for PHT are unsatisfactory. To investigate and to improve the results of surgery, a long-term stable animal model of PHT is needed. Partial portal vein ligation is a good method of producing a prehepatic PHT rat model. The technique is fast and economic. However, researchers have had different experiences in the long-term stability of the model. Some authors have suggested that the portal vein pressure (PVP) would drop to normal 2 wk after ligation, but others think that the models would be stable for more than a year.

### Research frontiers

Several types of PHT animal models have already been established. But focusing on the long-term stability of these models it is still a frontier of research.

### Innovations and breakthroughs

In their experiments, the prehepatic PHT rat model was proved to be stable for more than 10 wk. The anatomic features of this model were described in detail. Moreover, it is interesting that almost no adhesion in the abdominal cavity and no blood thrombosis in the mesenteric vein were found after repeated operations. Thus, it is possible to measure the PVP repeatedly in one rat, providing a better comparison in the same animal. This is superior to the method commonly used, i.e. a single measurement in each experimental rat.

### Applications

The prehepatic PHT rat model is quite useful in the study of PHT, as the alterations in the splanchnic circulation and the pathophysiology of the hyperdynamic circulation can be studied. It is a fast and economic model and can be widely used in this research field. In addition, it can also be used in clinical research, e.g. a new design for spleno-hepatopexy, and to explore new surgical techniques for PHT in children.

### Terminology

**Portal hypertension:** A series of syndromes with abnormal circulation and increased blood pressure in the portal system. According to its pathology, PHT can be divided into three types, prehepatic, intrahepatic and posthepatic.

**Varices:** Abnormally dilated/stretched veins, frequently caused by the development of portal collateral vessels as a result of portal hypertension. It often occurs in the portal system, such as the mesenteric vein and the splenic vein. The most clinically important varices in humans are found in the esophagus and stomach-submucosal varices of the lower esophagus or gastric fundus sub-mucosa.

**Spleno-hepatopexy:** Is a newly designed surgical procedure which allows communication of the spleen with the liver to establish a compensatory collateral circulation by-pass which crosses over the blockage of the portal vein. Thus, the blood in the distal portal system may be drawn into the liver, instead of into the systemic circulation allowing liver detoxification. This surgical technique would be beneficial for patients with prehepatic portal hypertension, especially in children.

### Peer review

The study by Dr. Wen et al, examines the natural history of portal hypertension in a rat model subjected to partial portal vein occlusion. It may be worth the authors effort as the paper provides valuable information on a common animal model of portal hypertension.

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# Significance and relationship between Yes-associated protein and survivin expression in gastric carcinoma and precancerous lesions

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node metastasis (76.9%), the positive rate of survivin was significantly higher than that in the group without lymph node metastasis (41.2%),  $P < 0.01$ . In 98 cases of gastric carcinoma, the expression of YAP and of survivin were positively correlated,  $r_s = 0.246$ ,  $P < 0.01$ .

**CONCLUSION:** YAP may play an important role as a carcinogenic factor and may induce survivin expression. Detecting both markers together may help in early diagnosis of gastric carcinoma.

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**Key words:** Apoptosis; Cell proliferation; Gastric cancer; Immunohistochemistry; Neoplastic processes; Survivin protein; Yes-associated protein

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## Abstract

**AIM:** To analyze the differences and relevance of Yes-associated protein (YAP) and survivin, and to explore the correlation and significance of their expression in gastric carcinoma and precancerous lesions.

**METHODS:** The PV9000 immunohistochemical method was used to detect the expression of YAP and survivin in 98 cases of normal gastric mucosa, 58 intestinal metaplasia (IM), 32 dysplasia and 98 gastric carcinoma.

**RESULTS:** The positive rates of YAP in dysplasia (37.5%) and gastric carcinoma (48.0%) were significantly higher than that in normal gastric mucosa (13.3%),  $P < 0.01$ . The positive rates of survivin in IM (53.4%), dysplasia (59.4%) and gastric carcinoma (65.3%) were significantly higher than in normal gastric mucosa (11.2%),  $P < 0.01$ . Survivin expression gradually increased from 41.7% in well differentiated adenocarcinoma through 58.3% in moderately differentiated adenocarcinoma to 75.6% in poorly differentiated adenocarcinoma, with significant Rank correlation,  $r_s = 0.279$ ,  $P < 0.01$ . The positive rate of survivin in gastric carcinoma of diffused type (74.6%) was significantly higher than that in intestinal type (51.3%),  $P < 0.05$ . In gastric carcinoma with lymph

## INTRODUCTION

Yes-associated protein (YAP) is a type of cellular adaptor protein and transcriptional co-activator, which was initially isolated by Sudol *et al*<sup>[1]</sup> in 1994, as a result of its binding to the Src family member non-receptor tyrosine kinase YES (Yes kinase-associated protein). In biological conditions, YAP was described as a target of the Hippo (Hpo)-Salvador-Warts pathway and was phosphorylated by the pathway to negatively regulate growth by simultaneously inhibiting proliferation and promoting apoptosis. In recent years, some investigators have found YAP to be overexpressed and highly activated in hepatic cancers and mammary cancers<sup>[2-4]</sup>, suggesting its carcinogenicity. Survivin is a new member of the inhibitor of apoptotic protein (IAP) family, and was initially cloned by the cDNA of effector cell protease receptor-1 in the human genomic library in 1997<sup>[5]</sup>. Many

investigations have found survivin to be overexpressed in most common tumors, but almost never in normal tissues<sup>[6]</sup>. The overexpression of survivin was closely related to tumorigenesis and progression, and was one of the strongest apoptotic inhibitors identified. In tumors, lack of, or mutation of, any factor(s) in the Hpo signaling pathway can lead to dephosphorylation and activation of YAP, which then induces a high expression of Ki67, c-myc, SOX4, H19, AFP, BIRC5/survivin, BIRC2/cIAP1 and other cellular proliferation-related genes and inhibitors of apoptosis. Of note is the massive induction (30-fold) of BIRC5/survivin, leading to breakdown in the balance of cellular proliferation and apoptosis, and an increase in the occurrence and development of tumors<sup>[7]</sup>.

Gastric cancer is one of the malignant diseases with the highest incidence and mortality rates, but its pathophysiology remains to be clarified. We measured the expression level of YAP and survivin in normal gastric mucosa, precancerous lesions and gastric carcinoma using an immunohistochemical (IHC) method in order to analyze the significance and correlations of these two factors with gastric tumorigenesis.

## MATERIALS AND METHODS

### Clinicopathological data

We collected gastric carcinoma specimens from the First Affiliated Hospital of China Medical University: 98 cases of gastric carcinoma, including 29 cases of early gastric carcinoma (EGC) and 69 cases of advanced gastric carcinoma (AGC), with matched normal gastric mucosa, 58 cases of intestinal metaplasia (IM), and 32 cases of dysplasia (DYS). There were 66 males and 32 females, mean age 60 years. Gross types were as follows: EGC cases: 18 cases of type I + IIc, 10 cases of type III, one case of extensive superficial type; AGC cases: seven cases of Borrmann I + II, 62 cases of Borrmann III + IV. According to the World Health Organization histological classification of GC, the 98 cases were classified as follows: two papillary adenocarcinoma, 12 well differentiated adenocarcinoma, 25 moderately differentiated adenocarcinoma, 41 poorly differentiated adenocarcinoma, two undifferentiated adenocarcinoma, seven signet ring cell carcinomas and nine mucinous adenocarcinoma.

### Tissue microarray construction and IHC staining

Samples were fixed in 10% formalin, embedded in paraffin, cut into 4  $\mu$ m thick sections and constructed in blocks for tissue microarray. All the samples were evaluated by two experienced pathologists for diagnosis.

Expression of YAP and survivin in gastric carcinomas, precancerous lesions and normal gastric mucosa were detected using an IHC method. A PV-9000 kit was purchased from Beijing Zhongshan Golden Bridge Biotechnology Company. Anti-human rabbit YAP polyclonal antibody was purchased from the Cell Signaling Technology Company (working dilution 1:25). Anti-human rabbit polyclonal antibody survivin (ready to use)

was purchased from Fuzhou Maixin Company (China). All procedures were implemented according to the manufacturer's instructions. For negative controls, sections were treated with 0.01 mol/L phosphate-buffered saline instead of primary antibodies.

### IHC staining evaluation

YAP was specifically located in the cytoplasm and nucleus of carcinoma cells; survivin was specifically located in the cytoplasm of carcinoma cells. Staining intensity (A) was classified as 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). The percentage of positive cells (B) examined in 200 cells were divided into 0 (< 5%), 1 (5%-25%), 2 (26%-50%), 3 (51%-75%) and 4 (> 75%). According to the product of A and B, the IHC result was classified as 0, negative (-); 1-4, weakly positive (+); 5-8, moderately positive (++) and 9-12, strongly positive (+++).

### Statistical analysis

Statistical analysis was performed using SPSS 11.5 Package,  $\chi^2$  test, Fisher's exact test and Kendall's  $\tau_{ab}$  test were used to differentiate the rates of different groups and test the correlation between the two factors.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Expression of YAP in normal gastric mucosa, IM, DYS and gastric carcinoma

The positive rates of YAP presence in dysplasia (37.5%) and gastric carcinoma (48.0%) were significantly higher than that in normal gastric mucosa (13.3%),  $P < 0.01$ ; there was no statistically significant difference between YAP expression in the normal gastric mucosa and IM (16/58, 27.6%), dysplasia and gastric carcinoma,  $P > 0.05$ . YAP expression showed an increasing trend from well differentiated adenocarcinoma (4/12, 33.3%), through moderately differentiated adenocarcinoma (11/25, 44.0%) to poorly differentiated adenocarcinoma (24/41, 58.5%), although without significant Rank correlation. The positive rate of YAP expression showed an increasing trend from gastric carcinoma without lymph node metastasis (5/17, 29.4%), to gastric carcinoma with lymph node metastasis (24/52, 46.2%), though without statistical significance,  $P > 0.05$ . There was no significant correlation of the expression of YAP with patients' gender, age, Borrmann's classification of gastric carcinoma or Lauren classification,  $P > 0.05$  (Tables 1 and 2, Figure 1).

### Expression of survivin in normal gastric mucosa, IM, DYS and gastric carcinoma

The positive rates of survivin in IM (53.4%), dysplasia (59.4%) and gastric carcinoma (65.3%) were significantly higher than that in normal gastric mucosa (11.2%),  $P < 0.01$ . The expression level gradually increased from well differentiated adenocarcinoma (41.7%), through moderately differentiated adenocarcinoma (58.3%) to poorly differentiated adenocarcinoma (75.6%), with significant Rank correlation,  $r = 0.279$ ,  $P < 0.01$ .

**Table 1 Correlation of Yes-associated protein (YAP) expression with normal gastric mucosa, intestinal metaplasia (IM), dysplasia (DYS) and gastric carcinoma (GC)**

Groups	n	YAP expression			Positive (%)	P	
		-	+	++	+++		
Normal mucosa	98	85	10	2	1	13.3	0.110 <sup>a</sup> /0.009 <sup>b</sup>
IM	58	42	12	3	1	27.6	0.625 <sup>c</sup> /0.083 <sup>d</sup>
DYS	32	20	9	3	0	37.5	0.653 <sup>e</sup>
GC	98	51	30	12	5	48.0	0.0001 <sup>f</sup>

Fisher's exact test. <sup>a</sup>Normal mucosa vs IM; <sup>b</sup>Normal mucosa vs DYS; <sup>c</sup>IM vs DYS; <sup>d</sup>IM vs GC; <sup>e</sup>DYS vs GC; <sup>f</sup>Normal mucosa vs GC.

**Table 2 Correlation of YAP expression with clinicopathologic features of gastric carcinoma**

Groups	n	YAP expression			Positive (%)	P
		-	+	++	+++	
Sex						0.309
Male	66	31	22	8	5	53.0
Female	32	20	8	4	0	37.5
Age						0.304
< 60	54	30	15	8	1	45.5
≥ 60	44	21	15	4	4	51.2
Gross type						
EGC						0.937
Type I + IIc	18	6	8	3	1	66.7
Type III	10	4	3	2	1	60.0
AGC						0.074
Type Bor I + II	7	2	2	2	1	71.4
Type BorIII + IV	62	38	17	5	2	38.7
WHO's histological types						r = 0.181
Well-diff. ade.	12	8	4	0	0	33.3
Moderately-diff. ade.	25	14	6	3	2	44.0
Poorly-diff. ade.	41	17	15	6	3	58.5
Undiff. ade.	2	2	0	0	0	0.0
Papillary ade.	2	0	1	1	0	100.0
Signet ring cell carcinoma	7	4	2	1	0	42.9
Mucinous ade.	9	6	2	1	0	33.3
Lauren types						0.669
Intestinal type carcinoma	39	23	11	4	1	41.0
Diffused type carcinoma	59	28	19	8	4	52.5
Lymph node metastasis						0.602
Yes	52	28	16	5	3	46.2
No	17	12	3	2	0	29.4

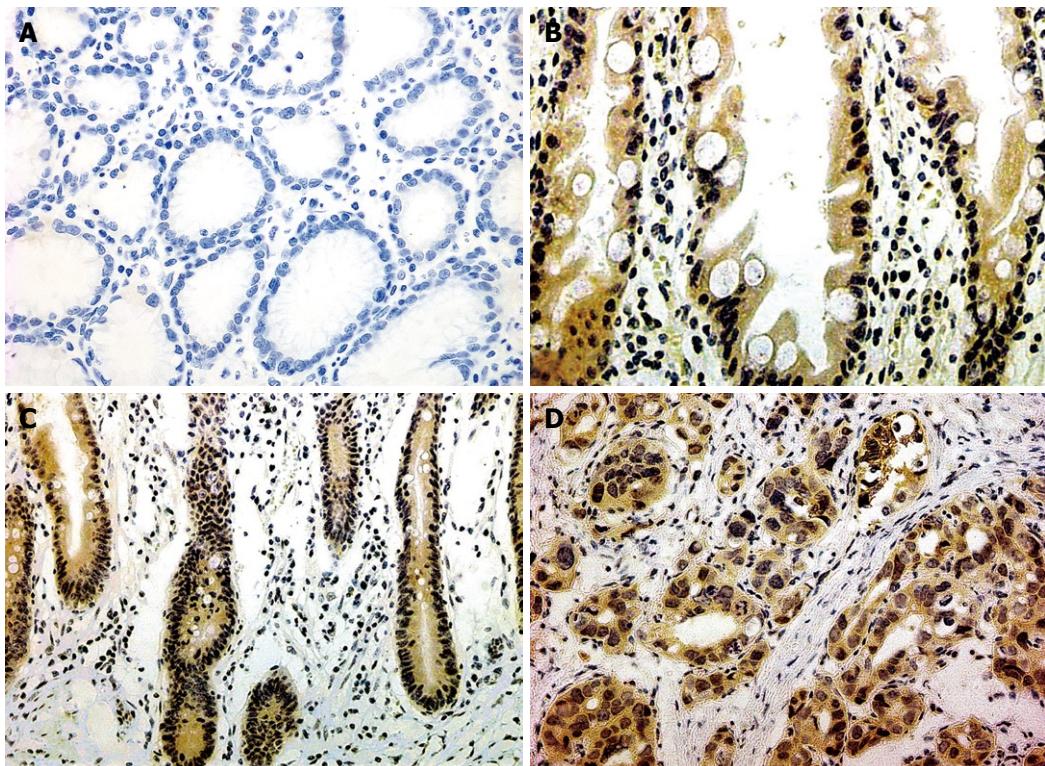
Fisher's exact test. ade.: Adenocarcinomas; diff.: Differentiated; EGC: Early gastric carcinoma; AGC: Advanced gastric carcinoma. <sup>a</sup>Well-diff. vs Moderately-diff. ade.; <sup>b</sup>Moderately-diff. vs Poorly-diff. ade.; <sup>c</sup>Well-diff. vs Poorly-diff. ade.

The positive rate of survivin in gastric carcinoma of diffused type (74.6%) was significantly higher than that in intestinal type (51.3%),  $P < 0.05$ . In gastric carcinoma with lymph node metastasis (76.9%), the positive rate of survivin was significantly higher than that in the group without lymph node metastasis (41.2%),  $P < 0.01$ . There was no relationship between gastric carcinoma and sex, age and gross type of carcinoma (Tables 3 and 4, Figures 2-4).

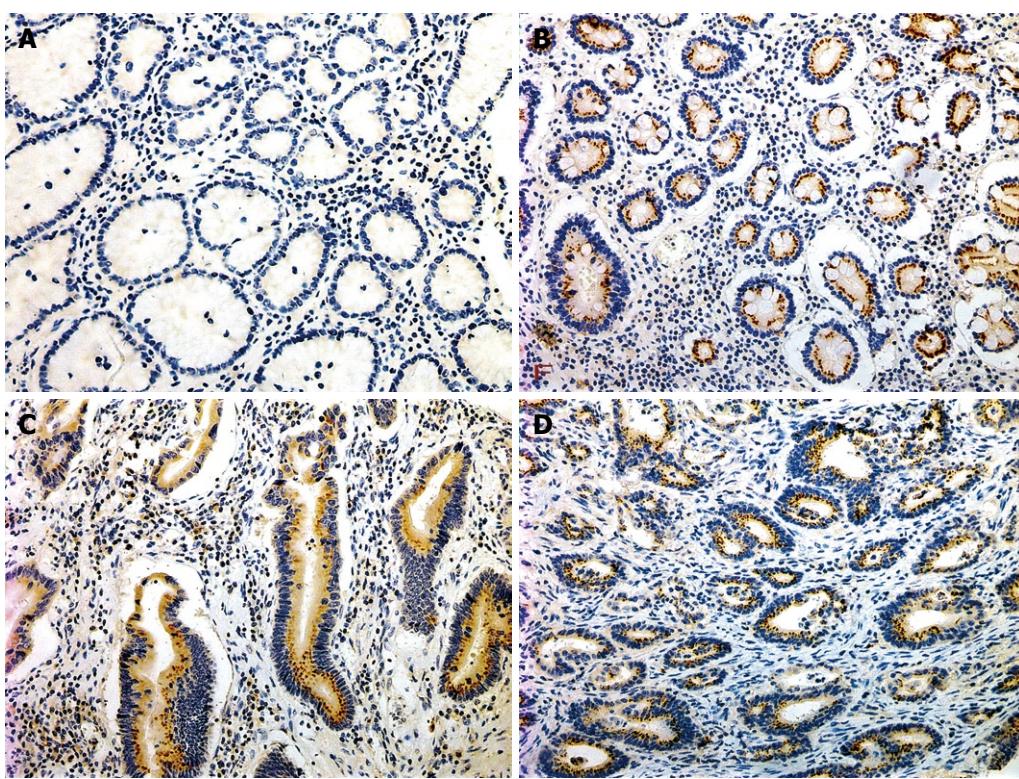
## DISCUSSION

The Hpo pathway was originally identified in *Drosophila* as a potent regulator of inhibition of cell growth and promotion of apoptosis. The pathway consists of a tumor suppressor kinase cascade which negatively regulates growth and results in inactivation of a transcriptional co-activator, Yorkie (Yki)<sup>[8]</sup>. The human ortholog of Yki,

YAP, has a 31% sequence homology and similar biologic activity. YAP is a 65 kDa phosphoprotein which is rich in proline. In biological conditions, YAP is phosphorylated by the Hpo signaling pathway, and is highly conserved with other components of this pathway, regulating the balance between cell proliferation and apoptosis to maintain the steady-state of the cellular environment<sup>[2,9,10]</sup>. Dysregulation of any factor(s) in this pathway can lead to tumorigenesis. Overholtzer *et al*<sup>[9]</sup> introduced the YAP gene by retroviral infection into the immortalized, but non-tumorigenic, human mammary epithelial cell line MCF10A and found that overexpression of YAP induced epithelial-to-mesenchymal transition, suppression of apoptosis, growth factor-independent proliferation, and anchorage-independent growth in soft agar, which suggests that YAP contributes to malignant transformation in cancers, and supports the potential significance of this pathway in human cancer.



**Figure 1 Expression of Yes-associated protein (YAP) in normal gastric mucosa (A), intestinal metaplasia (B), dysplasia (C) and gastric carcinoma (D). IHC PV9000,  $\times 200$ .**



**Figure 2 Expression of survivin in normal gastric mucosa (A), intestinal metaplasia (B), dysplasia (C) and gastric carcinoma (D). IHC PV9000,  $\times 200$ .**

Zhao *et al*<sup>[2]</sup> evaluated YAP expression in 115 cases of human hepatocellular carcinoma (HCC) samples by IHC staining of tissue microarrays. Among the 115 cases of human HCC samples examined, 54% showed strong YAP staining, while 95% of normal liver tissue samples (40 out of 42 cases) showed very weak staining, indicating a significant difference in YAP levels between normal and cancerous tissues. Similar observations were made in prostate cancer tissues. Up to now, we

have found only one report on the expression and role of YAP expression in gastric carcinoma, where YAP expression in 78 normal gastric mucosa was 14%, while in 55 gastric carcinomas and 92 gastric metastatic disease the expression was 30% and 35% respectively, significantly higher than that in normal gastric mucosa<sup>[11]</sup>. Our IHC investigation found that the expression of YAP in DYS and gastric carcinoma was significantly higher than in normal gastric mucosa, suggesting that an

**Table 3 Correlation of survivin expression with normal gastric mucosa, intestinal metaplasia, dysplasia and gastric carcinoma**

Groups	n	Survivin expression			Positive (%)	$\chi^2$	P
		-	+	++			
Normal mucosa	98	87	10	1	0	11.2	0.0001 <sup>1,a</sup> /0.0001 <sup>1,b</sup>
IM	58	27	19	8	4	53.4	2.683 0.486 <sup>1,c</sup> /0.443 <sup>d</sup>
DYS	32	13	8	6	5	59.4	1.584 0.663 <sup>e</sup>
GC	98	34	34	20	10	65.3	67.944 0.0001 <sup>f</sup>

<sup>1</sup>Fisher's exact test. <sup>a</sup>Normal mucosa vs IM; <sup>b</sup>Normal mucosa vs DYS; <sup>c</sup>IM vs DYS; <sup>d</sup>IM vs GC; <sup>e</sup>DYS vs GC; <sup>f</sup>Normal mucosa vs GC.

**Table 4 Correlation of survivin expression with clinicopathologic features of gastric carcinoma**

Groups	n	Survivin expression			Positive (%)	$\chi^2$	P
		-	+	++			
Sex						4.87	0.172
Male	66	20	27	14	5	69.7	
Female	32	14	7	6	5	56.2	
Age						2.74	0.434
< 60	54	20	15	13	6	63.0	
≥ 60	44	14	19	7	4	68.2	
Gross type							
EGC							0.310 <sup>1</sup>
Type I + II c	18	5	7	3	3	72.2	
Type III	10	6	1	2	1	40.0	
AGC							0.696 <sup>1</sup>
Type Bor I + II	7	3	2	1	1	57.1	
Type Bor III + IV	62	19	24	14	5	69.4	
WHO's histological types						$r_k = 0.279$	0.006
Well-diff. ade.	12	7	4	1	0	41.7	0.824 <sup>1,a</sup>
Moderately-diff. ade.	25	11	10	2	2	56.0	0.223 <sup>1,b</sup>
Poorly-diff. ade.	41	10	16	10	5	75.6	0.149 <sup>1,c</sup>
Undiff. ade.	2	1	1	0	0	50.0	
Papillary ade.	2	1	0	1	0	50.0	
Signet ring cell carcinoma	7	1	2	3	1	85.7	
Mucinous ade.	9	3	1	3	2	66.7	
Lauren types						8.61	0.035
Intestinal type carcinoma	39	19	14	4	2	51.3	
Diffused type carcinoma	59	15	20	16	8	74.6	
Lymph node metastasis							0.005 <sup>1</sup>
Yes	52	12	19	15	6	76.9	
No	17	10	7	0	0	41.2	

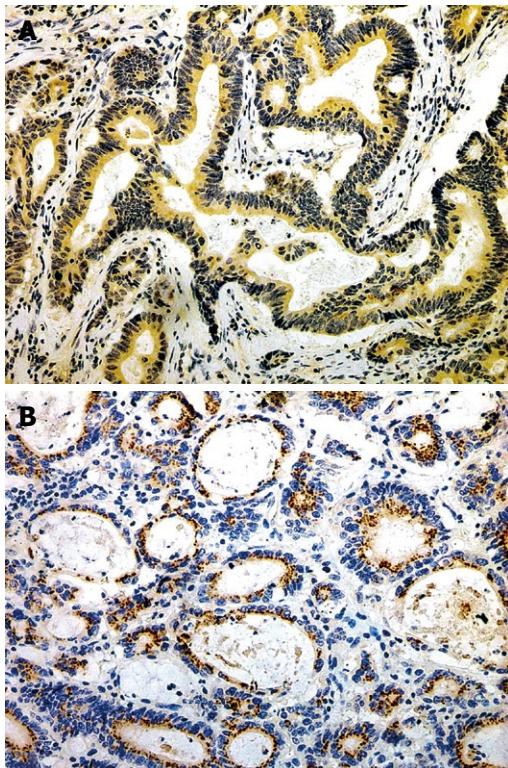
<sup>1</sup>Fisher's exact test. <sup>a</sup>Well-diff. vs Moderately-diff. ade.; <sup>b</sup>Moderately-diff. vs Poorly-diff. ade.; <sup>c</sup>Well-diff. vs Poorly-diff. ade..

abnormality of the Hpo pathway leads to overexpression of YAP, resulting in malignant transformation of the gastric mucosa. We speculate that YAP may play an important role as a tumorigenic factor and early gastric tumorigenic molecule during gastric carcinogenesis.

Survivin is a new member of the IAP family, and has been implicated to have a role in protection from apoptosis and regulation of mitosis<sup>[12]</sup>. The survivin gene has been located on the 17q25 chromosome, encoding a 16.5 kDa protein. Survivin is characterized by a unique structure with a single BIR on the N terminal and an  $\alpha$ -helix structure on the C terminal; the BIR structure is thought to play a role during anti-apoptosis, while the helix structure may participate in the microtubule binding structure<sup>[13,14]</sup>. Data from a large analysis of human transcripts revealed survivin as the fourth most highly expressed protein in human cancer tissue compared with normal tissue<sup>[15-18]</sup>. Xiao et al<sup>[19]</sup> found that the positive rates of survivin expression in tumors with metastases (in lymph node metastasis 86.2%, liver metastasis

100% and ovarian metastasis 100%) were significantly higher than that in tumors without metastasis (64.3%). Our data indicated that the positive rates of survivin in IM, atypical hyperplasia and gastric carcinoma were significantly higher than that in normal gastric mucosa. The expression level gradually increased from well differentiated adenocarcinoma, through moderately differentiated adenocarcinoma to poorly differentiated adenocarcinoma, with significant Rank correlation. The positive rate of survivin in gastric carcinoma of the diffused type was significantly higher than that in the intestinal type. In gastric carcinoma with lymph node metastasis, the positive rate of survivin was significantly higher than that in the group without lymph node metastasis, indicating that survivin may be involved in the occurrence, development and lymph node metastasis of gastric carcinoma. Survivin can act as a prognostic and predictive indicator for gastric carcinoma patients.

Dong et al<sup>[7]</sup> used microarray analysis to identify YAP-induced genes in murine livers. Selected genes

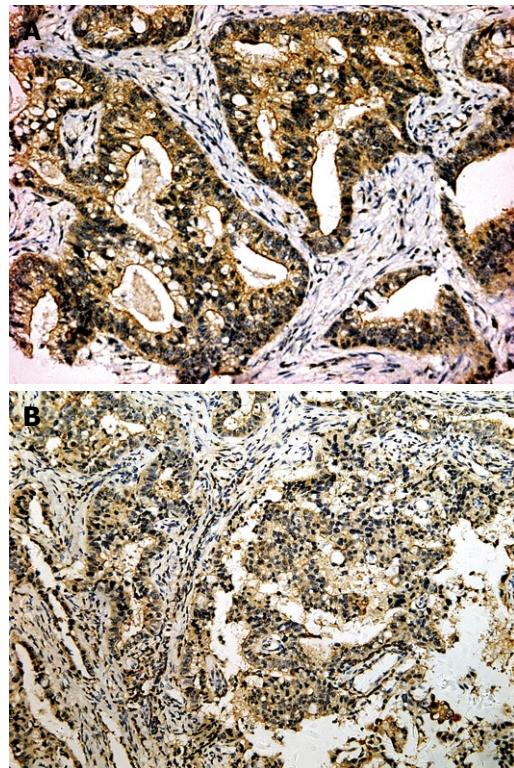


**Figure 3** The expression of survivin in gastric carcinoma without lymph node metastasis (A,  $\times 200$ ) and with lymph node metastasis (B,  $\times 100$ ). IHC PV9000.

from the microarray analysis were validated by real-time quantitative polymerase chain reaction analysis. YAP induced the transcription of many genes which are normally associated with hepatocyte proliferation, such as Ki67, c-myc, SOX4, H19, and AFP. It also induced the expression of several negative regulators of apoptosis, such as the IAP family members BIRC5/survivin and BIRC2/cIAP1, and the BCL2 family gene MCL1. Of note is the massive induction (30-fold) of BIRC5/survivin. To determine whether cIAP1 and YAP might cooperate during tumorigenesis,  $p53^{-/-}$ , myc liver progenitor cells were infected with either YAP and control vector or YAP plus cIAP1 and assayed for their ability to form tumors *in vivo*. Tumors arising from  $p53^{-/-}$ , myc hepatoblasts coexpressing cIAP1 and YAP grew faster than those expressing either oncogene alone, suggesting that they may collaborate to contribute to tumorigenesis and progression<sup>[20]</sup>.

Our investigation found that the expression of YAP and survivin in gastric carcinoma were positively correlated, and we speculate that YAP may induce a high expression of cell proliferation-related factors and apoptotic inhibitors, such as Ki67, cIAP1 and survivin. Survivin may participate in gastric carcinogenesis, progression and metastasis by inhibiting apoptosis of gastric carcinoma and regulating cellular mitosis. Whether YAP and survivin collaborate to contribute to gastric carcinogenesis and progression require further study.

Previous reports have found that YAP was an activator of cell death in mammalian cells. YAP was shown to activate apoptosis in response to DNA damage by



**Figure 4** The expression of survivin in primary gastric carcinoma (A) and relevant lymph node metastasis (B). IHC PV9000,  $\times 200$ .

interacting with p73 in several cancer cell lines<sup>[21,22]</sup>. This is in direct contrast to the results of our investigation and other previous reports. The roles of YAP in biological and pathological conditions remain to be clearly defined.

## COMMENTS

### Background

Yes-associated protein (YAP) is a type of cellular adaptor protein and transcriptional co-activator. In recent years, some investigators have found YAP to be overexpressed and highly activated in hepatic cancers and mammary cancers, suggesting its tumorigenicity. Survivin is a new member of the inhibitor of apoptotic protein (IAP) family, which was initially cloned by the cDNA of the effector cell protease receptor-1 in the human genomic library in 1997. The authors measured the expression of YAP and survivin in normal gastric mucosa, precancerous lesions and gastric carcinoma using an immunohistochemical method, to analyze the significance and correlations of the two factors with gastric carcinogenesis.

### Research frontiers

The Hippo (Hpo) pathway was originally identified in Drosophila as a potent regulator of inhibition of cell growth and promotion of apoptosis. The pathway consists of a tumor suppressor kinase cascade which negatively regulates growth and results in inactivation of a transcriptional co-activator, Yorkie (Yki). The human ortholog of Yki, YAP, has a 31% sequence homology and similar biologic activity. YAP is a 65 kDa phosphoprotein, rich in proline. In biological conditions, YAP is phosphorylated by the Hpo signaling pathway, and is highly conserved with other components of this pathway, regulating the balance between cell proliferation and apoptosis to maintain the steady-state of the cellular environment.

### Innovations and breakthroughs

Previous reports have found that YAP was an activator of cell death in mammalian cells. YAP was shown to activate apoptosis in response to DNA damage by interacting with p73 in several cancer cell lines. This is in direct contrast to the results of their investigation and other previous reports.

### Applications

The authors investigation found that the expression of YAP and survivin in

gastric carcinoma were positively correlated. They speculate that YAP may induce a high expression of cell proliferation-related factors and apoptotic inhibitors, such as Ki67, cIAP1 and survivin. Detecting YAP and survivin together may help in early diagnosis of gastric carcinoma. Whether YAP and survivin collaborate to contribute to gastric tumorigenesis and progression requires further study.

#### Peer review

The investigation found that the expression of YAP and survivin in gastric carcinoma were positively correlated, and the authors speculated that YAP may induce a high expression of cell proliferation-related factors and apoptotic inhibitors, such as Ki67, cIAP1 and survivin. Survivin may participate in gastric carcinogenesis, progression and metastasis by inhibiting apoptosis of gastric carcinoma cells and regulating cellular mitosis. The study is interesting and is worth further exploration.

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S- Editor Tian L L- Editor Cant MR E- Editor Zheng XM



BRIEF ARTICLES

## Evaluation of standard liver volume formulae for Chinese adults

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Shi ZR, Yan LN, Li B, Wen TF. Evaluation of standard liver volume formulae for Chinese adults. *World J Gastroenterol* 2009; 15(32): 4062-4066 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4062.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4062>

### Abstract

**AIM:** To evaluate different standard liver volume (SLV) formula and verify the applicability of the formulae for Chinese adults.

**METHODS:** Data from 70 cases of living donor liver transplantation (LDLT) performed at our transplantation centers between January 2008 and April 2009 were analyzed. SLV was estimated using our recently reported formula [the Chengdu formula: SLV (mL) = 11.5 × body weight (kg) + 334] and other reported formulae used for Chinese adults. Actual intraoperative liver volumes were obtained from a review of the patients' medical records.

**RESULTS:** The actual right liver volume was not significantly different from the estimated right liver volume determined by the Chengdu formula, but was significantly smaller than estimates using the Heinemann, Urata, Vauthey, and Lee formulae ( $P < 0.01$ ), and significantly larger than estimates using the Fan formula ( $P < 0.05$ ).

**CONCLUSION:** The Chengdu formula was demonstrated to be reliable by its application in LDLT.

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**Key words:** Standard liver volume; Living donor liver transplantation; Chinese adult; Liver volume formula

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### INTRODUCTION

Living donor liver transplantation (LDLT) has been used to alleviate the shortage of available liver donors. Accurate estimation of the standard liver volume (SLV) of the living donor and recipient is crucial. Overestimation of the donor's SLV may result in excessive hepatic resection leading to liver failure, while underestimation of the recipient's SLV may result in small-for-size graft syndrome<sup>[1-5]</sup>. Since 2001, our transplant centers have carried out 212 LDLTs. We estimated the SLV using computed tomography (CT) or reported formulae. However, there was a difference between these estimates and the actual liver volumes (ALVs) for Chinese adults. Recently, we developed a new formula (named the Chengdu formula) to estimate SLV using data from 115 LDLTs<sup>[6]</sup>. The formula is: SLV (mL) = 11.5 × body weight (kg) + 334. Using this formula, the SLVs were evaluated in 76 cases of LDLT performed from January 2008 to April 2009. Its accuracy was compared to that of other internationally reported formulae<sup>[7-10]</sup> to assess which formula is the most accurate for Chinese adults.

### MATERIALS AND METHODS

#### Patient selection

The data from 76 living donors were analyzed. Inclusion criteria were: (1) a healthy adult donor, aged 19-59 years; (2) right liver graft without middle hepatic vein; (3) adult-to-adult LDLT; (4) single donor; (5) no history of long term drinking. Exclusion criteria: (1) donor age < 18 or > 60 years; (2) left hepatic graft or left lateral lobe graft; (3) double donor grafts; (4) adult-to-child transplant; (5) donors who were hepatitis B or C carriers<sup>[11-14]</sup>.

#### Clinical data

Data of preoperative donors included age, sex, height

**Table 1** Reported formulae for ESLV

Author	Report date	Formula	Material used (race, number)
Urata et al <sup>[7]</sup>	1995	ESLV = 706.2 × BSA + 2.4	CT Volumetry (Japanese, 96)
Heinemann et al <sup>[8]</sup>	1999	ESLV = 1072.8 × BSA - 345.7	Autopsy (Caucasian 1332)
Vauthey et al <sup>[9]</sup>	2002	LV = 18.51 × BW + 191.8	CT volumetry (Western, 292)
Lee et al <sup>[5]</sup>	2006	ESLV = 691 × BSA + 95	LDLT (Korea, 311)
Fan et al <sup>[4]</sup>	2000	ESLW = 218.32 + BW × 12.29 + gender × 50.74 (M = 1, F = 0)	LDLT (Chinese, 159)
Chengdu <sup>[6]</sup>	2009	ESLV = 334.024 + 11.508 × BW	LDLT (Chinese, 115)

ESLV: Estimated standard liver volume; BSA: Body surface area; BW: Body weight; CT: Computed tomography; LDLT: Living donor liver transplantation.

**Table 2** Donor characteristics

Age (yr)	32.21 ± 10.07 (19-59)
Gender (Male:Female)	53:17
Body weight (kg)	62.97 ± 8.41 (42-87)
Body height (cm)	167.31 ± 8.15 (148-185)
Body mass index (kg/m <sup>2</sup> )	22.23 ± 2.44
Body surface area (m <sup>2</sup> ) by DuBois formula	1.7082 ± 0.14
Body surface area (m <sup>2</sup> ) by Mosteller formula	1.7081 ± 0.14
Total liver volume on CT (mL)	1189.53 ± 114.75
Right lobe graft volume on CT without MHV	658.98 ± 81.14
Right lobe volume without MHV to total liver volume on CT (%)	55.4 ± 3.7
Actual right liver volume (mL)	578.58 ± 72.33

MHV: Middle hepatic vein.

(BH, measured to the nearest 1 cm), body weight (BW, measured to the nearest 0.5 kg), and body surface area (BSA) calculated using the DuBois formula: BSA (m<sup>2</sup>) = BW (kg) 0.425 × BH (cm) 0.725 × 0.007184 or the Mosteller formula: BSA (m<sup>2</sup>) = square root BH (cm) × BW (kg)/3600. From the diaphragm to the superior mesenteric artery plane, the entire liver image was scanned using a 7 mm thick layer. In the Leonardo workstation, the LV was measured by venous phase images<sup>[15,16]</sup>. All preoperative CT examinations of donors were performed by a single radiologist and all donor procedures were performed by the same surgical unit. The volume of the grafts was measured by a 3 L beaker using a drainage method intraoperatively and the error was less than 10 mL<sup>[17,18]</sup>.

Right liver graft without middle hepatic vein reconstruction from a living donor was performed as described, with temporary occlusion of the right portal vein (PV) and right hepatic artery and use of ultrasonography to guide parenchymal transection. The right hepatic duct, right hepatic artery, right portal vein branch, and right hepatic vein were transected approximately 2-3 mm from the confluence<sup>[19,20]</sup>, leaving the donor's main PV and confluence intact. The graft was flushed with University of Wisconsin solution through the PV and hepatic artery<sup>[21,22]</sup>.

The volume of 70 livers was calculated using the Chengdu standard LV formula<sup>[6]</sup> as described above. The estimated right LV (ERLV) was obtained by multiplying the SLV by the proportion of the LV contributed by the right lobe on CT. The actual right LV (ARLV) was obtained by intraoperative measurement. The difference

between the ERLV and ARLV was statistically evaluated.

The formulae of Heinemann et al<sup>[8]</sup>, Urata et al<sup>[7]</sup>, Vauthey et al<sup>[9]</sup>, Lee et al<sup>[5]</sup>, and Fan et al<sup>[4]</sup> in addition to our own formula<sup>[6]</sup> were used to determine the estimated SLV (ESLV) of our donor livers. The previously reported formulae are shown in Table 1. For each liver, we calculated the difference between the ALV and volume estimated by each formula (ELV).

### Statistical analysis

After testing for normal distribution (kurtosis and skewness tests), descriptive statistics were calculated and data were expressed as means ± SD for age (year), BW (kg), BH (cm), body mass index (BMI), and BSA. The ERLV-ARLV and the ELV-ALV were compared by the 2-sided paired-samples *t*-test. *P* < 0.05 was considered statistically significant. All statistical analyses were performed using the SPSS (version 13.0) program.

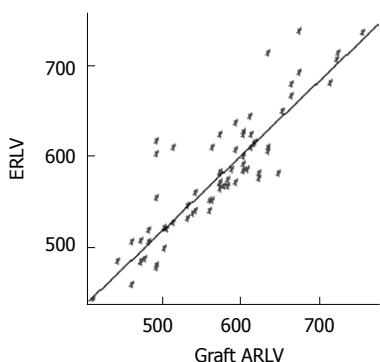
## RESULTS

Seventy donors (all Chinese; 53 men and 17 women; mean age, 32.21 ± 10.07; range, 19-57 years) met the selection criteria. All donors were related to the recipients.

The characteristics of donors are shown in Table 2. All donors were considered healthy on the basis of BMI. All but one donor with a BMI of 17 kg/m<sup>2</sup> had a BMI of 18-28 kg/m<sup>2</sup>. The mean volume of the right lobe on CT was 658.98 ± 81.14 mL and represented 55.4% ± 3.7% of the whole liver on CT.

The mean ELV and mean ERLV using the Chengdu standard formula were 1058.70 ± 96.74 mL and 586.15 ± 67.17 mL, respectively. The mean ARLV was 578.58 ± 72.33 mL. Differences for individual donors between ERLV and ARLV were not significant (*t* = -1.882, *P* = 0.064). A plot of the relationship of ARLV to the ERLV calculated using the Chengdu formula is shown in Figure 1.

The mean total LV determined preoperatively on CT was 1189.53 ± 114.75 mL. The mean RLV on CT without the middle hepatic vein was 658.98 ± 81.14 mL, and 55.4% ± 3.7% of the total LV. The ALV calculated from the volume of the graft and the ratio of the RLV to the total LV on CT (%) was 1050.10 ± 107.41 mL. The Heinemann, Urata, Vauthey, and Lee formulae significantly overestimated the LV (*P* < 0.01), while the Fan formula significantly underestimated the LV (*P* <

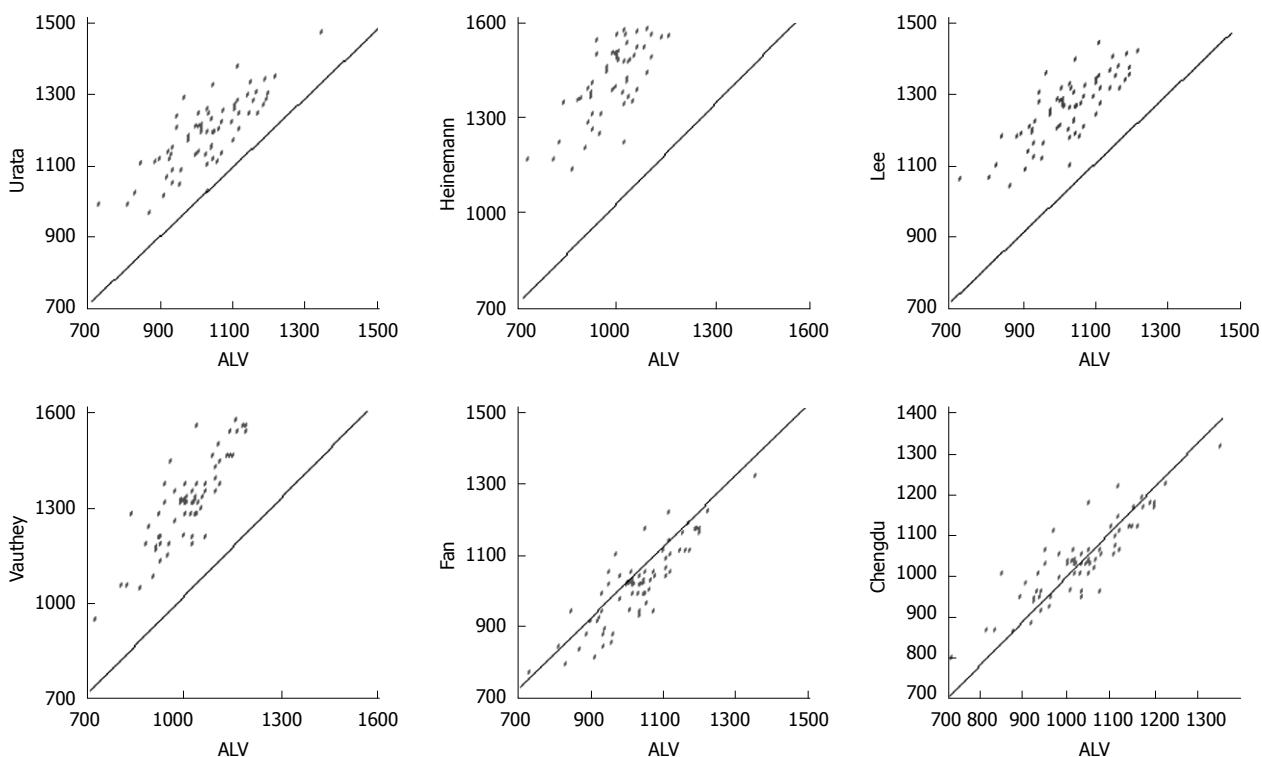


**Figure 1 Correlation between actual right liver volume (ARLV) and estimated right liver volume (ERLV) by the Chengdu formula.** When both were the same, a dot would be on the linear line.

**Table 3 Statistical analysis of estimated LV by each formula**

Formula	mean $\pm$ SD	t	P-value
Urata	1208.73 $\pm$ 99.92	-20.91	P < 0.01
Heinemann	1486.85 $\pm$ 151.78	-40.84	P < 0.01
Vauthey	1357.40 $\pm$ 155.60	-32.44	P < 0.01
Lee	1275.36 $\pm$ 97.77	-29.87	P < 0.01
Fan	1034.28 $\pm$ 111.61	2.465	P = 0.016
Chengdu	1058.70 $\pm$ 96.74	-1.417	P = 0.161
ALV	1050.10 $\pm$ 107.41	ND	ND

ALV: Actual liver volume; ND: Not determined.



**Figure 2 Correlation between actual liver volume (ALV) and estimated liver volume (ELV) by each formula.** When both were the same, a dot would be on the linear line. Formulae of Urata, Heinemann, Vauthey, and Lee overestimated LV with respect to ALV. The Fan formula underestimated LV and the Chengdu formula gave a good estimate of ALV.

0.05). There was no significant difference between ALV and ELV using the Chengdu formula (Figure 2).

## DISCUSSION

CT has become a standard method for assessing liver graft volume in living donors. Estimation of LV by CT (compared to actual volume) has a margin of error of 5%-25%<sup>[23,24]</sup>. In the present study, all donors had preoperative CT assessment of LV (mean total LV, 1189.53  $\pm$  114.75 mL and mean volume of right lobe graft without middle hepatic vein, 658.98  $\pm$  81.14 mL). The actual volume of the right liver was 578.58  $\pm$  72.33 mL. In the present study, the LV on CT was 10%-20% higher than the ALV<sup>[25-27]</sup>. The reasons may be as follows: (1) Preoperative CT measurement is carried out under normal blood flow conditions. Perioperatively, liver resection interrupts the blood supply causing a loss of liquid volume, collapse of supporting structures, and thereby reduction in the volume

of the liver. (2) Sources of error (partial volume effect, inter-observer variation, and respiratory movements) may account for this difference<sup>[28]</sup>.

The difference between the ERLV (using our formula) and ARLV was compared to the difference between ERLV, calculated using the formulae of Heinemann, Urata, Vauthey, Lee, and Fan, and ARLV in our 70 donors. The Heinemann, Urata, Vauthey, and Lee formulae overestimated LV (P < 0.01)<sup>[29]</sup>. The reasons may include: ethnic differences (patients in Europe and the United States were Caucasian). All except the Sheung Tat Fan and Chengdu formulae were used to estimate LV from CT LV or autopsy LV. Estimates of LV by CT were 5%-25% higher than the ALV<sup>[30]</sup>.

Statistical analysis showed that the Fan formula tends to underestimate LV. The weight and height of the donors in our study were higher than those in the Hong Kong group. This may be one of the reasons both results are very close (Table 3). Above all, we believe that

the Chengdu formula was demonstrated to be reliable by its application in LDLT. We were limited to use of single center data in the present study, but we hope to improve the formula by using national multicenter data in the future<sup>[31]</sup>.

## COMMENTS

### Background

With development of living donor liver transplantation (LDLT), especially improvement of right graft adult-to-adult LDLT, the danger of donating has been paid more and more attention. The exact liver volume is not only relevant for the recipient, but also for the donor to avoid dangerous life-threatening residual liver volumes.

### Research frontiers

Scholars of different countries established several standard liver volume (SLV) formulae from clinical data. The authors estimated the SLV using computed tomography or reported formulae. However, there was a gap between these estimates and the actual liver volumes for Chinese adults. Recently, they developed a new formula (named the Chengdu formula) to estimate SLV using data from 115 LDLTs.

### Innovations and breakthroughs

With the Chengdu formula, the SLVs were evaluated in 76 cases of LDLT performed from January 2008 to April 2009. Its accuracy was compared to that of other internationally reported formulae to assess which formula is the most accurate for Chinese adults.

### Applications

With national multicenter data in the future, the Chengdu formula for SLV can be improved. It may then be applied to the evaluation of donors for LDLT.

### Terminology

Standard liver volume: normal liver volume without disease affecting the volume of liver.

### Peer review

Very interesting manuscript dealing with a very hot topic: determination of optimal size matching between graft and recipient in LDLT by means of race-adapted calculation of liver volumes. The recently published liver volume formula for Chinese people (Chengdu formula) has been demonstrated to be more reliable than others and therefore it should be adopted especially in this particular form of LT.

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# Parvovirus B19 induced hepatic failure in an adult requiring liver transplantation

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## INTRODUCTION

Parvovirus B19 is a common infection that often occurs in childhood, with 50% of adolescents having antibodies to the virus by 15 years of age<sup>[1]</sup>. The infection is transmitted by respiratory droplets and through blood products derived from viremic donors. Most acute infections in children do not result in symptoms, but the clinical presentation can range from erythema infectiosum to non-specific febrile symptoms. In adults, a polyarthropathy that can resemble rheumatoid arthritis or systemic lupus erythematosus has been reported, particularly in middle aged women<sup>[2]</sup>. In patients with hemoglobinopathies the infection can cause a clinically relevant drop in hemoglobin, which may require transfusion. In the immunocompromised patient, this infection can result in bone marrow suppression and transient mild transaminase elevations commonly occur. In children there have been several reports of severe acute hepatitis and acute liver failure, which are most often self-limited<sup>[1]</sup>. There is, however, at least one reported case of parvovirus B19 induced fulminant hepatic failure in a child that required urgent liver transplantation<sup>[3]</sup>.

The majority of published cases of parvovirus B19 induced hepatitis in adults have suggested that hepatic involvement by the virus is less severe than in the pediatric population<sup>[4]</sup>. We report the case of a 55-year-old immunocompetent woman with no previous history of liver disease who developed fulminant hepatic failure secondary to acute infection with parvovirus B19 and required urgent liver transplantation.

## Abstract

Parvovirus B19 induced acute hepatitis and hepatic failure have been previously reported, mainly in children. Very few cases of parvovirus induced hepatic failure have been reported in adults and fewer still have required liver transplantation. We report the case of a 55-year-old immunocompetent woman who developed fulminant hepatic failure after acute infection with Parvovirus B19 who subsequently underwent orthotopic liver transplantation. This is believed to be the first reported case in the literature in which an adult patient with fulminant hepatic failure associated with acute parvovirus B19 infection and without hematologic abnormalities has been identified prior to undergoing liver transplantation. This case suggests that Parvovirus B19 induced liver disease can affect adults, can occur in the absence of hematologic abnormalities and can be severe enough to require liver transplantation.

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**Key words:** Parvovirus B19; Fulminant hepatic failure; Orthotopic liver transplant; Fulminant hepatitis

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## CASE REPORT

A 55-year-old immunocompetent woman who was born in Canada presented to her family physician and subsequently a local emergency room with a two week history of fatigue, general malaise, nausea with emesis,

anorexia, dark urine and pruritus. She was noted to be jaundiced and complained of mild arthralgias in her upper extremities. One week prior to developing these symptoms, she was a passenger on a commercial airplane flight and assisted an ill jaundiced passenger who had vomited. She was a retired registered nurse with no risk factors for viral hepatitis. She denied the use of herbal remedies and acknowledged minimal alcohol consumption. On initial assessment, she was afebrile with marked icterus, no hepatosplenomegaly or ascites and mild asterixis.

Initial laboratory investigations revealed a hemoglobin 161 g/L (normal: 115-155 g/L), white blood cell count  $6.6 \times 10^9/L$  (normal:  $4 \times 10^9$ - $11 \times 10^9/L$ ), platelet count  $236 \times 10^9/L$  (normal:  $150 \times 10^9$ - $400 \times 10^9/L$ ), creatinine 62  $\mu\text{mol}/L$  (normal: 40-95  $\mu\text{mol}/L$ ), aspartate aminotransferase 1838 U/L (normal: 10-38 U/L), alanine aminotransferase 1398 U/L (normal: 20-65 U/L), alkaline phosphatase 304 U/L (normal: 50-160 U/L),  $\gamma$ -glutamyl transpeptidase 179 U/L (normal: 10-55 U/L), direct bilirubin 166  $\mu\text{mol}/L$  (normal: 0-5  $\mu\text{mol}/L$ ) and total bilirubin 359  $\mu\text{mol}/L$  (normal: 0-18  $\mu\text{mol}/L$ ), international normalized ratio 1.9 (normal: 0.9-1.1), partial thromboplastin time 34 s (normal: 24-34 s), albumin 29 g/L (normal: 35-48 g/L). Hepatitis A IgG was positive and IgM was negative, hepatitis B surface antigen was negative and hepatitis C serology was negative and investigations excluded hepatitis E. Acetaminophen level was < 66  $\mu\text{mol}/L$  (normal: < 66  $\mu\text{mol}/L$ ). An urgent abdominal ultrasound demonstrated a normal appearing liver with no focal lesions.

Two days after admission to a local hospital she was transferred to the regional liver transplantation centre for assessment. On arrival, she remained icteric and demonstrated evidence of mild hepatic encephalopathy and ascites. Her liver enzymes continued to deteriorate and her liver function tests became increasingly abnormal. Her Model for End-stage Liver Disease (MELD) score was 28<sup>[5]</sup>. Further investigations revealed a serum ceruloplasmin of 301 mg/L (normal: 215-540 mg/L), IgA 3.24 g/L (normal: 0.7-4.0 g/L), IgG 13.4 g/L (normal: 6.7-15.2 g/L), IgM 1.09 g/L (normal: 0.4-2.3 g/L). The patient's anti-nuclear antibody, anti-smooth muscle antibody and anti-mitochondrial antibody were negative and iron studies were within normal limits. Serology for human immunodeficiency virus and cytomegalovirus were negative, Epstein Barr virus IgG was positive and IgM was negative. Serology for parvovirus B19 was positive, with both IgG and IgM being detected by ELISA (Biotrin).

Five days after transfer, she was placed on the waiting list for liver transplantation. Ten days after transfer she developed worsening encephalopathy and was moved to the top of the transplant list. Twelve days after her transfer she underwent an orthotopic liver transplant. One day post-transplant she was extubated and by the following day she was transferred to the solid organ transplant ward in stable condition.

Histopathologic analysis of the explanted liver

demonstrated massive hepatic necrosis consistent with viral induced fulminant hepatitis. A sample of the explanted liver was ground up with a mortar and pestle in a 2 mL volume of Minimal Essential Medium. After clarifying by centrifugation, the preparation was extracted for nucleic acid using the EasyMag platform (from bioMerieux). The extracted DNA was then tested by a parvovirus specific PCR using the forward primer CCAGGAATGACTACAAAAGGCAAATAC and the reverse primer GGTAATGCAGGGTTCTTG. The reaction was carried out for 40 cycles, each consisting of 95°C for 0 s, 52°C for 30 s and 72°C for 30 s. The PCR products were analyzed by electrophoresis on agarose gel containing ethidium bromide and visualized under UV light. A band corresponding to a 191 base pair amplicon, diagnostic for Parvovirus B19, was seen. The finding of Parvovirus B19 by PCR in the explanted liver therefore confirmed the serological diagnosis of acute Parvovirus B19 infection.

## DISCUSSION

Parvovirus B19 has been proposed as a causative agent of hepatitis, hepatitis-associated anemia and acute liver failure. There are several cases reported in the literature of patients with abnormal liver biochemistry, with and without associated anemia, caused by acute infection with parvovirus B19. One small series reported detectable parvovirus B19 DNA by polymerase chain reaction in liver tissue from 4 of 6 (67%) pediatric patients with acute liver failure accompanied by hepatitis-associated anemia and in 2 of 4 (50%) of those with acute liver failure in isolation<sup>[6]</sup>. Viral DNA was not detected in any of the patients' sera. A second small series found that parvovirus DNA was detected in 5 of 6 (83%) livers from patients with idiopathic non-A-E acute liver failure with hepatitis-associated anemia, 2 of 3 (67%) livers in patients with isolated acute liver failure and 1 of 6 (17%) livers from patients with acute liver failure of known non-parvovirus etiology<sup>[7]</sup>. So *et al*<sup>[8]</sup> (2007) have recently published a case report describing an 11-year-old boy who presented with fulminant hepatic failure secondary to acute parvovirus B19 infection who required urgent liver transplantation.

The majority of the available literature regarding acute parvovirus B19 induced fulminant hepatic failure has described cases involving children. Despite this, however, there are several published reports of acute parvovirus B19 infection in adults associated with the development of acute hepatitis<sup>[8-11]</sup>. Interestingly, in virtually every case reported the patients have had a complete and spontaneous remission. This has led to speculation that the syndrome in adults is not only less common than in children, but that it has a much less severe course with better patient outcomes. The case described in this report appears to be the first reported in which an adult patient has been recognized as having acute parvovirus B19 induced fulminant hepatic failure prior to liver transplantation. There remains a

remote possibility that she acquired the infection in childhood and that this acute episode was precipitated by an immune response to viral reactivation. In any event, it demonstrates that adults may also develop fulminant hepatic failure in the absence of hematologic abnormalities and may ultimately require liver transplantation as a potential life saving intervention.

It is worth noting that the notion of parvovirus B19 as a cause of acute viral hepatitis is not universally accepted and that there is also literature published that questions this association. A small study by Wong *et al*<sup>[12]</sup> documented the presence of parvovirus B19 DNA in the liver tissue of 4 of 15 (27%) patients with acute hepatitis as compared to 3 of 22 (14%) patients with non-viral liver disease. They concluded that no difference exists in the prevalence of parvovirus B19 in liver tissue in patients with acute liver failure or hepatitis-associated anemia as compared to those with chronic hepatitis B and C infection. Despite this study's findings, evidence continues to mount in favour of parvovirus B19 as a causative agent of acute hepatitis and fulminant hepatic failure.

In conclusion, there is growing evidence that Parvovirus B19 may cause acute viral hepatitis, which can result in fulminant hepatic failure requiring liver transplantation. Although this infection is most commonly acquired in childhood, adults who become acutely infected can develop liver dysfunction as a result. The liver disease can occur independently from the often-associated hematologic abnormalities, as illustrated by the case described in this report. Fulminant hepatic failure induced as a result of acute infection with parvovirus B19 remains a rare clinical entity, however it may be underreported due to infrequent testing that results from a lack of awareness about this syndrome. A wider recognition of parvovirus B19 as a potential cause of severe liver disease is expected to augment our ability to make a definitive diagnosis of the etiology underlying such severe clinical presentations.

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CASE REPORT

## "Pseudotumoral" hepatic pattern in acute alcoholic hepatitis: A case report

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### INTRODUCTION

Alcoholic liver disease (ALD) represents a common cause of morbidity and mortality in Europe and United States, its clinical manifestations ranging from fatty liver to end-stage cirrhosis. In this context, acute alcoholic hepatitis (AAH) is a serious complication, with a short-term mortality rate exceeding 50% in most severe cases<sup>[1-4]</sup>. Liver biopsy maintains its pivotal role in diagnosing AAH, in predicting its outcome and in the selection of patients suitable for treatment: steatosis, ballooning degeneration, Mallory bodies, perivenular polymorphonuclear inflammation and "chicken-wire" fibrosis represent the most frequent findings<sup>[5]</sup>.

In the setting of AAH, imaging studies do not confirm the presence of ALD, but can be used to evaluate hepatic parenchymal changes. Ultrasonography (US) scan, computed tomography (CT) scan, and magnetic resonance imaging (MRI) can be used to diagnose fatty changes, cirrhosis or neoplastic diseases of the liver.

On MRI, specific features suggestive for alcoholic cirrhosis versus virus-induced cirrhosis include a higher volume index of the caudate lobe, smaller size of regenerative nodules of the liver and more frequent visualization of the right posterior hepatic notch<sup>[6]</sup>. On US liver scan, the presence of "pseudoparallel channel signs" and of low hepatic artery resistivity index (RI) at duplex Doppler investigation have both been reported in AAH<sup>[7-9]</sup>. A "pseudotumoral" hepatic pattern at CT scan has also been reported in this setting, even if only scanty data are available<sup>[10]</sup>. Advanced fibrosis can be

### Abstract

In acute alcoholic hepatitis (AAH), a "pseudotumoral" appearance of the liver parenchyma on computed tomography (CT) scan has been reported. The main findings are hypervascularized areas closely similar to those observed in large hepatocellular carcinomas. We report a case of a patient affected by AAH with an unusual appearance of these "pseudotumoral" areas on CT scan, close resembling a metastatic cancer rather than a primary hepatocellular carcinoma. In fact, in contrast with previous reports, the picture was characterized by the presence of many inhomogeneous, hypoattenuated areas highlighted during both pre- and post-contrast phases. Moreover, we report the first description of "pseudotumoral" lesions on ultrasound scan. This patient was successfully treated with corticosteroids, even if many controversies still exist regarding their efficacy in this setting.

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**Key words:** Acute alcoholic hepatitis; Pseudotumoral hepatic lesions; Alcoholic liver disease; Computed tomography; Ultrasound

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determined using transient elastography<sup>[11]</sup>.

The present case report refers to an AAH patient with unusual pseudotumoral US and CT scan hepatic pattern, with favourable clinical course after alcohol withdrawal and steroid treatment.

## CASE REPORT

A 33-year-old man, immigrating from Bangladesh, was referred to our Gastrointestinal Unit on August 9, 2007 because of marked asthenia, nausea, vomiting, abdominal pain and weight loss of 11 kg (from 66 to 55 kg for 170 cm of height) during the previous month. Blood arterial pressure was 105/70 mmHg and heart rate 80 beats per minute. The state of consciousness was normal and the physical examination revealed a painful hepatomegaly, with the lower hepatic edge 20 cm below the right costal margin. His past history revealed heavy smoking, accounting for a lifetime packet sum of 1800, and daily alcohol intake of 60 g until 2005; alcohol intake was then denied until hospital admission. His laboratory tests are reported in Table 1 (left column). Past or current HBV, HCV and HIV infections were ruled out by determining HBsAg and anti-HBc (tested with commercial electrochemiluminescence immunoassay kits-Elecys HBsAg, anti-HBc; Roche Diagnostics, GMBH, Manheim, Germany), anti-HCV (Innotest-HCV-Ab IV; Innogenetics, Ghent, Belgium) and anti-HIV (tested with chemiluminescence immunoassay, Ag/Ab Combo-Architect, Abbott, Chicago, Illinois, USA). Serological and stool tests for parasitic infections were negative. Anti-nuclear, anti-mitochondrial and anti-LKM antibody were searched for by indirect immunofluorescence performed on 4 µm cryostat sections from rat liver, kidney and stomach tissues, at a sera dilution of 1:40. ECG and chest X rays were negative. US liver scan revealed a severe derangement of hepatic structure, characterized by multiple micro- and macronodular hyperechoic lesions; the biliary tree was not dilated and there were no signs of portal hypertension. Color-Doppler examination showed intrahepatic arterial dilation with pseudoparallel channel sign and low hepatic artery RI (Figure 1). At total body CT scan there was a marked liver enlargement, and multiple hypoattenuated areas were noted both with and without contrast medium (Figure 2); a diagnosis of metastatic liver disease was made. Transient elastography (Fibroscan®) was also performed and the observed value of 75 kPa (normal value < 8.0) was consistent with advanced liver fibrosis. Upper gastrointestinal tract endoscopy revealed esophageal varices (F1) and portal hypertensive gastropathy. To better define hepatic lesions, US-guided liver biopsy was obtained from both hypoattenuated areas and the surrounding parenchyma, and specimens routinely stained. At histology, main findings included a diffuse fibrosis surrounding regenerating nodules, intrasinusoidal collagen deposition, perivenular polymorphonuclear infiltration, focal fatty infiltration and ballooning degeneration with Mallory bodies, all features consistent with a final diagnosis of AAH on

**Table 1** Biochemical characteristics of the patient

Parameters (reference value)	August 9, 2007	November 22, 2007	November 12, 2008
Haemoglobin (g/dL) (13-16)	12.7	12.2	12
MCV (fL) (84-94)	86	86	82
White blood count ( $\times 10^3$ ) (5.5-8.5)	9.7	8	5.3
Platelets count ( $\times 10^3$ ) (150-350)	119	102	184
PT (%) / INR (70-100/1.0-1.2)	54/1.3	90/1.2	94/1.1
PCR (mg/dL) (< 0.5)	1.8	0.6	0.4
Total/direct bilirubin (mg/dL) (1.1/0.8)	11.9/-	4.5/2.2	1.3/0.9
AST/ALT (IU/L) (< 35)	318/73	70/36	32/28
GGT (IU/L) (< 50)	1.309	150	46
Serum iron (mcg/dL) (70-170)	212	-	141
Transferrin (mg/dL) (200-400)	183	-	293
Ferritin (ng/mL) (400-220)	2.78	665	213

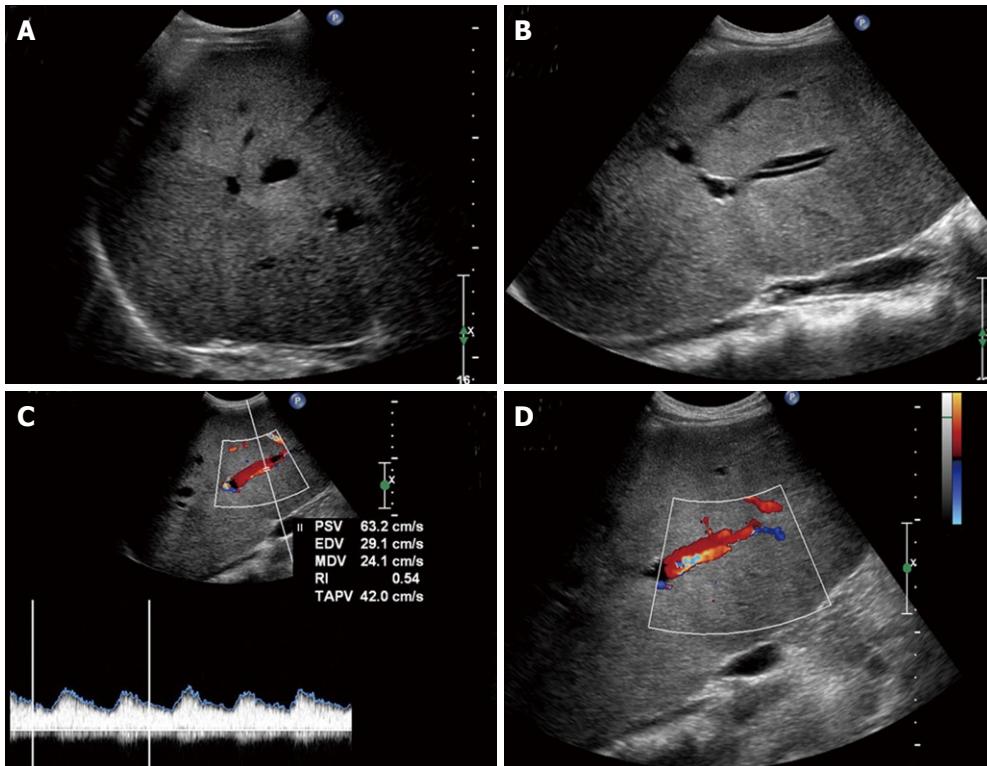
Patient's main laboratory test at enrolment (left column), 2 mo after complete alcohol withdrawal (central column) and at last control 1 year later (right column). MCV: Mean corpuscular value; PT: Prothrombin time; INR: International normalized ratio; CRP: C-reactive protein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT:  $\gamma$ -glutamyltransferase.

cirrhosis, with "pseudotumoral" areas. In the meantime, careful re-evaluation of usual daily alcohol intake, extended to patient's friends, allowed to estimate an actual daily intake of 250 g for many years.

According to a Maddrey's discriminating factor of 63, we instituted a treatment consisting in the daily administration of 40 mg of prednisolone, rapidly followed by clinical and laboratory improvement. The regimen was slowly tapered down and at the following clinical observation in November 2007, the patient was asymptomatic, he had stopped drinking, his body weight had increased by 13 kg (from 55 to 68) and his physical examination revealed a dramatic reduction in liver size, with the lower hepatic edge at 5 cm below the right costal margin. Laboratory tests at that time are summarized in Table 1 (central column). Moreover, a new determination of the transient liver elastography indicated a value of 51 kPa, accounting for a decrease of 24 kPa as compared to the previous measurement. A further control, performed on November 11, 2008, showed the complete normalization of both physical findings and blood tests (Table 1, right column). Interestingly, at this time, the score at transient elastography was 8.5 kPa. As mean corpuscular volume of red blood cells was unusually "normal" (86 fL, with reference value of 84-94 fL) in the setting of AAH, and, also considering the ethnic origin of the patient, we investigated a possible underlying hemoglobinopathy by HPLC (high performance liquid chromatography), analysing different Hb fractions (Variant, Bio-Rad, Milan, Italy). Findings were consistent with a heterozygous state for HbE.

## DISCUSSION

The present case report concerns a patient with AAH superimposed to established cirrhosis, with "pseudotumoral" hepatic areas. This unusual finding was first



**Figure 1** US scan showing a large hypoechoic area compared to surrounding parenchyma (A) and the image of “parallel channel” sign (B). Color-Doppler demonstrates the “pseudoparallel channel” sign, characterized by dilated intrahepatic arterial branch with an adjacent portal venous tract, and the low hepatic artery RI (C, D).



**Figure 2** CT scan showing a wide hypovascularized area in the pre-contrast phase (A) that remains hypovascularized during both early (B) and late (C) arterial phases.

described in two reports<sup>[9,10]</sup>, possibly involving the same single case. More recently, Colli *et al*<sup>[12]</sup> described both the CT and histological characteristics of “pseudotumoral” hepatic areas in five patients with AAH. These focal lesions were described as hypoattenuated areas when compared to the surrounding parenchyma, during the pre-contrast phase, becoming hyperattenuated during the post-contrast and late arterial phases, respectively, a pattern consistent with hypervasculated areas possibly related to a high-grade tissue regeneration. Interestingly, such lesions were closely similar to those observed in cases of large hepatocellular carcinoma<sup>[13]</sup>, accounting for possible misdiagnosis. Differently from what previously reported, in this case the finding of many inhomogeneous, hypoattenuated areas highlighted during both pre- and post-contrast phases was more similar to metastatic cancer rather than to primary hepatocellular carcinoma and was responsible for the initial misdiagnosis of hepatic metastases. This led to further

investigations, including a liver biopsy, which provided the correct diagnosis of AAH, ruling out any malignancy. This picture differs from the previously reported AAH CT pattern.

Occasionally, focal areas of normal parenchyma in an otherwise diffuse fatty liver may simulate mass lesions, described as “pseudolesions”, that may pose a difficult diagnostic problem<sup>[14]</sup>. These areas usually present a vascularisation similar to the surrounding parenchyma: in the present case, instead, the lesions appeared clearly different in each vascular phase, as compared to the liver. Moreover, pseudo-tumoral hepatic lesions were described in a variety of other benign conditions, such as inflammatory pseudotumors, parasitic infestations, tuberculosis infection, or areas of focal sparing in diffuse processes<sup>[15-19]</sup>, conditions ruled out in our patient.

An additional interesting finding, in the present case, was the presence of a typical alcohol-related duplex-Doppler image, called “pseudoparallel channel sign”,

reported in patients with AAH by Sumino *et al*<sup>7</sup> and characterized by dilated intrahepatic arterial branch with an adjacent portal venous tract. A dilation of hepatic artery, with increased peak systolic velocity, has also been described by Han *et al*<sup>9</sup>, who assumed that in AAH the presence of liver fibrosis increases sinusoidal resistance, blocking sinusoidal blood flow and ultimately portal blood flow in a retrograde manner. Therefore, in order to maintain hepatic perfusion, there is a dilation of hepatic artery leading to increased blood flow. A further interesting Doppler finding in this patient was the low hepatic artery RI, whose role in diagnosing AAH remains however controversial. Colli *et al*<sup>8</sup> reported a statistically significant decrease of hepatic artery RI in patients with AAH, as compared to both healthy and cirrhotic patients, a finding in contrast with the cirrhotic pattern observed in our patient. The possible relevance of the hepatic artery RI in AAH has also been challenged by Han *et al*<sup>9</sup>, who reported a high variability of this sign in patients with liver disease, accounting for a lack of a clear-cut distinction between AAH and cirrhosis.

To assess the severity of underlying liver disease and to properly take care of the patient, we assessed three main prognostic models, all validated for AAH [i.e. Maddrey's discriminating factor (mDF), model for end-stage liver disease and Glasgow for acute alcohol hepatitis score (GAHS)]<sup>[20-22]</sup>. Our case scored a total of 63, 14 and 9, respectively, compared to reference values of 32, 21 and 9. A mDF  $\geq 32$  and a GAHS score  $\geq 9$  identify patients with a very poor prognosis who have been reported to have had a good clinical response to corticosteroids<sup>[23]</sup>. Based on an mDF of 63 and a GAHS of 9, a corticosteroid regimen was instituted and then slowly progressive tapered down, on the basis of progressive clinical, laboratory and radiological improvement. At present, prognostic scores for AAH may orient the patient management, even if the use of steroids in this setting has recently been challenged by pertinent metanalytic data<sup>[24]</sup> while other treatments, such as anabolic steroids, pentoxifylline and infliximab, are still under investigation<sup>[25-27]</sup>.

To complete the liver disease staging and to obtain data useful in the follow up, our patient underwent also a transient elastography (FibroScan®). Values obtained in this case were very high, indicating not only a possible advanced fibrosis of the liver, but also confirming recent reports suggesting that transient elastography can be influenced by other parameters, such as the degree of necroinflammatory activity, especially during acute hepatitis<sup>[28]</sup>.

To summarize, in patients with AAH, "pseudotumoral" hepatic areas can appear at CT scan not only as hypervascular lesions similar to HCC, as previously described, but also as hypoattenuated lesions during all contrast phases, closely similar to liver metastases. This feature has to be carefully considered to avoid misdiagnosis.

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CASE REPORT

## An adult case of celiac sprue triggered after an ileal resection for perforated Meckel's diverticulum

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### INTRODUCTION

Celiac disease is an autoimmune enteropathy often seen in gluten sensitive patients<sup>[1]</sup>. It has two presentations in adults, namely the classical (diarrhea-predominant) type and the silent type<sup>[2]</sup>. The silent group includes atypical presentations. Some initiating factors, such as gluten overload, surgery, giving up smoking, and infections can trigger the disease, which can become apparent in an abrupt manner<sup>[3,4]</sup>.

Meckel's diverticulum is a common congenital anomaly of the small bowel. Ulcer, hemorrhage, intussusception, intestinal obstruction, perforation, and, very rarely, vesicodiverticular fistulae and tumors are complications of these diverticula<sup>[5]</sup>. We present a case of Meckel's diverticula that was diagnosed as celiac disease after surgery. This is the first reported case of Celiac disease that has been diagnosed after an ileal resection rather than upper abdominal surgery.

### Abstract

Celiac disease can be triggered by upper abdominal surgery, such as vagotomy, oesophagectomy, pancreaticoduodenectomy, and gastrojejunostomy. Here we report a case of a 24 year-old woman who developed celiac disease after an ileal resection for perforated Meckel's diverticula. This is the first reported celiac case that has been triggered, not by upper abdominal surgery, but after ileal resection for Meckel's diverticula.

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**Key words:** Celiac disease; Meckel's diverticula; Ileal resection

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Topal F, Akbulut S, Topcu IC, Dolek Y, Yonem O. An adult case of celiac sprue triggered after an ileal resection for per-

### CASE REPORT

A 24 year-old woman applied to the emergency service with abdominal pain, nausea, and vomiting. She did not have any bowel movements or flatus and her abdominal pain worsened after her hospitalization. There was tenderness and guarding on abdominal palpation. Her initial laboratory tests revealed a leukocytosis score of 14000/mm<sup>3</sup>.

Due to her worsening abdominal pain and a white blood cell count that progressively increased to 16.000/mm<sup>3</sup>, urgent surgery was performed for an acute abdomen. Perforated Meckel's diverticula, located 80 cm proximal to the ileocecal valve, were observed during the operation. Ten centimeters of small bowel segment including the Meckel's diverticulum was resected and an end-to-end anastomosis was performed. Pathological investigation of the surgical specimen revealed perforated Meckel's diverticula and segmental ileal resection. The patient was discharged 4 d after the surgery without any complications.



**Figure 1** Diffuse villous atrophy in the duodenum (HE,  $\times 10$ ).

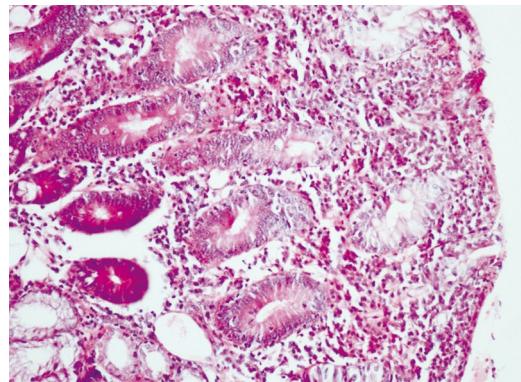
Twenty days after the discharge, the patient applied to the gastroenterology clinic with complaints of abdominal pain, flatulence and a loose stool 4-times/d. Her laboratory examination revealed Hb:10 g/dL, WBC: 5400/mm<sup>3</sup>, Plt: 458.000/mm<sup>3</sup>, vitamin B12: 119 ng/mL (180-914), AST: 14 U/L (5-45), ALT: 14 U/L (5-45), and ALP: 260 U/L (80-270). An upper gastrointestinal endoscopy was performed for her anemia, which showed antral gastritis and scalloping of duodenal mucosal folds. Endoscopic duodenal biopsy revealed diffuse atrophic villi with an increase in intraepithelial lymphocytes suggesting celiac disease (Figures 1 and 2). For confirmation of Celiac disease, gluten antibodies were determined as follows: anti-gliadin IgA, 88.2 U/mL (0-12); anti-gliadin IgG > 100 U/mL (0-12); anti-endomysial IgA antibody, (+++); anti tissue transglutaminase IgG, 51.2 U/mL (0-10); and anti tissue transglutaminase IgA, > 200 U/mL (0-10). Gluten was removed from the diet and thereafter her complaints of abdominal pain, flatulence, and diarrhea resolved. Her laboratory tests after a 2-mo gluten free diet were; Hb 12.2 g/dL and vitamin B12: 461 ng/mL (180-914).

## DISCUSSION

Celiac disease is an autoimmune enteropathy seen in gluten sensitive patients. It is a common genetic disorder with a prevalence of 1%-2%<sup>[6]</sup>. The disease can manifest itself by different clinical presentations. There are gastrointestinal symptoms, diarrhea and weight loss in the classical type, while extra intestinal findings are most common in the atypical or subclinical form<sup>[2-4]</sup>.

Our patient could have been in the silent form of the disease that became overt after the triggering effect of surgery. There are celiac disease patients in the literature that were triggered by upper gastrointestinal surgery, such as vagotomy, oesophagectomy, pancreaticoduodenectomy, and gastrojejunostomy<sup>[7-10]</sup>. Our case is the first report of celiac disease being triggered by ileal surgery.

The autoimmune activation mechanism triggered by the surgery is not yet known. However, it has been postulated that raised intestinal permeability might be involved in the pathogenesis of celiac disease<sup>[11]</sup>. Andersen



**Figure 2** Increased intraepithelial lymphocytes in the duodenum (HE,  $\times 50$ ).

et al<sup>[12]</sup> have shown by a triple sugar test that bowel permeability is increased in ileostomy patients. Perhaps this hyperpermeability could be the triggering factor in our patient. Another possible mechanism for the emergence of post-operative Celiac disease in our patient could be antigenic overload secondary to postoperative changes<sup>[9]</sup>.

Early diagnosis of Celiac disease in the postoperative period is important to prevent complications. A clinician should be aware of Celiac disease when the patient has refractory diarrhea, anemia, weight loss, and hypoalbuminemia after ileal surgery, not just after upper gastrointestinal surgery.

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# Pneumobilia, chronic diarrhea, vitamin K malabsorption: A pathognomonic triad for cholecystocolonic fistulas

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## Abstract

Cholecystocolonic fistula (CF) is an uncommon type of internal biliary-enteric fistulas, which comprise rare complications of cholelithiasis and acute cholecystitis, with a prevalence of about 2% of all biliary tree diseases. We report a case of a spontaneous CF in a 75-year-old diabetic male admitted to hospital for the investigation of chronic watery diarrhea and weight loss. Massive pneumobilia demonstrated on abdominal ultrasound and computerized tomography, along with chronic, bile acid-induced diarrhea and a prolonged prothrombin time due to vitamin K malabsorption, led to the clinical suspicion of the fistula. Despite further investigation with barium enema and magnetic resonance cholangio-pancreatography, diagnosis of the fistulous tract between the gallbladder and the hepatic flexure of the colon could not be established preoperatively. Open cholecystectomy with fistula resection and exploration of the common bile duct was the preferred treatment of choice, resulting in an excellent postoperative clinical course. The incidence of biliary-enteric fistulas is expected to increase due to the parallel increase of iatrogenic interventions to the biliary tree with the use of endoscopic retrograde cholangio-pancreatography and the increased rate of cholecystectomies performed. Taking into account that advanced imaging techniques fail to demonstrate the fistulas tract in half of the cases, and that CFs usually present with non-specific symptoms, our report

could assist physicians to keep a high index of clinical suspicion for an early and valid diagnosis of a CF.

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**Key words:** Cholecystocolonic fistula; Cholecystocolonic fistula; Bilioenteric fistula; Pneumobilia

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## INTRODUCTION

Internal biliary-enteric fistulas (IBFs) are very rare, comprising 0.4%-1.9% of all biliary tract diseases<sup>[1-3]</sup>. IBFs are detected in only 0.2%-0.9% of all biliary tract operations<sup>[4-6]</sup>. Depending on the site of communication with the extrahepatic biliary tree, several types of fistulas are recognized (cholecystoduodenal, choledochoduodenal, cholecystogastric, cholecysto-choledochal, cholecystocolonic, cholecystoduodenocolic)<sup>[1,2]</sup>. Peptic ulcer and malignancies of the stomach, gallbladder, pancreas, duodenum, colon and bile ducts have been identified as etiologic factors, with cholelithiasis being the predisposing factor<sup>[2]</sup>. Iatrogenesis may be responsible for an increasing incidence of IBFs in the near future<sup>[2]</sup>, as the use of endoscopic retrograde cholangio-pancreatography (ERCP) and the rate of cholecystectomies performed annually increase.

A cholecystocolonic (otherwise cholectystocolonic) fistula (CF) is an uncommon type of IBF, which forms a communication between the gallbladder and the hepatic flexure of the transverse colon (Figure 1)<sup>[7]</sup>. Theoretically, fistula formation was part of the natural history of acute cholecystitis prior to the era of cholecystectomy and antibiotics<sup>[8]</sup>. Its frequency among other types of IBF

ranges between 8% and 13.6%<sup>[1,9,10]</sup>. CFs are associated with several severe complications, such as acute cholangitis, biliary peritonitis and biliary cirrhosis<sup>[3,11,12]</sup>, leading to a global mortality rate between 10% and 15%<sup>[4,9,10]</sup>.

Since Courvoisier first discussed spontaneous biliary fistulas in 1890<sup>[5]</sup>, several authors have described interesting case-reports of CF in the literature<sup>[13-38]</sup>. However, diagnosis still remains a challenge, mainly because: (1) CF presents with varying and non-specific symptoms; (2) CF is very rare and is kept last in differential diagnosis; and (3) even advanced imaging techniques fail to demonstrate the fistulas tract in almost half of the cases<sup>[1,2,13]</sup>.

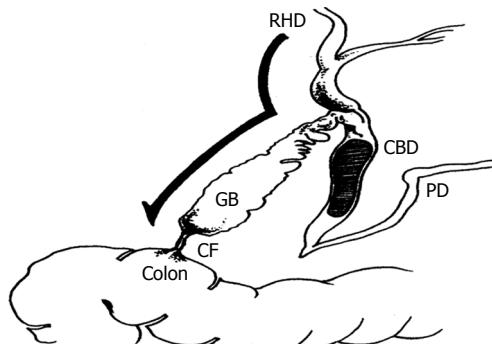
In this paper, we report a case of a spontaneous CF and review the literature. The aim of this paper is to elaborate on the clinical manifestation of a CF and describe the pathophysiological mechanisms. In conclusion, we suggest that the triad of pneumobilia, chronic diarrhea and malabsorption of vitamin K could assist physicians to keep a high index of clinical suspicion for CF, leading to an early and valid diagnosis.

## CASE REPORT

A 75-year-old male patient was referred to our university hospital for the investigation of chronic diarrhea and weight loss. The patient reported suffering from watery diarrhea of 3-4 bowel movements daily, lasting longer than 18 mo, and weight loss of 15 kg. The patient reported having no fever, nausea, vomiting, jaundice or abdominal pain. Medical history revealed the presence of mild diabetes mellitus type 2, which was controlled by diet, and a long-lasting smoking habit. The patient had already been subjected to colonoscopy prior to his admittance. Biopsies, including the terminal ileum, had been unrevealing. Routine laboratory tests of total white blood cell (WBC) count, erythrocyte sedimentation rate (ESR), aminotransferase levels and electrolytes had also been found within normal.

On admission, the patient was afebrile. No pathological signs were found on physical examination. Laboratory investigation demonstrated alkaline phosphatase of 228 IU/L (normal range 35-120 IU/L),  $\gamma$ -glutamyl transpeptidase of 120 IU/L (normal: 10-45 IU/L), C-reactive protein of 6.51 mg/dL (normal: 0-0.5 mg/dL) and a prolonged prothrombin time (PT) of 20.3 s (normal: 12.2 s), that was returned to normal after parenteral administration of vitamin K (10 mg subcutaneously). Total and differential WBC counts, haemoglobin, platelet count, ESR, aminotransferase levels, bilirubin, total protein, albumin and electrolytes ranged within normal values. Fecal examination included negative stool culture and negative examination of stool for ova and parasites. Stool examination for fat was negative, but fat determination of 24 h stool was unavailable.

Ultrasonographic examination of the liver and the extrahepatic biliary tree demonstrated pneumobilia and moderate dilatation of the intrahepatic bile ducts, with a normal common bile duct and a thick-walled gallbladder (Figure 2). Enhanced CT of the abdomen confirmed



**Figure 1 Schematic demonstration of a cholecystocolic fistula (modified from Benage et al<sup>[7]</sup>). GB: Gallbladder; CBD: Common bile duct; RHD: Right hepatic duct; PD: Pancreatic duct; CF: Cholecystocolic fistula.**



**Figure 2 Ultrasonographic examination revealing pneumobilia and moderate dilatation of the intrahepatic bile ducts, with a normal common bile duct and a thick-walled gallbladder.**

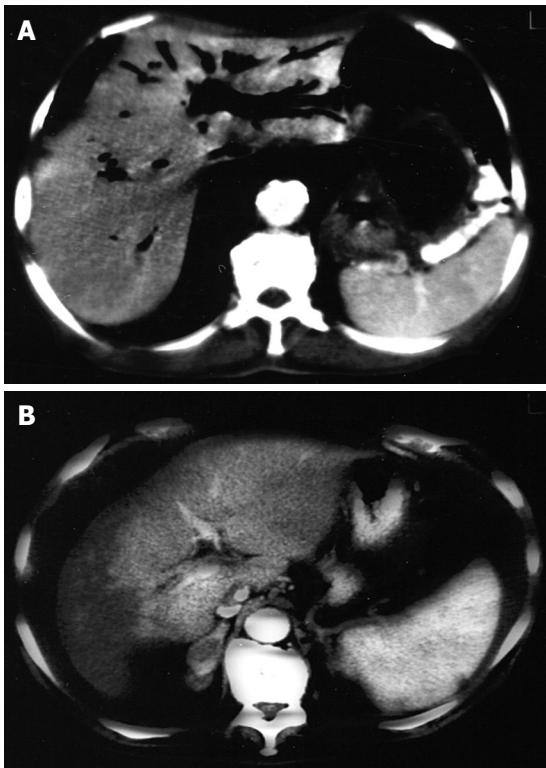
massive pneumobilia and absence of common bile duct dilatation (Figure 3A). Furthermore, the presence of air was detected in the gallbladder, which was neighboured to the hepatic flexure of the colon (Figure 3B). Neither the following barium enema nor the MR cholangio-pancreatography were able to detect any fistulous tract between the gallbladder and the colon.

The patient was subsequently referred to surgery for diagnostic laparotomy. During surgery a fistulous tract from the fundus of the gallbladder to the hepatic flexure of the colon was detected, along with an impacted large gallstone near Vater's villa. Cholecystectomy with fistula resection and bilio-enteric anastomosis after gallstone removal was undertaken. The patient had an excellent postoperative clinical course. Diarrhea stopped, and he regained his weight within a 6 mo period.

## DISCUSSION

We report a case of a spontaneous CF in a male diabetic patient, due to asymptomatic cholelithiasis. A combined triad of strong clues (pneumobilia, chronic, bile acid-induced diarrhea and malabsorption of fat-soluble vitamin K) provided high clinical suspicion for a CF, which was only confirmed during diagnostic laparotomy.

Several authors have reported their own experience of CFs<sup>[2,7,9,13-38]</sup>. Table 1 summarizes patients' demographics,



**Figure 3** Computed tomography scan. A: Massive pneumobilia ("air cholangiogram"); B: Presence of air detected in the gallbladder.

etiological factors, clinical presentation, presence of pneumobilia, means of fistula visualization and treatment option preferred. CF occurs mostly in the elderly, with a female preponderance<sup>[3,9]</sup>. In our case and in more than 90% of all cases, cholelithiasis is the main etiologic factor. Cholelithiasis may cause either recurrent episodes of acute cholecystitis/cholangitis or asymptomatic chronic calculous cholecystitis. The sequence of events that lead to the formation of the fistula include gallbladder inflammation, mechanical erosion by gallstones and gangrenous changes of both the gallbladder wall and the wall of the adjacent colon<sup>[14,17,39]</sup>. The gallstone found in the common bile duct in our patient could have affected the formation of the fistula by increasing the pressure in the biliary tree and thus enhancing the mechanical erosion of the gallbladder by another gallstone or gallstones that could easily have escaped through the fistulous tract.

CF usually has a benign clinical course, remaining asymptomatic for a long time, and can only be found incidentally<sup>[24,26,28]</sup> or be suspected by the presence of unexplained pneumobilia in patients with a history of episodes of right upper quadrant (RUQ) pain<sup>[17,22]</sup>. In other cases, varying symptoms are identified like fever, nausea and vomiting, attacks of abdominal pain localized mainly in the RUQ and jaundice<sup>[15,19,23,36]</sup>. These symptoms are non-specific and cannot be directly attributable to the fistula but rather to the main etiologic factor, cholelithiasis. In these cases, clinical presentations of acute cholecystitis, cholangitis or acute/chronic pancreatitis predominate.

A more specific symptom of a CF is chronic diarrhea<sup>[2,9,13,14,21,24,27,32,35]</sup>. CFs alter the normal enterohepatic

circulation of bile acids, leading to chronic watery diarrhea. This is a type 1, bile acid-induced diarrhea, which results from the stimulation of colonic mucosa by bile acids and the subsequent colonic secretion of water and electrolytes<sup>[35,40,41]</sup>. In other cases, especially when hepatic synthesis of bile acids is reduced, bypass of the distal ileum allows bile acids to escape absorption, leading to steatorrhea<sup>[7,9,35,36]</sup>. Bile salts are needed for micelle formation and subsequent absorption of fat and fat-soluble vitamins, while protein absorption is not affected. Fat malabsorption resulting from impaired micelle formation is not as severe as malabsorption resulting from pancreatic lipase deficiency, because fatty acids and monoglycerides can form lamellar structures, which to a certain extent can be absorbed<sup>[42]</sup>. Theoretically, malabsorption of fat-soluble vitamins (A, D, K and E) may be marked, because micelle formation is required for their absorption. However, malabsorption of fat-soluble vitamins A and E has never been reported in the literature, while malabsorption of vitamin D has only been described in one female patient with CF and osteoporosis/osteomalacia<sup>[7]</sup>. The deficiency of those vitamins has to be marked to provide clinical symptoms, and laboratory evaluation is difficult and outside routine clinical practice. On the other hand, vitamin K deficiency can easily be detected by a prolonged PT and can be fixed with parenteral administration.

Rare complications of CF reported in the literature are ectopic gallstone in the colon with obstruction<sup>[29,30,34,37,38]</sup> or hemorrhage of the lower gastrointestinal tract<sup>[20]</sup>, and extraperitoneal abscess with sepsis due to ascending contamination by colon bacteria<sup>[18,20,43]</sup>.

Barium enema<sup>[2,9,13,21,24,26,27,32]</sup>, <sup>99m</sup>Tc scintigram<sup>[24,43]</sup>, CT<sup>[29,44]</sup>, MRCP and ERCP<sup>[7,13-15,17,19,21,22,35,36]</sup> have all been used in order to demonstrate the fistula. However, non-visualization of the fistulous tract occurs in almost half of the cases<sup>[1,2,13,44]</sup>, and, subsequently, patients are subjected to exploratory laparotomy. The presence of pneumobilia, detected by plain abdominal films, abdominal ultrasound or CT, may provide presumptive evidence for the existence of a biliary-enteric fistula, but it is non-specific<sup>[2,45]</sup>. Other radiological features include a small atrophic gallbladder adherent to neighboring organs or a shrunken thick-walled gallbladder around gallstones<sup>[2,44]</sup>.

Open cholecystectomy with fistula resection and exploration of the common bile duct is the treatment of choice mostly preferred in order to avoid attacks of cholecystitis and/or cholangitis. However, successful cases using the laparoscopic approach are increasingly reported in the literature<sup>[15,23,26,28,31-33]</sup>. In non-surgical patients, either conservative treatment with antibiotics and supplementation of fat-soluble vitamins<sup>[9,16]</sup>, or ERCP sphincterotomy<sup>[36,46]</sup> to reduce biliary pressure are suggested.

In conclusion, especially in patients with a history of gallstone disease, the presence of chronic watery diarrhea and vitamin K malabsorption, combined with the radiological finding of pneumobilia, form a pathognomonic triad. Either internists/gastroenterologists

**Table 1 Systematic review of cases of cholecystocolic fistulas reported in the literature**

<b>Author (yr)</b>	<b>No pts</b>	<b>Age (yr)</b>	<b>Etiology</b>	<b>Main clinical manifestation</b>	<b>Presence of pneumobilia</b>	<b>Means of fistula visualization</b>	<b>Treatment option preferred</b>
Chatzoulis et al <sup>[14]</sup> (2007)	1M	52	Cholelithiasis (mirizzi syndrome type IV)	Diarrhea, RUQ pain, fever	+ (US, CT)	MRCP, ERCP	Cholecystectomy, fistula excision, Roux-en-Y bilio-enteric anastomosis
Wang et al <sup>[15]</sup> (2006)	1F	63	Gallbladder and CBD stone	RUQ pain	+	ERCP	Laparoscopic cholecystectomy, fistula closure
Singh et al <sup>[16]</sup> (2004)	1F	92	Gallstones	Melena	+	-	Conservative
Arvanitidis et al <sup>[17]</sup> (2003)	1M	72	Acute obstructive cholecystitis	Unexplained pneumobilia, history of RUQ pain	+ (US)	ERCP	Open cholecystectomy, fistula resection, exploration of CBD
Hussien et al <sup>[18]</sup> (2003)	1M	63	Xanthogranulomatous cholecystitis	Fever due to extraperitoneal abscesses	-	-	Cholecystectomy, fistula excision, closure of colon defect
Velayos Jiménez et al <sup>[9]</sup> (2003)	1F	79	Cholelithiasis	Chronic diarrhea, steatorrhea (?)	+ (US, CT)	BE	Conservative (antibiotics, vitamins)
De Keuleneer et al <sup>[19]</sup> (2002)	NR	NR	Cholelithiasis (Mirizzi syndrome type I)	Obstructive jaundice	-	ERCP	Cholecystectomy with Roux-en-Y hepato-enteric anastomosis
Ramos-De la Medina et al <sup>[20]</sup> (2002)	1F	48	Cholelithiasis	Lower gastro-intestinal bleeding	-	-	Open surgery
	1F	60	Gallbladder malignancy	Multiple hepatic abscesses	-	-	
Dutta et al <sup>[21]</sup> (2002)	1F	65	NR	Diarrhea, weight loss, malabsorption (?)	+	BE, ERCP	Fistectomy, cholecystectomy
Schoeters et al <sup>[22]</sup> (2002)	1F	81	NR	Incidental pneumobilia, RUQ pain	+ (PAF, US)	ERCP	NR
Fujitani et al <sup>[23]</sup> (2001)	1F	58	Cholelithiasis, chronic cholecystitis	RUQ pain	-	Intraoperatively	Laparoscopic cholecystectomy and fistula resection
Sam et al <sup>[24]</sup> (2001)	1F	75	Incidental (Crohn's disease?)	Diarrhea	- (Air in gallbladder)	BE, Tc-99m chole-scintigraphy	NR
Inal et al <sup>[2]</sup> (1999)	1F	72	Chronic cholecystitis	Diarrhea, weight loss	+ (US)	BE	Open cholecystectomy, repair of the fistula
Kuo et al <sup>[25]</sup> (1999)	1F	65	Chronic cholecystitis	Diarrhea, fever, RUQ, jaundice	+ (PAF)	BE, ERCP	Cholecystectomy, fistulas division
Hida et al <sup>[26]</sup> (1999)	1F	61	Cholelithiasis	Incidental finding (BE screening), RUQ pain	-	BE	Laparoscopic stamping technic
Holst et al <sup>[27]</sup> (1999)	1F	76	Gallstone disease	Diarrhea, RUQ pain	-	BE	Open cholecystectomy, fistula resection
Reddy et al <sup>[28]</sup> (1998)	1F	72	History of acute cholecystitis	Incidental finding during laparoscopic cholecystectomy	-	-	Laparoscopic approach, tube caecostomy
Bornet et al <sup>[29]</sup> (1998)	NR	NR	Gallstone disease	Colonic gallstone obstruction	+ (CT)	CT?	None
Hession et al <sup>[13]</sup> (1996)	1F	70	Acute or chronic cholecystitis	Diarrhea	-	BE	NR
	1F	78		Diarrhea	-	BE	
	1F	69		Diarrhea	-	BE	
	1F	73		Diarrhea	-	BE	
	1F	77		Colon ileus	+	BE	
	1F	85		RUQ pain, diarrhea	+	BE	
	1M	43		Melena	-	ERCP	
Pérez Morera et al <sup>[30]</sup> (1996)	2F	NR	Gallstone disease	Colonic gallstone obstruction	+ (postoperatively)	BE	Repair of the fistula at a later stage
Ibrahim et al <sup>[31]</sup> (1995)	1F	68	Cholelithiasis	Incidental finding during laparoscopic cholecystectomy, RUQ pain, diarrhea	-	Operative cholecystogramm	Laparoscopic approach
Gentileshci et al <sup>[32]</sup> (1995)	NR	NR	Gallstone disease	Severe diarrhea	-	BE	Laparoscopic approach
Prasad et al <sup>[33]</sup> (1994)	1F	67	NR	Incidental finding during laparoscopic cholecystectomy	-	Operative cholangiography	Laparoscopic approach
Swinnen et al <sup>[34]</sup> (1994)	NR	NR	Gallstone disease	Colonic gallstone obstruction	+ (US)	Colonoscopy & contrast fistulography	NR
Benage et al <sup>[7]</sup> (1990)	1F	78	Gallstone obstruction of CBD	Malabsorption (steatorrhea, osteomalacia, hypocalcemia)	-	ERCP	Open cholecystectomy, repair of the fistula, calcium & D3 supplementation
Sing et al <sup>[35]</sup> (1990)	1M	80	Cholelithiasis, CBD obstruction, (Billroth II?)	Diarrhea, weight loss, fat malabsorption	+ (US)	ERCP	Open cholecystectomy, fistula resection, T-tube in CBD

Caroli-Bosc et al <sup>[36]</sup> (1990)	2		Gallstones	Steatorrhea Jaundice, fever	- -	ERCP ERCP, cholescintigram	Open surgery ERCP sphincterotomy
Patel et al <sup>[37]</sup> (1989)	1F	NR	Gallstone disease	Colonic gallstone obstruction	-	?	?
Anseline et al <sup>[38]</sup> (1981)	1F	90	Gallstone disease	Colonic gallstone obstruction	+ (X-ray contrast)	Intraoperatively	None

M: Male; F: Female; NR: Not reported; RUQ: Right upper quadrant; CBD: Common bile duct; PAF: Plain abdominal film; BE: Barium enema; US: Ultrasound examination; CT: Computed tomography; MRCP: MR cholangio-pancreatography; ERCP: Endoscopic retrograde cholangiopancreatography.

or surgeons should keep a high clinical index for a valid preoperative diagnosis of CFs.

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CASE REPORT

# Suture granuloma of the abdominal wall with intra-abdominal extension 12 years after open appendectomy

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Augustin G, Korolija D, Skegro M, Jakic-Razumovic J. Suture granuloma of the abdominal wall with intra-abdominal extension 12 years after open appendectomy. *World J Gastroenterol* 2009; 15(32): 4083-4086 Available from: URL: <http://www.wjgnet.com/1007-9327/15/4083.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.4083>

## INTRODUCTION

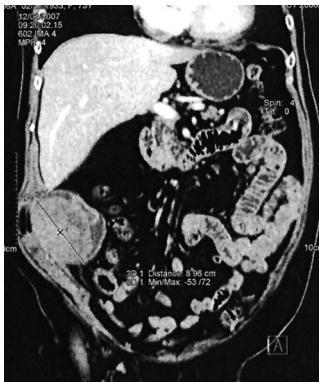
Appendicitis was recognized as a surgical disease when Reginald Heber Fitz correctly pointed out that the frequent abscesses in the right iliac fossa were often due to perforation of the veriform appendix, and he referred to the condition as appendicitis<sup>[1]</sup>. Since that discovery and the development of various surgical incisions and appendectomy techniques, many early and late postoperative complications and coincident conditions have become evident. One of these complications is a postoperative abdominal wall mass in the region of McBurney's muscle-splitting incision. The diagnosis and management of abdominal wall masses after open appendectomy are challenging because various conditions such as appendectomy-related, primary-local (appendectomy-unrelated) and primary-systemic could be the cause of abdominal wall masses postoperatively. This report presents the first known case of a suture granuloma with intra-abdominal extension as a cause of an abdominal wall mass after open (muscle-splitting) appendectomy.

## CASE REPORT

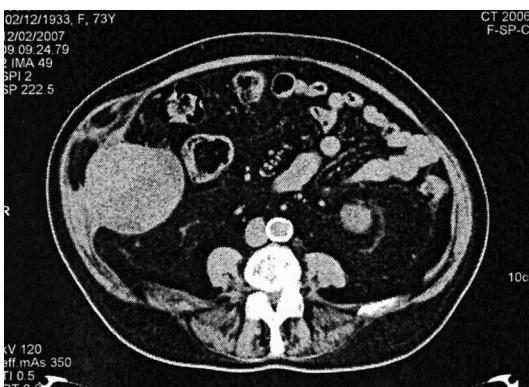
A 75 year-old woman presented with a slightly painful palpable mass in the right lower abdomen lasting for 6 mo. The pain in the right lower quadrant was described as continuous, nonradiating, mild and non-disturbing. There was no nausea or vomiting. Body temperature

## Abstract

Most complications after appendectomy occur within ten days; however, we report the unusual case of a suture granuloma 12 years after open appendectomy. The afebrile 75-year-old woman presented with a slightly painful palpable mass in the right lower abdomen. There was no nausea or vomiting and bowel movements were normal. She lost 10 kg during the 3 mo before presentation. The patient had undergone an appendectomy 12 years previously. Physical examination revealed a tender mass, 10 cm in diameter, under the appendectomy scar. The preoperative laboratory findings, tumor markers and plain abdominal radiographs were normal. Multi-slice computed tomography scanning showed an inhomogenous abdominal mass with minimal vascularization in the right lower abdomen 8.6 cm × 8 cm × 9 cm in size which communicated with the abdominal wall. The abdominal wall was thickened, weak and bulging. The abdominal wall mass did not communicate with the cecum or the ascending colon. Complete excision of the abdominal wall mass was performed *via* median laparotomy. Histopathological examination revealed a granuloma with a central abscess. This case report demonstrates that a preoperative diagnosis of abdominal wall mass after open appendectomy warrants the use of a wide spectrum of diagnostic modalities and consequently different treatment options.



**Figure 1** CT scan of the abdominal wall mass in the right lower abdomen protruding into the abdominal cavity dislocating small bowel loops.



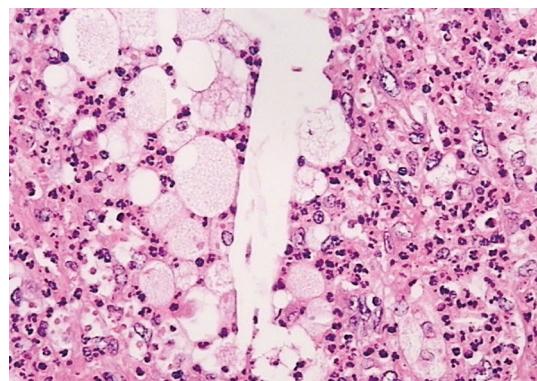
**Figure 2** CT scan of the lower abdomen showing the abdominal wall mass with thickened and bulging abdominal wall and no communication with the cecum.

was 36.8°C and bowel movements were normal. She lost 10 kg during the 3 mo before presentation. The patient had undergone open appendectomy (muscle-splitting incision) 12 years previously. Five years ago she had undergone vaginal hysterectomy with bilateral salpingo-oophorectomy for uterine leiomyomata. Following surgery the patient was in good health and without any symptoms or complaints. She did not take any medications. Physical examination revealed a slightly tender mass, 10 cm in diameter, under the appendectomy scar in the right lower abdomen. The swelling was elastic and poor in mobility. Other resistances were not found, and the rest of the physical examination was normal.

Preoperative laboratory findings and plain abdominal radiographs were normal. Tumor markers were as follows: CEA = 2.42 µg/L; AFP 1.24 µg/L; CA19.9 = 4.89 kU/L and CA 125 = 5.80 kU/L. Abdominal ultrasonography demonstrated a low-echoic mass lesion 8 cm × 8 cm just lateral to the cecum and in communication with the lateral abdominal wall. No peristalsis or communication with the bowel lumen was observed. Esophagogastroduodenoscopy revealed chronic gastritis and colonoscopy revealed sigmoid diverticulosis and normal mucosa in the cecum with normal ileocecal valve. Multi-slice CT scanning showed an inhomogenous abdominal mass with minimal vascularization in the right lower abdomen



**Figure 3** Excised abdominal wall mass.



**Figure 4** Histological image showing the abscess with polymorphonuclear leukocytes, histiocytes and cholesterol crystals (central part of the photograph). (HE,  $\times 400$ ).

which was 8.6 cm × 8 cm × 9 cm in size and communicated with the abdominal wall. The abdominal wall was thickened, weak and bulging (Figure 1). There was no communication between the cecum and the abdominal wall which was also confirmed by previous colonoscopy (Figure 2). From these findings an abdominal wall tumor was suspected and elective surgery was performed.

After a midline laparotomy and adhesiolysis, the greater omentum was detached from the mass in the right lower abdomen which was located intraperitoneally. There was no free intraperitoneal fluid or fibrin deposits. The elastic mass was adherent to the abdominal wall and there was no communication with the small and large bowel, retroperitoneal or vascular structures. After partial omentectomy, the mass was completely extirpated from the abdominal wall (Figure 3). Histopathological examination revealed a foreign-body granuloma with a central abscess (Figure 4). The patient's early postoperative course was uneventful and she left hospital on the 10th postoperative day. On several control examinations during the first 18 mo, the patient was completely symptomless.

## DISCUSSION

This case represents an unusual complication of a suture granuloma with intra-abdominal extension as a cause of an abdominal wall mass 12 years after open appendectomy.

To our knowledge (Medline search 1962-2007) this is the third case of such a complication after appendectomy. Abdominal wall abscesses after appendectomy were diagnosed by Matsuda *et al*<sup>[2]</sup> 11 years postoperatively and by Ichimiya *et al*<sup>[3]</sup> 25 years postoperatively.

A unique feature in our case was the intra-abdominal extension of the abdominal wall suture granuloma with a central abscess which complicated definitive diagnosis.

Since the development of various surgical incisions and appendectomy techniques, many early and late postoperative complications and coincident conditions have become evident. One of these complications is a postoperative abdominal wall mass in the region of McBurney's muscle-splitting incision. Morbidity associated with appendectomy can be as high as 25% in complicated cases<sup>[4]</sup>. Morbidity can be divided into early and late complications. Early complications are more common and mostly include wound hematoma, seroma, abscess or intra-abdominal abscess due to persistence of cavities between the muscle layers and the subcutaneous tissue which encourages fluid collections<sup>[5]</sup>. These complications can then result in cystic formations or masses that can simulate a tumor of the abdominal wall, if they are not readily resolved. For this reason, a meticulous surgical technique, careful hemostasis and placement of suction drains in the subcutaneous tissue are recommended, principally in obese patients<sup>[6]</sup>. Late complications are rare and can include obstruction due to adhesions, postoperative hernia or progression of inflammatory bowel disease not evident at operation for suspected appendicitis. Most of these complications can present as an abdominal mass. An abdominal mass as a primary pathology or postoperative finding always makes precise preoperative diagnosis difficult. A complete list of differential diagnoses of late presenting abdominal wall masses after open appendectomy is shown in Table 1.

Several points should be stressed. Firstly, abdominal wall masses could be: (1) appendectomy-related; (2) primary; (3) posttraumatic; and (4) related to other interventions in the surrounding area for other pathologic conditions. Thus, history taking and physical examination are crucial. The time interval from appendectomy is essential because it determines the difference between early and late complications of appendectomy. Furthermore, symptoms and signs of unrelated diseases (local or systemic) should be confirmed or ruled out (abdominal wall tumors, extension of intra-abdominal malignancy, endometriosis, lymphoproliferative disorders *etc*). Confirmation of invasive interventions in the surrounding area is very important. Open/laparoscopic surgery for intra-abdominal malignancy/infectious diseases, percutaneous or laparoscopic biopsy or percutaneous fine-needle aspiration for malignant hepatobiliary disease could be the cause of abdominal wall port site or incisional metastases or abscesses.

Secondly, by delineating the peritoneal line, the intraperitoneal or extraperitoneal location of the lesion can be determined. This is important for several reasons. First, the entrance into the peritoneal cavity

**Table 1 Differential diagnosis of late presentation abdominal wall masses after open appendectomy**

Suture granuloma
Rectus hematoma
Spontaneous
Traumatic
Postoperative
Wound hematoma (organized)
Abscess
Abdominal wall (various etiologies)
Intra-abdominal (extension)
Hernia
Incisional
Spigelian
Groin
Keloid
Traumatic neuroma
Heterotopic bone formation
Incisional
Traumatic
Abdominal wall tumors
Benign (various)
Malignant (various)
Metastatic
Hematogenous
Post-instrumentation
Port site/trocar metastases
Incisional site metastases
Percutaneous
Intra-abdominal malignancy (extension)
Urachal remnant/cyst/inflammatory mass
Uterine/extruterine (lipo) leiomyomas
Primary
Incisional
Endometriosis
Cutaneous (primary)
Surgical scar endometriosis
Mastocytosis
Systemic juvenile xanthogranulomatosis
Lymphoproliferative disorders (congenital/acquired)
Parasitic (abscess or granuloma)
Enterobius vermicularis
Hydatid cyst
Mycetoma (endemic)
Actinomycosis
Extension from intestinal actinomycosis
Abdominal wall (hematogenous)

could be avoided during surgery if the lesion is located extraperitoneally. Also if the abscess is the cause (acute or chronic) then the extraperitoneal route avoids spillage of contents into the abdominal cavity thus eliminating the possibility of intra-abdominal abscess as a postoperative complication.

Generally, early postoperative masses are easier to diagnose and treat, while late postoperative abdominal wall masses could be of various etiologies that warrant the use of a wide spectrum of diagnostic modalities and consequently different treatment options. All these facts signify the importance of preoperative diagnosis. Thus, abdominal ultrasound, contrast-enhanced multi-slice CT and other diagnostic modalities should be used according to clinical findings. It is concluded that late postoperative abdominal wall masses after open appendectomy can be of various etiologies that warrant

the use of a wide spectrum of diagnostic modalities and consequently different treatment options.

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LETTERS TO THE EDITOR

## No evidence demonstrating hepatotoxicity associated with hydroxycitric acid

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### TO THE EDITOR

In the Letter to the Editor by Lobb<sup>[1]</sup>, the author has hypothesized that the putative hepatotoxicity of the dietary supplement, Hydroxycut, may be due to one of its components, namely hydroxycitric acid (HCA) derived from *Garcinia cambogia*. However, it is important to note that, of the 14 different formulations of Hydroxycut that have been marketed, only 8 contain HCA. In general, these products are cocktails containing up to 20 different ingredients. In the case studies cited by the author<sup>[1]</sup>, information is not provided regarding the specific Hydroxycut products that were used in each of the case reports. This is of concern given that of the numerous ingredients present in these products, no specific reason was given for identifying HCA as the putative hepatotoxic agent other than as the possible hepatotoxic agent in some of the previous case reports. Because of the name of this product line, it is understandable that a possible word association might be made with HCA. Can the reported hepatotoxicity of these products (assuming it is correct) be attributed to an ingredient or combination of ingredients? No information or discussion was presented regarding the potential hepatotoxicity of any of the other ingredients or combinations of ingredients, and it is distracting to make inferences without sound research support.

The importance of the issues raised above is underscored by the numerous animal<sup>[2-8]</sup> and human<sup>[5,9-12]</sup> studies on the safety and efficacy of HCA (as Super CitriMax, HCA-SX). Regrettably, none of these studies was referenced and discussed by the author of this Letter to the Editor<sup>[1]</sup>. In experimental animal studies at up to 25X the human equivalency dose of HCA, no reports are available on hepatotoxicity or other adverse effects. A HCA dose of 2500 mg/kg, equivalent to 150 000 mg in a 60 kg individual, had no adverse effect in the tested animals<sup>[2-4]</sup>.

### Abstract

Although a number of cases of hepatotoxicity are associated with the use of Hydroxycut weight management products, it has been alleged that their effects are primarily due to the presence of hydroxycitric acid (HCA, as Super CitriMax) in the formulations. However, while these products contain up to 20 different ingredients, some do not contain HCA. Case studies reported to date have not considered in depth the literature on the numerous animal and human studies that have been conducted on the safety and efficacy of HCA. No HCA-associated hepatotoxicity or treatment-related adverse effects have been reported in these studies, and thus it is premature to make the assumptions presented in the recent case studies regarding Hydroxycut. If it is established in well controlled studies that the use of these formulations with and/or without HCA can result in the occurrence or progression of hepatotoxicity, additional studies should be conducted to characterize the causative factor(s).

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**Key words:** Hydroxycitric acid; Super CitriMax; Hydroxycitric acid-SX; *Garcinia cambogia*; Hydroxycut; Liver failure; Hepatotoxicity; Safety and efficacy

**Peer reviewer:** Xiao-Ming Yin, MD, PhD, Associate Professor of Pathology, Associate Director, Division of Molecular Diagnostics, Department of Pathology, University of Pittsburgh School of Medicine, Scaife Hall, 7th Fl, Room S739, 3550

Soni *et al*<sup>[5]</sup> have summarized the results of 15 HCA human clinical studies, 14 of them were double blind and placebo controlled, and one was a single arm, open trial. No treatment-related adverse effects have been reported in any of these studies. These authors concluded that HCA at a level up to 2800 mg/d is safe for human consumption. The combined data strongly suggest that HCA itself is not the culprit with respect to the case studies reporting hepatotoxicity associated with Hydroxycut use. In the Health Hazard Report on Hydroxycut by Mozersky *et al*<sup>[13]</sup>, the Board noted that it did not know what ingredient(s) present in these products can cause hepatotoxicity, assuming the products are indeed the causative agents. More studies are needed before a definitive conclusion can be made.

Interestingly, several animal studies have suggested that HCA may have hepatoprotective<sup>[14,15]</sup> and chemoprotective<sup>[16]</sup> properties. In addition, Kaats<sup>[17]</sup> has recently conducted a 60-d study on 25 human subjects using a product containing 4600 mg/d of HCA. The results showed no evidence of adverse effects and indicated hepatoprotection based on decreasing values for the hepatic enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

It should be noted that the dried fruit of *Garcinia cambogia*, a source of HCA, has been consumed for centuries throughout Southeast Asia<sup>[18]</sup> and is in the USDA's list of Perennial Edible Fruits<sup>[19]</sup>. Consistent with the above findings, HCA (as Super CitriMax) has been generally recognized as safe (GRAS) by the Burdock Group, one of the nation's leading food ingredient safety and toxicology groups.

Finally, a key issue that was not discussed by Lobb<sup>[1]</sup> is the possibility that other co-consumed substances, such as acetaminophen, alcohol, or a wide range of prescription drugs, may have been responsible for the hepatotoxicity. The referenced case study by Shim and Saab<sup>[20]</sup> does in fact note that acetaminophen was consumed along with aspirin. Acetaminophen toxicity is the leading cause for calls to Poison Control Centers in the United States and results in almost 500 deaths annually due to acute liver failure<sup>[21]</sup>.

There is no question that issues exist with respect to the regulation, quality control, and appropriate safety and efficacy studies of supplements, just as there are issues with numerous drugs, including acetaminophen, that cause extensive morbidity and mortality.

However, to point an accusatory finger at an ingredient that has been extensively studied and for which no adverse effects have been reported in animal and human studies, is counterproductive.

Given the widespread use of dietary supplements in the USA as well as in other countries, it is imperative that sound science should be used in the evaluation of the potential negative as well as the positive effects of these products. With respect to the potential negative effects of some of these products, an important step forward in this regard is the current requirement for adverse event reporting. However, it is important to note

that these reports typically reflect the associations, rather than the clear-cut cases of causality. When the associations are noted, they should be rigorously examined, and if the supplements are found to be the causative factors for the pathology reported, the true agents need to be firmly identified, along with the dose at which the negative effects are induced.

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LETTERS TO THE EDITOR

## Arterial embolization is the best treatment for pancreaticojejunal anastomotic bleeding after pancreateoduodenectomy

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### TO THE EDITOR

We read with great interest the recent article by Liu *et al*<sup>[1]</sup> published in the April issue of the "World Journal of Gastroenterology" comparing the results of transcatheter arterial embolization and open surgical hemostasis in the treatment of patients with massive pancreaticojejunal anastomotic hemorrhage after pancreateoduodenectomy. We have several comments. Transcatheter embolization is now accepted as the salvage treatment of choice for acute bleeding from the upper gastrointestinal tract. Many published studies have confirmed the feasibility of this approach and the high technical and clinical success rates, ranging from 91% to 100% and from 63% to 100%, respectively, in all case-series including more than 10 patients over the last decade<sup>[2,3]</sup>. First, we are surprised in this study that 6 (35.3%) of the 17 patients had no angiography prior to additional open surgical hemostasis. In our experience, arteriography plays the primary role in the initial investigation of active gastrointestinal bleeding after pancreateoduodenectomy and should be the first step of investigative procedure in such situations, even in hemodynamically unstable patients. It was reported that the gastroduodenal artery stump is one of the main sources of pancreaticojejunal anastomotic hemorrhage after pancreateoduodenectomy<sup>[3]</sup>, as confirmed in this study. Selective angiography of the celiac trunk and common hepatic artery allows in the majority of cases to detect extravasation of contrast medium. However, it is usually difficult to catheterize the gastroduodenal artery stump. Then, we think that coil embolization of the common or proper hepatic artery on either side of the bleeding point ("sandwich technique") is preferable to prevent retrograde filling<sup>[4]</sup>. It seems unlikely that this technique was used by the authors, probably explaining recurrent bleeding in 2 (20%) of the 10 patients treated with transcatheter arterial embolization. Liver failure rarely occurs when hepatic artery embolization is achieved with this technique. However, verification of portal venous flow and the absence of underlying liver disease prior to embolization are required. When

### Abstract

Massive pancreaticojejunal anastomotic bleeding, mainly from the gastroduodenal stump, is one of the most common complications of pancreateoduodenectomy. Selective angiography should be systematically the first step of investigative procedure in such situations. Phacoarteriography may be used if the bleeding point is not spontaneously identified, and allows safe and effective treatment with transcatheter arterial embolization compared to blind open surgical hemostasis. Coil embolization of the common or proper hepatic artery on either side of the bleeding point with "sandwich technique" is then the preferred technique to prevent retrograde filling. Surgery should be performed only as a last resort.

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**Key words:** Pancreateoduodenectomy; Complication; Anastomotic bleeding; Hepatic artery; Transcatheter arterial embolization

**Peer reviewer:** Paul E Sijens, PhD, Associate Professor, Radiology, UMCG, Hanzeplein 1, 9713GZ Groningen, The Netherlands

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the celiac trunk and common hepatic arteriograms are negative, selective catheterization of the superior mesenteric artery must be performed routinely to increase the probability of visualizing active bleeding, because the inferior pancreaticoduodenal artery sometimes supplies the pancreaticojejunal anastomosis. Furthermore, intraarterial anticoagulants, vasodilators, or fibrinolytic agents may be used during angiography to directly elicit contrast medium extravasation, thereby significantly facilitating embolization. In conclusion, we agree with the authors about the safety and efficacy of transcatheter arterial embolization for the treatment of acute hemorrhage after pancreatectoduodenectomy. Angiography should be performed first in such situations. In most cases, embolization obviates the need for surgery and is associated with lower complications and mortality rates than open surgical hemostasis.

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## Meetings

### Events Calendar 2009

January 12-15, 2009  
Hyatt Regency San Francisco, San Francisco, CA  
Mouse Models of Cancer

January 21-24, 2009  
Westin San Diego Hotel, San Diego, CA  
Advances in Prostate Cancer Research

February 3-6, 2009  
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)  
Second AACR Conference  
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009  
Hyatt Regency Boston, Boston, MA  
Translation of the Cancer Genome

February 8-11, 2009  
Westin New Orleans Canal Place, New Orleans, LA  
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009  
Hong Kong Convention and Exhibition Centre, Hong Kong, China  
19th Conference of the APASL  
<http://www.apasl2009hongkong.org/en/home.aspx>

February 27-28, 2009  
Orlando, Florida  
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009  
Vienna, Austria  
EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease  
[www.easl.ch/vienna2009](http://www.easl.ch/vienna2009)

March 13-14, 2009  
Phoenix, Arizona  
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009  
Marriott Wardman Park Hotel  
Washington, DC  
13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009  
Glasgow, Scotland  
British Society of Gastroenterology (BSC) Annual Meeting  
Email: [bsg@mailbox.ulcc.ac.uk](mailto:bsg@mailbox.ulcc.ac.uk)

April 8-9, 2009  
Silver Spring, Maryland  
2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009  
Colorado Convention Center, Denver, CO  
AACR 100th Annual Meeting 2009

April 22-26, 2009  
Copenhagen, Denmark  
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)  
<http://www.easl.ch/>

May 17-20, 2009  
Denver, Colorado, USA  
Digestive Disease Week 2009

May 29-June 2, 2009  
Orange County Convention Center Orlando, Florida  
45th ASCO Annual Meeting  
[www.asco.org/annualmeeting](http://www.asco.org/annualmeeting)

May 30, 2009  
Chicago, Illinois  
Endpoints Workshop: NASH

May 30-June 4, 2009  
McCormick Place, Chicago, IL  
DDW 2009  
<http://www.ddw.org>

June 17-19, 2009  
North Bethesda, MD  
Accelerating Anticancer Agent Development

June 20-26, 2009  
Flims, Switzerland  
Methods in Clinical Cancer Research (Europe)

June 24-27 2009  
Barcelona, Spain  
ESMO Conference: 11th World Congress on Gastrointestinal Cancer  
[www.worldgicancer.com](http://www.worldgicancer.com)

June 25-28, 2009  
Beijing International Convention Center (BICC), Beijing, China  
World Conference on Interventional Oncology  
<http://www.chinamed.com.cn/wcio2009/>

July 5-12, 2009  
Snowmass, CO, United States  
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009  
Aspen, CO, United States  
Molecular Biology in Clinical Oncology

August 1-7, 2009  
Vail Marriott Mountain Resort, Vail, CO, United States  
Methods in Clinical Cancer Research

August 14-16, 2009  
Bell Harbor Conference Center, Seattle, Washington, United States  
Practical Solutions for Successful Management  
<http://www.asge.org/index.aspx?id=5040>

September 23-26, 2009  
Beijing International Convention Center (BICC), Beijing, China  
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists (IASGO)  
<http://iasgo2009.org/en/index.shtml>

September 27-30, 2009  
Taipei, China  
Asian Pacific Digestive Week  
<http://www.apdwcongress.org/2009/index.shtml>

October 7-11, 2009  
Boston Park Plaza Hotel and Towers, Boston, MA, United States  
Frontiers in Basic Cancer Research

October 13-16, 2009  
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Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

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The Liver Meeting

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John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States  
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

November 21-25, 2009  
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[www.gastro2009.org](http://www.gastro2009.org)



### Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.



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- 3 Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of

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- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

**Patent** (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc, assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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